MYCN and the p53-MDM2/MDMX-p14^{ARF} network in neuroblastoma and response to MDM2-p53 antagonists

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Abstract

Background: MYCN-amplification is a major negative prognostic marker, occurring in 25-30% of neuroblastomas. MYCN plays contradictory roles in promoting cell growth and sensitizing cells to apoptosis, and we have recently shown that p53 is a direct transcriptional target of MYCN, and may be an important mechanism of MYCN-induced apoptosis. Although p53 mutations are rare in neuroblastoma at diagnosis, the p53/MDM2/$p14^{ARF}$ pathway is inactivated in 35% of cases through *MDM2*-amplification or $p14^{ARF}$ inactivation. Neuroblastoma is therefore an ideal target for p53 reactivation using MDM2-p53 antagonists. MDMX, a homologue of MDM2, is another negative regulator of p53 which is often overexpressed in cancers and has been shown to compromise the effects of MDM2-p53 antagonists in various cancer types. MDMX expression and the effect on MDM2-p53 antagonists has not been investigated in neuroblastoma.

Hypotheses 1) Reactivation of p53 by inhibition of its negative regulator MDM2, using the MDM2-p53 antagonists Nutlin-3 and MI-63, will result in p53-mediated growth arrest and apoptosis preferentially in *MYCN*-amplified cells 2) MDMX knockdown increases and $p14^{ARF}$ knockdown decreases the sensitivity of neuroblastoma cell lines to MDM2-p53 antagonists.

Methods: The effect of MYCN, MDM2, MDMX and $p14^{ARF}$ was investigated on the response to MDM2-p53 antagonists using siRNA in a panel of 21 neuroblastoma cell lines. Sensitivity was measured by growth inhibition, apoptosis assays including caspase activity and fluorescent activated cell sorting, and the effect on the p53 response measured by Western blotting.

Results: Using the SHEP Tet21N MYCN regulatable system, MYCN(-) cells were more resistant to both Nutlin-3 and MI-63 mediated growth inhibition and apoptosis compared to MYCN(+) cells and siRNA mediated knockdown of MYCN in 4 *MYCN*-amplified cell lines resulted in decreased p53 expression and activation, as well as decreased levels of apoptosis following treatment with MDM2-p53 antagonists. In a panel of cell lines treated with Nutlin-3 and MI-63, the sub-set amplified for *MYCN* had a significantly lower mean GI_{50} value and increased caspase 3/7 activity compared to the non-*MYCN*-amplified group of cell lines, but p53 mutant cell lines were resistant to the antagonists regardless of *MYCN* status.

Knockdown of MDM2 did not alter the apoptotic response to Nutlin-3 or MI-63 but surprisingly, knockdown of MDMX resulted in decreased levels of apoptosis. MDMX expression varied amongst the neuroblastoma cell lines and positively correlated with caspase 3/7 activity following MDM2-p53 antagonist treatment. $p14^{ARF}$ impaired cell lines underwent less apoptosis following MDM2-p53 antagonist treatment and following Nutlin-3 treatment, 3 of 4 $p14^{ARF}$ impaired cell lines underwent a pronounced G_1 arrest. $p14^{ARF}$ knockdown alone resulted in decreased caspase 3/7 activity, and following MDM2-p53 antagonist treatment there was decreased caspase 3 cleavage and activity, and decreased PARP cleavage.

Conclusions: Amplification or overexpression of MYCN sensitizes neuroblastoma cell lines with wildtype p53 to MDM2-p53 antagonists and these compounds may therefore be particularly effective in treating high risk *MYCN*-amplified disease. This data also suggests that neuroblastomas with high MDMX expression may be more susceptible to MDM2-p53 antagonist treatment, but that cells with inactivated $p14^{ARF}$ predominantly undergo a G_1 arrest which may protect them from apoptosis. MDMX and $p14^{ARF}$ status may therefore be important in addition to MYCN in determining the outcome of neuroblastomas treated with MDM2-p53 antagonists.

Declaration

I hereby declare that the work presented in this thesis is original and has not been previously submitted to any other academic institution.

Signed:

Date: 23rd September 2011

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Abbreviations

^{123I}I-MIBG	iodine-131-meta-iodobenzylguanidine
ADP	adenosine diphosphate
ALK	anaplastic lymphoma kinase
AML	acute myeloid leukemia
Amp	amplified
ANOVA	analysis of variance
Apaf-1	apoptotic protease activating factor 1
ARF	alternative Reading Frame
ARF-BP1	ARF binding protein 1
ASH	American Society of Hematology
ASK1	apoptosis signal-regulating kinase 1
ASN	antisense
ASPP	apoptosis-stimulating protein of p53
ATM	ataxia telangiectasia
AurKA	aurora kinase A
BAK	BCL2 homologous antagonist killer
BAX	BCL2 associated X protein
BCA	bicinchoninic Acid
BCL2	B-cell CLL/lymphoma 2
BCL-X_L	B-cell lymphoma-extra large
BH3-only	Bcl-2-homology domain 3 only
bHLH	basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
BMP	bone morphogenetic protein
BSA	bovine serum albumin
C-terminus	carboxy-terminus
Caspase	Cysteine-dependent Aspartate-Specific Proteases
CBP	creb-binding protein
Cdc	cell division cycle
CDK	cyclin-dependent kinase
cDNA	copy DNA
CEN	Centromere
CGH	comparative genomic hybridisation
Cip/Kip	CDK interacting protein/kinase inhibitory protein
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
Ct	number of cycles to reach threshold
CTBP2	C-terminal binding protein 2
Cys	Cysteine
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
dH_2O	distilled water
ddH_2O	double distilled water
DDK3	dickopf 3
DFMO	α-difluoromethylornithine

DMs	double minutes
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
dNTP	deoxyribonucleoside triphosphate
DR4	death receptor 4
DR5	death receptor 5
dsRNA	double stranded RNA
E2F	E2F family of DNA-binding transcription facotrs
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra acetic acid
FACs	fluorescence activated cell sorting/flow cytometry
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanate
FL1	fluorescent detector 1
FL2	fluorescent detector 2
FL2-A	FL2-area
FL2-H	FL2-height
FL2-W	FL2-width
FL3	fluorescent detector 3
FRET	fluorescence resonance energy transfer
FSC-H	forward scatter - height
G_1	Gap 1
G_2	Gap 2
G_0	Gap 0
GI_{50}	the concentration at which a compound reduces the growth of the cell population by 50%
GADD45	growth arrest on DNA damage
GD-2	disialoganglioside
GM-CSF	granulocyte-macrophage colony-stimulating factor
GCS	glucosylceramide synthase
Gy	gray
H_2O	water
HAUSP	Herpes virus-associated ubiquitin-specific protease
HCl	hydrochloric acid
HDAC1	histone deacetylase 1
Her2	human epidermal growth factor receptor 2
HIF1α	hypoxia-inducible factor-1α protein
HIPK2	Homeodomain-interacting protein kinase 2
hMOF	human orthologue of Drosophila melanogaster MOF
HRP	horseradish peroxidase
HSRs	homogeneously staining regions
I-type	intermediate-type
IAP	inhibitor of apoptosis
IC_{50}	Concentration of an inhibitor at which 50% inhibition of the response is seen
ID2	inhibitor of DNA binding
IgG	immunoglobulin G
IF	immunofluorescence
IHC	immunohistochemistry
IL-2	interleukin 2

INPC	International Neuroblastoma Pathology Classification
INSS	International Neuroblastoma Staging System
INRG	International Neuroblastoma Risk Group
IR	irradiation
JNK	c-Jun N-terminal kinase
KAP1	KRAB-ZFP-associated protein 1
Leu	leucine
LL	lower left
LOH	loss of heterozygosity
LR	lower right
M-phase	mitosis-phase
MAX	Myc associated factor x
MC	monoclonal
MCS	multiple cloning site
MDM2	mouse double minute 2
MDMX	mouse double minute 4 homolog
MEF	mouse embryonic fibrolast
$MgCl_2$	magnesium chloride
Miz-1	Myc-interacting zinc finger protein
MRD	minimal residual disease
MRP-1	multidrug resistance protein 1
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MYC/N	members of the MYC family
N-terminus	amino-terminus
N-type	neuronal-bearing type
NES	nuclear export signal
NF-κB	nuclear factor kappa b
NMI	N-myc interactor
NMP	nucleophosmin
NoLS	nucleolar localisation signal
Non-amp	non-amplified
O/N	overnight
OD	optical density
OgD	oligomerisation domain
ODC	ornithine decarboxylase
OPTI	optiMEM-glutamax serum free media
P1	promoter 1
P2	promoter 2
$p21^{WAF1}$	p21 wildtype activated fragment 1
p53 pSer 15	p53 phosphorylation at serine 15
p53AIP	p53-regulated apoptosis-inducing protein
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly(ADP-Ribose) polymerase
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PCMV	human cytomegalovirus promoter
P-gp	p-glycoprotein
Phe	phanylalanine
Phox2a/2b	paired-like homeobox 2a/2b
PI	propidium iodide
PIG3	p53-induced gene 3

$Pmin_{hCMV}$	minimal promoter of human CMV
PNET	primitive neuroectodermal tumour
PS	phosphatidylserine
PUMA	p53-upregulated modulator of apoptosis
Rb	retinoblastoma protein
RING	really interesting new gene
RISCs	RNA-induced silencing complexes
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
RT-PCR	reverse transcription PCR
S-phase	synthesis-phase
S-type	substrate adherent type
SCR	scrambled
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	serine
shRNA	short-hairpin RNA
siRNA	short interfering RNA
SKP2	S-phase kinase associated protein 2
SN	sense
SNP	single nucleotide polymorphism
SRB	Sulforhodamine B
SSC-H	Side Scatter - Height
STR	short tandem repeat
TAD	transactivation domain
TAF1	TBP-associated factor 1
TBE	Tris/Borate/EDTA
TBP	TATA-binding protein
TBS	Tris Buffered Saline
TCA	Trichloroacetic Acid
Tet	Tetracycline
*tet*O	TET operator
*tet*R	Tet respressor protein
TFIIB	transcription initiation factor B
TFIID	transcription initiation factor D
TFIIH	transcription initiation factor H
TH-MYCN	MYCN transgenic mouse
Thr	threonine
TIP49	TBP-interacting protein 49
TIP60	TBP-interacting protein 60
TNF	tumor necrosis factor
TP53INP1	Tumor protein p53-inducible nuclear protein 1
TRAIL	TNF-related apoptosis-inducing ligand
TRE	Tetracycline Responsive Promoter Element
Trk	tyrosine kinase
Trp	tryptophan
TRRAP	transformation/transcription domain-associated protein
TSG	tumour suppressor gene
tTA	Tetracycline-controlled Transactivator

UK	United Kingdom
UL	Upper Left
ULF	ubiquitin ligase of ARF
UR	Upper Right
USA	United States of America
UT	untreated
UV	Ultra Violet
VP16	Virion Protein 16
w/v	weight/volume
Wt	wildtype
WWP1	WW domain containing E3 ubiquitin ligase 1
XIAP	x-linked inhibitor of apoptosis protein
YY1	ying-yang 1

Chapter 1. Introduction

1.1 Cancer

Cancer forms as a result of multiple genetic mutations which lead to uncontrollable cell growth, invasion and metastases, and is influenced by both genetic and environmental factors (Pelengaris and Kahn, 2006). In a normal cell, a physiological balance between tumour suppressors and oncogenes carefully controls cell proliferation. In a cancer cell, evidence points towards a multistep process of sequential alterations in oncogenes and tumour suppressor genes (TSGs) that result in the acquirement of capabilities known as the hallmarks of cancer (Figure 1.1) (Hanahan and Weinberg, 2011). The increasing genomic instability within a cancer cell leads to heterogeneity, where a tumour possesses cytogenetically different clones that contribute to differences in clinical behaviour and treatment response in the same diagnostic tumour types.

As shown in Figure 1.1, compounds targeting each of the ten hallmarks of cancer have been developed over the last decade, and the list continues to grow.

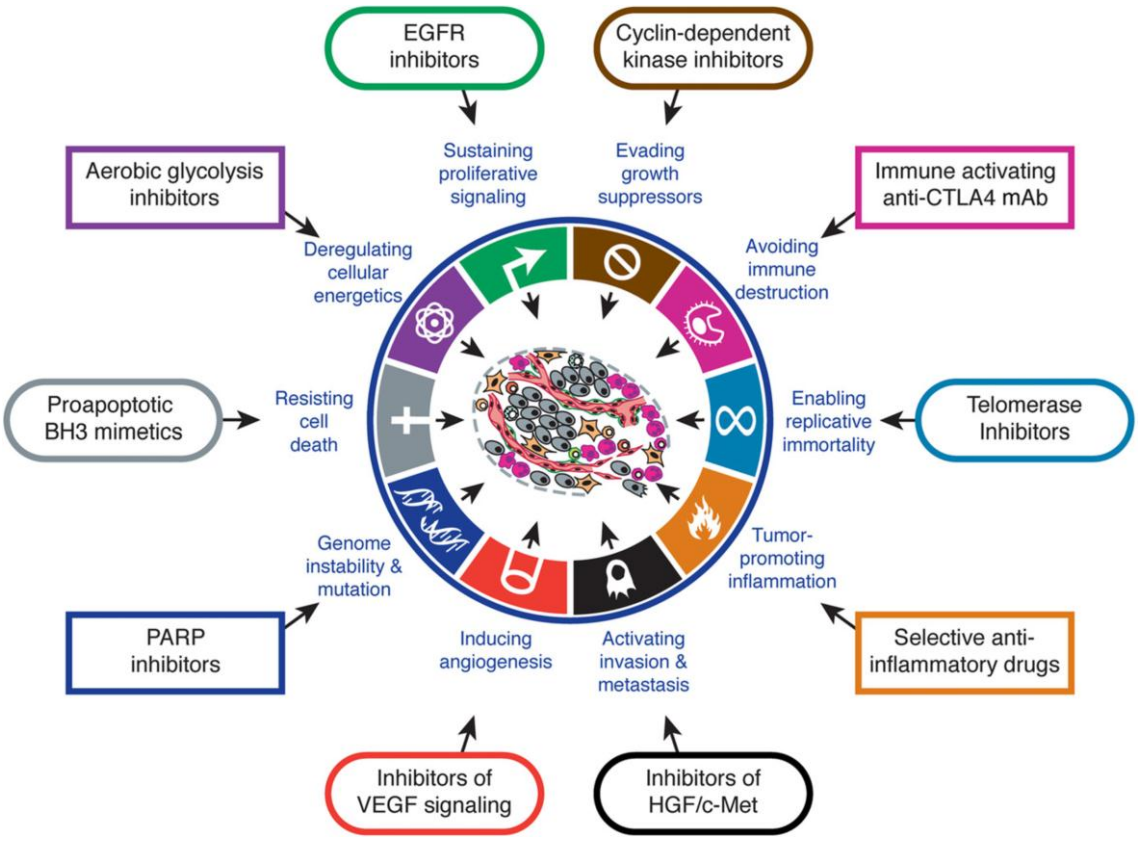


Figure 1.1. **The hallmarks of cancer (Hanahan and Weinberg, 2011).**

1.2 Oncogenes

Gain-of function mutations in oncogenes promote cancer formation. Oncogenes encode proteins that are involved in regulating cell proliferation and apoptosis, and have the potential to cause cancer by increasing cell survival and/or promoting cell proliferation. Proto-oncogenes can be activated to oncogenes by a number of mechanisms; 1) Structural alterations in chromosomes including inversions and translocations or juxtaposition of enhancer elements. These changes may increase or deregulate the transcription of an oncogene, for example via an alternative promoter. 2) Gain of function mutations which increase protein activity directly, or alter the structure of the protein, enhancing activity. 3) Overexpression or amplification (Croce, 2008; Konopka et al., 1985; Tsujimoto et al., 1985). These alterations confer growth advantages and increase survival of these cells, as a result of alteration in structure, or deregulation of expression (Bishop, 1991).

There are 6 classes of oncogene products: transcription factors, growth factors, growth factor receptors, signal transducers, chromatin remodelers and apoptosis regulators. Oncogenes focused on in this thesis include the MYCN transcription factor, mouse-double minute 2 (MDM2) and the MDM2 paralogue, MDMX.

Oncogene proteins can be targeted by small molecules. A well-known example is Imatinib, used for the treatment of chronic myelogenous leukemia (CML) (Goldman and Melo, 2001). In this thesis, the use of small molecule MDM2 inhibitors in neuroblastoma is investigated.

1.3 Tumour suppressor genes (TSGs)

Loss of function mutations within TSGs predispose to cancer. In normal cells TSGs inhibit cancer development and oppose oncogene function. There are 2 types of TSGs, 'gatekeepers' and 'caretakers' (van Heemst et al., 2007; Sherr, 2004; Kinzler and Vogelstein, 1997). Gatekeepers inhibit proliferation and promote apoptosis, regulating the growth of tumours, whereas caretakers control cellular processes that repair genetic alterations and therefore play key roles in maintaining genomic integrity. Mutations of caretakers cause genomic instability and an overall increase in mutation rate within a cell. TSGs investigated in this study include the p53 gatekeeper, commonly referred to as the 'guardian of the genome', and $p14^{ARF}$.

Familial cancers usually result from an initial germline mutation of one allele of a TSG followed later by either a somatic mutation, loss of a second allele (loss of heterozygosity), or transcriptional silencing through hypermethylation of the second allele (Taneja et al., 2011).

1.3.1 Knudson's two-hit cancer model

The Knudson two-hit hypothesis for cancer development was formulated in 1971 by Alfred Knudson (Knudson, 1971). He proposed that the initiation of cancer requires the loss of both wildtype alleles of a TSG and the resulting 'loss-of-function'. As shown in Figure 1.2, individuals with both wildtype alleles of the TSG require 2 'hits' for cancer development. However, if an individual has a germline mutation in one allele, only one 'hit' is required and these individuals therefore have increased susceptibility to cancer development. It is now thought that four or more individual mutations affecting critical cellular signalling pathways are required for tumourigenesis (Vogelstein and Kinzler, 2004).

1.4 Neuroblastoma

Neuroblastoma is the most common solid extracranial cancer in children, representing 7% of all solid childhood tumours and 15% of childhood cancer related deaths (Hildebrandt and Traunecker, 2005; Maris and Matthay, 1999; Gurney et al., 1997). In Western countries, the incidence of neuroblastoma is 10.9 children per million under the age of 15 (Spix et al., 2006). The mean age of diagnosis is 20 months, and 90% of cases are diagnosed by the age of 6 years (Ora and Eggert, 2011). It is an extremely heterogeneous disease, characterised by its diverse clinical behaviour. Cases range from a relentlessly progressive highly malignant form of the disease, that responds poorly to treatment and usually relapses, to low risk localised tumours with excellent prognosis (Hildebrandt and Traunecker, 2005). Neuroblastoma is also unique in that it has a high rate of spontaneous regression in infants (Castleberry, 1997). Over 50% of cases are high risk and difficult to cure, with long term survival rates remaining below 40% despite intense multimodal therapies (Figure 1.3).

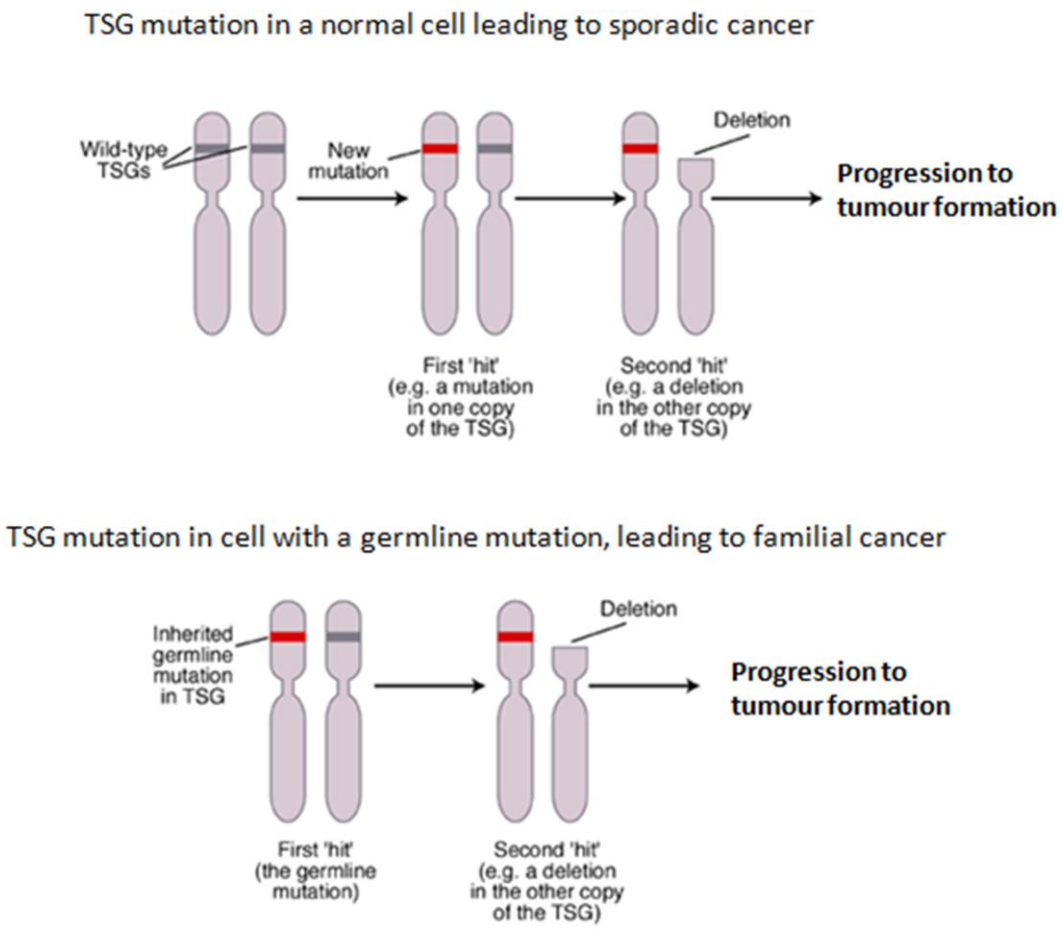


Figure 1.2. **Knudson's two hit hypothesis model for tumour formation (Richards, 2001).**

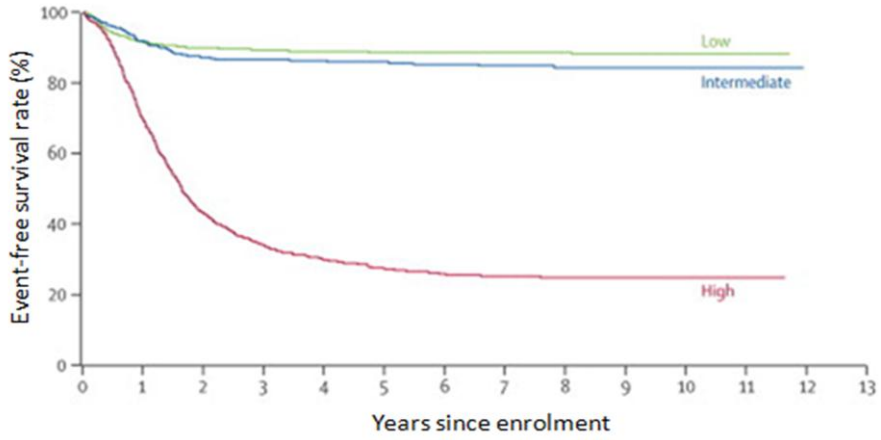


Figure 1.3. **Survival of patients with neuroblastoma based on risk group. High risk groups have a significantly lower survival rate than low and intermediate groups (Maris et al., 2007).**

1.4.1 Neural Development

Neuroblastoma is a cancer of the peripheral nervous system that arises from developing neural crest cells that go on to form the sympathetic nervous system. The pluripotent sympathogonia form the sympathetic ganglia, the chromaffin cells of the adrenal medulla and the paraganglia (Gestblom et al., 1999). Due to the origin of neural crest cells, neuroblastomas develop anywhere in the sympathetic nervous system, such as the paraspinal sympathetic ganglia, abdomen, neck, chest and pelvis (Maris et al., 2007; Castleberry, 1997). About 65% of tumours occur in the abdomen, often the adrenal medulla.

During neural development, neural crest cells migrate and differentiate into several lineages such as melanocytes, sensory neurons, enteric ganglion cells and sympathetic neurons as shown in Figure 1.4. Bone morphogenetic proteins (BMPs) and their receptors are the first signalling molecules and they are responsible for inducing differentiation or migration of neural crest cells (Huber et al., 2002). Differentiation into sympathetic neurons is linked with transient expression of basic helix-loop-helix (bHLH) transcription factors which include MASH1, HES1, MYCN, HIF1α and HuD, in addition to homeobox genes such as *Phox2a* and *Phox2b*, and also the p53 paralogue, p73. Some of these genes are upregulated or amplified in aggressive neuroblastoma (Nakagawara and Ohira, 2004). Terminal differentiation to mature sympathetic cells is strongly regulated by members of the neurotrophin family and their receptors (Nakagawara and Ohira, 2004; Nakagawara, 2001). Other genetic aberrations associated with neuroblastoma have been mapped to specific regions or genes involved in regulating neuron differentiation and development, and many genes involved in these pathways are expressed at high levels in favourable neuroblastoma (Nakagawara, 2005; Nakagawara and Ohira, 2004; Nakagawara, 2001).

MYCN is transiently expressed and defines the direction of neuronal differentiation. It functions by inducing ID2 proteins which generally function as differentiation inhibitors and positive regulators of proliferation in neuronal development (Iavarone and Lasorella, 2004; Lasorella et al., 2000). The ID2 proteins inhibit the retinoblastoma protein (Rb) and other proteins such as HES1. MYCN has been shown to induce ID2 in neuroblastoma, stimulating cell proliferation through Rb inhibition (Lasorella et al., 2000).

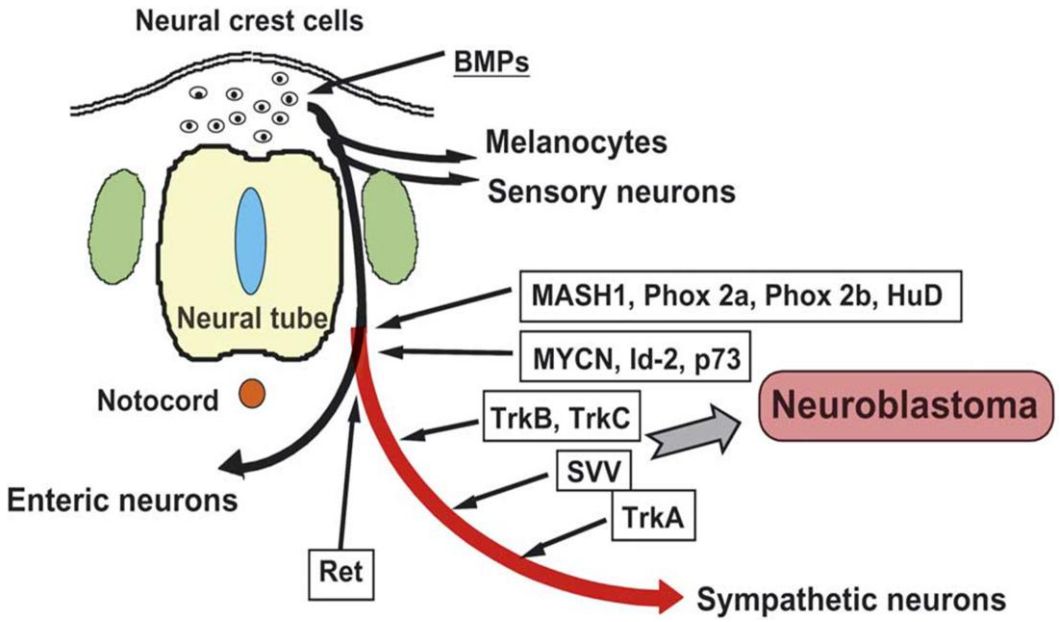


Figure 1.4. **The differentiation pathway of sympathetic neurons (Nakagawara and Ohira, 2004).**

1.4.2 Tumour cell biology of neuroblastoma

Neuroblastomas belong to the 'small blue round cell' neoplasm of childhood, and to the group of peripheral neuroblastic tumours (Triche, 1986), which comprise neuroblastoma, ganglioneuroblastomas (nodular and intermixed) and benign ganglioneuromas shown in Table 1.1.

Schwann cells are sheath cells of the central and peripheral nervous system, which support neurones and are derived from the neural crest (Brodeur, 2003). They are involved in regulating both differentiation and apoptosis, and also play a role in blocking angiogenesis and cell growth (Liu et al., 2005). The number of Schwann cells and the differentiation status of the neuroblastic component affect the phenotype of the tumour, have prognostic significance and are used to assign treatment in some subtypes of neuroblastoma.

Tumour group	Description
Ganglioneuromas (Figure 1.5a)	• Most differentiated form of neuroblastoma • Schwannian stroma dominant • Contains neurophils • Mostly mature ganglia cells neurones (no neuroblasts) • Benign
Ganglioneuroblastoma (Figure 1.5b)	• Undifferentiated tumours or partially differentiated tumours • Composed of neuroblasts (small round cells) • Schwannian stroma rich (intermixed) or a mixture of Schwannian stroma rich and Schwannian stroma poor (nodular). • Malignant and benign parts
Neuroblastoma	• Undifferentiated or poorly differentiated tumours with large dense nuclei • Little cytoplasm • Schwannian stroma poor • Malignant

Table 1.1. **Categories of neuroblastic tumours according to the International Neuroblastoma Pathology Criteria (Shimada et al., 1999).**

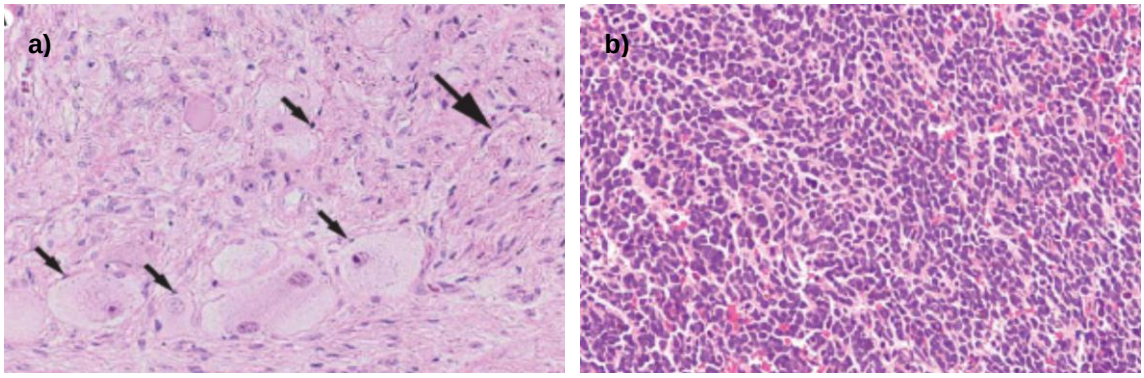


Figure 1.5. **Neuroblastoma tumour cells with varying degrees of differentiation. a)** Schwann cells and ganglion cells, the latter indicated by the arrows are prominent in stroma-rich differentiated neuroblastoma. **b)** Undifferentiated neuroblastoma consists of densely packed small round blue cells with little cytoplasm (Maris et al., 2007).

1.4.3 Cell Type

Consistent with its origins from multi-potent neural crest cells, neuroblastoma cell lines are composed of a number of cell types which are classified into 3 categories based on their morphological and biological characteristics; N-type (neurite-bearing), S-type (substrate-adherent) and the intermediate I-type (Table 1.2). Cell type may have

prognostic relevance, as in response to certain morphogens they migrate along specific neural crest lineages (Ross et al., 2003). Studies have found that a) S-type cells have increased $p21^{WAF1}$ and MDM2 levels following cytotoxic drug treatment compared to N-type cells b) N-type cells have increased apoptosis following DNA damage compared to S-type cells, and c) that whilst some N-type cells fail to G_1 arrest, all S-type cells G_1 arrest in response to DNA damage (Carr-Wilkinson et al., 2011; Rodriguez-Lopez et al., 2001; Isaacs et al., 1998). I-type cells are associated with more malignant and progressive disease than N- and S-type (Ross et al., 1995).

Cell Type	Description
I-type	• Stem cells with morphological features of N-type and S-type neuroblastoma cells. They attach equally well to both cell and substrate and have a round prominent nucleus.
N-type	• Neuroblastic/neuroendocrine precursors or immature neuroblasts. They attach better to cell than substrate and have a round prominent nucleus that sometimes has neurites. The cell bodies are small and refractile and have a high nuclear:cytoplasmic ratio, contain neurites and have a high saturation density. • Present at various differentiation states.
S-type	• These are Schwannian/metabolic precursors that are non-neuronal. They adhere tightly to substrates, have no neurites, form monolayers in culture and have contact inhibition of growth • May be tumour derived.

Table 1.2. **The 3 cell types that appear in neuroblastoma tumour cell lines. Adapted from (Ross et al., 2003).**

1.4.4 Predisposition to neuroblastoma

Most cases of neuroblastoma occur sporadically through somatic changes including allelic gains and losses, oncogene activation, tumour suppressor inactivation and changes in cell ploidy (Brodeur, 2003). Only 1-2% of cases of neuroblastoma occur in children with an inherited predisposition to the disease (Maris and Matthay, 1999). Familial neuroblastomas have an autosomal dominant pattern of inheritance, with incomplete penetrance, arising from a germline mutation in one allele (Maris and Matthay, 1999). The disease has the same diverse clinical behaviour as somatic neuroblastoma, ranging from aggressive progression to spontaneous regression (Maris et al., 1997).

Very recently, two genes have been identified as playing a role in familial neuroblastoma; the *PHOX2B* gene and the *anaplastic lymphoma kinase* (ALK) gene (Mosse et al., 2008; Mosse et al., 2004). The *PHOX2B* gene is associated with

differentiation of the sympathetic nervous system and the synthesis of catecholamines. However, somatic mutations of this gene are very rare (Raabe et al., 2008; van Limpt et al., 2004). The *ALK* gene has been identified as predisposing to neuroblastoma, with germline mutations present in neuroblastoma pedigrees (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). ALK is a transmembrane receptor tyrosine kinase that is preferentially expressed in the central and peripheral nervous system (Chiarle et al., 2008).

1.4.5 Clinical presentation and diagnosis

Presentation at diagnosis ranges from a painless mass, to a rapidly growing and expansive tumour that gives rise to life threatening symptoms. About half of neuroblastoma patients present with metastatic disease, and usually have haematological metastases arising in the bone marrow, cortical bone, liver, skin and lymph nodes and less commonly, lungs and central nervous system (Friedman and Castleberry, 2007). Symptoms may be non-specific, but include fever, pallor, anorexia, bone pain, and retro-orbital and orbital metastases. Spontaneously regressing disease makes up around 5% of detected cases, and the tumours are usually small and localised with metastases usually occurring in the bone marrow, liver and skin (4s disease) (Maris et al., 2007; Brodeur, 2003).

A diagnosis of neuroblastoma is based on both an increase in catecholamines and catecholamine metabolites in urine and serum, and histological diagnosis of tumour specimen or bone marrow aspirate, with or without immunohistochemistry (Brodeur et al., 1993). A ^{123}I-MIBG scan is often carried out to identify potential metastases.

1.4.6 Genetics of sporadic neuroblastoma

A number of genetic aberrations occur in neuroblastoma, some of which have prognostic significance and impact on disease severity. Common cytogenetic alterations include loss of chromosome 1p, deletions in chromosomes 2p, 3p, 4p, 9q, 11q, 14q, gain of 17q and *MYCN*-amplification. Allelic imbalances have also been described at 5q, 9p and 19q (Caren et al., 2010; Attiyeh et al., 2005; Schwab et al., 2003; Caron et al., 2001; Plantaz et al., 2001; Bown et al., 1999; Schleiermacher et al., 1994). At regions of genetic loss and gain, there is likely to be oncogene activation and/or tumour suppressor inactivation. Recently, high-resolution array CGH has allowed comprehensive examination of aberrations in neuroblastoma tumours and cell lines (Janoueix-Lerosey et al., 2009; Carr et al., 2007; Michels et al., 2007; Mosse et al., 2005;

Chen et al., 2004). It is a combination of these genetic abnormalities rather than a single one that has been shown to be prognostic, and together they are called segmental chromosomal aberrations.

1.4.6.1 MYCN amplification

The MYCN oncogene is located on the distal short arm of chromosome 2, at locus 2p24 (Schwab et al., 1983). It is amplified in 22-30% of primary neuroblastomas, usually with 50-500 copies per cell as shown in Figure 1.6, with correspondingly high protein levels in most cases (Seeger et al., 1988; Brodeur et al., 1984). Amplification occurs in the form of homogeneously staining regions (HSRs) at random chromosome regions, or as double minute chromatin bodies (DMs), followed by integration as HSRs (Kohl et al., 1983).

MYCN-amplification is a major marker of adverse prognosis, and is strongly associated with rapidly progressive advanced stage disease (see Section 1.5). It is often associated with other genetic abnormalities and most *MYCN*-amplified tumours have either allelic loss of 1p or 17q gain, or both (Bown et al., 1999; Fong et al., 1989). Some *MYCN*-amplified tumours have non-syntenic co-amplification of *MDM2* (Corvi et al., 1995b).

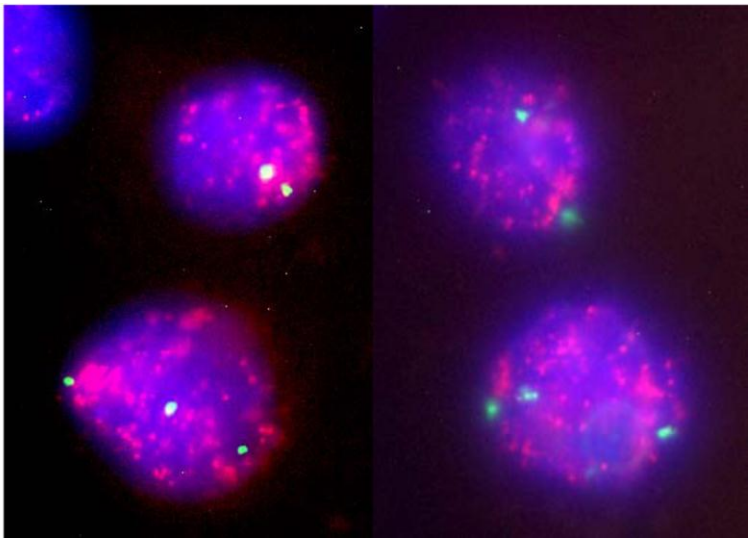


Figure 1.6. **FISH detection of *MYCN*-amplification in neuroblastoma cells.**

1.4.6.2 1p deletion

LOH or deletion of the short arm of chromosome 1 (1p) is significantly associated with aggressive disease, and occurs in 23-35% of neuroblastoma (Maris et al., 2000; Caron et al., 1996). 1p36 deletion was predictive of survival in multivariate analyses, and it is likely there are one or more TSGs present in this region, including p73. The most

common cause of 1p loss is the translocation and unbalanced gain of 17q (Bown et al., 2001). In addition, 62% of tumours with 1p deletion have *MYCN*-amplification and 1p deletion is associated with di- or tetraploidy (Bown, 2001; Maris et al., 1995; Fong et al., 1989).

Some studies found increased relapsed rates were associated with 1p36 deletion in low and intermediate risk neuroblastoma. However, the use of more intensive treatment was successful in treating these patients (Cohn et al., 2009).

1.4.6.3 Gain of 17q

An unbalanced gain of 17q occurs in more than 50% of neuroblastomas and is strongly indicative of poor prognosis, whilst whole chromosome gains of chromosome 17 are seen in 40% of triploid cases and may be favourable (Bown et al., 2001; Bader et al., 1991). The prognostic significance of 17q gain is not strong enough, nor independent enough to be included in clinical trials on its own. Survivin is located at 17q25, and is a member of the Inhibitor of Apoptosis Protein (IAP) family. In neuroblastoma, Survivin expression is associated with unfavourable histology and aggressive tumours (Islam et al., 2000; Adida et al., 1998).

1.4.6.4 Loss of 11q

Loss at 11q23 occurs in 26-44% of neuroblastomas (Maris et al., 2001; Mertens et al., 1997) and is associated with unfavourable prognosis in non-*MYCN*-amplified tumours (Attiyeh et al., 2005; Spitz et al., 2003). It inversely correlates with *MYCN*-amplification. Allelic loss of 11q is an independent marker of decreased event-free survival in all risk groups (Canete et al., 2009; Simon et al., 2006; Spitz et al., 2003).

1.4.6.5 Ploidy

Gains and losses of one or more chromosomes of the diploid genome occur regularly in neuroblastoma. Near diploid lesions contribute a risk factor for patients with metastatic disease between 12 and 18 months of age without *MYCN*-amplification (George et al., 2005; Bowman et al., 1997; Look et al., 1991). Localised tumours with *MYCN*-amplification and hyperploidy are associated with better outcome (Bagatell et al., 2009; Schneiderman et al., 2008).

1.4.6.6 ALK mutations

Somatic and activating mutations in *ALK* have been identified in 8% of neuroblastomas (Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al.,

2008). The link between aberrations and tumour biology have not been fully identified but as of yet, no consistent correlations between *ALK* mutations and aggressive disease have been identified.

1.4.6.7 Trk receptor tyrosine kinases

Neurotrophins and their receptors play roles in regulating both differentiation and survival of neural cells during development, and are implicated in the pathogenesis of neuroblastoma (reviewed by (Schramm et al., 2005)). Neurotrophin function is mediated through the high affinity TrK family of tyrosine kinase receptors; TrKA, TrKB and TrKC. TrKA and TrKC expression are associated with favourable neuroblastoma and good prognosis whereas TrKB expression is associated with unfavourable neuroblastoma and *MYCN*-amplification (Brodeur et al., 1997; Nakagawara et al., 1994; Nakagawara et al., 1993; Nakagawara et al., 1992).

1.4.6.8 Conceptual model of neuroblastoma development

A model of biological and genetic features which characterise the development of neuroblastomas into low and high risk tumours is shown in Figure 1.7. This model assumes all neuroblastomas have a common precursor and that a commitment defining risk group is made shortly after tumour initiation (Kushner and Cheung, 2005).

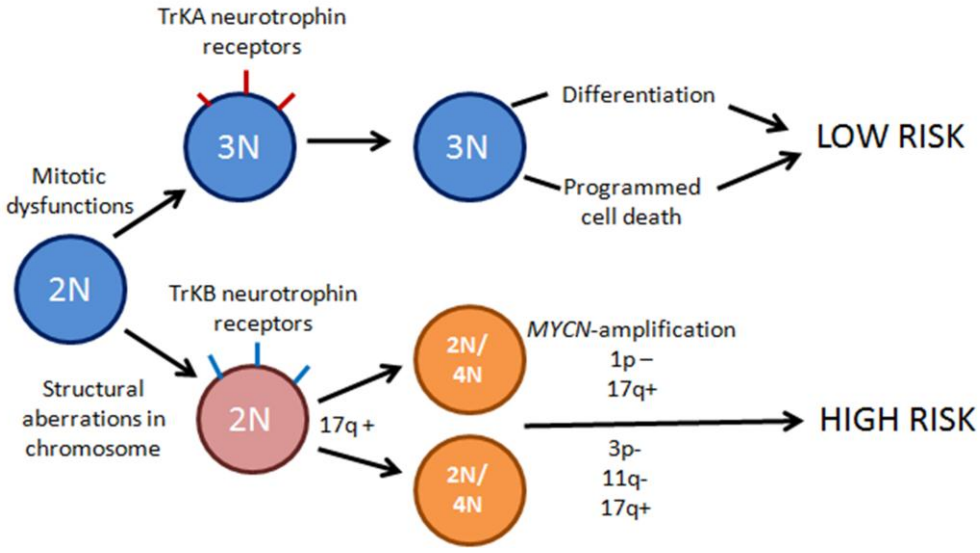


Figure 1.7. **A proposed model for the biological and genetic abnormalities involved in neuroblastoma development, and risk group categorisation (Kushner and Cheung, 2005).**

1.4.7 Risk stratification and staging

In recent years, survival rates and patient outcome for low and intermediate risk groups have improved dramatically due to tailoring of treatment to risk group, resulting in less intensive treatment (Baker et al., 2010; De Bernardi et al., 2009; Modak et al., 2009; Hero et al., 2008). However, survival rates for patients with high-risk disease remain below 40% despite intense multimodal treatment (Haupt et al., 2010; Pearson et al., 2008; Maris et al., 2007). Survivors of high risk disease often relapse and become resistant to conventional chemotherapeutic drugs, or have complications from the intense therapy later in life (Perwein et al., 2011; Laverdiere et al., 2009).

Genetic factors, tumour histology and stage all contribute to the likelihood of disease progression and a system is needed to categorise patients into different groups to allow for treatment to be tailored to tumour behaviour (Maris and Matthay, 1999).

The International Neuroblastoma Staging System (INSS), developed by Brodeur *et al* classifies patients into stages 1-4s, and is shown in Table 1.3 (Hildebrandt and Traunecker, 2005; Brodeur, 2003). The INSS takes into account the local and distant extent of the disease, and the resectabiliy of the tumour, but not any genetic or biochemical markers.

The International Neuroblastoma Pathology Classification (INPC) is a histopathological classification system based on age, the presence and absence of Schwannian stroma, the degree of differentiation and the mitosis-karyorrhexis index (Shimada et al., 1999). Over the past two decades, INSS stage, patient age and amplification of *MYCN* have been used as the 3 major prognostic markers for treatment stratification in clinical trials worldwide but it is possible that additional prognostic markers will help. To make risk grouping uniform worldwide and to establish an international consensus approach for current pretreatment risk stratification, the International Neuroblastoma Risk Group (INRG) was created by the international experts and defines the risk groups as shown in Table 1.4 (Cohn et al., 2009). The INSS and the INPC form the basis of the INRG which incorporates the basic histopathological categories and tumour cell differentiation to achieve a global stratification system, and successfully distinguishes patients with highly metastatic disease requiring aggressive multimodal therapy from those who may be cured from just surgery or observation alone. Thirteen variables were analysed in 8800 patients diagnosed between 1990 and 2002 and a schema developed with 16 pretreatment designations and the age cut-off was increased to 18 months (Moroz et al.,

2011; Cohn et al., 2009; London et al., 2005). The INRG uses radiological characteristics of the primary tumour to allow successful surgery, and predicts the extent of the disease through bone marrow morphology and imaging studies (Maris et al., 2007).

Stage	Description	Incidence
1	Localised with complete gross excision with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour could be positive)	5%
2A	Localised tumour with or without complete gross excision, with ipsilateral non-adherent lymph nodes negative for tumour microscopy	10%
2B	Localised tumour with or without complete gross excision, with ipsilateral non-adherent lymph nodes positive for tumour microscopy. Enlarged contralateral lymph nodes should be negative microscopically.	
3	Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; or localised unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement.	20%
4	Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, or other organs (except as defined by 4S) Distant metastatic disease.	60%
4S	Localised primary tumour in infants younger than 1 year (as defined for stages 1, 2A, or 2B) with dissemination limited to skin, liver or bone marrow (<10% malignant cells)	5%

Table 1.3. **INSS staging of neuroblastoma (Maris et al., 2007; Hildebrandt and Traunecker, 2005).**

1.4.8 Current treatment (reviewed by (Ora and Eggert, 2011) and (Tweddle, 2009))

1. Observation. Most stage 4s neuroblastomas spontaneously regress, therefore patients without symptoms or favourable prognostic markers are observed closely. In a study using the 'wait-and-see' approach for these patients, 47% of tumours spontaneously regressed (Hero et al., 2008), and in another study 17 of 53 required treatment (Tanaka et al., 2010).

2. Surgery. Surgery is the main treatment in localised disease, and is sometimes performed after chemotherapy to shrink tumour size.

3. Chemotherapy. The majority of patients present with metastases at diagnosis and require chemotherapy. Chemotherapeutic agents used in the treatment of neuroblastoma include alkylating agents such as cyclophosphamide, platinum analogues such as cisplatin and carboplatin, vinca-alkoids such as vincristine, epipodophyllotoxins such as etoposide and anthracyclines such as doxorubicin. These compounds have well established activities and efficacies against neuroblastoma. Clinical trials are currently in place with combination therapies involving topotecan, irinotecan and temozolomide. The chemotherapy course in high risk disease is as follows:

INRG stage	Age (months)	Histologic Category	Grade of Tumour Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					Very Low
L1		Any, except GN maturing or GNB intermixed		Not amp			Very Low
				Amp			High
L2	<18	Any, except GN maturing or GNB intermixed		Not amp	No		Low
					Yes		Intermediate
	≥18	GNB nodular; neuroblastoma	Differentiating	Not amp	No		
					Yes		Low
			Poorly differentiated or undifferentiated	Not amp			Intermediate
				Amp			High
M	<18		Any	Not amp		Hyper-diploid	Low
	<12		Any	Not amp		Diploid	Intermediate
	12 to <18			Not amp		Diploid	Intermediate
	<18		Any	Amp			High
	≥18						High
MS	<18		Favourable Any Unfavourable Any	Not amp	No		Very Low
					Yes		High
				Amp			High

Table 1.4. **The International Neuroblastoma Risk Group developed by the international experts (Cohn et al., 2009). GN – ganglioneuroma, GNB – ganglioneuroblastoma.**

Dose intensive induction, aimed at reducing tumour burden. This involves treatment with combinations of chemotherapeutic drugs, used at high doses in high risk patients. Topotecan is used if initial treatment response is insufficient.

Consolidation treatment to remove residual tumour and metastases. High dose myeloablative chemotherapy with combinations of busulfan, melphalan, carboplatin and etoposide. Autologous stem cell rescue is being trialled in high risk patients and has improved patient outcome (Matthay et al., 2009; Matthay et al., 1999).

Maintenance treatment to eliminate minimal residual disease (MRD). Often local or systemic relapses occur due to MRD. To eliminate MRD, differentiation inducing therapies are used such as retinoid derivatives (e.g. 13-cis-retinoic acid) and immunotherapy with monoclonal antibodies and cytokines such as IL-2. Disialoganglioside (GD2) is a surface glycolipid antigen present on neuroblastoma cells and normal neurons but does not affect neurons in normal tissues that are protected by the blood-brain barrier. Recent trials with combined GD2, IL-2 and GM-CSF have improved the 2-year event free survival of patients with high risk neuroblastoma from 46% to 66% (Simon et al., 2011; Yu et al., 2010; Gilman et al., 2009; Klingebiel et al., 1998).

4. Radiotherapy. Neuroblastomas are radiosensitive but radiotherapy is not usually used in low and intermediate risk patients, unless the disease progresses. In high risk patients, radiotherapy is usually given at the site of tumour during the consolidation phase of treatment.

1.4.8.1 Treatment of recurrent disease

For relapse from low and intermediate risk disease, second resection may be performed, with or without moderately intensive chemotherapy.

Relapse of high risk disease is extremely difficult to treat, and there are no treatment regimes that can offer a long-term cure (Garaventa et al., 2009). Treatment of high risk disease is also associated with dose-related toxicity (Laverdiere et al., 2005).

1.5 The MYCN oncogene

The *MYCN* gene encodes a 60-63kDa nuclear phosphoprotein (Schwab et al., 1983). In common with all MYC family members, the N-terminus contains the transcriptional

activation domain and a MYC box, and the C-terminal domain contains a basic helix-loop-helix/leucine zipper (bHLH/LZ) with a role in protein dimerisation and induction of sequence specific DNA binding and transcriptional regulation (Wenzel and Schwab, 1995). Like other members of the MYC family, MYCN is a transcription factor that plays a critical role in a wide range of cellular functions including cell growth and proliferation, differentiation and apoptosis (Brodeur, 2003; Galderisi et al., 1999).

MYCN and MYCC share 30% homology, and whereas MYCC is expressed at all stages of development, MYCN is only expressed during early embryogenesis and organogenesis in undifferentiated cells of the nervous system, lung, heart, kidney and spleen (Hurlin, 2005; Slamon et al., 1986). More is known about MYCC than MYCN but knockout of either results in embryonic lethality (Charron et al., 1992).

1.5.1 MYCN and embryogenesis

Expression of MYCN is essential during normal neural crest development, but is downregulated as tissues terminally differentiate (Thomas et al., 2004). MYCN is expressed at high levels during embryogenesis, and is expressed in migrating neural crest progenitor cells where expression is restricted to cells undergoing neuronal differentiation (Edsjo et al., 2004; Galderisi et al., 2003). MYCN may accumulate in the nucleus to regulate proliferation and differentiation, but translocates to the cytoplasm when no longer required (Galderisi et al., 2003). MYCN knockout mice had a reduced number of neurons in sympathetic ganglia, and abnormalities in organogenesis, insufficient proliferation and did not develop into an embryo (Hurlin, 2005; Sawai et al., 1993; Charron et al., 1992; Stanton et al., 1992). Amplification of *MYCN* disrupts cell cycle exit, and the terminal differentiation that occurs during normal neuroblast development (Grimmer and Weiss, 2006).

1.5.2 MYCN in neuroblastoma

MYCN is frequently amplified in advanced stage neuroblastoma, and requires aggressive treatment despite age or stage (Cohn and Tweddle, 2004; Brodeur, 2003; Seeger et al., 1985; Brodeur et al., 1984). Infants under 18 months with *MYCN*-amplified tumours have an event-free survival of 26% compared to 83% for infant stage 4 patients without *MYCN*-amplification (Cohn et al., 2009). The status of MYCN is routinely used in clinical practice to assign treatment.

The biology of high risk neuroblastoma is influenced by the subsequent overexpression of MYCN oncoprotein, and its targets include telomerase and those functioning in ribosome biogenesis and protein synthesis (Boon et al., 2001; Mac et al., 2000). When MYCN expression is deregulated through overexpression or amplification, the result is autocrine growth factor activity and uncontrollable cell proliferation (Schweigerer et al., 1990), enhanced tumour cell motility and metastases and invasion (Goodman et al., 1997), genomic instability through disruption of centrosome replication (Slack et al., 2007; Sugihara et al., 2004), reduced cell adhesion (Tanaka and Fukuzawa, 2008), diminished expression of angiogenic inhibitors (Hatzi et al., 2002; Fotsis et al., 1999) and promotion of immune escape in neuroblastoma by inhibition of the chemoattraction of natural killer T cells (Song et al., 2007). Increased MYCN expression correlates directly with the growth potential of neuroblastoma cells and MYCN co-operates to transform primary cells, and to transform established cells to malignancy (Negroni et al., 1991; Schweigerer et al., 1990). The role of MYCN in tumour formation is highlighted in transgenic mice, where targeted expression of MYCN in the neuroectoderm resulted in neuroblastoma development several months after birth with a phenotype very similar to human neuroblastoma (Weiss et al., 1997). The pro-apoptotic function of MYCN was exploited when induced MYCN expression in non-*MYCN*-amplified cell lines resulted in apoptosis and growth suppression (Kang et al., 2006; Nesbit et al., 1999). Caspase-3 action was activated and Bcl-X_L and Bcl-2 levels reduced, activating BAX and sensitising cells to apoptosis from genotoxic stress (Cole and McMahon, 1999).

1.5.3 MYC-mediated transcription and transrepression

MYC proteins can transcriptionally activate and repress many target genes. Many MYCC target genes have been identified, as described at http://mycancergene.org/site/mycTargetDB.asp (Zeller et al., 2003), but less is known about MYCN.

MYC proteins carry out their transcriptional regulatory function by forming heterodimers with MAX, a ubiquitously expressed nuclear phosphoprotein, through their bHLH-LZ domain. MYC-MAX dimers bind DNA at E-box sequences in the promoters of target genes where transcriptional co-factors are then recruited (Figure 1.8). The canonical E-box is 5’ CACTGT, and non-canonical E-boxes are 5’ CANNTG. Surprisingly, almost 40% of MYCC target genes do not contain E-boxes (Zeller et al., 2006), and it is suggested that a better indicator of MYC binding sites is detection of histone H3 – lysine 4 methylation (Guccione et al., 2006).

MYC family members are highly regulated, and for that reason have a short half-life. MAX on the other hand is constitutively expressed, has a long half-life and is in excess to MYC. The formation of MYC-MAX dimers is therefore limited by the levels of MYC (reviewed by (Grandori et al., 2000)). MAX also acts as a cofactor for a family of MYC antagonists; Mad, Mnt and Mxi1 (reviewed by (Adhikary and Eilers, 2005)). In quiescent cells, MAX is present at high levels and dimerises with both itself and MYC antagonists to repress transcription through binding to E-box sequences at the same site that MYC-MAX dimers initiate transcription (Nikiforov et al., 2003; Amati et al., 1992) (Figure 1.8). An increase in MYCN through cell cycle entry or as a result of amplification in neuroblastic tumours results in increased levels of MYC-MAX dimers, which dominate in proliferating cells (Adhikary and Eilers, 2005).

MYCN also interacts with some proteins via the C-terminal domain, including the N-MYC interactor (NMI), transformation/transcription associated protein (TRRAP) and TBP interacting protein 49 (TIP49) (reviewed by (Schwab, 2004)). MYCC can also recruit DNA methyltransferases to promoter sequences resulting in methylation and repression of target genes (Brenner et al., 2005).

MYC mediated transcriptional repression is independent of E-boxes, and involves recruitment of MYC to target gene promoters by Miz-1, blocking the recruitment of transcription factors as occurs for repression of $p21^{WAF1}$ (Seoane et al., 2002; Staller et al., 2001).

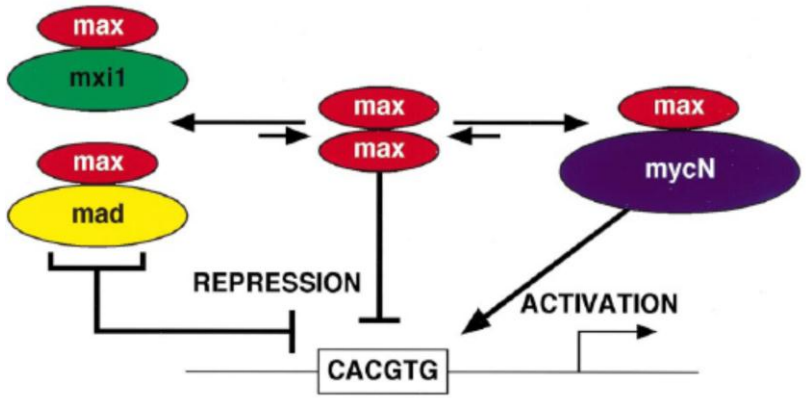


Figure 1.8. **MAX and its binding partners, including MYCN, and their role in the repression and activation of target genes through binding of E-box sequences (Maris and Matthay, 1999).**

1.5.4 The MYCN paradox

MYCN has a dual function in driving both cellular proliferation, and inducing apoptosis. MYC induced apoptosis may be an important mechanism during embryonic development, or as a failsafe mechanism to prevent abnormally elevated levels of MYC (Nilsson and Cleveland, 2003).

Ectopic MYCN expression increases DNA synthesis and proliferation, and drives cell cycle progression (Lutz et al., 1996) and also potently sensitises neuroblastoma cells to enhanced apoptosis in response to a variety of stimuli including TNF-related apoptosis-inducing ligand (TRAIL), chemotherapy, and irradiation (Petroni et al., 2011; Bell et al., 2006; Cui et al., 2005; Fulda and Debatin, 2004; Fulda et al., 2000; Lutz et al., 1998). To provide a selective advantage for the tumour, defects in apoptotic pathways are proposed as a mechanism by which *MYCN*-amplified neuroblastoma cells evade MYCN-induced apoptosis (Hogarty, 2003).

1.5.4.1 Regulation of genes associated with cell cycle progression

MYCN affects the G_1 checkpoint through p53-dependent and p53-independent mechanisms (Bell et al., 2007), and several genes are involved in these processes:

MDM2. MDM2 was identified as a direct transcriptional target of MYCN (Slack et al., 2005a), which binds to the P2 region of the MDM2 promoter. Overexpression of MDM2 as a result of *MYCN*-amplification may be a mechanism by which *MYCN*-amplified tumours inhibit p53 function and avoid apoptosis (Slack et al., 2005b). MYCN expression in the regulatable SHEP Tet21N system caused centrosome amplification, and MDM2 plays a functional role in MYCN-mediated centrosome amplification (Slack et al., 2007; Sugihara et al., 2004).

ODC. Ornithine decarboxylase (ODC) is located at 2p24-25 and is co-amplified with *MYCN* in 19% of *MYCN*-amplified neuroblastomas (Hogarty et al., 2008). It is the rate limiting enzyme in the polyamine biosynthesis pathway and is also a direct transcriptional target of MYCN. Difluoromethyl ornithine (DFMO), the ODC suicide inhibitor, slows growth in *MYCN*-amplified neuroblastomas, and increases $p21^{WAF1}$, and the G_1 population of cells (Rounbehler et al., 2009). TH-MYCN transgenic mice treated with DFMO have a significantly longer survival than control mice (Rounbehler et al., 2009; Hogarty et al., 2008).

SKP2. S-phase associated kinase 2 (SKP2) is part of a ubiquitin ligase complex, and acts at the G_1-S checkpoint (Nakayama and Nakayama, 2006). Its primary targets for degradation are negative regulators of the cell cycle, including $p21^{WAF1}$ and $p27^{KIP1}$. Following MYCN knockdown, SKP2 levels are decreased, alongside a reduction in TP53INP, a protein that has been shown to cause a G_1 arrest and apoptosis, and increases p53-dependent upregulation of $p21^{WAF1}$ (Bell et al., 2007; Tomasini et al., 2005; Tomasini et al., 2003).

DKK3. Dickkopf 3 (DKK3) is a member of the DKK family of secreted WNT antagonists, and is a tumour suppressor in various cancer types (Kuphal et al., 2006; Hoang et al., 2004; Hsieh et al., 2004). MYCN represses DKK3 indirectly, and ectopic expression of DKK3 in neuroblastoma inhibits cell proliferation (Koppen et al., 2008; Bell et al., 2007).

ID2. ID2 is an inhibitor of the Rb protein which inhibits differentiation and promotes cell proliferation. It antagonises the growth suppressive functions $p16^{INK4a}$ by binding hypo RB and releasing E2F which then promotes transcription of S-phase proteins driving the cell through the cell cycle and enhancing cell proliferation (Lasorella et al., 2002; Lasorella et al., 2001; Lasorella et al., 2000). The link between MYCN and ID2 in neuroblastoma remains unclear.

E2F1. E2F proteins are important regulators of cell cycle progression and are negatively regulated by the Rb pathway. E2F proteins directly activate and postiviely regulate MYCN in neuroblastoma, in a cell cycle specific manner (Strieder and Lutz, 2003). In addition, E2F1 is a direct downstream target of MYCC, although this has not been confirmed for MYCN (Fernandez et al., 2003).

1.5.4.2 MYCN and cell cycle arrest

MYCN-amplified and non-*MYCN*-amplified cells respond differently to DNA damage (Bell et al., 2006; Tweddle et al., 2001b). *MYCN*-amplification is associated with reduced expression of $p21^{WAF1}$ and hypoRb, and failure to G_1 arrest. MYCN may not directly repress $p21^{WAF1}$, but may act via H-TWIST to impair p53 function following irradiation. H-TWIST and MYCN expression strongly correlate and H-TWIST has been reported to attenuate p53 function (Valsesia-Wittmann et al., 2004). Recently we have shown that it is a combination of MYCN and neuroblastoma cell type (N and S) which determine the response to DNA damage (Carr-Wilkinson et al., 2011).

1.5.4.3 Regulation of genes associated with apoptosis

MYCN plays a role in inducing apoptosis. There is a high mitosis-karyorrhexis index in *MYCN*-amplified tumours (Saha et al., 2010; Altungoz et al., 2007; Goto et al., 2001; Shimada et al., 1999; Shimada et al., 1995) and MYCN transgenic mouse tumours (Moore et al., 2008). In the MYCN transgenic mouse model, tangible body macrophages were detected, reflecting higher levels of apoptosis (Moore et al., 2008). In addition, forced expression of MYCN in non-*MYCN*-amplified neuroblastoma cells induced apoptosis (Tang et al., 2006a).

Mechanisms by which MYC family members induce apoptosis are not fully understood (Adhikary and Eilers, 2005). MYCC was shown to induce apoptosis (reviewed by (Meyer et al., 2006)) via CD96/Fas (Hueber et al., 1997), TNF (Klefstrom et al., 1994) and TRAIL (van Noesel et al., 2003) receptor signalling, direct activation of BAX (Mitchell et al., 2000), cytochrome C release (Juin et al., 1999) and induction of pro-apoptotic BIM (Egle et al., 2004). Other MYC regulated proteins involved in the modulation of apoptosis include:

p53. The p53 promoter contains a non-canonical E-box and is a direct transcriptional target of MYCN (Chen et al., 2010b). p53 induces cell cycle arrest and apoptosis (see Section 1.6), and MYCN-induced p53 transcription may be an important mechanism by which MYCN sensitises cells to apoptosis.

p14^{ARF}. MYCC transcriptionally activates p14^{ARF}, a protein involved in activating p53 in response to oncogenic stress (Gregory et al., 2005; Zindy et al., 1998), and also induces apoptosis via p53-independent mechanisms upon MYCC overexpression (Qi et al., 2004). However, there have been no studies reporting that MYCN sensitises cells to apoptosis by p14^{ARF}. In addition p14^{ARF} binds directly to MYCC/N, and may repress expression of anti-apoptotic genes through inhibition of transcriptional activity and sequestration in the nucleolus (Amente et al., 2007; Qi et al., 2004).

H-TWIST. H-TWIST is an oncogenic transcription factor with anti-apoptotic activity that co-operates with MYCN and is often overexpressed in *MYCN*-amplified neuroblastoma. H-TWIST may inhibit the p14^{ARF}-p53 pathway (Valsesia-Wittmann et al., 2004).

PUMA. PUMA is a BH3-only, BCL2 family member which is a direct target gene of p53, and is a principle mediator of p53-induced apoptosis via the mitochondrial

pathway (Jeffers et al., 2003). PUMA is required for the p53-dependent apoptotic response to c-MYC (Jeffers et al., 2003; Yu and Zhang, 2003). MYCN may sensitise cells to apoptosis via p53-mediated upregulation of PUMA. In *MYCN*-amplified cell lines increased levels of apoptosis have been reported (Bell et al., 2006) and may be mediated via pro-apoptotic proteins PUMA and PIG3 as has been described for MYCC (Seoane et al., 2002).

BCL2. MYCC induces a pathway that indirectly suppresses BCL-X_L or BCL-2 anti-apoptotic function and expression and activates BAX (Eischen et al., 2001). MYCN is likely to have a similar function. We have previously reported an inverse correlation between BCL2 and MYCN in neuroblastoma (Tweddle, 2002).

In addition MYCN helps to determine the choice between the cytostatic and apoptotic response to p53 induction. MYCN is directly recruited to the $p21^{WAF1}$ promoter by Miz-1, which blocks $p21^{WAF1}$ induction. Therefore MYCN switches the response in favour of apoptosis (Strieder and Lutz, 2003).

1.5.4.4 Evasion of apoptosis

High levels of MDM2 in *MYCN*-amplified neuroblastoma are proposed as a mechanism by which neuroblastomas evade apoptosis. In addition, *Survivin* is expressed at significantly higher levels in *MYCN*-amplified cells (Miller et al., 2006), and BIN-1, a MYC interacting adaptor protein involved in inhibition of MYC-mediated transformation and apoptosis, is downregulated in *MYCN*-amplified cells (Tajiri et al., 2003; Hogarty et al., 2000). Caspase 8 is sometimes deleted or silenced though hypermethylation, and is associated with *MYCN*-amplification (Teitz et al., 2000), but the effects of this are inconsistent (Fulda et al., 2006; Casciano et al., 2004; Iolascon et al., 2003).

1.6 The p53 tumour suppressor protein

The p53 tumour suppressor plays a critical role in maintaining genomic integrity within a cell and is the central component in a complex network. The importance of p53 is demonstrated by its absence or mutation in over 50% of human cancers.

1.6.1 p53 function

p53 is a transcription factor that regulates an array of different genes, encoding both proteins and microRNAs, and efficiently inhibits cell proliferation. As shown in Figure

1.9, p53 functions as a key integrator within the cell, responding to a number of diverse stress signals and translating them into different cellular outcomes which include cell cycle arrest, apoptosis, block in DNA synthesis, DNA repair, senescence, and differentiation (Haupt et al., 2003; Vogelstein et al., 2000). Less well-studied responses include autophagy (Amaravadi and Thompson, 2007; Crighton et al., 2006), cell migration (Roger et al., 2006), embryo implantation (Hu et al., 2007b), regulation of metabolism (Bensaad et al., 2006; Matoba et al., 2006) and angiogenesis (Teodoro et al., 2006). In total p53 can transactivate and transrepress over 2500 genes to trigger antiproliferative programs (Zilfou and Lowe, 2009; Lohrum and Vousden, 1999; Agarwal et al., 1998; Giaccia and Kastan, 1998), and there are many other genes whose expression is indirectly affected.

Under normal cellular conditions, p53 levels are tightly regulated and remain low. Upon activation by cellular stress, the protein accumulates in the nucleus where it binds DNA response elements in a sequence specific tetrameric form, or as a dimer of dimers, to two cognate half sites in DNA (Kitayner et al., 2006; Oren, 1999; Agarwal et al., 1998). In addition to upregulated translation, the p53 half-life increases up to 5 times (usually 30 minutes) (Giaccia and Kastan, 1998). The manner in which p53 responds to different stresses, and decides on its biological response is strongly dependent on the tissue and cell type, in addition to the strength and nature of the stress (including type of damage and duration of stress signal), and the environment of the cell (reviewed in (Murray-Zmijewski et al., 2008)). A simple model is proposed that assumes low levels of stress and reparable damage results in a survival response, whereas high levels of sustained stress and irreparable damage results in cell death or senescence (Vousden and Prives, 2009). In addition, there are DNA-sequence specific differences in the contacts made between the p53 protein surfaces, which could be involved in determining the level of induction of a specific target gene (Kitayner et al., 2006).

Many models of p53 function assume apoptosis is key to eliminating cancer cells, but there is growing evidence that other functions of p53 are equally important in preventing tumour formation. p53 can retain tumour suppressive function even in the absence of a robust apoptotic response. Mice with a p53 mutation preventing apoptosis but maintaining other p53 functions were reasonably well protected from tumour development (Liu et al., 2004a).

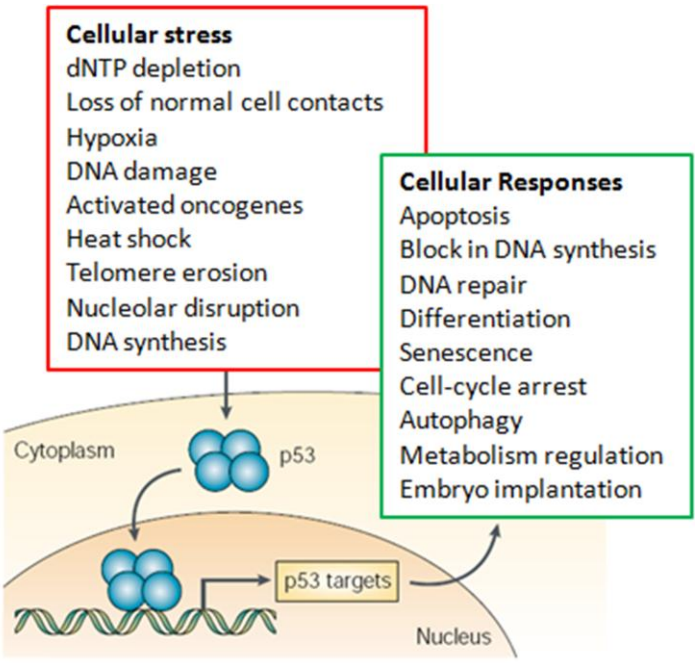


Figure 1.9. **p53 reacts to a variety of stress signals and initiates a number of responses including apoptosis and cell cycle arrest.**

1.6.2 The structure of p53

p53 is a 53kDa nuclear phosphoprotein, encoded by the *TP53* gene which spans 11 exons and is located at the 17p13.1 locus on the short arm of chromosome 17 (reviewed by (Mercer et al., 2007)). The structure of p53 and some common modification sites associated with particular responses are shown in Figure 1.10. The protein contains 4 principle functional domains:

1. **The N-terminal transactivation domain with a nuclear export signal (residues 1-42).** At the N-terminus there are two highly acidic transactivation domains; TA1, and TA2 (Candau et al., 1997). Each has distinct interacting partners and exhibit independent transactivation activity (Jenkins et al., 2009). Together, they mediate the interaction of p53 with several general transcription factors including TBP, TAF1, TFIIB and TFIIH, to stimulate gene transcription (Laptenko and Prives, 2006; Espinosa et al., 2003).
2. **A proline-rich domain containing 5 PXXP motifs which allow for rapid protein-protein interaction (residues 61-94).** This region also plays a role in p53 stability, regulated by MDM2 (Sakamuro et al., 1997).
3. **A core DNA binding domain (residues 102-292).** The core domain comprises of an immunoglobulin scaffold with a DNA binding surface formed by a loop-

sheet-helix motif, and two β-turns, tethered by a single zinc atom (Cho et al., 1994). The core domain exhibits sequence specific DNA binding activity within the nucleus and also has a cytoplasmic role in Bax activation, leading to the permeabilisation of the outer mitochondrial membrane and initiation of the caspase cascade. 80% of mutations occur in the core domain, and affect cell fate by regulating DNA binding activity and interfering with protein interactions.

4. **A C-terminus containing a tetramerisation domain (residues 324-355) flanked by nuclear localisation signals and a strongly basic regulatory domain (residues 363-393) (Mercer et al., 2007).** This tetramerisation domain mediates intermolecular formation of four p53 monomers to form a tightly packed tetramer, which is biologically active and efficiently binds to p53 response elements. The regulatory domain is subject to extensive post-translational modifications, and is implicated in both p53 transactivation and DNA binding activities (Ho et al., 2006; An et al., 2004; McKinney et al., 2004; Merrill et al., 1999).

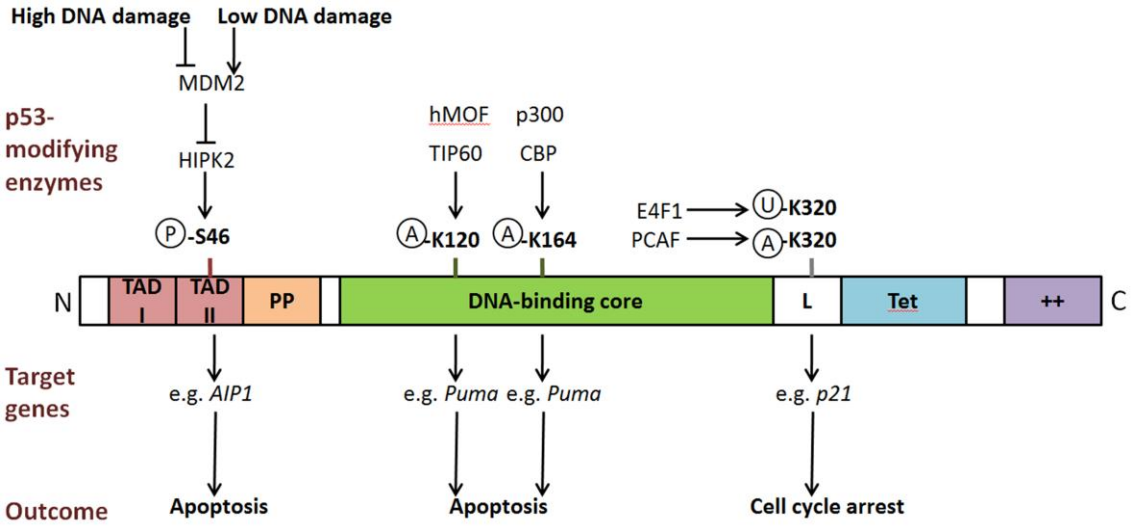


Figure 1.10. **The structure of the 53kDa p53 protein, and some key residues subject to modification and their subsequent responses (Vousden and Prives, 2009).**

1.6.3 p53-mediated cell cycle arrest

1.6.3.1 The cell cycle

The cell cycle is a series of events that lead to the division and replication of a cell and has several distinct phases (shown in Figure 1.11):

1. **Gap 0 (G_0) phase.** Both quiescent and senescent cells enter the G_0 'resting' phase, when they are no longer dividing. This is common in fully differentiated cells such as neurones.
2. **Gap 1 (G_1) phase.** G_1 phase, also known as 'growth phase' is when cells increase in size and synthesise enzymes required for S phase. Here the G_1 checkpoint control mechanism ensures everything is ready for DNA synthesis.
3. **Synthesis (S) phase.** DNA replication occurs during S phase, and the amount of DNA in the cell doubles, though the ploidy of the cell remains the same.
4. **Gap 2 (G_2) phase.** During the gap between S and M phase, significant biosynthesis occurs, involving the production of microtubules required for mitosis. A G_2 checkpoint control mechanism ensures cells are ready to enter M phase.
5. **Mitosis (M) phase.** Cell growth stops and cells divide into two daughter cells. A checkpoint in the middle of mitosis (metaphase checkpoint) ensures that the cell is ready to complete division.

Cyclins and cyclin-dependent kinases (CDKs) determine a cell's progress through the cell cycle (Nigg, 1995). Cyclins form the regulatory subunits and CDKs the catalytic subunit of an activated heterodimer. CDKs phosphorylate target proteins to co-ordinate entry into the next phase of the cell cycle, and different cyclin-CDK combinations determine the downstream target protein. Cyclins are synthesised at specific stages in the cell cycle in response to various molecular signals (Kumar et al., 2004).

The first cell cycle complex to form is cyclin D-CDK4, which phosphorylates the Rb protein releasing E2F and activating transcription of target genes, including cyclin E which binds CDK2 and pushes the cell from G_1-S phase. Cyclin B forms a complex with cdc2 to initiate the G_2-M phase transition. The nuclear envelope breaks down, and the prophase stage of mitosis is initiated (Kumar et al., 2004).

1.6.3.2 Cell-cycle arrest

p53 can block cell cycle progression through transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$, and also GADD45 and 14-3-3σ (el-Deiry, 1998).

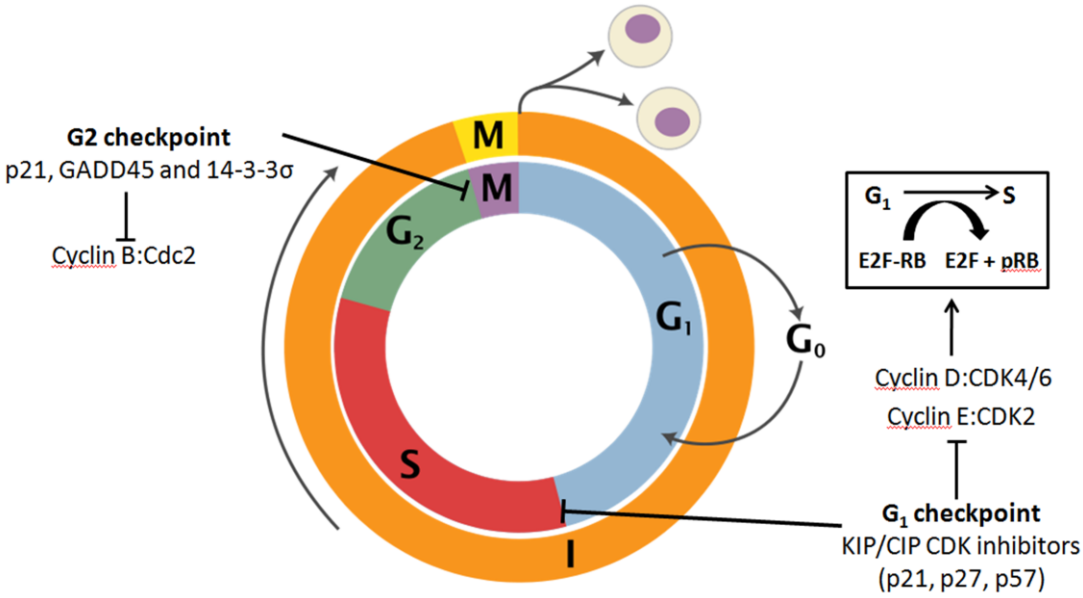


Figure 1.11. **The cell cycle, and key proteins involved in preventing G_1-S, and G_2-M progression at the G_1 and G_2 checkpoints. I – interphase, M- mitosis phase (cell division).** *Adapted from http://aranzazu17.wordpress.com/2011/05/13/cell-cycle-mitosis/.*

1.6.3.3 p21^{WAF1}

The G_1-S checkpoint is regulated through transcriptional stimulation of p21^{WAF1}, which functions to block G_1 to S progression, subsequently inducing a G_1 arrest (Giono and Manfredi, 2006; Vousden and Lu, 2002). p21^{WAF1}, encoded by the *CDKN1A* gene, is a member of the Cip/Kip family of CDK inhibitors, and inhibits the function of CDK2, CDK3, CDK4, and CDK6 which have direct roles in the transition between cell cycle phases (Gartel and Tyner, 2002). Forced p21^{WAF1} expression has been shown to arrest the cell cycle at G_1/S and G_2/M borders (Dulic et al., 1998). In the nucleus, p21^{WAF1} binds to and inhibits the activity of CDK1 and CDK2 and blocks transition from G_1-S phase, or from G_2-M phase after DNA damage, enabling repair of damaged DNA. Inhibition of the cyclin E/CDK2 complex prevents phosphorylation of Rb, and subsequent release of E2F transcription factors (Sherr and Roberts, 1999) as shown in Figure 1.11. The result is inhibition of S-phase progression and cells arrest in G_1 until either DNA is repaired, or apoptosis is induced (Sherr and Roberts, 1999; Xiong et al., 1993).

p21^{WAF1} is very sensitive to even low levels of p53 induction, and may provide a temporary block induced by mild damage or stress, allowing cells to survive until the

damage is resolved or stress removed. However, this transient cell cycle arrest could be risky in cells with oncogenic potential that cannot be repaired and may resume proliferation.

$p21^{WAF1}$ is also important for induction of replication senescence as well as stress-induced premature senescence. In addition, $p21^{WAF1}$ is also reported to positively or negatively regulate apoptosis. When localised in the cytoplasm, $p21^{WAF1}$ has an anti-apoptotic effect. It is able to bind to and inhibit caspase 3, ASK1 and JNK (apoptotic kinases) (Abbas and Dutta, 2009; Janicke et al., 2007; Gartel and Tyner, 2002).

The function of $p21^{WAF1}$ in response to DNA damage probably depends on the extent of the damage. A model is proposed where low levels of damage induce $p21^{WAF1}$ and subsequent cell cycle arrest, as well as anti-apoptotic functions whereas extensive DNA damage results in reduced $p21^{WAF1}$ and apoptosis. $p21^{WAF1}$ therefore acts as a tumour suppressor, but also acts as an oncogene by preventing apoptosis.

1.6.3.4 Gadd45 and 14-3-3δ

p53 transcriptionally activates Gadd45 and 14-3-3δ that participate in G_2 arrest (Colman et al., 2000; Zhan et al., 1999). Gadd45 prevents the G_2/M transition, through inhibition of Cdc2 (CDK1), and subsequent inhibition of the cyclin B-Cdc2 interaction and kinase function (Taylor and Stark, 2001; Wang et al., 1999; Zhan et al., 1999). 14-3-3δ, a scaffold protein, removes cyclin B/Cdc2 from its site of action in the nucleus (Hermeking et al., 1997). Repression of cyclin B through p53 also arrests cells in G_1 (Taylor and Stark, 2001).

1.6.4 p53-mediated apoptosis

Apoptosis results from a number of mechanisms including the activation of apoptotic genes, repression of anti-apoptotic genes and through non-transcriptional mechanisms. As shown in Figure 1.12, there are two main apoptotic pathways regulated by p53; the extrinsic pathway and the intrinsic pathway. It is proposed that the intrinsic pathway is primarily utilised in p53-mediated apoptosis, whereas the extrinsic pathway is used to augment the apoptotic response (Fridman and Lowe, 2003). Both pathways converge and induce a caspase cascade which results in apoptosis.

1.6.4.1 The extrinsic/death receptor pathway

p53 target genes involved in the extrinsic pathway include the death receptors Fas/CD95 (O'Connor et al., 2000), DR4 (Liu et al., 2004b), and DR5 (Takimoto and El-

Deiry, 2000) which are located at the plasma membrane. In response to stress, these receptors repress Inhibitor of Apoptosis Proteins (IAPs) (Takimoto and El-Deiry, 2000) and induce caspase-mediated apoptosis (Haupt et al., 2003).

1.6.4.2 The intrinsic/mitochondrial pathway

The intrinsic pathway is the main apoptotic pathway in response to DNA damage. Upon activation, p53 interacts with the Bcl-2 family of proteins in the cytosol. These include the pro-apoptotic proteins Bcl-2-associated X protein (BAX), Bak and Bcl-X1, which antagonise the pro-survival function of Bcl-2 and Bcl-X_L (Mihara et al., 2003). These proteins translocate to the mitochondria, inducing mitochondrial outer membrane permeabilisation and cytochrome c release (Cory and Adams, 2002; Marchenko et al., 2000; Korsmeyer, 1999).

p53 also induces expression of pro-apoptotic 'BH3-only' proteins including BH3-interacting death agonist (BID), Bad, Noxa (Latin for 'damage'), p53-upregulated apoptosis inducing protein (p53AIP1), and p53-upregulated modulator of apoptosis (PUMA) (Haupt et al., 2003; Han et al., 2001; Nakano and Vousden, 2001b). PUMA binds to anti-apoptotic Bcl-2 and BCL-X_L (Yu et al., 2001a) promoting BAX translocation to the mitochondria and subsequent cytochrome c release (Ming et al., 2006; Chipuk et al., 2005). Studies have suggested a strong dependence of p53-mediated apoptosis on the presence of PUMA (Vousden, 2005; Jeffers et al., 2003; Yu and Zhang, 2003). In addition, PUMA can also exhibit p53-independent apoptotic functions (Jeffers et al., 2003). Interestingly, both PUMA and NOXA have been shown to mediate neural precursor cell death (Akhtar et al., 2006).

Cytochrome c release promotes the formation of the apoptosome complex with apoptotic protein-activating factor-1 (Apaf-1) and caspase 9 (Nakano and Vousden, 2001a; Yu et al., 2001b) which induces a caspase cascade and ultimately apoptosis.

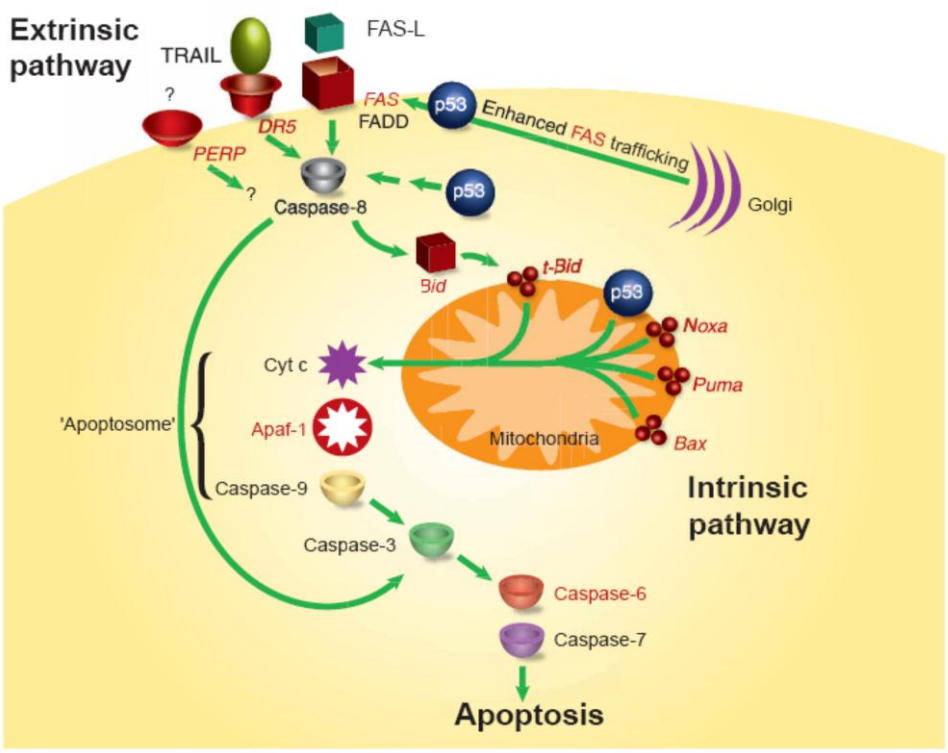


Figure 1.12. **The p53-induced intrinsic and extrinsic apoptotic pathways. The extrinsic pathway involves death receptors and the formation of the death inducing complex, inducing apoptosis through a cascade caspase. The intrinsic pathway is induced in response to DNA damage, resulting in mitochondrial depolarisation and the release of cytochrome c, also followed by a caspase cascade (Haupt et al., 2003). The final caspase cascade for both the intrinsic and extrinsic pathway involves caspase 3, 6 and 7.**

1.6.5 p53-mediated cellular senescence

p53 plays a key role in induction of cellular senescence, or irreversible cell cycle arrest. Senescence is a major anticancer function and may be a reason why tumours arise so easily when p53 is lost. Senescence is likely to result from changes in the expression of a number of genes such as plasminogen inhibitor 1 (PAI-1) and the key mediator, $p21^{WAF1}$ (Leal et al., 2008; Kortlever et al., 2006; Brown et al., 1997). In apoptotic-defective cells, which retain other functions of p53, $p21^{WAF1}$ activation induces senescence (Cosme-Blanco et al., 2007; Van Nguyen et al., 2007). Mice with this mutant were crossed with $p21^{WAF1}$-null mice and the result was loss of cell cycle response and cancer formation (Barboza et al., 2006). Therefore p53-dependent activation of $p21^{WAF1}$ is important in senescence-dependent tumour suppression. However, lack of $p21^{WAF1}$ does not strongly correlate with tumour development (Choudhury et al., 2007).

1.6.6 p53 and cancer

Inactivation of p53 is considered essential for the development of most human cancers, and approximately 50% harbour p53 inactivating mutations (Hollstein et al., 1991). In the other 50%, upstream and downstream defects are believed to account for loss of p53 activity. p53-mutated or p53-null cancer cells have inactivated growth inhibitory pathways (McDermott et al., 2005; Wu and El-Deiry, 1996).

p53 status usually correlates with the responsiveness of a cancer cell to radiation and chemotherapeutic agents. In addition radiation therapy and other DNA damaging stresses that activate p53 select for p53 mutant cells and therefore secondary cancers are often associated with therapy-induced mutations in p53 and are resistant to treatment. Reactivation of p53 in mouse models leads to regression in many tumour types (Ventura et al., 2007; Xue et al., 2007b; Martins et al., 2006), and this is discussed in Section 1.17.

1.6.7 Types of mutations

There are a number of ways in which DNA can change, resulting in different types of mutations, including:

1. ***Single-base substitutions, or point mutations:*** when a single base is substituted for another. These can be:

   a) *missense mutations.* The new nucleotide alters the codon, producing an altered amino acid in the protein product.

   b) *nonsense mutations.* The new nucleotide changes the codon to a STOP codon, resulting in a truncated protein product.

   c) *silent mutation.* The nucleotide substitution results in a codon which produces the same amino acid as the wildtype nucleotide, and results in no change to the protein product.

   d) *splice-site mutation.* Nucleotide signals at splice sites guide the enzymatic activity responsible for removal of intron sequences. If this is altered, introns may be translated into the protein product.
2. **Insertions and deletions**: addition or removal of base pairs results in a frameshift mutation, encoding an entirely different amino acid sequence, and can also result in a premature STOP codon.
3. **Duplications:** the doubling of a section of the genome. During meiosis, crossing over between sister chromatids that are out of alignment can produce one chromatid with a duplicated gene.

4. **Translocations:** the transfer of a piece of one chromosome to a nonhomologous chromosome.

1.7 Negative Regulation of p53: MDM2 and MDMX

For controlled activation of p53, tight control of activity and levels of mouse double minute- 2 (MDM2) and the MDMX paralogue are necessary (Wade et al., 2010; Wang et al., 2009). MDM2 is considered the principle negative regulator of p53 (Haupt et al., 1997; Momand et al., 1992), but perhaps less recognised but equally important is MDMX. In addition MDM2 is a direct transcriptional target of p53, forming a negative feedback loop (Deb, 2003). This loop is essential in maintaining the balance of the two proteins, and prevents accumulation of either under normal conditions but allows for rapid changes of both in response to cellular stress. Unlike MDM2, MDMX is not a transcriptional target of p53 (Wang et al., 2001). Despite their similarities, neither protein can substitute for the loss of the other; analyses of knockout mice show MDMX and MDM2 suppress p53 in a nonredundant yet synergistic manner (Marine et al., 2006). MDMX-null mice die *in utero* in a p53-dependent manner, but can be rescued upon p53 knockout (Parant et al., 2001), whereas MDM2 knockout is lethal during early embryogenesis in mice as a result of hyperactive p53, but can also be rescued by p53 knockout (Montes de Oca Luna et al., 1995).

1.7.1 Structure and function

MDMX was originally identified as a paralogue of MDM2, and the proteins share substantial sequence homology and have similar structures as shown in Figure 1.13 (Saha et al., 2010; Shvarts et al., 1997; Shvarts et al., 1996).

The *MDM2* gene spans 12 exons, is located at locus 12q14.3-q15 and encodes a 90kDa protein. MDM2 is an E3 ubiquitin ligase that targets p53 for ubiquitination and degradation. MDM2 variants result from alternative internal splice sites within the gene, and each variant contains 2 promoters; P1 upstream of exon 1, and P2 between exons 1 and 2, to which p53 can bind in order to rapidly activate MDM2 transcription and expression (Zauberman et al., 1995; Chen et al., 1994). P1 is constitutively active at low levels, whereas P2 has multiple transcription factor binding sites and an E-box (Zauberman et al., 1995). The MDMX gene is located at locus 1p12, and encodes a 70kDa protein.

Both proteins bind p53 at a short α-helical stretch within the N-terminus, via their most conserved N-terminal hydrophobic region (Bottger et al., 1999). Other conserved domains include the RING finger, a Zn-finger and a central acidic domain as shown in Figure 1.13. The RING finger and acidic region of MDM2 are essential for p53 ubiquitination. However, corresponding regions of MDMX do not have this function (Kawai et al., 2003b; Meulmeester et al., 2003). p53 levels are mainly controlled at the post-translational level through stabilisation and degradation, but can also be controlled at the transcriptional level. Under normal cellular conditions, p53 levels are kept low and p53 is constantly targeted for degradation by MDM2.

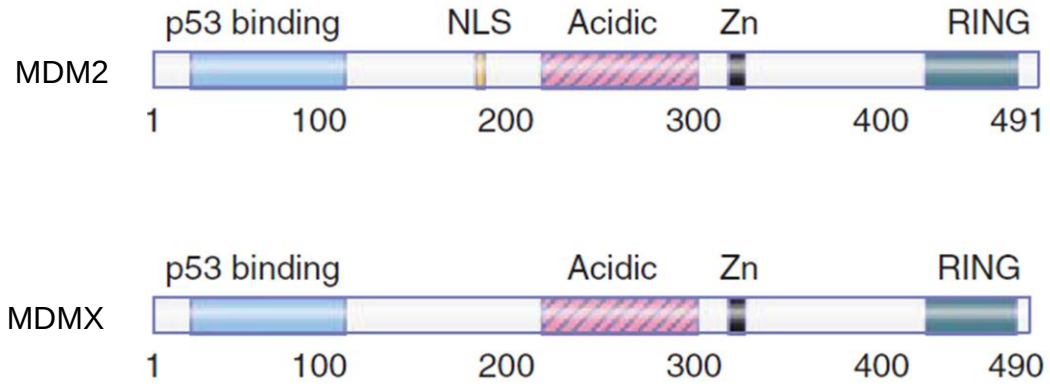


***Figure 1.13*. Domain structure of homologies MDM2 and MDMX. The N-terminus of both proteins is responsible for p53 binding. The central acidic domain of MDM2 but not MDMX binds ribosomal proteins, and the RING finger is required for MDM2-MDMX heterodimerisation (Perry, 2010).**

1.7.2 MDM2-mediated control of p53

Multiple MDM2 activities inhibit p53 function. MDM2 can regulate both the transcriptional activity and the half-life of p53 (Honda et al., 1997). There are 3 main mechanisms by which MDM2 achieves this (shown in Figure 1.14):

1. MDM2 has E3 ubiquitin ligase activity within the RING domain, and is the main mediator of endogenous p53 ubiquitination. MDM2 functions as an adaptor protein, binding the N-terminus of p53 and an E2 conjugating enzyme (Linke et al., 2008). The recruited E3 ligase directly transfers ubiquitin molecules to lysine residues within the C-terminus of p53, targeting p53 for proteosomal degradation in the cytoplasm by the 26S proteosome (Honda et al., 1997; Wu et al., 1993). Cysteine 464 within the RING finger domain is required for the ubiquitin ligase activity of MDM2 (Honda et al., 1997).

2. MDM2 has a nuclear export signal (NES) and has the ability to bind p53 and shuttle it from its site of action in the nucleus to the cytoplasm where it cannot bind DNA, and therefore cannot transcriptionally activate target genes (Tao and Levine, 1999). Export of the MDM2-p53 complex occurs via the CRM1-dependent export pathway shown by studies whereby p53 stability was increased through inhibition of this pathway (Haupt et al., 1997). Rapid shuttling of p53 between the cytoplasm and nucleus is important for the regulation of p53 by MDM2 (Tao and Levine, 1999; Roth et al., 1998).
3. Upon interaction MDM2 binds the transactivation domain within the N-terminal of p53, directly inhibiting p53-mediated transcription (Wadgaonkar and Collins, 1999; Momand et al., 1992). MDM2 binds p53 at the same residues that it binds the TFIID complexes required for transcriptional activation of target genes (Lin et al., 1994), and therefore MDM2 and TFIID compete for this site. Also MDM2 has been reported to inhibit p53 transcriptional activity by promoting conjugation of the ubiquitin-like protein NEDD8 to p53 (Xirodimas et al., 2004).

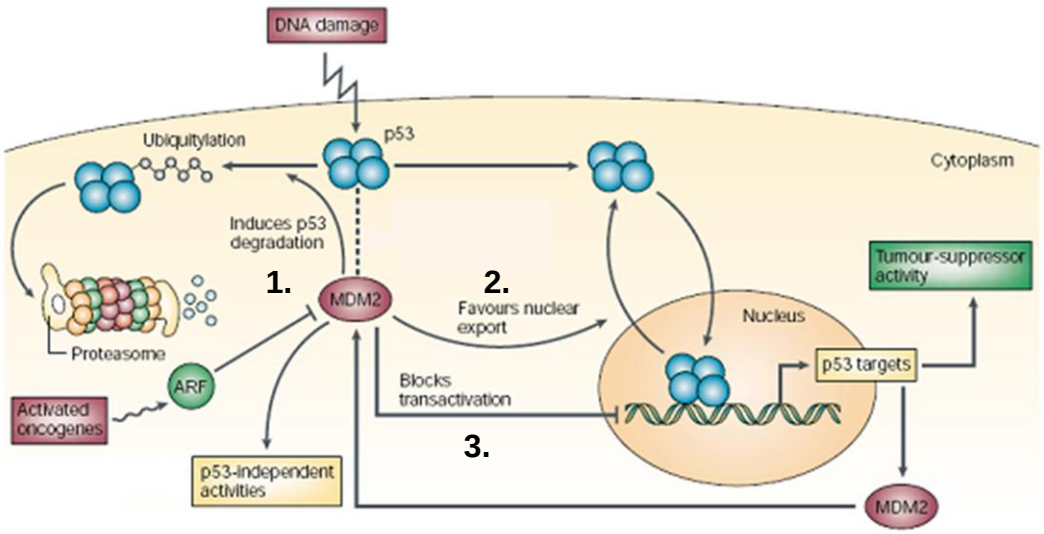


Figure 1.14. **The regulation of p53 by MDM2. MDM2 expression induces; 1) p53 degradation through ubiquitination, 2) exportation of p53 from the nucleus to the cytoplasm and 3) MDM2 also blocks p53 transactivation. These functions of MDM2 diminish the tumour suppressor activities of p53. (Chene, 2003).**

MDM2 also promotes ubiquitination of histone proteins within the vicinity of p53-responsive promoters, resulting in transcriptional repression (Xirodimas et al., 2004) and may also contribute to p53 inactivation by recruiting several corepressor proteins

such as HDAC1, CTBP2, YY1 and KAP1 (Sui et al., 2004; Mirnezami et al., 2003; Ito et al., 2002).

1.7.3 MDM2 and MDMX mediated control of p53

1.7.3.1 MDMX and MDM2 interactions

There are two models as to how MDMX and MDM2 regulate p53:

1. MDM2 and MDMX function independently. MDMX binds tightly at the N-terminal transcriptional activation domain of p53, inhibiting p53 transactivation function (Shvarts et al., 1996). Binding of MDMX here prevents interaction of p300, which results in reduced acetylation of p53, a modification required for p53 activation (Danovi et al., 2004; Sabbatini and McCormick, 2002). MDM2 inactivates p53 primarily by working as an E3 ligase to control p53 turnover (Marine et al., 2007).
2. There is interplay between MDM2 and MDMX in p53 regulation (Wade et al., 2010; Kruse and Gu, 2009; Vousden and Prives, 2009; Kawai et al., 2007; Poyurovsky et al., 2007; Uldrijan et al., 2007; Marine et al., 2006; Gu et al., 2002).

More evidence points towards the second model, and structurally, formation of the MDM2-MDMX heterocomplex is favoured over MDM2 homocomplexes (Linke et al., 2008). MDMX does not homodimerise. In addition, the two proteins were found to exist in cells mainly as heterocomplexes (Kawai et al., 2007). MDM2 and MDMX interact via their C-terminal RING finger domains (Linke et al., 2008; Sharp et al., 1999). MDMX does not have E3 ubiquitin ligase activity but can modulate p53 levels through control of MDM2 levels (Linke et al., 2008).

The ratio of MDMX and MDM2 has been shown to strongly determine the outcome on p53 stability. Some studies have shown that high MDMX binds p53, preventing negative regulation by MDM2 and leading to p53 stabilisation (Gu et al., 2002), whereas others have shown that high MDMX favours heterodimerisation with MDM2 and initiates p53 degradation. It may be that under different circumstances, MDMX can either stimulate or inhibit the E3 ubiquitin ligase activity of MDM2 (Wade et al., 2010). In the absence of exogenous stress, MDM2 promotes balanced turnover of itself, MDMX and p53 through ubiquitination and autoubiquitination (Stommel and Wahl,

2004; de Graaf et al., 2003). MDM2 is the only known ubiquitin ligase of MDMX, and when bound it ubiquitinates MDMX at K422 (Xia et al., 2008; Stommel and Wahl, 2004). At low MDMX levels, MDM2 is a relatively ineffective ubiquitin ligase for p53, and undergoes autoubiquitination and degradation (Linke et al., 2008; Kawai et al., 2007; Poyurovsky et al., 2007; Uldrijan et al., 2007). At increased levels of MDMX, heterodimerisation results in reduced autoubiquitination of MDM2, shifting the MDM2 substrate from itself to p53 as shown in Figure 1.15 (Okamoto et al., 2009; Gilkes et al., 2008; Linke et al., 2008; Kostic et al., 2006; Linares et al., 2003; Gu et al., 2002). The MDM2-MDMX complex is a more active ubiquitin ligase than MDM2 homodimers, and provides an extended interaction motif with the E2 protein (Linke et al., 2008; Kawai et al., 2007; Poyurovsky et al., 2007). Even when the functional ubiquitin ligase activity of MDM2 is removed, MDMX promotes p53 ubiquitination, and therefore the main reason for heterodimerisation appears to be to promote MDM2 stability (Linke et al., 2008). This interaction also stabilises MDMX, but compared to MDM2, MDMX has much less impact on p53 levels.

In response to stress, both MDMX and MDM2 are eliminated. MDM2 and MDMX are phosphorylated at residues outside of the RING domain, and p53 is phosphorylated reducing the affinity for MDM2 and MDMX. MDMX is degraded in an MDM2-dependent manner, and MDM2 is eliminated through autoubiquitination. Once DNA damage signals return to normal, kinase inhibition and phosphatase activation removes the pool of phosphorylated MDM2 and MDMX, leading to their stabilization.

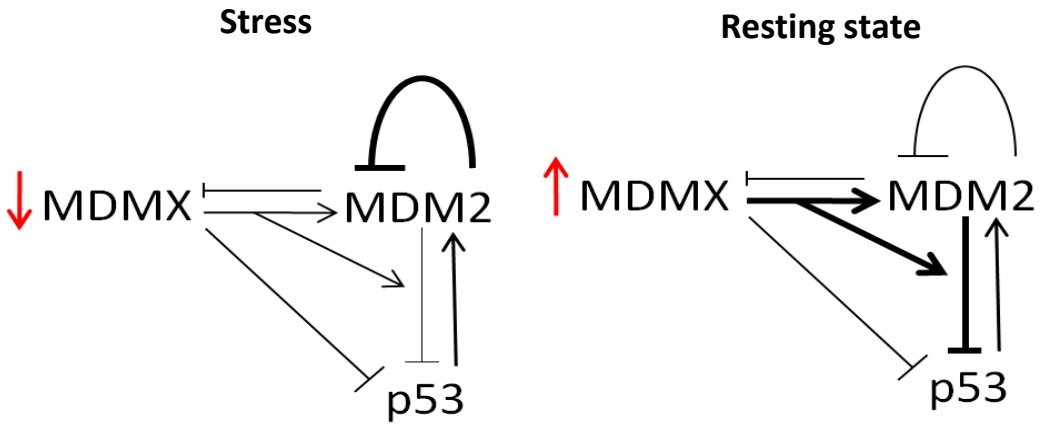


Figure 1.15. **Levels of MDMX are important in determining the role of MDM2. In response to stress, MDMX ubiquitination by MDM2 is upregulated, as is MDM2 autoubiquitination, and the negative control on p53 is eliminated.**

1.8 The DNA damage response

Under normal conditions, both MDM2 and p53 have short half-lives whereas MDMX is relatively stable (Gilkes et al., 2008). In response to DNA damage or stress, p53 activity and stability are modulated through post-translational modifications including phosphorylation and acetylation. These modifications generally suppress MDM2 binding, reducing MDM2-mediated degradation and enhancing p53 transcriptional activity as a result of stabilisation, and accumulation in the nucleus (Vousden and Lu, 2002; Oren, 1999; Brooks and Gu, 2003; Xu, 2003). p53 is phosphorylated by DNA-damage induced DNA-PK and ATM at serine 15 and serine 37 within the N-terminus, repressing the ability of MDM2 to inhibit p53 transcription, perhaps due to conformational change. In addition phosphorylation of p53 at serine 20 by ATM-activated Chk2 inhibits MDM2 binding (Shieh et al., 1997). MDM2 is also phosphorylated by ATM, impairing its ligase activity and ability to degrade p53 (Freedman et al., 1999).

DNA damage also induces phosphorylation of MDMX by ATM, Chk1 and Chk2, at several C-terminal residues, generating a docking site for 14-3-3σ (Gilkes et al., 2008; Li et al., 2002). This stimulates nuclear translocation of endogenous MDMX which is then degraded in an MDM2-dependent manner (Gilkes et al., 2008; Hu et al., 2006; de Graaf et al., 2003; Kawai et al., 2003a; Pan and Chen, 2003). MDM2-mediated degradation of MDMX is strongly stimulated upon ATM-dependent phosphorylation of three serine residues S342, S367 and S403. These phosphorylations have multiple effects including nuclear accumulation of MDMX in a 14-3-3σ dependent manner (LeBron et al., 2006; Pereg et al., 2006; Chen et al., 2005b; Okamoto et al., 2005).

Evidence suggests that p53 integrates stress signals via post-translational modifications, adapting its response by co-ordinating with different protein partners, enabling the p53-mediated stress response to be specific (Vousden and Prives, 2009). Some examples are shown in Figure 1.10. Acetylation at K164 by transcriptional co-activators p300 and CREB-binding protein (CBP) appear to be important for the activation of the majority of p53 target genes (Tang et al., 2008). Skp2 prevents p300 from binding to and acetylating p53 with consequent reduced expression of p53 targets such as $p21^{WAF1}$ and PUMA, implicating p300 and CBP as critical for p53-dependent arrest and apoptosis (Kitagawa et al., 2008). Serine 46 phosphorylation by HIPK2 (homeodomain interacting protein kinase 2) correlates with induction of pro-apoptotic p53AIP1 (D'Orazi et al., 2002). After moderate DNA damage, MDM2 induces HIPK2

degradation, but severe DNA damage results in decreased MDM2, allowing HIPK2 to stabilise and phosphorylate p53 at S46 (Rinaldo et al., 2007). Several other kinases also phosphorylate S46. PUMA levels are induced by acetylation at K120 (Sykes et al., 2006; Tang et al., 2006b).

If DNA damage is repaired, the loop is reset and the cell re-enters the cell cycle (Freedman et al., 1999). MDM2 levels increase in a p53-dependent way, and antagonise p53, and MDM2 and p53 levels return to normal. The p53-mediated induction of MDM2 under normal conditions limits the duration and intensity of a non-lethal stress response.

1.8.1 Co-factors of p53

Co-factors of p53 are important in determining the p53 response. p300 is an acetyl transferase important in the activation of many p53 target genes. Once bound to DNA, p300 influences p53 stability through acetylation, but does not exhibit any known promoter selectivity (Dornan et al., 2003). Another family of cofactors, Apoptosis Stimulating Protein of p53 (ASPP) selectively regulate p53's activity, and are able to selectively affect transcriptional activity of p53 promoters including BAX and p53-inducible gene-3 (PIG3). (Vives et al., 2006; Slee et al., 2004; Samuels-Lev et al., 2001). Recently, both ASPP1 and ASPP2 were found to bind p300 and cooperate with p300 to enhance p53 transcriptional activity, whilst maintaining ASPP promoter selectivity (Gillotin and Lu, 2011).

1.9 p53 localisation and transcription independent functions

To function as a transcription factor, p53 must be localised in the nucleus where it is able to bind DNA. However, p53 has transcription-independent functions outside the nucleus.

Cytoplasmic p53

Whereas MDM2-mediated polyubiquitination of p53 regulates p53 stability, in conditions of low MDM2, monoubiqutination of p53 induces relocalisation from the nucleus to the cytoplasm (Li et al., 2003). This may occur in unstressed cells although one study found p53 was distributed equally between the nucleus and cytoplasm before DNA damage (Wang et al., 2007). Ubiquitin ligases Cullin-7, Parc and WWP1 (WW domain containing E3 ubiquitin ligase 1), mediate cytoplasmic localisation of p53

(Mulhall et al., 2010; Laine and Ronai, 2007; Andrews et al., 2006),. Parc and Cullin-7 do not ubiquitinate p53 whereas WWP1 directly binds and ubiquitinates p53 which then remains inactive in the cytoplasm.

Mitochondrial p53

In stressed cells, low levels of MDM2 promote monoubiquitination of p53. Monoubiqutinated p53 accumulates at the mitochondria where is interacts with pro- and anti-apoptotic members of the Bcl-2 family, resulting in release of factors from the mitochondria that drive apoptosis (Vaseva and Moll, 2009; Mihara et al., 2003). At the mitochondria p53 is activated by stress-induced HAUSP, a p53-deubiquitinating enzyme (Marchenko et al., 2007), and interacts directly with pro-apoptotic Bak and Bax proteins, and anti-apoptotic Bcl-X_L. High levels of MDM2 promote polyubiquitination which results in degradation of p53 (Lee and Gu, 2010).

1.9.1 Aberrant localisation of p53 in neuroblastoma

Aberrant cytoplasmic localisation of p53 has been proposed as a mechanism for p53 inactivation in neuroblastoma cells. Increased activity of MDM2, or dysfunction of HAUSP results in cytoplasmic retention of p53 in neuroblastoma (Van Maerken et al., 2009c). p53 localisation in neuroblastoma is a subject of debate. In an initial study p53 was cytoplasmic in 96% of undifferentiated, but not differentiated neuroblastomas (Moll et al., 1995). Other studies report a predominantly nuclear localisation of p53 in undifferentiated neuroblastomas and both cytoplasmic and nuclear p53 in differentiated neuroblastoma (Chen et al., 2007; Tweddle et al., 2001b; Wolff et al., 2001). There are also conflicting reports in cell lines, with reports that p53 is cytoplasmic, cytoplasmic and partly nuclear, equally cytoplasmic and nuclear, and only nuclear (Nikolaev et al., 2003; Tweddle et al., 2003; Wolff et al., 2001; Smart et al., 1999; Goldman et al., 1996; Moll et al., 1996). Several studies within our group found p53 to be predominantly nuclear and functional in neuroblastoma (Chen et al., 2007; Tweddle et al., 2001b).

Cytoplasmic retention of wildtype p53 may be either an infrequent anomaly, or a relative block on p53 that can be overcome by appropriate p53-inducing stimuli (Van Maerken et al., 2009c). Proposed mechanisms for abnormal p53 accumulation in the cytoplasm of neuroblastoma cells include hyperactive nuclear export of p53, cytoplasmic tethering of p53, resistance of proteosomal degradation and impaired nuclear re-import of p53.

1.10 p53-independent roles of MDM2

MDM2 overexpression promotes cancer and resistance to treatment, even in cancer patients with deficient p53 (Zhang and Zhang, 2005; Jones et al., 1998). MDM2 interacts with molecules other than p53, and is able to bind to some RNA molecules (Anderson et al., 2007; Lai et al., 1998; Elenbaas et al., 1996). MDM2 can bind the mRNA of the apoptosis regulator XIAP through the C-terminal RING, mediating translation, which may promote resistance to anticancer drugs (Gu et al., 2009). MDM2 also activates hypoxia inducible factor-1α (HIF1α) (Lee et al., 2009; Chen et al., 2003). Hypoxia is a defining characteristic of solid tumours and HIF-1α plays a central role in tumour adaptation to hypoxia through transcription of a variety of genes such as VEGF and glycolytic enzymes (Gordan and Simon, 2007).

1.11 MYCN and MDM2

MYCN-mediated direct transcriptional upregulation of MDM2 was reported by Slack *et al*, and has since been supported by another study (Westermann et al., 2008; Slack et al., 2005a). Chen *et al.* found MDM2 is required for suppression of p53 activity during MYCN driven tumourigenesis in TH-MYCN transgenic mice (Chen et al., 2009). siRNA mediated knockdown of p53 in Tet21N MYCN+ cells led to abrogated MDM2 expression, suggesting that higher MDM2 in these cells is due to increased p53 rather than MYCN (Chen et al., 2009). MYCN may directly upregulate MDM2 to counteract p53 induction. Very recently, MYCN has been identified as a translational target of MDM2 (Gu et al., 2011).

1.12 MDM2 and MDMX in cancer

1.12.1 MDM2 and cancer

MDM2 is overexpressed in many cancer types (Fadok et al., 2001; Freedman et al., 1999; Momand et al., 1998; Haupt et al., 1997; Kubbutat et al., 1997). *MDM2*-amplification occurs in over one third of sarcomas (Leach et al., 1993; Oliner et al., 1992), and although less frequent, also occurs in other cancers including glioblastomas (Reifenberger et al., 1993), leukaemia's (Bueso-Ramos et al., 1993), oesophageal carcinomas (Shibagaki et al., 1995), breast carcinomas (Marchetti et al., 1995) and neuroblastoma (Carr-Wilkinson et al., 2010; Tweddle et al., 2001b; Corvi et al., 1995a;

Corvi et al., 1995b). These tumours are usually p53 wildtype and *MDM2*-amplification is proposed as a mechanism of p53 inactivation.

MDM2 can enhance the tumourigenic potential of cells (Fakharzadeh et al., 1991). In transgenic mouse models and human tumours, MDM2 overexpression results in insufficient p53 activity and contributes to tumour formation (Alkhalaf et al., 1999; Momand et al., 1998). MDM2 haploinsufficiency prevented tumourigenesis in Eμ-myc mouse models of lymphoma, and B-cell apoptosis was enhanced by MDM2 deficiency resulting in reduced tumour formation (Eischen et al., 1999).

1.12.2 MDMX and cancer

MDMX is overexpressed in nearly 30% of human cancers (Toledo and Wahl, 2007) including 18-19% of breast, lung and colon cancers (Danovi et al., 2004), 50% of head and neck squamous carcinomas (Valentin-Vega et al., 2007) and 65% of retinoblastomas (Laurie et al., 2006). MDMX overexpression is mainly a result of aberrant transcription (Gilkes et al., 2008) and is mutually exclusive of MDM2 overexpression in cancer cells (Danovi et al., 2004) suggesting deregulation of either of these repressors is sufficient to inactivate p53. Amplification of the *MDMX* gene (located on chromosome 1q32) also occurs in some cancer types including gliomas (Riemenschneider et al. 1999) and retinoblastomas (Laurie et al., 2006). As in MDM2 overexpressing or amplified cancers, p53 is usually wildtype in MDMX overexpressing or amplified cancers.

1.13 The $p14^{ARF}$ tumour suppressor gene

The *CDKN2A* gene contains the INK4A locus at 9p21-22, which encodes 2 genes; $p14^{ARF}$ and $p16^{INK4a}$ (Quelle et al., 1995; Stone et al., 1995). They share common second and third exons, but have a distinct first exon as shown in Figure 1.16. They are read in alternate reading frames, and therefore are not isoforms, share no sequence homology and have distinct functions (reviewed by (Gallagher et al., 2006)). Both $p14^{ARF}$ and $p16^{INK4a}$ act as tumour suppressors and play important roles in the prevention of cell proliferation and cancer development (Quelle et al., 1995). $p16^{INK4a}$ is a Cdk inhibitor, which prevents the phosphorylation of the Rb protein, promoting E2F binding and inhibiting cell cycle entry into S-phase (Serrano et al., 1993). The most well-known role of $p14^{ARF}$ is in regulating the p53 pathway, as shown in Figure 1.17 in response to hyperproliferative signals.

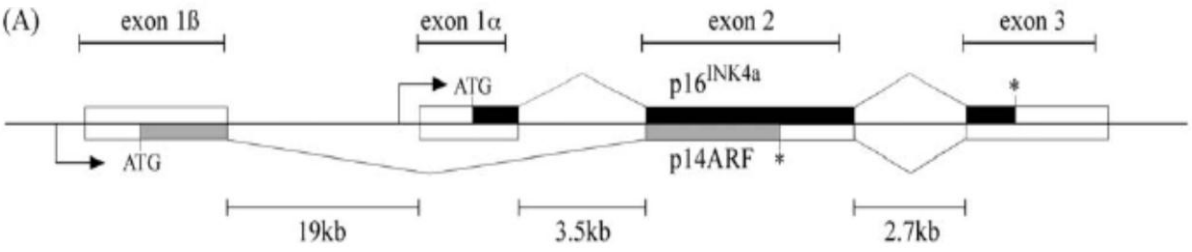


Figure 1.16. **The INK4A locus. The INK4A locus encodes two genes, $p16^{INK4A}$ and $p14^{ARF}$, each with a unique exon 1 but common exons 2 and 3. They are read in alternative reading frames. (Gallagher et al., 2006).**

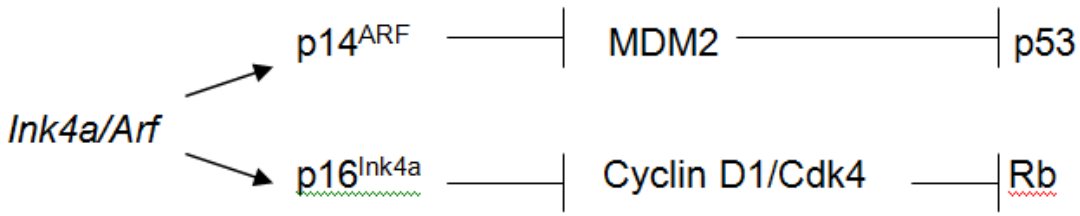


Figure 1.17. **The 2 products of the *INK4a/ARF* locus, $p16^{Ink4a}$ and $p14^{Arf}$ indirectly regulate the Rb and p53 pathways (Sherr, 2001).**

1.13.1 Structure of human $p14^{ARF}$ and comparison to murine $p19^{ARF}$

$p14^{ARF}$ is an arginine rich protein, containing over 20% arginine residues, and is therefore highly basic with hydrophobic properties. $p14^{ARF}$ is composed of 132 amino acids and is 13.9kDa, whereas the murine version, $p19^{ARF}$ has 169 amino acids and is 19.2kDa (reviewed by (Gallagher et al., 2006)). These proteins are poorly conserved, with only 50% identity and no homology to other proteins. There are no recognisable structural motifs and it may be that to become folded and neutralised, $p14^{ARF}$ must form complexes with other molecules. Human and mouse ARF have 11 out of 14 identical amino acids in the N-terminal, the region of most known functions (Gallagher et al., 2006). These functions include nuclear localisation, MDM2 binding and the ability to induce cell cycle arrest. The C-terminal of $p14^{ARF}$ contains a nuclear localisation sequence but this is not conserved and its function is uncertain (Gallagher et al., 2006).

Full length *$p14^{ARF}$* contains 2 nucleolar localisation signals (NoLS) and preferentially localises in the nucleoli, whereas $p19^{ARF}$ has just one NoLS (Weber et al., 1999). The

first $p14^{ARF}$ NoLS is located within exon 1β at the N-terminus, and is important for MDM2 binding. It plays an antiproliferative role and its deletion inhibits the ability to induce cell cycle arrest (Rizos et al., 2000). The second NoLS is located within exon 2, and is involved in $p14^{ARF}$ mediated SUMOylation of its binding partners (Xirodimas et al., 2002).

There are major differences between $p19^{ARF}$ and $p14^{ARF}$. Mouse cells lacking $p19^{ARF}$ do not undergo senescence in culture and $p19^{ARF}$ has been shown to increase as MEFs near senescence (reviewed by (Sharpless, 2005)). However, $p14^{ARF}$ does not appear to be required for senescence in humans as levels remain low as cells near senescence and $p14^{ARF}$ depleted cells can still undergo senescence when challenged by Ras (Sharpless, 2005). These results highlight the difference between $p19^{ARF}$ and $p14^{ARF}$, and indicate that properties of $p19^{ARF}$ should not be assumed to be present with $p14^{ARF}$.

1.13.2 Function and regulation of $p14^{ARF}$

$p14^{ARF}$ is a key sensor of hyper-proliferative signals generated by activated oncogenes, and engages both p53-dependent and p53-independent pathways to protect cells from malignant transformation (Sherr, 2006). $p14^{ARF}$ expression is induced by mitogenic stress as a result of ectopic expression of a variety of oncogenes such as Myc, E1A, E2F1 and Ras, but not DNA damage (Sherr, 2006; Sharpless, 2005) as part of a checkpoint response that limits cell cycle progression in response to hyperproliferative signals. $p14^{ARF}$ expression is also induced after exposure to some common cancer treatments including radiation and genotoxic drugs, and contributes to the elimination of damaged cells from the proliferative pool (Sherr, 2006). $p19^{ARF}$ is also induced by viral infection, and acts to reduce viral infectivity (Garcia et al., 2006). The best known function of $p14^{ARF}$ is in regulating p53 in response to oncogenic stress or aberrant growth signals (Xia et al., 2008), but it has both p53-dependent and p53-independent functions.

$p14^{ARF}$ is relatively stable, with a half-life of 1-6 hours (Gallagher et al., 2006). The mechanisms that regulate $p14^{ARF}$ turnover are unclear, but two residues within exon 1β are critical for stability (Serrano et al., 1996). $p14^{ARF}$ undergoes N-terminal ubiquitination independently of p53 or MDM2 (Pollice et al., 2008). A ligase specific for $p14^{ARF}$ has been identified, known as ULF (ubiquitin ligase of ARF), and oncogenic stress abrogates ULF-mediated $p14^{ARF}$ ubiquitination (Chen et al., 2010a).

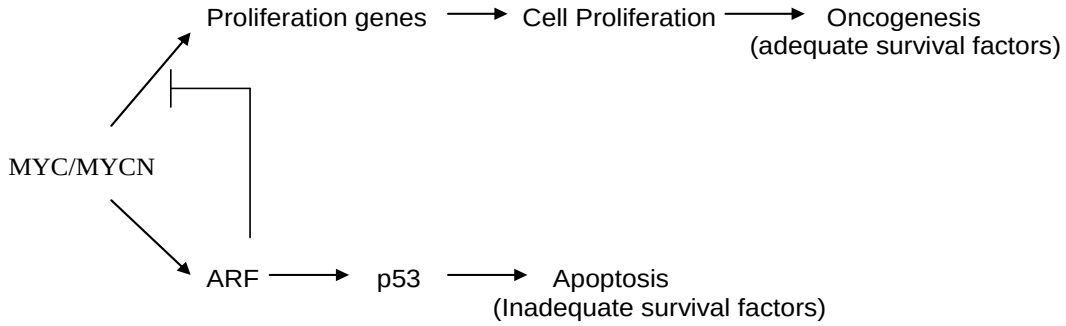


Figure 1.18. **The dual role of p14^{ARF} in activating the tumour suppressor pathway.** p14^{ARF} is induced by MYCN, and activates p53, and also inhibits MYC mediated proliferation (Datta et al., 2004; Zindy et al., 1998).

1.13.3 The role of p14^{ARF} in ribosomal RNA transcription

ARF predominantly resides in the nucleolus where is binds nucleophosmin (NPM) also known as B23 as shown in Figure 1.19 (Bertwistle et al., 2004; Itahana et al., 2003). NPM is an abundant nucleolar protein whose expression correlates directly with the proliferative state of the cell. NPM is a protein that shuttles between the nucleus and cytoplasm, and is involved in several cellular processes including ribosome biogenesis and centrosome duplication (Grisendi et al., 2006; Lindstrom and Zhang, 2006). The ARF-NMP interaction modulates ARF stability, and there are reduced levels of ARF in cells lacking NPM. In response to oncogenic stress, ARF enters the nucleolus and forms a stable complex with NPM, but the biological consequences of these complexes are not completely understood (Sherr, 2006).

1. ARF may exert growth inhibitory effects in the nucleolus, retarding rRNA transcription and processing, and interfering with NPM nucleocytoplasmic shuttling and therefore impeding ribosome export to the cytoplasm.
2. ARF sequestration by NPM in the nucleolus could hold ARF inactive, and its nucleoplasmic translocation promotes MDM2 inhibition. NPM and MDM2 may compete for ARF association at the same domains.

ARF's ribosome function may inhibit cell growth through binding with NPM in the nucleolus, and may regulate p53 activity through binding with MDM2 and ARF-BP1 in the nucleoplasm (reviewed by (Ozenne et al., 2010)).

1.13.4 p53-dependent functions of $p14^{ARF}$

$p14^{ARF}$ is a key activator of the p53 pathway and is predominantly nucleolar (Sharpless, 2005). It is able to regulate p53 following aberrant growth or oncogenic stress signals such as MYC or Ras. It is suggested that $p14^{ARF}$ is stored in the nucleolus with NPM, regulating ribosome biogenesis, and displaced in the nucleoplasm by stress-induced nucleolar perturbation, where it then counteracts MDM2 (Gjerset and Bandyopadhyay, 2006; Korgaonkar et al., 2005; Llanos et al., 2001). $p14^{ARF}$ inhibits MDM2 function by a) binding to MDM2 antagonising the E3 ubiquitin ligase activity and thereby preventing p53 degradation and b) by relocalising MDM2 to the nucleoli, releasing nucleoplasmic p53 from MDM2 (shown in Figure 1.19) (Sherr, 2006; Wang et al., 2001; Honda and Yasuda, 1999; Tao and Levine, 1999; Zhang et al., 1998). Reports suggest that nucleolar sequestration of MDM2 is required for p53 activation (Korgaonkar et al., 2005; Lin and Lowe, 2001; Midgley et al., 2000) whereas other studies show p53 stabilisation and induction of cell cycle arrest without relocalisation of endogenous MDM2 to the nucleoli (Korgaonkar et al., 2002; Llanos et al., 2001).

$p14^{ARF}$ also regulates p53 independently of MDM2. It inhibits another E3 ubiquitin ligase of p53, ARF-binding protein 1 (ARF-BP1), also known as Mule, neutralising the p53-antagonising NF-κB pathway (Chen et al., 2005a; Rocha et al., 2003) as shown in Figure 1.19.

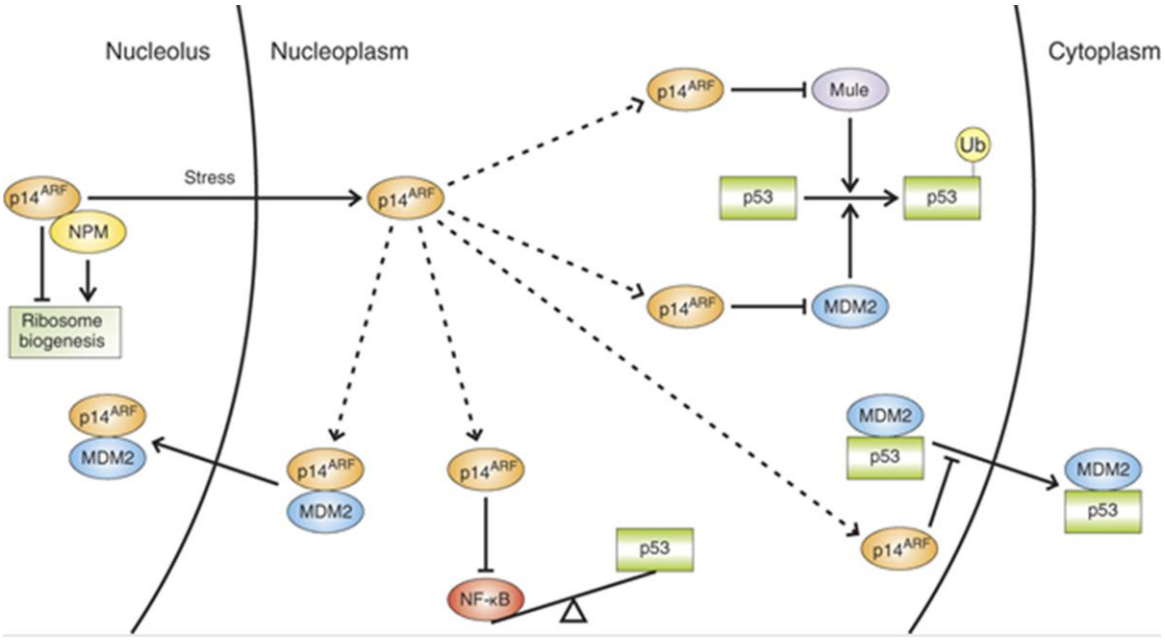


Figure 1.19. **A model for the mechanism of $p14^{ARF}$ in activation of p53. NMP binds $p14^{ARF}$ in the nucleolus and $p14^{ARF}$ binds ARF-BP1 (Coll-Mulet et al.) and MDM2 in the nucleoplasm to inhibit their E3 ubiquitin activities (Gallagher et al., 2006).**

1.13.5 p53-dependent regulation of $p14^{ARF}$

In p53 null cells, $p14^{ARF}$ is ubiquitously expressed and has increased levels, suggesting that when activated, p53 downregulates and inhibits $p14^{ARF}$ expression (Mascaux et al., 2008; Sharpless, 2005).

1.13.6 p53-independent functions of $p14^{ARF}$

Mice lacking $p19^{ARF}$, p53 and MDM2 are more tumour prone than mice lacking p53 and MDM2 only, and $p19^{ARF}$ -/- mice and +/- mice develop a broader spectrum of tumours than p53-null mice (Weber et al., 2000; Kamijo et al., 1999). ARF has been reported to interact with multiple proteins other than MDM2 and ARF-BP1, including E2F1, MDMX (see Section 1.13.8), HIF-1α, topoisomerase 1, MYC (see Section 1.13.7) and nucleophosmin (NPM) (see Section 1.13.3).

ARF is able to inhibit cell proliferation independently of p53. Overexpression of $p19^{ARF}$ induced G_1 cell cycle arrest in p53-null MEFs (Carnero et al., 2000; Weber et al., 2000) and $p14^{ARF}$ can alter cells in S-phase and trigger apoptosis by p53-independent mechanisms (Hemmati et al., 2002; Yarbrough et al., 2002). In p53-null lung tumours $p14^{ARF}$ inhibited tumour growth through G_2 arrest and apoptosis, and in nude mice $p14^{ARF}$ expression prevented tumour growth and induced regression (Eymin et al., 2003; Itahana et al., 2003; Eymin et al., 2001). Other studies show that $p14^{ARF}$ interacts with and antagonises the transcriptional function of MYC and E2F1 independently of p53 which may also be evidence of a p53-independent negative feedback mechanism (Sherr, 2006).

1.13.7 MYCN and $p14^{ARF}$

$p14^{ARF}$ has been found to bind and inhibit c-MYC-mediated activation of target genes, hyperproliferation and oncogenic transformation (Amente et al., 2006; Gregory et al., 2005; Datta et al., 2004; Qi et al., 2004). Amente *et al*. found that $p14^{ARF}$ can also directly bind and inhibit the transcriptional activity of MYCN, and promotes relocalisation of MYCN to the nucleolus rendering it inactive (Amente et al., 2007). In addition, MYCC has been shown to induce $p14^{ARF}$ expression. There are no reports that MYCN acts in the same way but due to the similarity to MYCC, it is possible to speculate that it does. However, like MYCC, MYCN also indirectly inhibits $p14^{ARF}$ through directly activating the HTWIST transcription factors, which impairs $p14^{ARF}$ activity (Valsesia-Wittmann et al., 2004; Maestro et al., 1999). This may be a

mechanism by which *MYCN*-amplified neuroblastomas escape from MYCN-dependent apoptosis.

1.13.8 MDMX and p14^{ARF}

MDM2 is reported to undergo p14^{ARF}-dependent SUMOylation with subsequent MDM2 stabilisation (Xirodimas et al., 2002). When co-expressed with p14^{ARF}, MDMX undergoes p14^{ARF}-mediated sumoylation, and inhibits sumoylation of MDM2 in a dose-dependent manner (Ghosh et al., 2005). This induces MDM2 ubiquitination and degradation. MDMX can therefore affect MDM2 stability through interaction with p14^{ARF} (Ghosh et al., 2005).

In contrast, Wang *et al.* found that p14^{ARF} stabilised p53 when co-expressed with MDMX (Wang et al., 2001), but this may be a result of p14^{ARF} interacting with endogenous MDM2 independently of MDMX.

There is also evidence that like MYCN and MDM2, MDMX binds to and is sequestered in the nucleolus by p14^{ARF}, resulting in increased p53 transactivation in response to cellular stress (Jackson et al., 2001).

1.13.9 p14^{ARF} and cancer

Mice null for *p19^{ARF}* are highly tumour prone, suggesting an important role in carcinogenesis and for p14^{ARF} as a tumor suppressor (Kamijo et al., 1997). Previous studies have shown that *CDKN2A* mutations induce chemoresistance by disabling p53 (Schmitt et al., 1999) and that loss of p19^{ARF} limits the therapeutic response to Imatinib (Williams et al., 2006). The INK4a/ARF locus is frequently deleted in human tumours, and is mutated in 20-40% of multiple case melanoma families (Kefford et al., 1999).
Mice defective for either *p19^{ARF}* or *p16^{INK4A}* have increased susceptibility to spontaneous tumour development, but to different degrees. *p19^{ARF}* deficient mice develop spontaneous tumours within 9.5 months of life, and mouse embryonic fibroblasts (MEFs) have a high proliferation rate. Mice with *p16^{INK4a}* deletion develop spontaneous tumours within 17 months and have a normal proliferation rate (Sharpless, 2005; Krimpenfort et al., 2001; Kamijo et al., 1999; Kamijo et al., 1997).

1.14 Summary of p53/MDM2/p14ARF interactions and MYCN

A summary of the interactions between the p53-MDM2/X-p14^{ARF} network and MYC/N is shown in Figure 1.20, along with transcriptional targets and cellular responses.

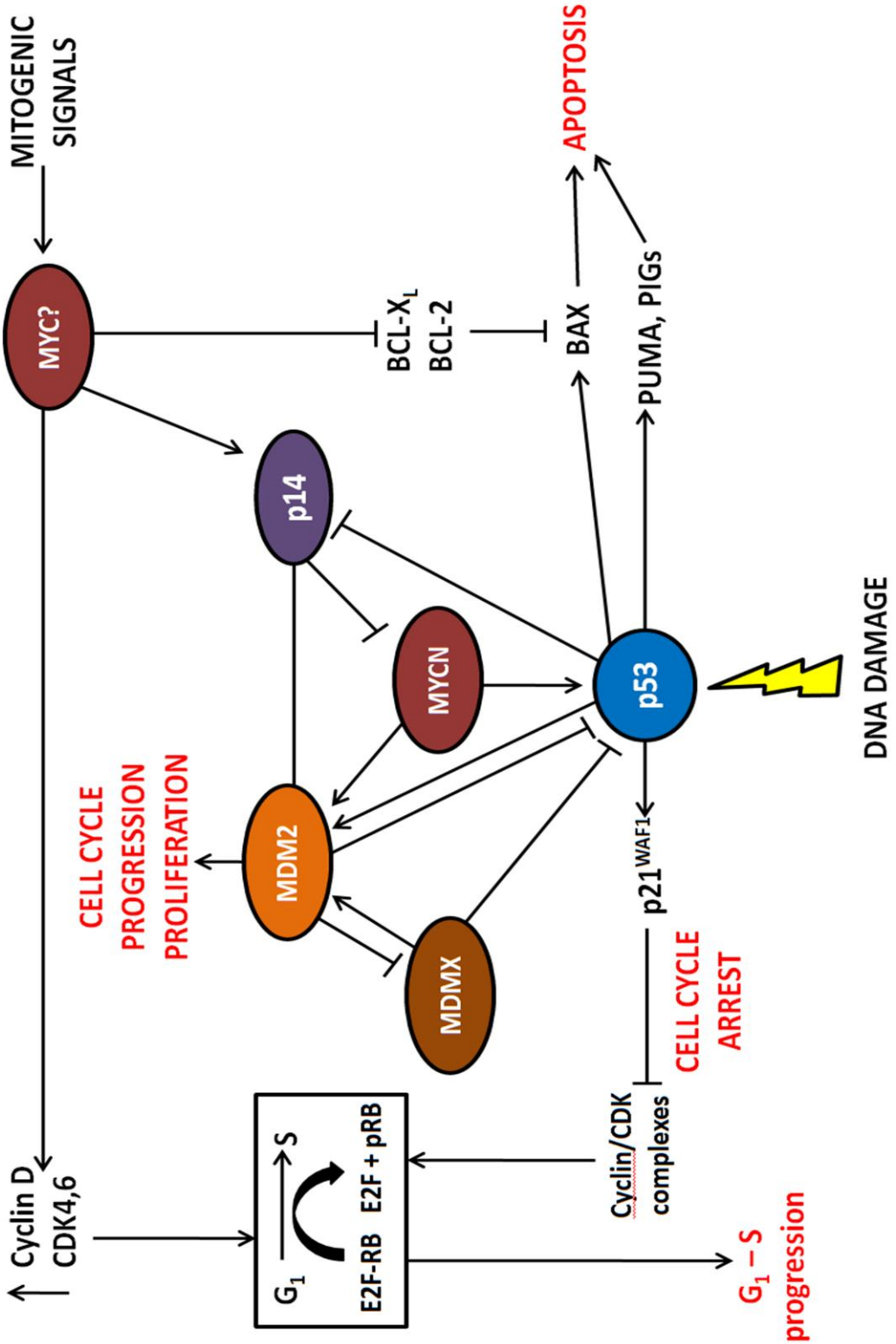


Figure 1.20. **The p53-MDM2/X-p14^{ARF} network and interaction with MYC/N and some common target genes and cellular responses.**

1.15 The p53-MDM2/X-p14^{ARF} pathway and MYCN in neuroblastoma

Since MYCN induces both proliferation and apoptosis, defects in apoptotic pathways are thought to occur in the presence of *MYCN*-amplification. These include both defects in apoptotic pathways and activation of survival or proliferative pathways (reviewed by (Hogarty, 2003)).

Most neuroblastomas respond well to initial therapy, but relapse with chemoresistant disease that correlates with the intensity of the therapy (Keshelava et al., 2001). A high proportion of relapsed neuroblastomas have upstream defects in the p53 pathway. The p53/MDM2/p14^{ARF} pathway is frequently abrogated in neuroblastoma. In studies by Carr-Wilkinson et al, the p53/MDM2/p14^{ARF} pathway was inactivated in 53% of neuroblastoma cell lines established at relapse (Carr et al., 2006) and in 49% of relapsed neuroblastoma tumours (Carr-Wilkinson et al., 2010). Inactivation of the p53/MDM2/p14^{ARF} pathway develops during treatment, and contributes to relapse and loss of p53 function contributes to multidrug resistant disease (Xue et al., 2007a; Keshelava et al., 2001).

1.15.1 p53

p53 mutation is independently prognostic for overall survival in neuroblastoma (Carr-Wilkinson et al., 2010). However, the frequency of mutations is low in both diagnostic and relapsed neuroblastoma, occurring at a frequency of <2% and 15% respectively (Carr-Wilkinson et al., 2010; Carr et al., 2006; Tweddle et al., 2003; Keshelava et al., 2001). The seven fold increase in mutation at relapse suggests that mutations may be acquired during chemotherapy and malignant progression of neuroblastoma (Carr-Wilkinson et al., 2010; Xue et al., 2007a; Kotchetkov et al., 2005; Tweddle et al., 2003; Tweddle et al., 2001a). A recent study by van Maerken *et al.* found p53 mutations in 27% (9/33) of cell lines, most of which were derived from progressive or relapsed tumours, and in the majority of other cell lines p53 was functional (Van Maerken et al., 2011).

Many studies have shown normal DNA-binding and transactivation functions of the p53 protein and an intact p53 signal transduction pathway in neuroblastoma with wildtype p53 suggesting that evasion of the p53 response relies on inappropriately increased activity of inhibitors of p53 signalling, or loss of positive regulators of p53 activity (Chen et al., 2007; Xue et al., 2007a; Van Maerken et al., 2006; Tweddle et al., 2003; Goldman et al., 1996).

The G_1 checkpoint function and apoptotic activity of p53 may be impaired through cytoplasmic sequestration in some neuroblastomas, although there are conflicting reports about p53 localisation in neuroblastoma (see Section 1.9) (Wang et al., 2003; Rodriguez-Lopez et al., 2001; Moll et al., 1996). There is evidence that wildtype p53 in neuroblastoma cells may be in the wrong conformation to integrate into transcriptional complexes, resulting in reduced transcriptional activity (Wolff et al., 2001) but on the whole the evidence supports an intact p53 pathway in neuroblastoma.

1.15.2 MDM2

Upstream suppression of p53 via MDM2 might be important for neuroblastoma tumourigenesis. MDM2 is sometimes overexpressed or amplified in neuroblastoma (Momand, 1998). In one study, *MDM2*-amplification occurred at a frequency of 24% in neuroblastoma cell lines derived from relapse, and only in the presence of *MYCN*-amplification (Carr et al., 2006; Tweddle et al., 2003). In tumour samples, 13% of diagnostic and relapsed tumour samples harboured *MDM2*-amplification and this occurred independently of *MYCN*-amplification in 2 tumour samples (Carr-Wilkinson et al., 2010). Keshelava *et al*. showed that elevated MDM2 expression is associated with multidrug resistance in some neuroblastoma cell lines, and that MDM2 ubiquitin ligase activity is rate limiting in the degradation of p53 in neuroblastoma (Keshelava et al., 2001).

A recent line of evidence supporting a role for the activity of MDM2 in the development and malignant behaviour of neuroblastoma stems from epidemiological studies of a T>G single nucleotide polymorphism in the MDM2 promoter (SNP309). This polymorphism leads to increased binding of the transcriptional activator Sp1 and enhanced MDM2 transcription and expression (Perfumo et al., 2009; Cattelani et al., 2008; Perfumo et al., 2008). SNP309 is associated with poor survival and individuals with SNP309 have an increased risk of neuroblastoma. Patients homozygous for SNP309 had a worse overall survival rate after relapse than homozygous patients, and heterozygous individuals showed an intermediate survival rate (Cattelani et al., 2008; Perfumo et al., 2008; Bond et al., 2004). MDM2 plays a role in MYCN induced tumourigenesis (Chen et al., 2009). There is evidence that MYCN-driven expression of MDM2 contributes to evasion of p53-directed apoptosis in neuroblastoma (Slack et al., 2005b). MDM2 haploinsufficiency in TH-MYCN transgenic mouse models delays tumourigenesis due to higher levels of p53 (Chen et al., 2009). MYCN has also been shown to require MDM2 to overcome p53 suppression for MYCN-directed centrosome

amplification, and genomic instability (Slack et al., 2007). This data suggests MDM2 is important for MYCN to overcome the tumour suppressive functions of p53 during neuroblastoma tumourigenesis, and this may be through MYCN-mediated upregulation of MDM2.

Amplification of 12q sequences encompassing the *MDM2* gene have been described mostly in neuroblastoma tumours that have *MYCN*-amplification, although a microarray study found 12q amplification in 5/95 neuroblastomas, and only 1 of these tumours had more than 10 copies of MYCN (Su et al., 2004). *MDM2*-amplification in neuroblastoma cell lines is associated with attenuated p53 transcriptional function and multidrug resistance (Keshelava et al., 2001; Tweddle et al., 2001b; Corvi et al., 1995a; Corvi et al., 1995b; Van Roy et al., 1995).

1.15.3 $p14^{ARF}$

The *CDKN2A* locus is the most frequent target of homozygous deletion in both neuroblastoma cell lines and primary tumours, and is also silenced by methylation (Caren et al., 2008; Thompson et al., 2001; Takita et al., 1997). Homozygous deletion affects both $p14^{ARF}$ and $p16^{INK4a}$, whereas in the study by Carr *et al*, methylation only affected $p14^{ARF}$ (Carr et al., 2006). Upstream inactivation of p53 via $p14^{ARF}$ abnormalities occurs at an increased frequency compared to *MDM2*-amplification and was detected at diagnosis and relapse in 9 of 12 paired cases of neuroblastoma (29% of cases) and 24% of cell lines. In two studies, methylation of $p14^{ARF}$ was observed at a frequency of 7% (Carr-Wilkinson et al., 2010) and 14% (Gonzalez-Gomez et al., 2003). Carr-Wilkinson *et al.* found homozygous deletion in a higher proportion of cases (22%) than found in previous reports (Bassi et al., 2004; Omura-Minamisawa et al., 2001; Thompson et al., 2001; Diccianni et al., 1996). There was a lack of $p19^{ARF}$ expression in *MYCN* transgenic tumours with MDM2 haploinsufficiency suggesting these tumours have selective pressure to silence the $p19^{ARF}$ locus and low $p14^{ARF}$ expression is important in development and progression of wildtype p53 neuroblastomas (Chen et al., 2009). In mouse models the cancer protective activity of p53 is lost in the absence of $p19^{ARF}$ (Christophorou et al., 2005).

This data suggests that an altered p53/MDM2/$p14^{ARF}$ axis, particularly increased MDM2 activity from amplification or $p14^{ARF}$ inactivation, is a critical mediator of p53 inactivation in neuroblastoma. Genetic aberrations of the *MDM2* locus as well as

epigenetic disruption of the *CDKN2A* locus may account for inactivation of the p53 pathway in a subset of neuroblastomas, both at diagnosis and relapse.

1.15.4 MDMX and neuroblastoma

MDMX expression in neuroblastoma has not been studied, and the effect of MDMX on response to cytotoxic or novel therapies in neuroblastoma has not been investigated.

MDMX is involved in the cytoplasmic tethering and inactivation of p53 (Ohtsubo et al., 2009). Both cytoplasmic MDM2 and MDMX were shown to cooperate in inhibiting p53 activity through ubiquitination and subsequent cytoplasmic localisation and tethering of p53, which may be mediated by formation of a stable complex. shRNA-mediated knockdown of MDMX expression in neuroblastoma cell lines resulted in decreased cytoplasmic p53 and a subsequent increase in nuclear p53 and increased transcriptional activity (Ohtsubo et al., 2009).

1.16 Other p53 family members: p63 and p73

p63 and p73 genes encode proteins that share strong structural, biochemical and biological similarities. Both have significant amino acid sequence homology with p53, and a similar function (Kaghad et al., 1997). In response to DNA damage and other conditions that activate p53, both p63 and p73 can bind specifically to DNA at conserved p53 response elements to induce a number of p53 responses including cell death, cell cycle arrest and cellular senescence, and target genes include p21, PUMA, NOXA, BAX and MDM2 (Stiewe et al., 2007; Harms et al., 2004,; Fontemaggi et al., 2002; Melino et al., 2002; Yang et al., 2002). In addition, p63 and p73 play roles in regulating proliferation and differentiation. The extent to which p63 and p73 regulate p53 target genes remains unclear.

Like p53, both p63 and p73 contain an N-terminal transactivation domain, a DNA binding domain (with 70% homology to p53 and conservation of all essential DNA contact residues) and a C-terminal oligomerisation domain (reviewed by Murray-Zmijewski et al., 2006). Many post-translational modifications that regulate p53 also target p63 and p73, and several p53 modulators and binding partners also signal to p63 and p73 (Collavin et al., 2010). Both p63 and p73 contain 2 promoters within the N-terminal; promoter 1 (P1) and promoter 2 (P2). Transcription from P1 results in full-length transactivating p73 (TAp73) and p63 (TAp63) which function as tumour suppressors, and can induce cell cycle arrest and apoptosis (Wang et al., 2007; Muller et

al., 2005). Transcription from P2 results in a truncated p63 (ΔNp63) or p73 (ΔNp73) protein which function as oncogenes, promoting cell survival (Grob et al., 2001). In addition, both isoforms of p63 and p73 undergo alternative C-terminal splicing, which generates up to 7 variants of each. It is thought that p53, p63, p73 and their isoforms co-operate to regulate cell development and to respond to cellular stress (Murray-Zmijewski et al., 2006).

ΔNp73 can block the tumour suppressive RB, p53 and TAp73 pathways, and promote expression of anti-apoptotic genes (Stiewe et al., 2003; Zaika et al., 2002; Grob et al., 2001). ΔNp73 isoforms are dominant negative inhibitors of both TAp73 and p53 by competing for promoter binding and therefore overexpression of ΔNp73 could block p53 activity and inhibit the transcriptional role TAp73 plays in inducing apoptosis and differentiation (Peirce et al., 2009; Deyoung et al., 2007; De Laurenzi et al., 2000). In addition both TAp73 and p53 promote ΔNp73 transcription, forming a negative feedback loop. The various p73 isoforms also bind to MDM2 and MDMX resulting in inactivation but not rapid degradation of p73 (Ongkeko et al., 1999; Zeng et al., 1999).

1.16.1 p73 in neuronal development

p73 is involved in neuronal development, and plays essential roles in regulating neural stem cell self-renewal and maintenance (Talos et al., 2010, Agostini et al., 2010; Fujitani et al., 2010). Complete p73 knockout mice have developmental defects in the central nervous system in both embryonal and adult neurogenesis, with 100% penetrance and mice die 5-6 weeks after birth (Talos et al., 2010, Yand et al., 2000; Yang et al., 1999).

A ΔNp73 isoform specific knockout mouse model confirmed the pro-survival role of ΔNp73 which is expressed in differentiated mature post-mitotic neurons (Wilhelm et al., 2010; Tissir et al., 2009; Pozniak et al., 2000), and there is evidence of neurodegeneration in $\Delta Np73^{-/-}$ mice (Yankner et al., 2008). Isoform specific knockout of TAp73 results in defects in neurogenesis (Tomasini et al., 2008) and there is evidence that TAp73 is required for neural stem cell proliferation (Fujitani et al., 2001). No human genetic disorders are associated with germline mutations in the p73 gene (reviewed by Khoury and Bourdon, 2011).

1.16.2 p73 in tumourigenesis and neuroblastoma

Studies have shown that TAp73$^{-/-}$ mice which retained ΔNp73 exhibit increased genomic instability associated with enhanced aneuploidy and have an increased incidence of spontaneous tumour formation (Tomasini et al., 2008, Yang et al., 2000). In addition, p73 +/- mice and p73+/-:p53+/- mice developed a more aggressive tumour phenotype compared to p73+/+ and p73+/+:p53+/+ mice (Flores et al., 2005), indicating a role for p73 in preventing cancer progression. On the other hand, ΔNp73$^{-/-}$ mice show impaired tumour formation in nude mice (Wilhelm et al., 2010).

Compared to p53, p73 is rarely mutated in cancers but its expression is often deregulated (Melino et al., 2002; Irwin et al., 2001). Mechanisms of p73 deregulation in a number of cancer types including neuroblastoma (both in cell lines and primary tumours) include: 1) hypermethylation of the TAp73 P1 promoter, 2) increased expression of the ΔNp73 isoform as a result of demethylation of the P2 promoter, and 3) loss of heterozygosity at the p73 locus, 1p36 (reviewed in Rufini et al., 2011). There is also evidence that p73 is a major determinant of chemosensitivity in human tumours (Lunghi et al., 2009). Overexpression of ΔNp73 is usually associated with treatment failure and chemoresistance, metastases and invasion, correlating with poor prognosis and reduced survival (reviewed in Bisso et al., 2011). It was found to be expressed at high levels by real-time PCR in 30% of primary neuroblastoma tumours and correlates with poor overall and progression-free survival in neuroblastoma patients (Casciano et al., 2002). It is a negative prognostic marker regardless of age, primary tumour site, stage and *MYCN* status.

Both *MYCN* amplification and 1p36 deletion are associated with poor patient survival in neuroblastoma. Neuroblastomas lacking *MYCN* amplification frequently contain relatively small 1p36 deletions and therefore inactivation of p73 may contribute to neuroblastoma formation. Tumours with *MYCN* amplification usually contain larger deletions that extend proximally beyond the p73 locus. MYCN has been shown to inhibit TAp73 expression; *MYCN* amplified tumours have reduced expression of p73, and transfection of cell lines with MYCN reduces p73 expression (Zhu et al., 2002). In addition, overexpression of TAp73 downregulates MYCN, which may induce differentiation (Watanabe et al., 2002; De Laurenzi et al., 2000).

1.16.3 p63 in epithelial differentiation and cancer

p63 is involved in epithelial differentiation. Genetic experiments in mice show that p63 is critical for epidermal morphogenesis and limb development, and that impairment of p63 results in several disorders. Mice with complete p63 knockout are born skinless, and have severe limb deformities (Yang et al., 1999). Unlike p73, p63 levels have been found to be very low in the central nervous system and it is not implicated in the formation of neuroblastoma (Jacobs et al., 2005). It is however, involved in other cancer types and ΔNp63 expression has been shown to correlate with chemoresistance (Rocco et al., 2006). In squamous cell carcinomas expression levels of the ΔNp63 isoform are significantly increased (Hibi et al., 2000).

1.17 Inhibiting the MDM2-p53 interaction

Genetic studies involving transgenic mouse models have demonstrated that p53 inactivation is critical for both tumour formation, and subsequent maintenance of the tumour (Ventura et al., 2007; Xue et al., 2007a; Martins et al., 2006). Many cancers that maintain wildtype p53 have inactive p53 function as a result of upstream defects including MDM2 and MDMX overexpression or $p14^{ARF}$ inactivation. In these p53-wildtype tumours, reactivation of p53 is an area of intense investigation.

Potential methods of p53 reactivation include inhibition of the MDM2-p53 interaction, lowering MDM2 levels, or blocking cytoplasmic shuttling (Vassilev et al., 2004). Genetic restoration of p53 activity in experimental mouse models resulted in inhibition of rapid and extensive tumour progression (Ventura et al., 2007; Xue et al., 2007a; Martins et al., 2006). This thesis focuses on p53 reactivation using low molecular weight compounds that block the interface of the MDM2-p53 binding site, known as MDM2-p53 antagonists (Chene, 2003).

1.17.1 The MDM2-p53 binding site

The p53 binding site of MDM2 is well characterised, and antagonists mimicking p53 can be created to block this interaction (Chene, 2003).

The MDM2-p53 interaction occurs within the 118 amino acid N-terminal transactivation domain of MDM2, and initiates the inhibitory effects of p53 (Oliner et al., 1993). 14 hydrophobic aromatic amino acids on the surface of MDM2 form a deep hydrophobic cleft in which p53 binds, composed of 2 helices forming the sides, 2 shorter helices that make up the bottom and two three-stranded β-sheets that cap each

end (Kussie et al., 1996). The minimal binding site for MDM2 on the p53 protein is also located at the N terminus, between residues 18-23, TFSDLW (Picksley et al., 1994). The binding domain of p53 forms an amphipathic α-helix , of which two and a half turns are involved in the binding of MDM2, and is followed by an extended region of 3 residues (Kussie et al., 1996). 3 hydrophobic and aromatic amino acids of p53; Phe19, Trp23 and Leu26 insert deep into the MDM2 cleft, and Thr18 is important for the stability of the helix (Vassilev et al., 2004; Massova and Kollman, 1999; Kussie et al., 1996). As well as Van der Waals interactions, three hydrogen bonds are involved in MDM2-p53 binding and Trp23 of p53 forms the strongest of these bonds. The p53-MDM2 interaction is shown in Figure 1.21.

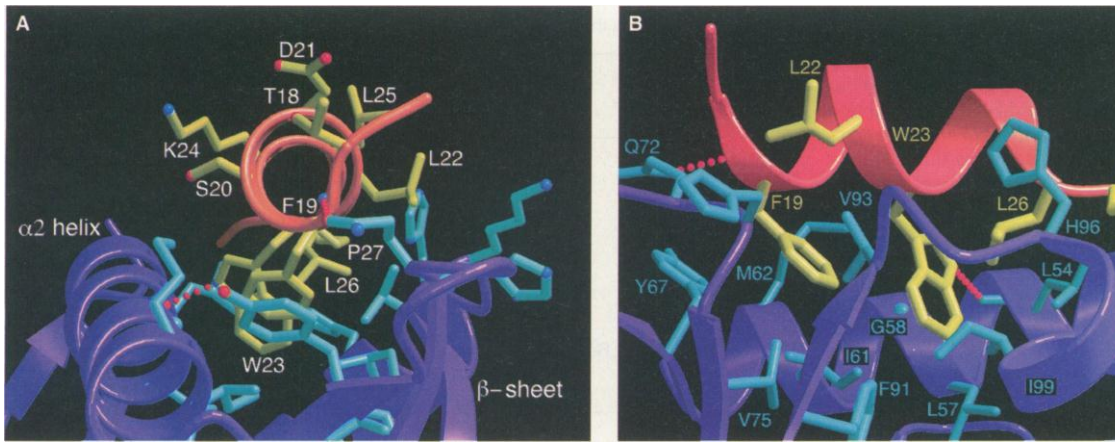


Figure 1.21. **The structure of the p53-MDM2 complex. Key residues are shown on both p53 and MDM2 and A, and B, show the interaction from different angles (Freedman et al., 1999).**

Molecules have been developed which mimic the three p53 amino acids involved in binding, and the Trp23 hydrogen bond should be preserved to ensure good binding affinity (Chene, 2003).

1.17.2 MDM2-p53 antagonists

The hydrophobic p53-binding pocket of MDM2 is ideal for developing low molecular weight compounds that prevent p53 binding (Vassilev, 2004). These need to be potent and selective, and small molecules will contribute to increased oral bioavailability (Vassilev, 2004). However, due to the hydrophobic nature of the pocket, oral bioavailability is slightly compromised as the compounds need to be lipophilic to improve binding energy (Chene, 2003).

The most well-known and studied MDM2 antagonist is the cis-imidazoline analogue Nutlin-3. Another cis-imidazoline analogue RG7112 is in phase I clinical trials for patients with advanced solid tumours (http://clinicaltrials.gov/show/nct00559533). Other MDM2 antagonists include the spirooxinodoles such as MI-63 and MI-219, the latter of which is expected to enter clinical trials (Millard et al., 2011; Shangary and Wang, 2009), the isoindolinones (Hardcastle et al., 2005), and chromenotriazolopyrimidines (Allen et al., 2009). JNJ-26854165, a novel tryptamine derivative that blocks MDM2-p53 proteosome interaction is in clinical development (Millard et al., 2011).

1.17.2.1 Nutlins

Nutlins selectively disrupt the MDM2-p53 interaction in an enantiomer specific manner by competing with p53 for the hydrophobic binding pocket of MDM2. The backbone sits in the same position as the p53 helical backbone within MDM2, and projects 3 groups into MDM2 which mimic the p53 amino acids (Vassilev et al., 2004). Nutlin-3 binds MDM2 with a K_i of 36nM (Michael and Oren, 2003). The structure of Nutlins 1-3 are shown in Figure 1.22, alongside their IC_{50} values.

Nutlins were used to validate the MDM2-p53 antagonist theory *in vitro* and *in vivo* and have been found to have antiproliferative effects in a variety of adult tumour types. Initial investigations with Nutlin-1 showed a dose-dependent antiproliferative and cytotoxic effect within cells containing wildtype p53, compared to cells with non-functional p53 (Tovar et al., 2006; Vassilev et al., 2004). Nutlin-3 inhibited the ability of MDM2 to drive ubiquitination and exportation of p53, resulting in nuclear accumulation of p53 and subsequent increased transcriptional activity and initiation of the DNA damage response (Tovar et al., 2006; Vassilev et al., 2004). This results in non-genotoxic p53 stabilisation and activation of growth arrest and apoptosis (Carvajal et al., 2005; Vassilev, 2004; Vassilev et al., 2004), and also sensitisation to conventional chemotherapies (Barbieri et al., 2006; Cao et al., 2006; Coll-Mulet et al., 2006). Nutlin inhibited growth of human tumour xenografts in nude mice (Vassilev et al., 2004).

Interestingly, whereas Nutlin induced apoptosis in leukaemia cells (Saha et al., 2010; Vassilev, 2007), only cell cycle arrest occurred in a wide array of solid tumours in both preclinical and clinical studies (Demidenko et al., 2010; Huang et al., 2009; Tovar et al., 2006). Upregulation of $p21^{WAF1}$ was associated with altered expression of pro-apoptotic genes, and may explain why some tumours undergo a reversible cell cycle arrest in

response to Nutlin, instead of apoptosis. A recent study however, found that the increased $p21^{WAF1}$ levels did not protect solid cancers against Nutlin induced apoptosis (Xia et al., 2011).

The Nutlin-3 analogue, RG7112, is currently in phase I clinical trials for solid tumours (http://clinicaltrials.gov/show/nct00559533).

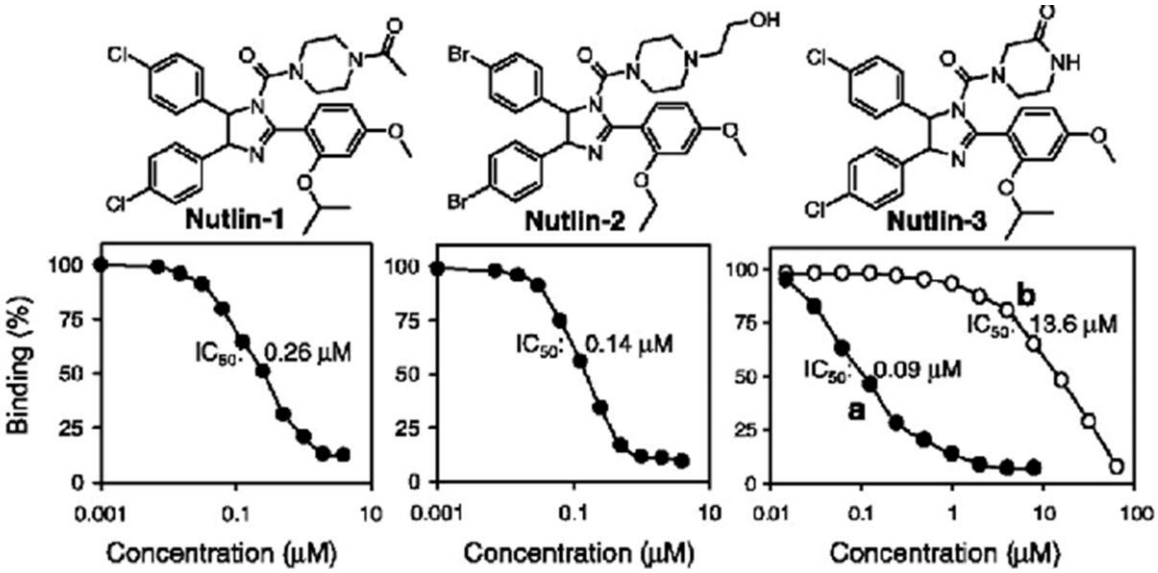


Figure 1.22. **Structure of Nutlins 1-3, and binding against concentration of each Nutlin to recombinant human MDM2 displayed alongside their IC-50. Nutlin-1 and Nutlin-2 were used as racemic mixtures, whereas Nutlin-3a and Nutlin-3b were separated on a chiral column (Vassilev et al., 2004)**.

1.17.2.2 Spirooxinodoles

Spiooxinodoles are another class of potent, selective, cell permeable, nonpeptidic small molecule inhibitors of the MDM2-p53 interaction (Shangary et al., 2008; Ding et al., 2006; Ding et al., 2005). MI-63 is one of these molecules and binds MDM2 with a K_i of 3nM, 2000 times more potent than the natural p53 peptide (Ding et al., 2006).

MI-63 was highly effective at activating wildtype p53 and inhibiting cancer cell growth (Ding et al., 2006), and induced apoptosis in rhabdomyosarcoma and AML (Samudio et al., 2010; Canner et al., 2009). MI-63 synergised with doxorubicin and AraC to induce apoptosis and in comparison with Nutlin was a more potent inhibitor of cell proliferation and viability (Samudio et al., 2010; Canner et al., 2009).

Unfortunately MI-63 has a poor pharmacokinetic profile, and modest bioavailability so is not a suitable candidate for drug development (Ding et al., 2006). Analogues of MI-63 have been developed that are small and potent with excellent bioavailability (Yu et al., 2009); MI-219 and MI-147. In a SJSA-1 xenograft mouse model, MI-147 was highly effective at inhibiting tumour growth and reducing tumour volume with no weight loss or toxicity to normal tissues (Yu et al., 2009). In combination with irinotecan, tumour growth was completely inhibited. MI-219 has a K_i for MDM2 of 5nM and rapidly but transiently stimulated p53 activation and p53-dependent growth arrest and apoptosis in tumour cells and tumour xenograft tissues (Shangary et al., 2008). Whilst p53 was activated in normal cells there was no apoptosis, and *in vivo*, was selectively toxic to tumour tissues (Shangary et al., 2008). The structure of MI-63, MI-147 and MI-219 are shown in Figure 1.23.

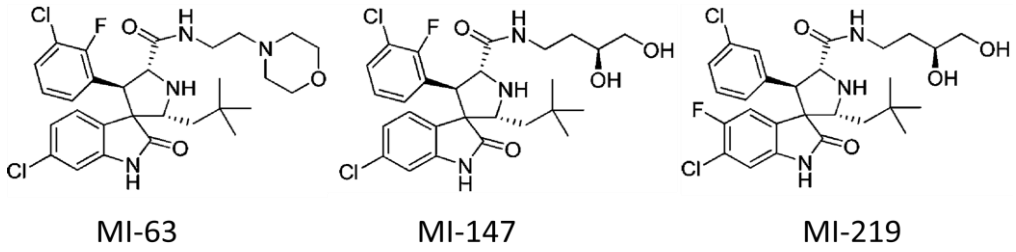


Figure 1.23. **The structures of the spirooxinodole analogues MI-63, MI-147 and MI-219 (Yu et al., 2009).**

1.17.3 MDM2-p53 antagonists in neuroblastoma

Unlike most cancers, most cases of diagnostic and relapsed neuroblastomas have wildtype p53, making reactivation of p53 an attractive therapeutic target. In addition, downstream functions are intact, and p53 is capable of inducing a normal response to stress or DNA damage (Fesik, 2005; Hogarty, 2003; Tweddle et al., 2003; Keshelava et al., 2000; Goldman et al., 1996; Hosoi et al., 1994; Vogan et al., 1993). Therefore upstream inactivation of p53 is necessary for evasion of cell cycle arrest or apoptosis in the face of on-going stress of rapidly proliferating neuroblastoma cells.

The use of MDM2-p53 antagonists for the treatment of neuroblastoma is under investigation. In comparison to other cancers including CLL, multiple myeloma, lung cancer and osteosarcoma, neuroblastomas had much more rapid and robust levels of p53 induction and rates of apoptosis after 24-48 hour treatment, and apoptosis was induced in all cell lines tested (Barbieri et al., 2006; Cao et al., 2006; Kojima et al., 2006;

Stuhmer et al., 2005; Vassilev et al., 2004). Most other solid cancers only undergo cell cycle arrest in response to Nutlin.

Nutlin-3 inhibits the growth of both chemosensitive and chemoresistant neuroblastoma *in vitro* and *in vivo* in a p53 dependent-manner (Van Maerken et al., 2009a; Van Maerken et al., 2009b; Barbieri et al., 2006). Nutlin induced antitumour effects in neuroblastoma cells and xenografts, and induced apoptosis in a dose and time-dependent manner in addition to limiting cell proliferation (Van Maerken et al., 2009a; Barbieri et al., 2006; Van Maerken et al., 2006). Cells that survived treatment either underwent a G_1 arrest, cellular senescence or neuronal differentiation (Van Maerken et al., 2006). Nutlin-3 induced cell cycle arrest and apoptosis in multidrug resistant cell lines (Van Maerken et al., 2009a).

1.17.3.1 The p53/MDM2/p14^{ARF} network and MYCN

p53 mutation renders neuroblastoma cells unresponsive to Nutlin treatment (Van Maerken et al., 2009a). 23 of 25 cell lines with wildtype p53 responded to Nutlin suggesting upstream defects of p53 in the majority of cell lines, and it is likely that high levels of MDM2 are responsible for p53 inactivation in neuroblastoma (Van Maerken et al., 2009a).

Previous reports provide conflicting data about the role of MDM2 on the response to MDM2-p53 antagonists in various cancer types, with some studies pointing towards sensitisation in the presence of MDM2-amplification or overexpression (Gu et al., 2008a; Tovar et al., 2006; Kojima et al., 2005), and other studies indicating no effect, including in neuroblastoma (although only 1 cell line was tested) (Liu et al., 2009; Kojima et al., 2006; Van Maerken et al., 2006). MDM2 knockdown had minimal effect on MI-219 induced growth suppression in other cancer types (Zheng et al., 2010). To investigate the role of MYCN on the response to MDM2-p53 antagonists, Barbieri *et al.* investigated the effect of Nutlin-3 in two MYCN-inducible cell lines, MYCN3 and Tet21N, both of which are derived from SHEP cells. No significant difference in IC_{50} values were observed, although there was a trend towards increased sensitivity in Tet21N cells (Barbieri et al., 2006). A report by Van Maerken *et al.* also found that Nutlin-3 initiates a response regardless of *MYCN* or *MDM2* status (Van Maerken et al., 2006). Interestingly, Petroni *et al.* found that Nutlin-3 induced apoptosis more efficiently in *MYCN*-amplified neuroblastoma cells in response to clastogenic agents,

and Fulda *et al.* found that MYCN increased sensitivity to chemotherapeutic drugs (Petroni et al., 2011; Fulda et al., 2000).

Overexpression of $p14^{ARF}$ pointed towards a stimulatory effect of $p14^{ARF}$ expression on the Nutlin response. Downregulation or impairment of $p14^{ARF}$ in neuroblastoma resulted in lower levels of apoptosis and decreased cell viability, whereas overexpression decreased cell survival and increased apoptosis (Van Maerken et al., 2011).

1.17.4 The role of HIPK2 in neuroblastoma and Nutlin-3 treatment

In solid tumours of non-neuroectodermal origin Nutlin-3 promoted p53 accumulation and induced cell cycle arrest but not apoptosis (Tovar et al., 2006). Nutlin 3 treatment decreased HIPK2 expression and accumulation of monophosphorylated p53 in U2OS cells undergoing growth inhibition. Nutlin-3 efficiently induced apoptosis in *MYCN*-amplified neuroblastoma cells and sensitised them to DNA damaging drugs, via an unexpected and possibly tumour type specific pro-apoptotic regulator, HIPK2 (Petroni et al., 2011). HIPK2 accumulated in SMSKCNR and IMR32 cells undergoing apoptosis in response to Nutlin-3. HIPK2 depletion in SMSKCNR cells, which are *MYCN*-amplified, resulted in reduced levels of apoptosis suggesting a role for HIPK2 in Nutlin-3 mediated apoptosis in neuroblastoma.

1.17.5 p73 and Nutlin-3

Nutlin-3, an MDM2 inhibitor, and chemotherapeutic agents such as cisplatin, have been shown to induce TAp73, as well as activating p53. In a p53-null and doxorubicin-resistant neuroblastoma cell line, Nutlin treatment increased TAp73 expression in an E2F-dependent manner, resulting in an increase in the ability of doxorubicin to block cell proliferation and induce apoptosis in a TAp73-dependent manner (Peirce et al., 2009). In addition, Nutlin has been shown to disrupt the MDM2-TAp73 interaction in cancer cell lines, increasing TAp73 transcriptional activity (Lau et al., 2008).

1.17.6 Combination therapy

In wildtype p53 cell lines, response to genotoxic drugs is significantly enhanced upon disruption of the MDM2-p53 loop. Therefore MDM2-p53 antagonists may be useful as an adjuvant to chemotherapeutic drugs (Barbieri et al., 2006). Combined therapy is appealing as patients would be subjected to lower doses of genotoxic drugs.

Many studies have shown a synergistic effect of Nutlin combined with chemo or radiotherapy (Barbieri et al., 2006; Cao et al., 2006; Coll-Mulet et al., 2006). A number of cell lines tested with this combined therapy were extremely sensitive to treatment. Nutlin-3 in combination with cisplatin induced growth inhibition and a 8-10 fold reduction in IC_{50} compared to cisplatin alone in neuroblastoma (Barbieri et al., 2006). Nutlin-3 in combination with bevacizumab lead to a significant repression in tumour cell growth compared to nutlin-3 alone both *in vivo* and *in vitro*, and in xenograft models. This was associated with a decrease in metastases and metastatic burden, and inhibition of angiogenesis (Patterson et al., 2011). Barbieri *et al.* demonstrated Nutlin-3 induction of apoptosis resulted in limited proliferation, and an enhanced response to genotoxic drugs such as etoposide and cisplatin (Barbieri et al., 2006). Petroni *et al*. found that Nutlin-3 induced apoptosis more efficiently than and cooperatively with bleomycin, and sensitised to other cytotoxic drugs, inducing high levels of apoptosis (Petroni et al., 2011).

1.17.7 The effect of MDMX on the response to MDM2-p53 antagonists

Nutlin-3 fails to induce apoptosis in cancer cells that overexpress MDMX. Despite the similarities between MDM2 and MDMX, Nutlin is ineffective at interrupting the transcription-repressive MDMX-p53 complex, and does not bind MDMX with high enough affinity to affect its interaction with p53 (Wade et al., 2006). MDMX may therefore continue to suppress Nutlin-induced p53, and be a major hindrance to MDM2-p53 antagonists (Wade et al., 2006).

Many human cancer cell lines overexpress MDMX and inhibit p53 transcriptional activity (Hu et al., 2006; Patton et al., 2006; Wade et al., 2006; Ramos et al., 2001). Inactivation of p53 by overexpressed MDMX is oncogenic (Marine et al., 2006). In addition, a number of studies have shown that the cellular activity of MDM2 inhibitors is decreased by MDMX, and Nutlin-3 does not induce apoptosis in cancer cells that express high levels of MDMX protein such as MCF-7 (Hu et al., 2006; Patton et al., 2006; Wade et al., 2006). Apoptosis was restored upon siRNA-mediated MDMX knockdown in these cells, or upon treatment with a peptide that disrupted both the MDM2 and MDMX association with p53 (Wade et al., 2006).

In addition, recent studies have shown that inhibition of MDMX enhances the response to MDM2-p53 antagonists (Vaseva et al., 2011; Wang et al., 2011). The apoptotic efficiency of Nutlin-3 in solid tumours *in vitro* and in xenografts is dramatically

enhanced when combined with the heat-shock-protein 90 inhibitor 17AAG, which interferes with MDMX-p53 complex formation and induces MDMX degradation. A 2.5 fold increase in transcriptional activity was observed compared to Nutlin-3 only. Another small molecule inhibitor of MDMX expression, XI-006 (a benzofluroxan derivative), increased p53 activity and induced proapoptotic target genes in various cancer cells, displaying an additive rather than synergistic effect (Wang et al., 2011). MCF-7 cells underwent apoptosis when treated with XI-006 in combination with nutlin-3, when previously they only arrested, and this enhanced Nutlin's effect on cell viability. Another small molecule that disrupts MDMX interaction had an additive rather than synergistic effect (Reed et al., 2010). This inhibitor transactivated proapoptotic genes in MCF7 overexpressing cells, resulting in apoptosis.

1.18 Hypothesis and Aims

Hypotheses:

The status of MYCN and the p53-MDM2/MDMX-$p14^{ARF}$ network influences the response to MDM2-p53 antagonists in neuroblastoma.

Aims:

1) To investigate the effect of MYCN on the growth inhibitory and apoptotic response to the MDM2-p53 antagonists Nutlin-3 and MI-63, using a MYCN regulatable neuroblastoma cell line (Tet21N), MYCN siRNA and a panel of *MYCN*-amplified and non-*MYCN*-amplified neuroblastoma cell lines.
2) To determine MDMX protein expression levels in neuroblastoma cell lines, and to investigate the effect of MDM2 and MDMX on the growth inhibitory and apoptotic response to Nutlin-3 and MI-63 using MDM2 and MDMX siRNA, and a panel of neuroblastoma cell lines. In addition, growth inhibition in *MDM2*-amplified neuroblastomas and *MDM2*-amplified sarcomas are to be compared.
3) To investigate the effect of $p14^{ARF}$ impairment and $p14^{ARF}$ knockdown on the cell cycle response, and levels of apoptosis following MDM2-p53 antagonist treatment, and to determine the mechanism of increased $p14^{ARF}$ levels in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines.
4) To test 2 cell lines for their response to MDM2-p53 antagonists and to sequence the p53 gene.

Chapter 2. Materials and Methods

2.1 Cell culture

Cell culture was carried out under sterile conditions in class II containment hoods (Biomat^2, Medair Technologies, MA, USA). All cell lines used in this study are displayed in Table 2.1, alongside their *MYCN*, *MDM2*, *p14^{ARF}* and *p53* status as well as their cell type. Cell lines were validated upon receipt using cytogenetic analysis courtesy of Dr Nick Bown, Institute of Human Genetics, Newcastle University, UK. Cell lines were routinely checked for mycoplasma using Mycoalert® Detection Kit (Lonza, Basel, Switzerland).

2.1.1 Passaging and seeding of cells

Cell lines were cultured as adherent monolayers in RPMI 1640 medium (Sigma, Dorset, UK) supplemented with 10% fetal calf serum (FCS) and grown in humidified incubators (Incu Safe, Sanyo, IL, USA) at 37°C and 5% CO_2. MYCN regulatable SHEP Tet21N cells were grown in 200µg/ml of G-418 (Calbiochem, Nottingham, UK), and 900µg/ml Hygromycin B (Invitrogen, Paisley, UK), and vector only Tet21 cells were grown 200µg/ml of G-418. Cells were passaged at ~70-80% confluency and routinely grown in 25cm^2, 75cm^2 or 175cm^2 flasks (Corning, Amsterdam, Netherlands). Cells were first washed with 10ml of phosphate-buffered saline (PBS), and then 2-4mls of 1x trypsin-EDTA (Sigma) added and incubated for approximately 5 minutes to detach cells from the surface of the flask/plate. At least an equal volume of medium was added to tripsinised cells to neutralise the trypsin. An aliquot of the cell culture was then either used to seed a new flask, or the cells counted so that a desired number of cells could be seeded for an experiment.

2.1.2 Counting cells

The concentration of cells was estimated using an Improved Neubauer haemocytometer (Hawksley, Sussex, UK). Cells were first dispersed using a 5ml COMBITIP® PLUS syringe (Eppendorf, Cambridge, UK) and 50µl of cells mixed with an equal volume of Carnoy's solution (Appendix 1). A 15µl volume of cell suspension was added to each side of the haemocytometer, drawn under the coverslip by capillary action, and the total number of cells in each 5x5 grid counted. Each grid has a total volume of 0.1mm^3 (1mm^2 (area) x 0.1mm (depth)), and is equivalent to 1x10^{-4}ml. The average number of cells from the 2 grids was calculated, and the cell count multiplied by 2 to take into account the 1 in 2 dilution with Carnoy's. To obtain cells/ml, the final cell count was multiplied by 10^4. The required volume of cell suspension was then calculated and cells

added to 6-well or 96-well plates (Corning) in RPMI 1640 medium (10% FCS) and incubated for at least 24 hours at 37°C, 5% CO_2, to allow cells to adhere before treatment.

Cell Line	*MYCN* status	*MDM2* status	*p14^{ARF}* status	*p53* status	Cell Type	References
NGP	Amp	Amp	Wt	Wt	N	(Brodeur et al., 1997)
LS	Amp	Amp	Wt	Wt	N	(Rudolph et al., 1991)
NB1691	Amp	Amp	Wt	Wt	N	(Thompson et al., 1997)
TR14	Amp	Amp	Wt	Wt	N	(Cowell and Rupniak, 1983)
IMR32	Amp	Non-amp	Wt	Wt	N	(Tumilowicz et al., 1970)
NBLW	Amp	Non-amp	Wt	Wt	N>S	(Foley et al., 1991)
SMSKCNR	Amp	Non-amp	Wt	Wt	N	(Reynolds et al., 1986)
LAN5	Amp	Non-amp	Wt	Wt	N	(Seeger et al., 1982)
PER108	Amp	Non-amp	Methylated	Wt	N	(McRobert et al., 1992)
CHLA136	Amp	Non-amp	Wt	Wt	N	(Keshelava et al., 2000)
SHSY5Y	Non-amp	Non-amp	Wt	Wt	N	(Biedler et al., 1978)
NBLS	Non-amp	Non-amp	Wt	Wt	S	(Cohn et al., 1990)
SJNB1	Non-amp	Non-amp	Wt	Wt	S>N	(McKenzie et al., 1999; McPake et al., 1998)
SHEP	Non-amp	Non-amp	Deletion	Wt	S	(Biedler et al., 1978)
NB69	Non-amp	Non-amp	Wt	Wt	N>S	(Feder and Gilbert, 1983)
GIMEN	Non-amp	Non-amp	Methylated	Wt	S>N	(Cornaglia-Ferraris et al., 1990)
SKNRA	Non-amp	Non-amp	Wt	Wt	S	(Reynolds et al., 2000)
LAN6	Non-amp	Non-amp	Deletion	Wt	N	(Wada et al., 1988)
SKNBe2C	Amp	Non-amp	Wt	Mutant	I	(Biedler et al., 1973)
IGNR91	Amp	Non-amp	Wt	Mutant	N	(Ferrandis et al., 1994)
SKNAS	Non-amp	Non-amp	Wt	Mutant	S	(Sugimoto et al., 1984)
NB100*	Non-amp	Non-amp	Wt	Mutant	-	(Luque et al., 1994)
SHEP Tet21N	Non-amp	Non-amp	Deletion	Wt	S	(Lutz et al., 1996)
Tet21 vector	Non-amp	Non-amp	Deletion	Wt	S	(Lutz et al., 1996)
SJSA-1**	Non-amp	Amp	Unknown	Wt	-	(Roberts et al., 1989)
MHM-N**	Non-amp	Amp	Unknown	Wt	-	(Müller CR, 2007)
RH18**	Non-amp	Amp	Unknown	Wt	-	(Hazelton et al., 1987)
T449**	Non-amp	Amp	Unknown	Wt	-	(Müller CR, 2007)
T778**	Non-amp	Amp	Unknown	Wt	-	(Müller CR, 2007)
BCH-N-AD	Amp	Unknown	Unknown	Mutant	?	(Peet et al., 2007)
BCH-N-NS	Amp	Unknown	Unknown	Mutant	?	(Peet et al., 2007)

Table 2.1. **The panel of cell lines used in this study.** All are neuroblastoma cell lines with the exception of NB100* (PNET cell line) and SJSA** (osteosarcoma), MHM-N** (osteosarcoma), RH-18** (rhabdomyosarcoma), T449** and T778** (liposarcomas). For cell type, N = neurite-bearing, S = substrate-adherent, I = mixture of N and S type.

References are to studies that the cell lines were first published in. The p53/MDM2/p14^{ARF} status of all neuroblastoma cell lines (except BCH-N-AD and BCH-N-NS) has been determined previously (Carr et al., 2006; Keshelava et al., 2001; Tweddle et al., 2001a). Wt – wildtype, Amp – amplified, non-amp – non-amplified.

2.1.3 Resurrecting and freezing down cells

Neuroblastoma cell lines were stored either in liquid nitrogen at -180°C. To resurrect cells, vials were defrosted in a waterbath, then transferred to a sterile universal tube and centrifuged at 1200rpm for 5 minutes to remove media containing Dimethyl Sulfoxide (DMSO) (Invitrogen). The cell pellet was resuspended in fresh medium and transferred to a 25cm^3 flask to be incubated at 37°C, 5% CO_2. Cells were passaged at least twice before experiments were performed. To replace frozen stocks, 75cm^3 or 175cm^3 flasks of cells were tripsinised and centrifuged at 1200rpm for 5 minutes. The cell pellet was resuspended in the appropriate amount of freeze media (10% DMSO, 20% FCS and 70% 1640 RPMI), and 1ml added to a labelled cryogenic vial (NUNC^{TM}, Rochester, NY, USA). The cryovial was then placed into the -80°C freezer and once frozen, transferred to the liquid nitrogen cryostore (Biosystem, Cryostor).

2.1.4 Nutlin-3/MI-63 treatment

Nutlin-3 was purchased from Enzo Life Sciences (Exeter, UK) and MI-63 was kindly provided by Siena Biotech (Siena, Italy) as part of a Framework Programe 6 DePPICT consortium collaboration. Both compounds were dissolved in DMSO (Sigma) to a 10mM stock solution and stored at -20°C. Cells treated with Nutlin-3 or MI-63 had equal volumes of DMSO added within an experiment. The structures of Nutlin-3 and MI-63 are displayed in Chapter 1.17.

2.2 SHEP Tet21N MYCN expression system

The SHEP Tet21N MYCN expression system allows the effects of conditional MYCN expression to be investigated, and has a Tet-OFF mechanism (Lutz et al., 1996). The Tet-OFF system was first developed in 1992 (Gossen and Bujard, 1992), and is switched on by the removal of tetracycline, which in the case of Tet21N cells, induces expression of MYCN. SHEP cells normally express no detectable levels of MYCN protein, and low mRNA. The SHEP Tet21N cells are an S-type clone of SKNSH cells (Biedler et al., 1973) and although they are generated from the least tumourigenic neuroblastoma cell type they are very chemoresistant (Rodriguez-Lopez et al., 2001;

Tweddle et al., 2001b; Jasty et al., 1998). Vector only Tet21 cells were used when appropriate as a control for tetracycline.

2.2.1 Generation of SHEP Tet21N MYCN regulatable cells

Tet-OFF SHEP Tet21N cells were generated by Lutz et al. (1996) using plasmids pUHD15-1 (Figure 2.1a), and pUHD10-3/*MYCN* (Figure 2.1b). The TET-off system employs a tetracycline controlled transactivator (tTA). This is composed of the Tet respressor protein (*tet*R) (from the tetracycline resistance operon of *E. coli*) fused with the C-terminal transactivation domain of Virion Protein 16 of Herpes Simplex Virus. The tTA is contained within the pUHD15-1 plasmid, expression of which is under the control of the human cytomegalovirus promoter (PCMV). In the absence of tetracycline, the tTA binds to the tetracycline responsive promoter element (TRE), made up of seven copies of the TET operator (*tet*O) sequence which fused upstream of the minimal promoter of human CMV (*P*min$_{hCMV}$), and is located in the second pUHD10-3 plasmid. This initiates gene transcription. In the presence of tetracycline, the tTA is unable to bind the TRE, resulting in no gene transcription.

In the generation of the SHEP Tet21N cells, the *MYCN* sequence (lacking the non-coding exon 1) was cloned into the *Eco*R1 site of pUHD10-3. The pUHD15-1 and pSV2neo plasmids were co-transfected into SHEP cells and transfected cells selected for by addition of G-418 antibiotic 12 hours after transfection. Clones were then co-transfected with the pUHD10-3/*MYCN* and pHMR272 plasmids. Addition of G-418 and hygromycin to growth media selected for transfected clones and clones that expressed MYCN in the absence of tetracycline, but not in the presence were maintained in media containing G-418 and hygromycin (Lutz et al., 1996). Further details on the generation of this cell line are described by Lutz *et al.* (Lutz et al., 1996)

2.2.2 Culturing of SHEP Tet21N and Tet21 cells

SHEP Tet21N cells were routinely cultured in RPMI 1640 with 10% FCS and 200µg/ml G-418 (Calbiochem) and 900µg/ml Hygromycin B (Invitrogen). 1µg/ml of tetracycline (Sigma) was added to the medium for at least 48 hours before experiments were set up. Tetracycline was stored at a stock solution of 1mg/ml in sterile ddH_2O and stored at -20°C. Medium was changed every 48 hours due to the short half-life of tetracycline.

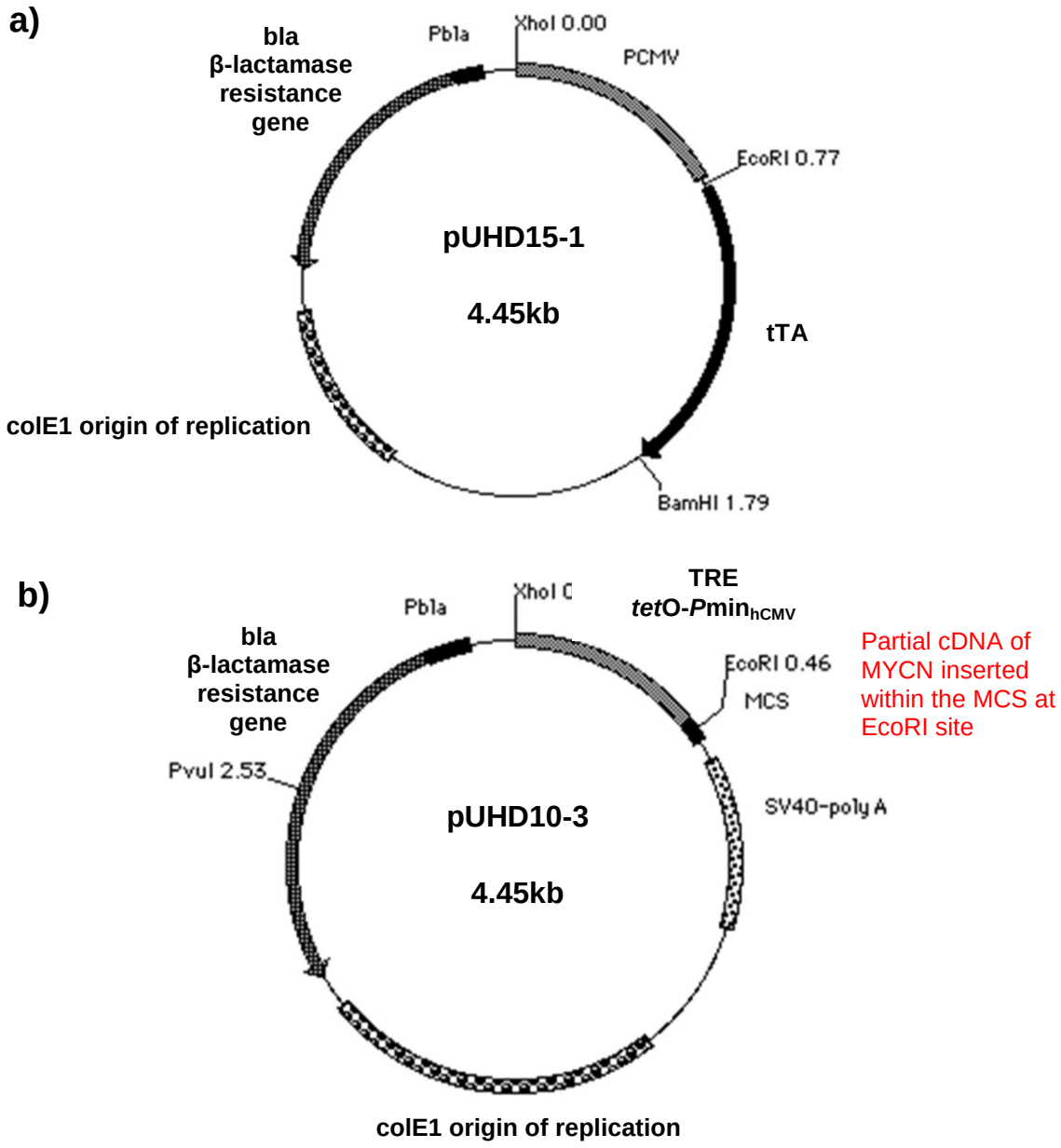


Figure 2.1. **Plasmid maps of a) pUHD15-1 and b) pUHD10-3 used to generate the Tet21N cells.** http://www.zmbh.uni-heidelberg.de/bujard/tTA/pUHD15-1.html and http://www.zmbh.uni-heidelberg.de/bujard/reporter/pUHD10-3.html

2.3 RNA interference

RNA interference is a mechanism of post-transcriptionally regulating gene expression. Discovered in 1998, RNA interference was a major technological advance in molecular biology. RNA interference is a method of silencing gene expression, specifically targeting double stranded RNA (dsRNA) for degradation and is triggered by non-coding small interfering RNAs (siRNAs). RNA interference occurs naturally in many organisms, where long dsRNA can be used to silence expression of target genes. The mechanism of RNA interference is shown in Figure 2.2. The long dsRNAs enter the RNA interference pathway, where they are first processed into 20-25 nucleotide small interfering RNAs (siRNAs) by an enzyme called Dicer. The siRNAs then assemble on to the RNA-induced silencing complexes (RISCs), where they unwind, and subsequently guide the RISCs to complementary RNA molecules where they cleave and destroy the cognate RNA.

Synthetic siRNAs are an easy and efficient way of achieving RNA interference. They are typically made of 19 RNA nucleotides with symmetric 2 nucleotide 3' overhangs (usually DNA bases). The use of siRNAs in mammalian cells has been shown to effectively bypass the antiviral response which is normally triggered upon introduction of long dsRNA, and specifically silences gene expression.

2.3.1 siRNA design and synthesis

siRNAs were synthesised by Eurogentec (Southampton, UK); their universal negative control was used throughout (cat no. SR-CL000-005) and is referred to as SCR (scrambled). All siRNAs were designed using siRNA design software based on published methods (Andrew et al., 2007; Elbashir et al., 2001a; Elbashir et al., 2001b). Previously described siRNA sequences for MYCN (Bell et al., 2006), p53 (Armstrong et al., 2007) and $p14^{ARF}$ (Xiong and Epstein, 2009) were used (Table 2.2). MDM2 and MDMX siRNAs were designed by Eurogentec (3 of each, sequences shown in Table 2.2), using siRNA design software, based on published methods for designing siRNA (Elbashir et al., 2001b). BLAST™ searches were performed against the human genome to ensure MDM2 and MDMX sequences had no cross-reactivity with other genes.

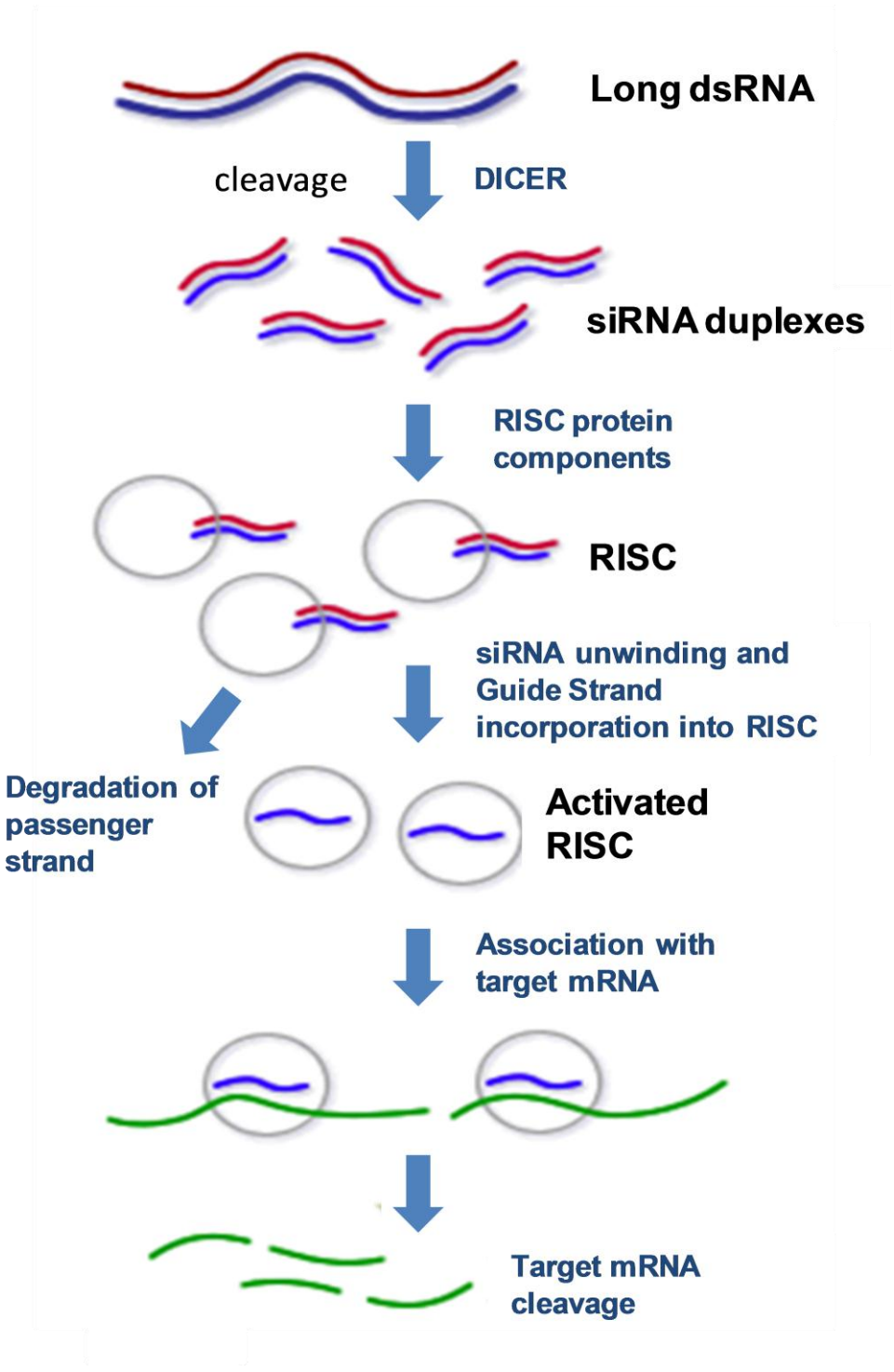


Figure 2.2. **The mechanism of RNA interference.** Image adapted from www.ambion.com/techlib/append/RNAi_mechanism

siRNA target	siRNA sequence (sense)
MYCN	5′UGAUCUGCAAGAACCCAGAtt 3′
p53	5′GACUCCAGUGGUAAUCUACtt 3′
MDM2 siRNA 1	5′GGGCUUUGAUGUUCCUGAUtt 3′
MDM2 siRNA 2	5′CGCCACAAAUCUGAUAGUAtt 3′
MDM2 siRNA 3	5′GCUUCACAAUCACAAGAAAtt 3′
MDMX siRNA 1	5′GGAGCAGCAUAUGGUAUAUtt 3′
MDMX siRNA 2	5′GGAUCACAGUAUGGAUAUUtt 3′
MDMX siRNA 3	5′GCAGUUAGGUGUUGGAAUAtt 3′
$p14^{ARF}$	5′GAACAUGGUGCGCAGGUUCtt 3′

Table 2.2. **siRNA sequences targeting MYCN, p53, MDM2, MDMX and $p14^{ARF}$.**

2.3.2 siRNA transfection

Cells were seeded in 6-well plates at a density of $4x10^5$ cells/well, in RPMI 1640 (10% FCS) with no antibiotics, 24 hours prior to transfection. Lipofectamine (Invitrogen) was used as the transfection reagent, and OptiMEM-glutamax (OPTI) serum free media (Gibco, Invitrogen). siRNAs were stored at stock concentrations of 20µM. The following calculations were used to determine the volume of siRNA and OPTI (400µl/well) required:

siRNA volume = N x 0.12 x nM

OPTI volume = 400µl x N

N = no. wells, nM = final siRNA concentration

Separately, Lipofectamine was diluted in OPTI and the volume required calculated as follows:

Lipofectamine volume = N x 0.15 x nM

OPTI volume = 400µl x N

The two solutions were gently mixed and incubated separately for 10 minutes at room temperature. An equal volume of siRNA solution was added to the lipofectamine solution, gently mixed and incubated at room temperature for a further 30 minutes to allow liposomes containing siRNA to form. Meanwhile, medium was aspirated off the cells (in 6-well plates), and 1ml of OPTI added to wash cells and remove any remaining FCS. 1.6ml of OPTI was added to each well. Following the 30 minute incubation, 800µl of the siRNA-lipofectamine solution was added to each well and incubated at 37°C, 5% CO_2. After 24 hours, the siRNA-lipofectamine solution was removed and

cells either harvested, or medium replaced with 2mls of fresh RPMI 1640 media for collection at a later time point (10% FCS).

2.4 Western blotting

2.4.1 Principles of Western blotting

Western blotting is a technique used to assess the protein expression levels of specific proteins in complex protein mixtures, such as cell or tissue samples. The main processes of Western blotting are 1) sodium dodecyl sulphate gel electrophoresis (SDS-PAGE), to separate proteins based on molecular weight under denaturing conditions (lysates are boiled with SDS and reducing agent β-mercaptoethanol to break disulphide bonds within the protein and coat the proteins uniformly with negative charge), 2) transfer of the proteins onto a nitrocellulose membrane, 3) the use of specific antibodies conjugated to horseradish peroxidise (HRP) and chemiluminescence to detect and visualise proteins.

2.4.2 Harvesting cells and protein concentration estimation

For collection of adherent cells only (in 6-well plates), medium was removed, cells washed in 1ml PBS and 30µl of lysis buffer (see Appendix 1) added. Cells were then harvested using a cell scraper and lysates placed in a 1.5ml microfuge tube (Eppendorf). Samples could be stored at -20°C until required. To harvest both adherent and non-adherent cells, the cell media was placed in a 20ml universal tube (Corning), cells were washed in PBS (also collected) and tripsinised as previously described (Section 2.1.1). Tripsinised cells were neutralised with media then added to the universal tube. The Universal tube was then centrifuged at 1200rpm, supernatant removed and replaced with PBS and cells pelleted again. The appropriate amount of lysis buffer was added (equivalent of 30µl/well), and lysates added to a microfuge tube (Eppendorf). Lysis buffer contains SDS, an anionic detergent which denatures the proteins, and gives the proteins a negative charge approximately proportional to the length of the protein.

2.4.3 Pierce Protein Estimation

The lysed samples were boiled for 10 minutes at 100°C, and since the samples can be viscous due to the presence of high molecular weight DNA, they were sonicated on full power for 2 x 5 seconds to fragment DNA. The samples were then mixed by vortexing and 5µl added to 45µl water to make a 1:10 dilution. Albumin standards 0.2, 0.4, 0.6,

0.8, 1.0 and 1.2mg/ml were made up from 2mg/ml stock, and 10µl of sample was pipetted into wells in a 96 well plate set up as shown in Appendix 1, alongside water as a negative control. Per plate, 20ml of Reagent A was mixed with Reagent B from the Pierce® BCA Protein Assay kit (Pierce, Rockford, IL, USA) and 190µl added to each well. This assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium, with the highly sensitive and selective colorimetric detection of the Cu^{1+} by bicinchoninic acid (BCA). The samples were gently mixed by agitation on a plate shaker, and incubated at 37°C for 30 minutes. The optical densities of the samples were then measured on a Spectromax 250 (Molecular Devices, Berkshire, UK) at 562nm, which was set up to automatically generate a standard curve and give concentrations of unknowns. These values were multiplied by 10 to take into account the 1:10 dilution, and the volume of sample required for a specific amount of protein calculated (between 15-50µg).

2.4.4 SDS-PAGE

Novex® 4-20% Tris-Glycine polyacrylamide gels (Invitrogen) were placed in Invitrogen Mini-Cell gel electrophoresis tanks and filled with 1x electrode buffer (Appendix 1). 5µl of loading buffer containing β-mercaptoethanol and bromophenol blue (Appendix 1) was added to each sample (containing 15-50µg of protein), which was boiled for 10 minutes, centrifuged briefly, and loaded into wells on the gel (a 10-well gel takes 50µl sample, 12 or 15 well gels take 20µl of sample). SeeBlue™ prestained molecular weight markers (Invitrogen) were used on each gel. The gels were run at 150V for approximately 1.5 hours until the dye front reached the bottom of the gel.

2.4.5 Transfer

Proteins from the polyacrylamide gel were transferred by electrophoresis to nitrocellulose Hybond™ C membrane (Amersham, Buckinghamshire, UK). Transfer electrophoresis tanks were filled with transfer buffer (Appendix 1) and set up according to manufacturer's instructions. Hybond™ C membrane, filter paper and glass fibre pads were soaked for 10 minutes in transfer buffer. Cassettes were set up in the following order (black side first): fibre glass pad, filter paper (Whatman, Kent, UK), gel, membrane, filter paper, fibre glass pad. The cassettes were closed and placed in transfer tanks, with the black side of the cassette facing the black anode and electrophoretic transfer carried out at 30V overnight or at 60V for 2 hours.

2.4.6 Blocking

Background staining on the nitrocellulose membrane was reduced by blocking non-specific binding using 5% w/v non-fat milk powder (Marvel) dissolved in 1x TBS Tween (Appendix 1), for 1 hour at room temperature, on a shaker.

2.4.7 Primary antibodies

Primary antibodies alongside their optimal dilutions, incubation times and incubation temperatures are shown in Table 2.3. Membranes were placed in 50ml Falcon tubes (BD Biosciences, San Jose, CA, USA) and antibodies added to 3ml 5% milk (dissolved in TBS Tween) except Phospho-p53(Ser15), which was added to 5% BSA as milk contains casein, a phosphoprotein, which is detected by the phospho-specific antibody, causing high background. Falcon tubes were then placed on a roller.

Protein	Antibody	Dilution	Incubation Time	Incubation Temp	Antibody Type	Manufacturer
MYCN	OP13	1:100	1 hour	RT	Mouse MC	Calbiochem
p53	DO-7	1:200	1 hour	RT	Mouse MC	Novocastra
MDM2	OP40	1:100	1 hour	RT	Mouse MC	Calbiochem
MDMX	A300-287A	1:1000	1 hour	RT	Rabbit PC	Bethyl
$p21^{WAF1}$	OP68	1:100	2 hours	RT	Mouse MC	Calbiochem
Phospho-p53(Ser15)	9284	1:1000	1 hour	RT	Rabbit PC	Cell signalling
PUMA	PC686	1:1000	1 hour	RT	Mouse MC	Calbiochem
$p14^{ARF}$	NA70	1:100	O/N	4°C	Mouse MC	Calbiochem
$p14^{ARF}$	RB1554P	1:100	2 hours	RT	Mouse MC	Lab Vision
Cleaved caspase 3	Asp175	1:1000	O/N	4°C	Rabbit PC	Cell Signalling
PARP-1/2	H-250	1:200	1 hour	RT	Rabbit PC	Santa Cruz
actin	AC-40	1:500	1 hour	RT	Mouse MC	Sigma-Aldrich

Table 2.3. **Primary antibodies used in this study.**
MC - monoclonal, PC – polyclonal, O/N – overnight, RT – room temperature.

2.4.8 Secondary Antibodies

Following the primary antibody incubation, the membrane was washed for 3x 5 minutes in 1x TBS Tween, leaving the membrane in the 50ml Falcon tube to minimise contact. Goat anti-mouse IgG and goat anti-rabbit IgG (Dako, Glostrup, Denmark) secondary antibodies which are conjugated to horseradish peroxidise (HRP), were used at a 1:1000 dilution in 3ml of 5% milk-TBS Tween in the same 50ml Falcon tube, and incubated at room temperature for 1 hour. The membrane was then washed as before for 3x 10 minutes on the roller.

2.4.9 Enhanced chemiluminescence protein detection

The Enhanced Chemiluminescence (ECL) kit (Amersham) was used to detect proteins. Detection Reagent 1 and Reagent 2 were mixed at a 1:1 ratio and the membranes covered in ECL solution for 1 minute. When SuperSignal® West Dura Kit (Thermo Scientific, Leicestershire, UK) was used for $p14^{ARF}$ detection, again Reagent 1 was mixed with an equal volume of Reagent 2, but added to the membrane for 5 minutes. ECL was drained off well and the membrane was wrapped in cling film and placed in an autoradiography cassette (Genetic Research Instrumentation, Essex, UK). In the dark room, x-ray film (Kodak) was placed on the membrane for varying lengths of time and subsequently developed and fixed using a Mediphot 937 (Colenta, Austria) automated film processor.

2.4.10 Densitometry

Densitometry was performed to semi-quantitatively measure protein expression levels compared to an actin loading control. A Fuji-Las Pro (FUJIFILM Life Science, Stamford, CA, USA) scanner was used to capture an electronic image of bands on the x-ray film and AIDA image analyser (Raytest, Straubenhardt, Germany) used to estimate size and density of the bands. The densitometry values for each protein band were measured and values normalised to those of the actin loading control. Fold induction of protein was calculated relative to control, and converted to % control.

2.5 Fluorescence activated flow cytometry

Two flow cytometers were used in this study; the FACscan and the FACs Calibur (both Becton Dickinson, BD Biosciences). A beam of light of a single wavelength is directed onto a hydrodynamically-focused single stream of fluid containing cells. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter), and several perpendicular to it (Side Scatter), and 3 fluorescent detectors (FL1, FL2 and FL3), all shown in Figure 2.3. Here the FL2 parameter was used to detect propidium iodide (PI), a fluorescent dye that binds stoichiometrically to DNA, and intercalates between the bases in DNA. PI is excited by UV or blue light to emit a red fluorescence (Ormerod, 2000). The single flow of cells pass through the flow cell where light from the argon laser beam excites the fluorescent PI dye bound to DNA. Photodetectors detect the scattered light and dichromic mirrors at right angles to the beam of light reflect specific wavelengths of light on to the

detectors. This light is converted into an electronic signal that is recorded (Ormerod, 2000).

PI binds to DNA and provides a quantitative measure of cellular DNA content, so the stage of the cell cycle the cells are in can be determined. Cells undergoing mitosis in the G_2 phase of the cell cycle have a DNA content double that of G_1 or G_0 cells, as cells have divided, whereas S-phase cells have DNA content between G_1 and G_2 as cells replicate in this phase (the cell cycle is discussed in Chapter 1.6.3). Cells are also treated with RNAase before incubation with PI, to eliminate any binding of PI to RNA.

2.5.1 Preparation of samples

Samples were seeded in 6-well plates (densities are specified in chapter specific methods). Following treatment, both adherent and non-adherent cells were harvested, washed with ice-cold PBS (spun at 1200rpm for 5 minutes) and fixed with 4:1 Methanol:Acetone. Samples were stored at 4°C for up to 2 weeks at this stage. Cells were washed twice in PBS, and then 100µl of RNAse (10x RNAse – 15.5mg in 10ml PBS) (Sigma) added for 20 minutes at 37°C, followed by 350µl propidium iodide (100 µg/ml) (Sigma) for 30 minutes and incubated at 37°C. After this time, samples were stored at 4°C.

2.5.2 FACscan/Calibur

Before running samples through the flow cytometer, they were passed through a syringe and needle to remove clumps as 2 cells stuck together would have the same DNA content as G_2 phase cells. Samples were measured and data acquired using the FACScan or FACs Calibur machine (Becton Dickinson), together with CellQuest Software (Becton Dickinson). Scatter plots of SSC-H vs. FSC-H, and FL2-A vs. FL2-W were set up to optimise instrument settings using an untreated control (see Appendix 1), as well as a histogram of counts vs. FL2-H where the G_1 peak (one diploid complement of DNA) was set to 200 on a linear scale, and the G_2 peak at 400. Cell aggregates and doublets could be identified on the FL2-A vs. FL2-W scatter plot. For each sample, 10000 events excluding doublets were collected and all events saved.

2.5.3 Analysis

Flow cytometry data was analysed using Windows Multiple Document Interface for Flow Cytometry 2.8 software (WinMDI 2.8 software (TSRI)). FL2-W versus FL2-A scatter plots were generated and cells were gated to exclude doublets/cell aggregates.

For cell cycle analysis, the sub G_1 cells were also excluded. The gated data was used to generate corresponding FL2-H histograms, and the proportion of cells in G_1, S, G_2, and/or sub G_1 phases was determined by marking the various phases of the cell cycle with markers M1, M2, M3 and M4 (shown in Figure 2.4), and generating a table of statistics. An increase in the G_1/S ratio defines a G_1 arrest, representing an increase in proportion of cells in G_1, and a decrease in the proportion of cells in S-phase.

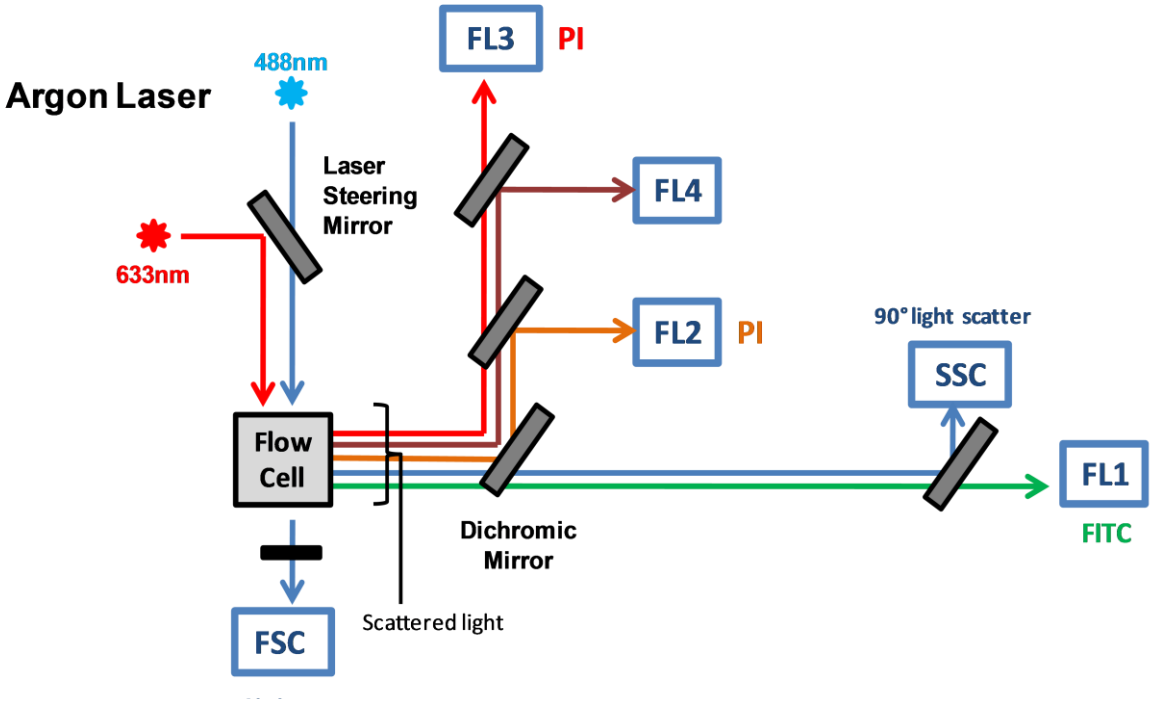


Figure 2.3. **The detector arrangement for the FACScan family of cytometers. Adapted from http://facs.scripps.edu/facslab.html.**

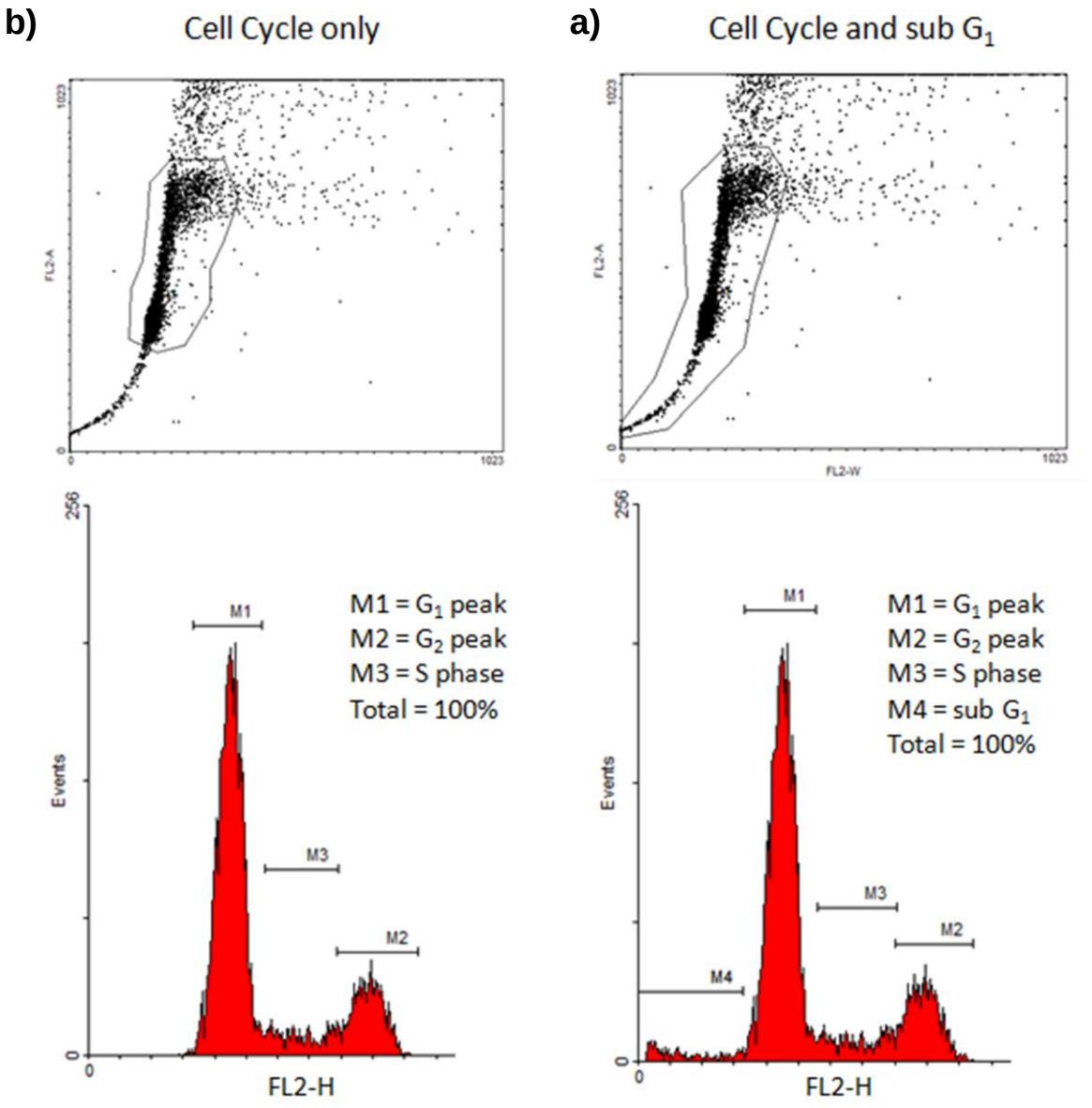


Figure 2.4 **FL2-W versus FL2-A scatter plots and histograms gated for a) cell cycle and b) apoptosis. Histograms have markers which represent the phases of the cell cycle, and the percentage of cells within the region the marker covers can be determined.**

2.6 Growth Inhibition assays

Growth inhibition assays allow a GI_{50} value to be determined, which is the concentration at which a compound reduces the growth of the cell population by 50% compared to untreated control cultures. The sulforhodamine B (SRB) assay was used. This is a colorimetric assay using a protein dye with absorbance measured at 590nM and essentially gives a measure of the protein in the well of a culture dish to indicate the number of cells, and takes into account both cell cycle arrest and apoptosis. A major limitation to this assay is that non-adherent cells are lost, and some cell lines are semi-adherent, or take longer than others to adhere. This may lead to some inaccuracies in the final results. However the appropriate controls should take this into account. Untreated cells were fixed and readings taken at day 0, and at each further time point, each plate had wells seeded with half the number of cells, and compared to untreated wells seeded with the full number of cells. These wells should have an absorbance value of half the value of the wells seeded with the full cell number, indicative that cells are growing in log phase.

2.6.1 Determining optimal cell density

The optimal number of cells was determined by plating out cells in 96-well plates increasing from 0-10000 (in 1000 intervals) and incubating to allow growth and division for 96 hours to generate growth curves from the SRB absorbance data. A cell seeding density was chosen where cells were growing in exponential growth phase, and had neither plateaued (suggesting cells were confluent) nor were still in lag phase.

2.6.2 Growth Inhibition assays

Cells were seeded in 96-well plates at the previously determined optimal cell density. They were given 24 hours to attach (in some cases 48 hour), and were then treated with a range of drug concentrations; Nutlin-3 - 20μM, 10μM, 4μM, 2μM, 1μM, 0.5μM, and 0.25μM, MI-63 - 10μM, 5μM, 2μM, 1μM, 0.5μM, 0.25μM and 0.125μM. Plate layout is shown in Appendix 1 and 3 wells per treatment were set up. A Day 0 control was also plated, and a half seeding density control to check that the SRB absorbance was proportional to cell number (i.e. would be half the value of the untreated normal plating density control at 72 hours post-treatment). Plates were incubated with Nutlin-3 or MI-63 for 72 hours then fixed with 25μl of 50% trichloroacetic acid (TCA) (Sigma), and stored at 4°C for at least 1 hour.

2.6.3 Sulforhodamine B assays

The sulforhodamine B (SRB) assay was developed by Skehan *et al* (Skehan et al., 1990). The SRB dye binds to the basic amino acid residues of proteins in cells, and the optical density of the SRB-bound protein recorded at 564nM is linearly proportional to the number of cells in the well.

2.6.4 SRB staining protocol

Cells that have been fixed in 50% TCA were allowed to come to room temperature, and then the TCA was washed off with 5 rinses in distilled H_2O. 100µl of SRB dye was added to each well and incubated at room temperature for 30 minutes. Plates were then washed 5 times in 1% acetic acid and left to dry overnight. 100µl of 10mM Tris-HCl (pH10.5) was added to each well, and placed on a shaker for 10 minutes. Once the SRB dye had dissolved, a SpectroMax 250 (Molecular Devices) 96-well plate densitometer was used to scan the plates at 590nM.

2.6.5 Analysis

GI_{50} values were calculated using GraphPad Prism Version 4.0 software (GraphPad Software, Inc.). The percentage cell number relative to control (untreated cells) was determined for each concentration of Nutlin-3 or MI-63, and data plotted using Prism statistical software. GI_{50} values were determined by transforming X values (concentrations) using X=log(X), and performing a nonlinear regression (curve fit) analysis with a sigmoidal dose-response, displaying 95% confidence intervals. This analysis automatically generates a GI_{50} value.

2.7 Caspase 3/7 activity assays

The Caspase-Glo® 3/7 Assay (Promega, Southampton, UK) is a luminescent assay that measures caspase-3 and -7 activities in cultures of cells. The assay provides a proluminescent caspase 3/7 substrate which contains the tetrapeptide sequence DEVD. The substrate is cleaved by the caspases to release aminoluciferin, a substrate of luciferase used in the production of light which can be detected by photomultiplier luminometry. The Caspase-Glo® 3/7 Reagent has been previously optimised for caspase activity, luciferase activity and cell lysis by the manufacturers.

2.7.1 Caspase-Glo® 3/7 assay protocol

Cells were seeded at 5000 cells/well in 96-well plates and with a final volume of 50µl/well prior to caspase reagent addition. The Caspase 3/7 kit was defrosted and the buffer added to the tablet and allowed to come to room temperature. A 1:1 volume of caspase reagent (50µl) was added to each well and incubated for 1 hour at room temperature. During this time cells lyse, and release activated caspase to cleave the substrate and generate a 'glow-type' luminescence. After 1 hour, the resulting solution from each well was transferred to a white-welled 96-well plate and analysed on a microplate Luminometer (Berthold Technologies, Herefordshire, UK). Luminescence readings were normalised and plotted relative to control.

2.8 Statistical Analyses

All statistical tests were performed using GraphPad Prism Version 4.0 software (GraphPad Software, Inc.). Tests were performed using log values and all t-tests were two-sided and paired or unpaired where stated. The type of statistical test used is specified in figure legends, alongside p-values. A p-value of $p < 0.05$ was considered to be statistically significant.

Chapter 3: *MYCN*-amplification or overexpression sensitises neuroblastoma cells to the effects of MDM2-p53 antagonists in neuroblastoma

3.1 Introduction

This chapter focuses on the effect of MYCN on the growth inhibitory and apoptotic response to the MDM2-p53 antagonists Nutlin-3 and MI-63. *MYCN* gene amplification is a major marker of adverse prognosis, occurring in 25-30% of neuroblastomas and is strongly associated with progressive disease and treatment failure (Cohn and Tweddle, 2004). Infants under 18 months with stage 4 disease have an event-free survival of 83% for *MYCN*-amplified neuroblastoma, compared to 26% for non-*MYCN*-amplified disease (Cohn et al., 2009). MYCN expression alone, targeted to developing neural crest tissue, has been shown to directly result in neuroblastoma tumour formation in transgenic mice (Weiss et al., 1997). There is evidence that MYCN expression sensitises neuroblastoma cells to apoptosis induced by cytotoxic drugs (Petroni et al., 2011; Hogarty, 2003; Fulda et al., 2000). However, since patients with *MYCN*-amplified tumours have such an inferior outcome, acquired aberrations in the apoptotic pathway are thought to be associated with *MYCN*-amplification and to be essential for tumour progression.

3.1.1 p53 inactivation in neuroblastoma

In neuroblastoma, p53 mutations are rare, occurring in <2% of cases at diagnosis and ~15% at relapse (Carr-Wilkinson et al., 2010). However, in 35% of cases in a study of diagnostic and relapsed tumours, p53 was found to be inactivated via other mechanisms, resulting in destabilisation of p53 or disruption of p53 activity (Carr-Wilkinson et al., 2010). In neuroblastoma, other mechanisms of p53 inactivation include amplification of the E3 ubiquitin ligase gene *MDM2*, or impairment of $p14^{ARF}$ (Carr-Wilkinson et al., 2010; Carr et al., 2006). Non-syntenic co-amplification of *MDM2* and *MYCN* has been reported in neuroblastoma cell lines and tumours, resulting in constant negative regulation of p53 (Carr-Wilkinson et al., 2010; Corvi et al., 1995b). More commonly in tumours, $p14^{ARF}$ function is impaired through methylation or homozygous deletion of the gene (Carr-Wilkinson et al., 2010). $p14^{ARF}$ negatively regulates MDM2 and therefore $p14^{ARF}$ inactivation drives cell survival through increased MDM2 activity.

3.1.2 MYCN and the p53/MDM2/$p14^{ARF}$ network

MYCN is a central modulator of the p53/MDM2/$p14^{ARF}$ network. There is evidence that both p53 and MDM2 are direct transcriptional targets of MYCN (Chen et al., 2010b; Slack et al., 2005a), and that p53 may be important for MYCN-induced apoptosis (Chen et al., 2010b). However, whereas both p53 and MDM2 are expressed at high levels in

neuroblastomas that express high MYCN (He et al., 2011; Chen et al., 2010b), induced expression of MYCN results in increased p53 expression but not MDM2 (He et al., 2011). In addition Chen *et al.* found that the increased MDM2 detected in the presence of MYCN decreased upon p53 knockdown suggesting that increased expression in these cell lines may be as a result of MYCN-driven p53 expression and not MYCN-driven MDM2 expression (Chen et al., 2010b).

MDM2 haploinsufficiency in mice has been shown to suppress MYCN-driven neuroblastoma tumourigenesis (Chen et al., 2009), and there is evidence that MDM2 may be the critical oncogene by which *MYCN*-amplified neuroblastomas acquire an aggressive phenotype (Slack and Shohet, 2005). Overactive MDM2 as a result of p53 being a direct transcriptional target of MYCN, and a transcriptional target of MDM2 may drive tumour formation in *MYCN*-amplified neuroblastomas. On the other hand, Carr-Wilkinson *et al* found that *MDM2*-amplification was not associated with survival in neuroblastoma tumour samples (Carr-Wilkinson et al., 2010). Since *MYCN*-amplification is thought to be associated with defects in activating or executing apoptotic pathways it is possible to speculate that *MYCN*-amplified tumours may be more susceptible to compounds that reactivate p53. Several studies have shown that the downstream apoptotic pathway of p53 is generally intact in neuroblastoma (Van Maerken et al., 2011; Hogarty, 2003; Tajiri et al., 2003; Tweddle et al., 2001a; Tweddle et al., 2001b; Goldman et al., 1996).

It has also been reported that $p14^{ARF}$ is activated by MYCC (Zindy et al., 1998), and although not investigated, due to the similarities between MYCN and MYCC, MYCN could also activate $p14^{ARF}$. In addition, MYCC/N indirectly inactivates $p14^{ARF}$ through activation of TWIST1, which is overexpressed in *MYCN*-amplified neuroblastomas and impairs $p14^{ARF}$ activity (Valsesia-Wittmann et al., 2004; Maestro et al., 1999). TWIST1 is proposed as a mechanism by which tumours escape MYCN-dependent apoptosis.

3.1.3 MYCN and the use of MDM2-p53 antagonists

In neuroblastoma cell lines with wild-type p53, Nutlin-3 has been reported to induce cell cycle arrest and apoptosis, and surviving cells underwent senescence or neuronal differentiation in the cell lines tested (Van Maerken et al., 2006). In this study, a limited number of 7 p53 wildtype cell lines were tested, 3 of which were *MYCN*-amplified, and no significant difference in cell viability or the apoptotic response to Nutlin-3 was found in *MYCN*-amplified compared to non-*MYCN*-amplified cell lines.

In another study, IC_{50} values for Nutlin-3 in 2 MYCN regulatable cell lines (both SHEP derivatives) were determined, and it was found that in Tet21N cells, there was increased sensitivity in MYCN(+) cells compared to MYCN(-) cells, both for Nutlin-3 alone and in combination with cisplatin (Barbieri et al., 2006). In SHEP MYCN3 cells, there was no difference in IC_{50} values for MYCN(+) compared to MYCN(-) cells following Nutlin-3 treatment, but there was increased sensitivity to combined Nutlin-3 and cisplatin treatment in MYCN(+) cells.

Although not previously investigated in neuroblastoma, or in relation to MYCN, another MDM2-p53 antagonist MI-63 has also been shown to induce apoptosis in cell lines with wildtype p53 (Canner et al., 2009).

3.1.4 Manipulating MYCN expression: Tet21N cells

SHEP cells, which have barely detectable endogenous MYCN, were used to generate a synthetic inducible system (SHEP-Tet21N, described in Chapter 2.2) to reversibly express MYCN. This allows the contribution of the MYCN protein to be investigated in a neuroblastoma cell line using an isogenic system, and has previously been used in many studies. MYCN induction has been shown to increase DNA-synthesis and the proliferation rate (Lutz et al., 1996), MYCN(+) Tet21N cells were more sensitive to cytotoxic drugs (Fulda et al., 2000), and MYCN(+) cells have previously been found to have lower IC_{50} values following Nutlin-3 treatment compared to MYCN(-) cells (Barbieri et al., 2006). The SHEP cells used to generate the Tet21N cells are an S-type clone of the SKNSH cell line (Biedler et al., 1973), and whilst this is the least tumourigenic neuroblastoma cell type (Cell Types are described in Chapter 1.4) it is conversely the most drug resistant compared with SHSY5Y (Rodriguez-Lopez et al., 2001; Tweddle et al., 2001b; Jasty et al., 1998). In addition, *MYCN*-amplified S-type cells have been found to have lower levels of MYCN expression compared to other cell types (Carr-Wilkinson et al., 2011; Foley et al., 1991). In this study, the Tet21N system was used to investigate the effect of MYCN on the growth inhibitory and apoptotic response to the MDM2-p53 antagonists Nutlin-3 and MI-63.

3.1.5 Manipulating MYCN expression: RNA interference

RNA interference allows post transcriptional silencing of genes, and is a way of studying the effect of manipulating a single gene to assess function and importance. siRNA or shRNA mediated knockdown of MYCN has been previously used in neuroblastoma. MYCN silencing has been shown to induce apoptosis and suppress cell

growth but to also induce neuronal differentiation (Nara et al., 2007; Kang et al., 2006). It was also associated with decreased Bcl-x_L protein and caspase 3 activation (Kang et al., 2006). The siRNA sequences used in this study have been previously used by our group to confirm that p53 as a direct transcriptional target of MYCN (Chen et al., 2010b), and in a study investigating the role of MYCN in the failure of neuroblastoma cell lines to G_1 arrest following DNA damage (Bell et al., 2006). In this study, the effect of MYCN knockdown on the response to MDM2-p53 antagonists has been investigated in several p53 wildtype cell lines.

3.1.6 Detecting apoptosis

This chapter mainly focuses on the effects of the MDM2-p53 antagonists Nutlin-3 and MI-63 on the induction of cell cycle arrest and apoptosis. MDM2-p53 antagonists, particularly Nutlin-3, have been shown to induce apoptosis in many cancer cell types (Gu et al., 2008b; Kojima et al., 2006; Tovar et al., 2006; Carvajal et al., 2005; Vassilev, 2004). Apoptosis is the most commonly studied form of programmed cell death, involving the activation of caspases. Cells undergo apoptosis in normal development and morphogenesis, homeostasis and to remove damaged or dangerous cells (Vaux and Korsmeyer, 1999). The mechanisms of apoptosis are described in more detail in Chapter 1.6, and involve 2 pathways; the intrinsic and the extrinsic pathways.

Several methods were used to detect apoptosis in the studies described in this chapter, including caspase 3/7 activation, caspase 3 and PARP cleavage, annexin V staining, induction of the p53 target gene and apoptotic marker PUMA, and measure of sub G_1 DNA populations. Since many features of apoptosis and necrosis overlap, and since there are many other forms of cell death, several methods have been used to confirm cell death by apoptosis. Sub G_1 DNA takes into account all types of cell death, as it measures fragmented DNA, and growth inhibition assays take into account any form of cell death (in addition to cell cycle arrest), as any adherent cells that have either become unattached from the surface, or that have disintegrated are lost compared to the control. The various techniques used in this chapter to assess apoptosis are described below.

3.1.6.1 Caspase 3/7 activity and cleavage

Caspases are highly conserved, Cysteine-dependent Aspartate-Specific Proteases involved in the initiation and execution of both intrinsic and extrinsic apoptotic pathways. Initiator caspases (caspases 2, 8, 9 and 10) are responsible for activating effector caspases through proteolytic cleavage (caspases 3, 6 and 7). The active

effector caspase then proteolytically degrades an array of intracellular proteins to carry out the cell death programme. Caspase 3 is considered the most important effector caspase, and the proenzyme is activated by cleavage by all initiator caspases. Following cleavage, the two subunits dimerise to form the active enzyme. Cleaved caspase 3 also cleaves and activates caspases 6 and 7. Caspase 3 and 7 have some overlapping functions but also some distinct roles in apoptosis and both are common to both intrinsic and extrinsic apoptotic pathways. Caspase 3 controls DNA fragmentation and morphological changes in apoptosis (Lakhani et al., 2006; Slee et al., 2001), whereas caspase 7 may be more important in the loss of cell viability (Lakhani et al., 2006). Activation of caspases results in cleavage of many substrates involved in DNA replication, DNA repair and protein synthesis, in addition to cytoskeletal reorganisation and disintegration of the cell into apoptotic bodies. Caspase 3/7 activity assays described in this chapter were used as a way of detecting apoptosis, allowing for rapid and quantifiable measurements of apoptotic activation. Cleaved caspase 3 (activated caspase 3) was also detected by Western blot to support caspase 3/7 enzymatic activity data.

3.1.6.2 PARP cleavage

Full length PARP is involved in DNA repair, differentiation and chromatin structure formation. PARP is efficiently cleaved by caspase 3 and 7, with stronger affinity for caspase 7 (Germain et al., 1999; Lazebnik et al., 1994), so whilst caspase activation does not guarantee that apoptosis will occur, just that caspase activity is initiated, cleavage of PARP suggests that the cells are in fact undergoing apoptosis. PARP cleavage produces a 89kDa fragment, containing the catalytic domain, and a 24kDa fragment containing the DNA binding domain which retains activity for strand breaks, inhibiting DNA repair, ADP-ribose polymer formation and transcription. PARP cleavage is detectable by Western blot, and in this study, the 89kDa fragment was detected.

3.1.6.3 PUMA induction

PUMA, a BH3-only protein, is a direct transcriptional target of p53, and plays an important role in p53-mediated apoptosis. It is a pro-apoptotic member of the Bcl-2 family and functions by binding and inhibiting the anti-apoptotic Bcl-2 proteins (Certo et al., 2006; Chen et al., 2005c), and may also activate pro-apoptotic BAX and BAK (Gallenne et al., 2009; Kim et al., 2009). Overexpression of PUMA has been shown to be associated with increased BAX expression, BAX conformational change,

translocation to the mitochondria, cytochrome c release and reduction in the mitochondrial membrane potential (Liu et al., 2003).

3.1.6.4 Annexin-V staining

An early feature of apoptosis is the translocation of the membrane phospholipid phophatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. This exposes PS to the external cellular environment and facilitates non-inflammatory recognition by phagocytic cells, allowing for the early uptake and disposal of damaged cells (Fadok et al., 2001). The process of early and efficient uptake with no release of cellular constituents results in essentially no inflammatory response.

Annexin-V is a recombinant and specific PS-binding protein (van Engeland et al., 1998). The increase in PS residues exposed on the surface of the plasma membrane during apoptosis can be detected by annexin-V labelled with a FITC fluorochrome (Bossy-Wetzel and Green, 2000), a sensitive probe for identification and quantification of apoptotic cells using flow cytometry.

Annexin-V is used in conjunction with PI which intercalates DNA but can only enter the cell upon loss of membrane integrity. PI is therefore excluded by early apoptotic cells, but allows for identification of later stages of apoptosis and necrosis.

3.1.6.5 Propidium iodide (PI) staining

Apoptotic cells, or cells undergoing cell death are characterised by DNA fragmentation (a later event in apoptosis compared to caspase activity). Unlike for annexin V and PI co-staining, cells analysed for PI staining alone are fixed, allowing PI to enter all cells and bind cellular DNA. The sub G_1 peak is determined, and is a measurement of fragmented DNA which can occur in apoptosis, necrosis and other forms of cell death. PI staining is also used to analyse the cell cycle at the same time, as the amount of DNA within an intact cell is determined.

3.1.6.6 Growth inhibition assays

GI_{50} values from growth inhibition assays are measured using colour intensity to quantify the proportion of cells that remain adherent to 96-well plates following treatment relative to control. This assay therefore takes into account both cells that have undergone cell death and dislodged from the surface, and cell cycle arrest.

3.1.7 Other forms of cell death

Since MDM2-p53 antagonists have been previously shown to induce apoptosis, the studies in this thesis have focused on measuring apoptosis following Nutlin-3 and MI-63 treatment. However other mechanisms of cell death include; activation of apoptosis inducing factor (AIF), and endonuclease G independent of caspases; paraptosis (Sperandio et al., 2000), driven by an alternative form of caspase 9 activity that is Apaf-1 independent; and autophagy, characterised by sequestration of cytoplasm and organelles in double or multimembrane vesicles and delivery to lysosomes for subsequent degradation. Nutlin-3 has not been found to induce other forms of cell death, although data presented at the 2010 ASH meeting has shown that Nutlin-3 induces autophagy in leukemia cells in a p53-dependent manner, and that this goes hand in hand with apoptosis (Ruvolo et al., 2010).

3.2 Hypotheses and Aims

Hypotheses:

- Since p53 is a direct transcriptional target of MYCN, *MYCN*-amplification or overexpression sensitises neuroblastoma to the apoptotic and growth inhibitory effects of the MDM2-p53 antagonists Nutlin-3 and MI-63.
- p53 mutant cell lines are resistant to MDM2-p53 antagonists regardless of *MYCN* status.

Aims:

1. To examine the effect of MDM2-p53 antagonists in the MYCN regulatable SHEP Tet21N cell line on the growth inhibitory response, levels of apoptosis and induction of the p53 response following Nutlin-3 and MI-63 treatment.
2. To determine the effect of *MYCN* knockdown on the p53 and apoptotic response to MDM2-p53 antagonists in 5 *MYCN*-amplified neuroblastoma cell lines.
3. To determine GI_{50} values (concentration required for 50% growth inhibition) and caspase 3/7 activity following Nutlin-3 and MI-63 treatment in a panel of *MYCN*-amplified and non-*MYCN*-amplified neuroblastoma cell lines, including p53 mutant, $p14^{ARF}$ impaired and *MDM2*-amplified cell lines.

3.3 Specific Materials and Methods

3.3.1 Cell Lines

22 neuroblastoma cell lines were used to examine the effect of MYCN on the response to MDM2-p53 antagonists. *MYCN*-amplified neuroblastoma cell lines used were NGP, LS, TR14, NB1691, IMR-32, NBLW, SMSKCNR, LAN5, PER-108, CHLA136, and the p53 mutant cell lines SKNBe2C and IGRN91. Non-*MYCN* amplified cell lines used were SHSY5Y, GIMEN, SJNB1, NB69, LAN6, SKNRA, SHEP, NBLS and the p53 mutant cell line SKNAS. Details of cell lines are found in Chapter 2.1. The conditional MYCN-expressing SHEP Tet21N cell line was used and cells cultured for at least 48 hours in 1µg/ml of tetracycline (Sigma) to switch off MYCN. Tet21 vector only cells were used as a control (Lutz et al., 1996). All cell lines were cultured in RPMI medium (Sigma) supplemented with 10% FCS. 200µg/ml of G-418 antibiotic was added to Tet21N and Tet21 media, and 900µg/ml Hygromycin B to Tet21N media.

3.3.2 siRNA-mediated knockdown of MYCN

Cells were seeded at $4x10^5$ cells/well in 6-well plates (Corning). At 30-50% confluency, siRNA duplexes against MYCN were transfected into cells with Lipofectamine™ reagent (Invitrogen) as described in Chapter 2.3 using previously described sequences shown in Table 2.2 (Bell et al., 2006). When siRNA-mediated knockdown was performed in 96-well plates, concentrations and volumes were calculated as for 6-well plates, then everything was divided by 16, with a final volume of 150µl in each well (50µl Lipofectamine:siRNA complexes; 100µl OptiMEM).

3.3.2.1 Optimisation of MYCN knockdown

The optimal concentrations of MYCN siRNA were determined for NGP, TR14, IMR32 and LS cells. NGP, TR14 and IMR32 cells were treated with 30nM, 40nM and 50nM of MYCN siRNA or SCR siRNA for 24 and 48 hours and densitometry performed to determine optimal knockdown. LS cells were treated with 25nM and 40nM siRNA for 24 hours. Optimal knockdowns were as follows: NGP 40nM 24 hours, TR14 30nM for 24 hours, IMR32 40nM for 24 hours and LS 40nM for 24 hours (Figure 3.1). For LAN5 cells a previously optimised concentration of 50nM for 24 hours was used (Chen et al., 2010b).

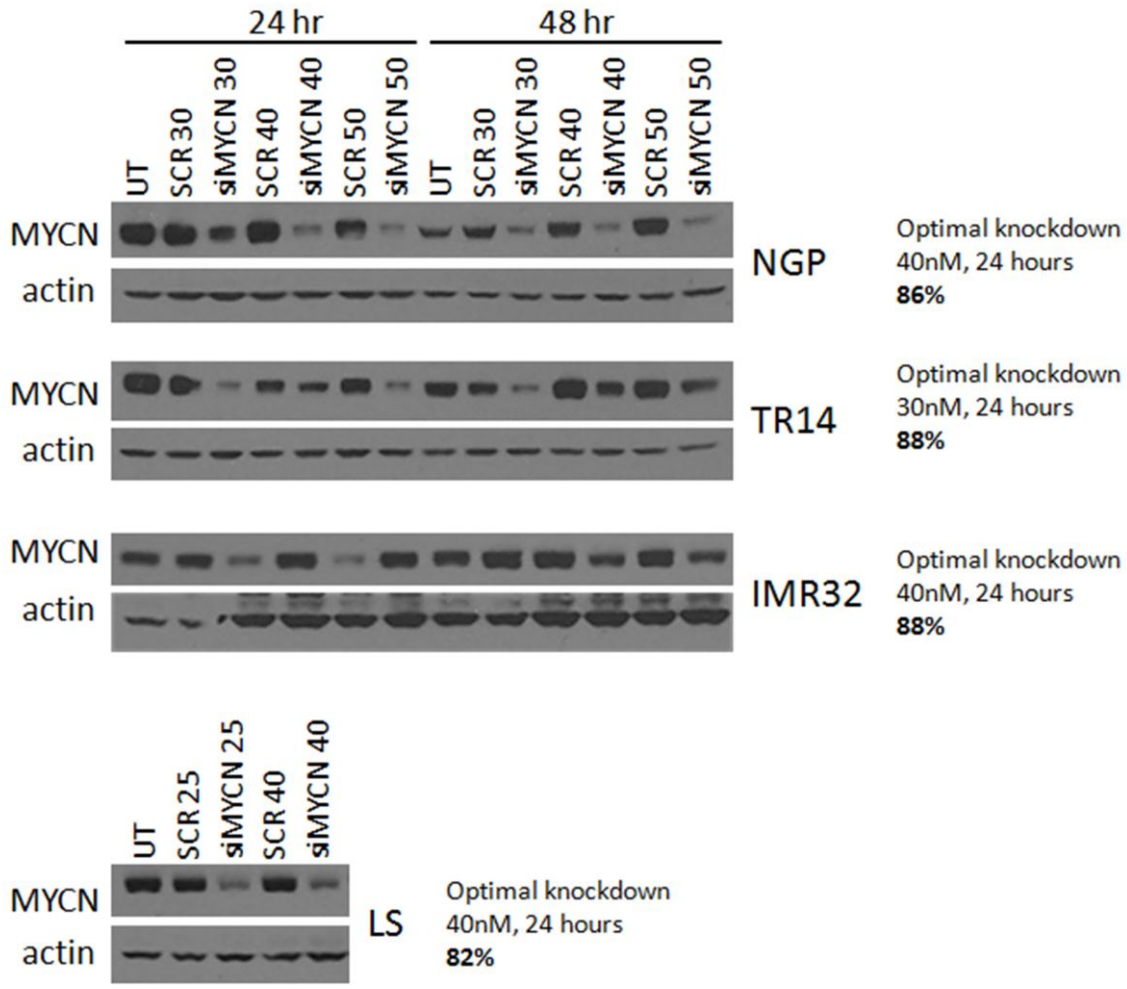


Figure 3.1. **Optimisation of MYCN knockdown using siRNA in NGP, TR14, IMR32 and LS cells.**

3.3.3 Flow cytometry

3.3.3.1 Propidium iodide staining

For cell cycle and sub-G_1 analysis, adherent and non-adherent cells were harvested, washed in PBS and fixed in 4:1 Methanol:Acetone. FACs analysis was performed using methods described in Chapter 2.5. Tet21N cells were seeded at $2x10^5$ cells/well, and all other cell lines at $4x10^5$ cells/well in 6-well plates. Instrument settings for LAN5 cells are shown in Appendix 1, and the FL2-H adjusted slightly for each cell line so that the G_1 peak was at 200 on a linear scale.

3.3.3.2 Annexin-V staining

3.3.3.2.1 Preparation of samples for Annexin-V FACs

NGP cells were seeded at a density of $4x10^5$ cells/well in 6-well plates (1 well per repeat). After 24 hours, cells were treated with SCR or MYCN siRNA as previously described (Section 3.3.2) for 24 hours. Cells were treated with 10µM Nutlin-3 or MI-63, or DMSO for 24 hours. 400µl of cells were transferred to microfuge tubes (Eppendorf) and pelleted in a microcentrifuge at 3000rpm for 5 minutes. Pellets were resuspended in 200µl sterile PBS and pelleted again at 3000rpm for 5 minutes. The cell pellet was then resuspended in 1x Binding Buffer from the Annexin-V-FITC Apoptosis Detection kit 1 (BD Biosciences) to a final concentration of $2x10^5$ cells/100µl. 100µl of cell suspension was transferred to a FACs tube, and 5µl of annexin-V, and 5µl of PI added, mixed gently and stored in the dark for 15 minutes. 400µl of 1x binding buffer was added and samples immediately analysed on the FACs machine. For each experiment 3 controls were set up; cells only, PI only, and annexin-V only to help with calibrations.

3.3.3.2.2 Data acquisition

Cell only samples (no PI or annexin-V staining) were first analysed on the FACs machine and SSC and FSC adjusted so that cells were clustered in the lower left (LL) quadrant (Shown in Figure 3.2). Annexin-V only controls were then analysed and parameters FL1 altered until cells formed 2 clusters in the LL and lower right (LR) quadrants. PI only stained cells were loaded into the machine and %FL2 in FL1 compensation altered to form two clusters in lower right (LR) and upper right (UR) quadrants. Finally, annexin-V and PI stained samples were analysed. 10,000 events were counted for each sample.

3.3.3.2.3 Data Analysis

Quadrants were placed on the dot plot so that viable cells (LL) were separated from cells in early (LR) or late apoptosis (UR), or necrosis (UL), and the percentage of cells in each quadrant calculated using the statistics tool. Examples of these for Nutlin-3 treatment alongside the DMSO control are shown in Figure 3.2.

3.3.3.2.4 Problems with annexin V staining in neuroblastoma cell lines

Despite a number of publications using annexin-V staining in neuroblastoma (Werdehausen et al., 2009; Fang et al., 2008; Ryu et al., 2005) the cell lines used in this study had high basal levels of annexin-V staining. Annexin-V staining was therefore not an ideal method of investigating apoptosis and was not taken further in these studies. Non neuroblastoma cell lines were used as a control for the technique.

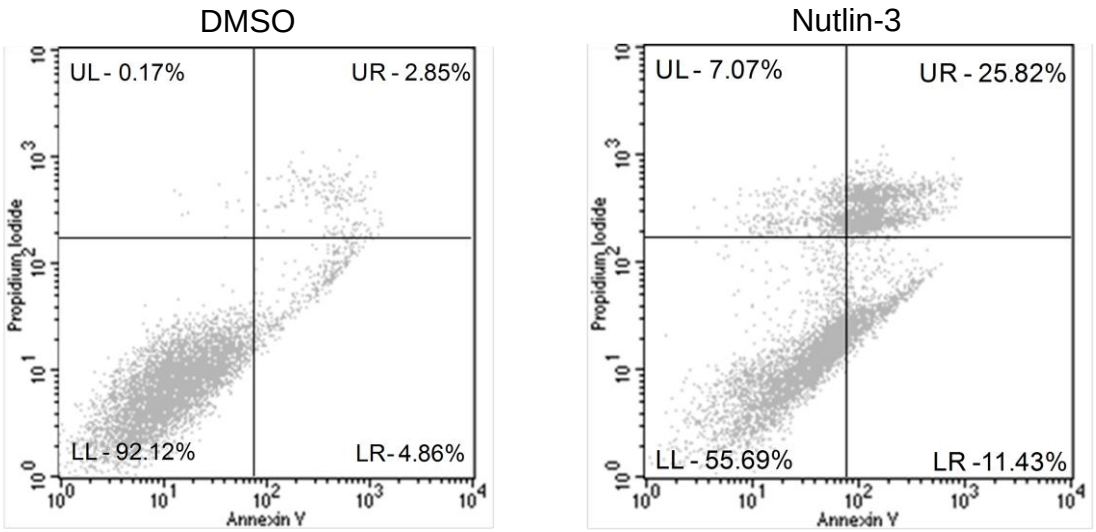


Figure 3.2. **Annexin-V/PI staining of cells treated with DMSO and cells treated with Nutlin-3.** Cells in quadrants lower right (LR) and upper right (UR) are considered apoptotic, cells in upper left (UL) are considered necrotic and cells in lower left (LL) are considered viable.

3.3.4 Clonogenic assays

SHEP Tet21N and Tet21 vector only cells (tetracycline+ and tetracycline-) were assessed for their colony forming ability, and were seeded at varying cell densities. Once optimal cell densities had been determined (300, 500 and 800 for untreated cells) cells were seeded in 6-well plates at a density of $2x10^4$ cells/well, treated with 0-40µM Nutlin-3 or MI-63 for 24 hours, then cells counted and seeded in $10cm^2$ petri dishes at 3 densities to assess their long term survival. Following 2 weeks incubation, colonies

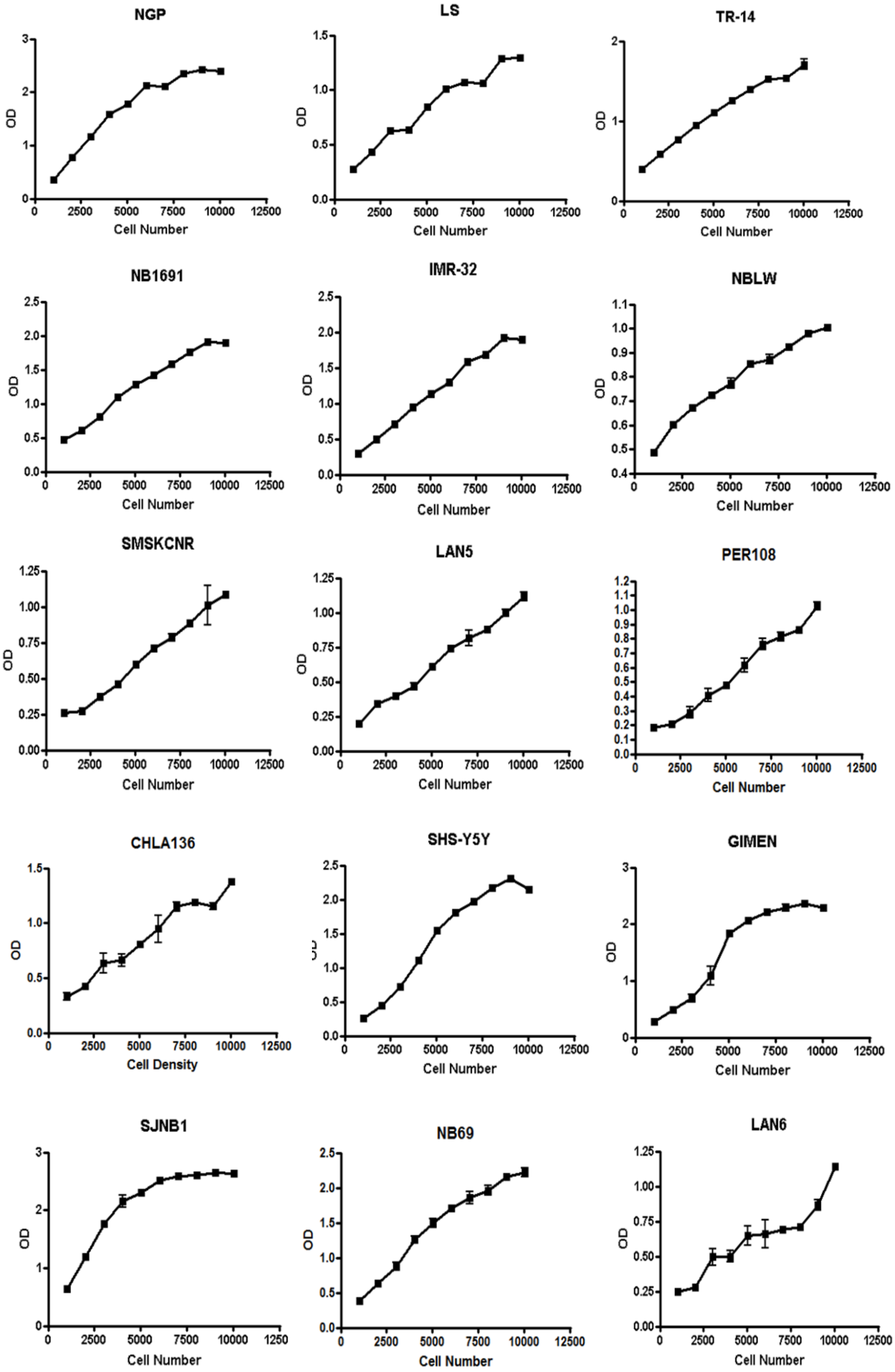
were fixed with Carnoy's solution (Appendix 1), and stained with crystal violet (0.5% w/v) for 10 minutes, before washing and leaving to dry. Colonies were counted with an automated colony counter (ColCount, Oxford Optronics Ltd., Oxford, UK) and the plating efficiency relative to DMSO control determined.

3.3.5 Examining apoptosis following double knockdown of MYCN and p53, following irradiation treatment

MYCN-amplified NGP cells were seeded at a density of $4x10^5$ cells/well (6-well plates) and after 24 hours were treated with MYCN siRNA (40nmol/L), p53 siRNA (50nmol/L), p53 and MYCN siRNA (35nmol/L of each siRNA), or SCR siRNA (70nmol/L) for 48 hours prior to irradiation-induced DNA damage. X-irradiation was used to induce DNA damage and a dose of 4Gy was chosen because it was previously used and shown to be sufficient to induce a p53-mediated DNA damage response in neuroblastoma cell lines (Tweddle et al., 2001b). NGP cells were treated with 4Gy of X-irradiation from a RS320 irradiation system (Gulmay Medical, Surrey, UK), and harvested at 48 hours for Western analysis. The same experiment was set up simultaneously in 96-well plates to assess caspase 3/7 activity using the Caspase-Glo 3/7 assay (Promega), and performed as previously described (Chapter 2.7).

3.3.6 Growth inhibition assays

The cell density was determined as shown in Figure 3.3 and Table 3.1, whereby cells were seeded and allowed to grow for 96 hours, and cell densities chosen when cells were in exponential phase of growth, as described in Chapter 2.6.1. SRB assays were performed and growth inhibition curves generated as described in Chapter 2.6, and GI_{50} values calculated.


NGP
LS
TR-14
NB1691
IMR-32
NBLW
SMSKCNR
LAN5
PER108
CHLA136
SHS-Y5Y
GIMEN
SJNB1
NB69
LAN6
OD
Cell Number
Cell Density

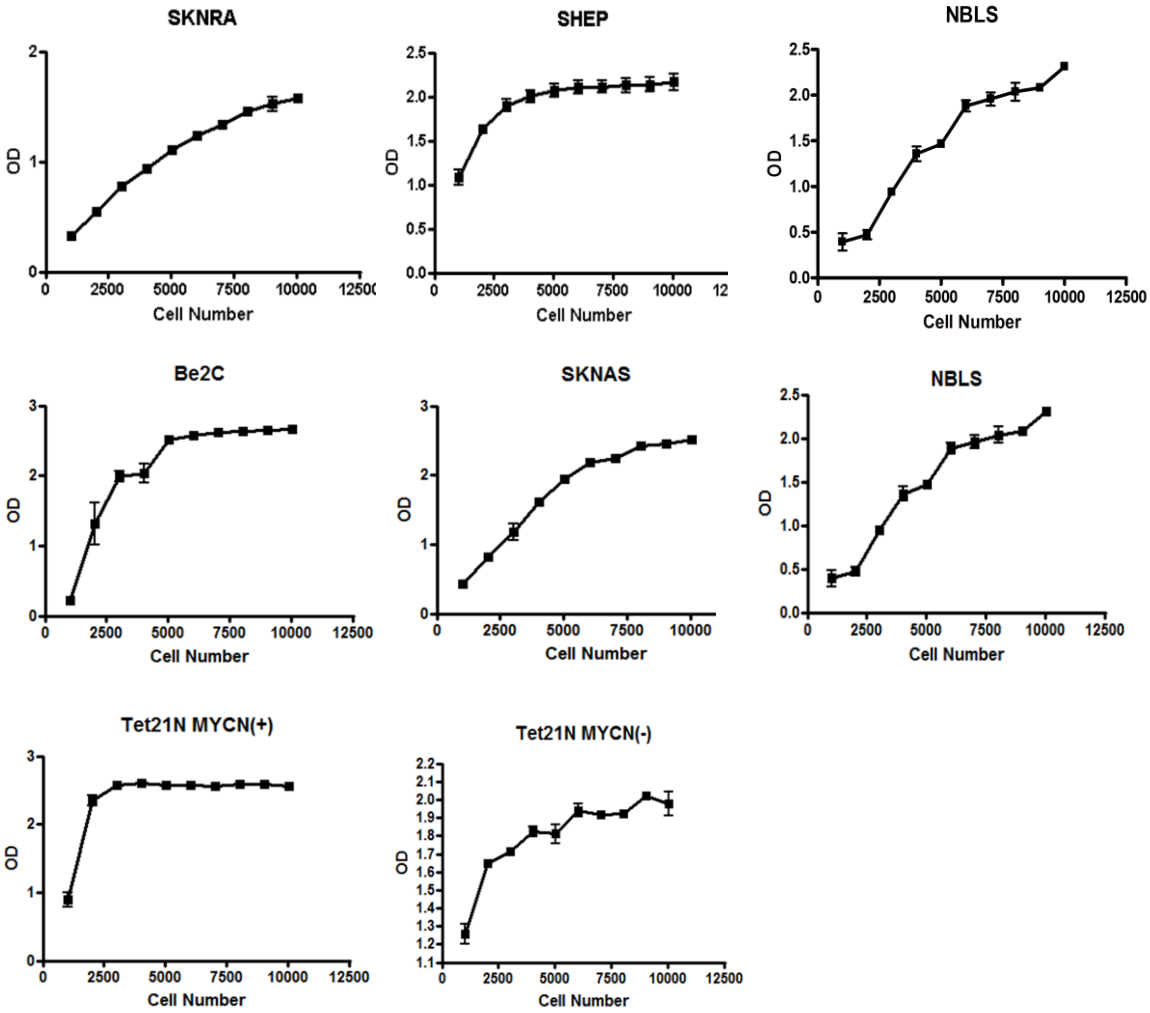


Figure 3.3. **Growth curves to determine cell density for growth inhibition assays.**

Cell Line	Cell Density	Cell Line	Cell Density
NGP	5000	NB69	5000
LS	5000	LAN6	5000
NB1691	5000	SKNRA	5000
TR14	5000	SHEP	2000
IMR32	5000	NBLS	5000
NBLW	5000	Be2C	2000
SMSKCNR	5000	SKNAS	4000
LAN5	5000	IGNR91	4000
PER108	6000	Tet21N(tet+)	2000
CHLA136	5000	Tet21N(tet-)	1500
SHSY5Y	4000	Tet21(tet+)	2000
GIMEN	4000	Tet21(tet-)	1500
SJNB1	3000		

Table 3.1. **Cell Densities used for growth inhibition assays.**

3.4 Results

3.4.1 Tet21N MYCN(+) cells are more sensitive to MDM2-p53 antagonist mediated growth inhibition and have decreased cell survival compared to MYCN(-) cells

Tet21N MYCN regulatable cells were treated with the MDM2-p53 antagonists Nutlin-3 and MI-63 in the presence (MYCN-) or absence (MYCN+) of tetracycline. Growth inhibition assays were performed with 0-20μM Nutlin-3 and 0-10μM MI-63 for 72 hours. As shown in Figure 3.4a and 3.4b MYCN(+) cells had significantly lower GI_{50}s compared to MYCN(-) cells ($p=0.02$ for Nutlin-3, and $p=0.0008$ for MI-63). GI_{50} values are shown in Table 3.2 alongside GI_{50} values for a panel of *MYCN*-amplified and non-*MYCN*-amplified neuroblastoma cell lines. As a control, growth inhibition assays were performed under the same conditions for Tet21 vector only transfected cells and no difference in GI_{50} was observed indicating no effect of tetracycline alone.

As shown in Figure 3.5a, clonogenic survival curves were generated using 0-40μM Nutlin-3 or MI-63 for 24 hours followed by a 14-day incubation. After 14 days, MYCN(-) cells formed more colonies, taking into account plating efficiency, indicating increased long term cell survival in these cells compared to MYCN(+) cells. Interestingly, the colony appearance between untreated MYCN(+) and MYCN(-) cells was quite different, with MYCN(+) forming larger and denser staining colonies compared to MYCN(-) cells (Figure 3.5b). It was previously reported that MYCN(+) cells have a doubling time of 78.5 hours compared to 90.4 hours for MYCN(-) cells (Bell et al., 2006), and this is reflected in the denser colonies formed by MYCN(+) cells compared to the MYCN(-) cells. Since the colony appearance of MYCN(+) and MYCN(-) cells following Nutlin-3 or MI-63 treatment remains the same as in the untreated controls, this suggests that whilst MYCN(+) cells form less colonies than MYCN(-) cells following MDM2-53 antagonist treatment, the surviving cells still have an ability to replicate. Vector only Tet21 cells were assessed for their colony forming ability in the presence and absence of tetracycline and there was no difference in colony appearance (Figure 3.5c) indicating that the difference in colony appearance seen in Tet21N cells is a result of manipulation of MYCN, and not an effect of tetracycline. Surprisingly, the colonies formed by Tet21 cells were very different from those formed by both Tet21N MYCN(+) and MYCN(-) cells.

3.4.2 Both MYCN(+) cells and MYCN(-) cells undergo a G_1 arrest, although MYCN(-) cells have an increased basal proportion of cells in G_1 population

The shorter doubling time of 78.5 hours in MYCN(+) compared to 90.4 hours for MYCN(-) cells (Bell et al., 2006) is reflected in the higher proportion of MYCN(-) cells in G_1 compared to MYCN(+) cells, which have a greater proportion of cells in S and G_2 phases under normal growth conditions (Figure 3.6, UT controls). Upon 2.5μM Nutlin-3 and MI-63 treatment, both MYCN(+) and MYCN(-) cells G_1 arrest, but this effect is more pronounced in MYCN(+) cells due to the lower G_1 baseline proportions. Interestingly, there is an increase in the proportion of cells in G_2 at increasing concentrations of both Nutlin-3 and MI-63 in MYCN(+) and MYCN(-) cells.

3.4.3 MYCN(+) cells have increased levels of apoptosis compared to MYCN(-) cells

The proportion of sub G_1 DNA and the levels of caspase 3/7 activity were used as measures of the degrees of apoptosis following Nutlin-3 or MI-63 treatment in Tet21N cells. DNA fragmentation is a later event in apoptosis compared to caspase activation, so the proportion of sub G_1 DNA was measured at 24, 48 and 72 hours compared to just 24 hours for caspase activity. As shown in Figure 3.7a, there was an increase in the percentage of sub G_1 DNA following Nutlin-3 and MI-63 treatment (0-20μM) in MYCN(+) cells compared to MYCN(-) cells at all time points and this increase was mostly significant (24 hours: Nutlin-3 $p<0.0001$, MI-63 $p=0.2150$l; 48 hours: Nutlin-3 $p<0.0001$, MI-63 $p<0.0001$; 72 hours: Nutlin-3 $p=0.0185$, MI-63 $p=0.2053$; 2-way ANOVA). An increase in the induction of caspase 3/7 activity at increasing concentrations of Nutlin-3 ($p<0.0001$) and MI-63 ($p<0.0001$) was observed in MYCN(+) compared to MYCN(-) cells (Figure 3.7b).

3.4.4 Induction of p53, p53 response and apoptotic markers in SHEP Tet21N cells

As shown in Figure 3.8, following 24 hours treatment with 5, 10 and 20μM Nutlin-3 or MI-63, p53 protein, p53 phosphorylation at serine 15 and p53 target gene products ($p21^{WAF1}$, MDM2 and PUMA) were induced in both MYCN(+) and MYCN(-) cells. Higher basal levels of p53 were detected in MYCN(+) cells and after Nutlin-3 treatment, increased p53 induction was observed in MYCN(+) cells compared to MYCN(-) cells. Both compounds induced greater levels of p53 phosphorylation at

serine 15 in MYCN(+) cells. In agreement with the sub G_1 DNA fraction and caspase activity, levels of the p53 target and apoptotic marker PUMA was increased in MYCN(+) compared to MYCN(-) cells following both Nutlin-3 and MI-63 treatment, providing further evidence that Tet21N cells are more sensitive to apoptosis in the presence of MYCN.

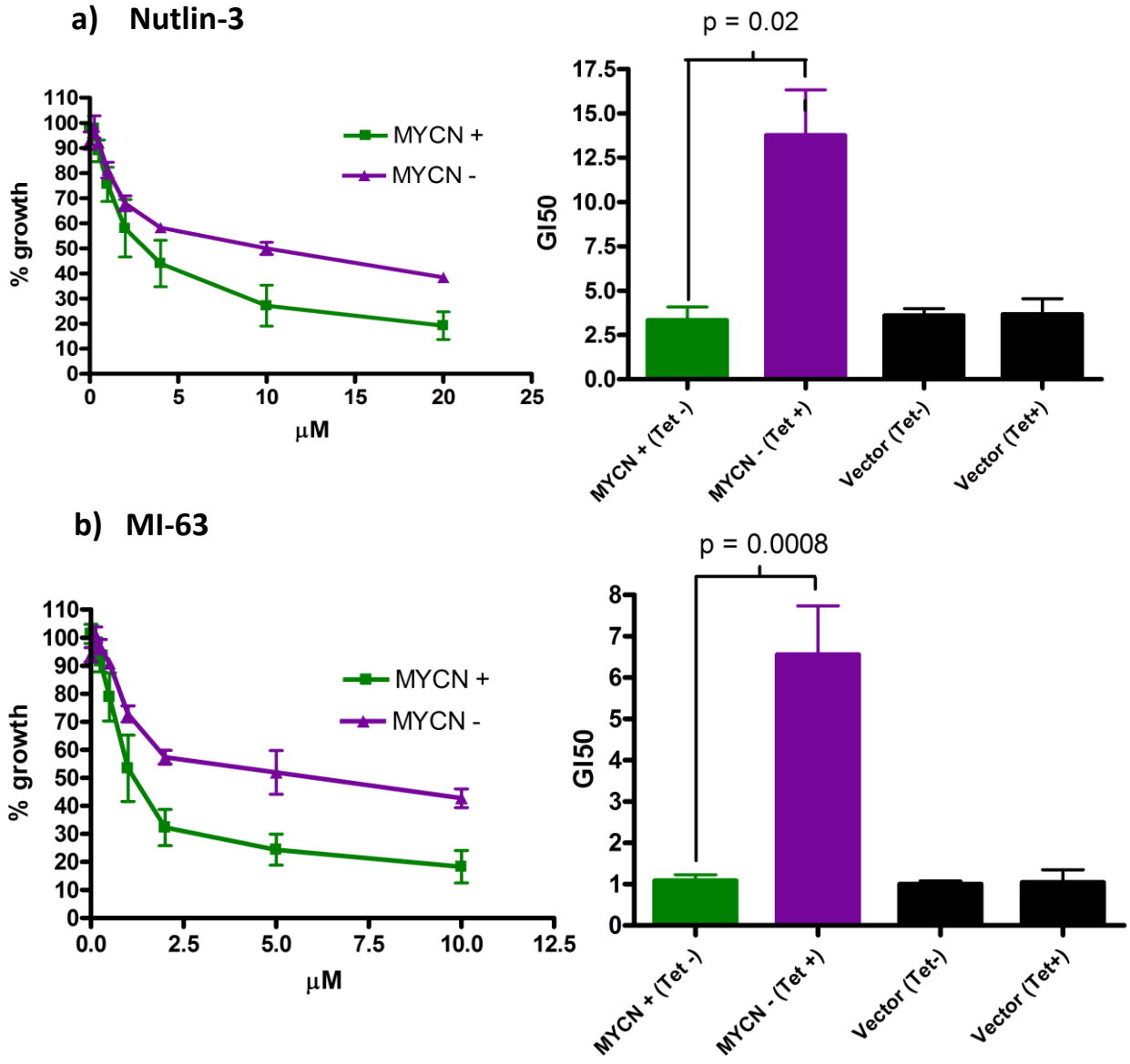


Figure 3.4. **MYCN(+) Tet21N cells are more sensitive to MDM2-p53 antagonist mediated growth inhibition than MYCN(-) Tet21N cells.** Growth inhibition assays for **a)** Nutlin-3 and **b)** MI-63 were performed after a 72 hour drug exposure. MYCN(+) cells have a significantly lower mean GI_{50} compared to MYCN(–) cells (p=0.02 for Nutlin-3 and p=0.0008 for MI-63, paired t-test). No difference in GI_{50}s was observed between the control vector only SHEP Tet21 cells in the presence and absence of tetracycline.

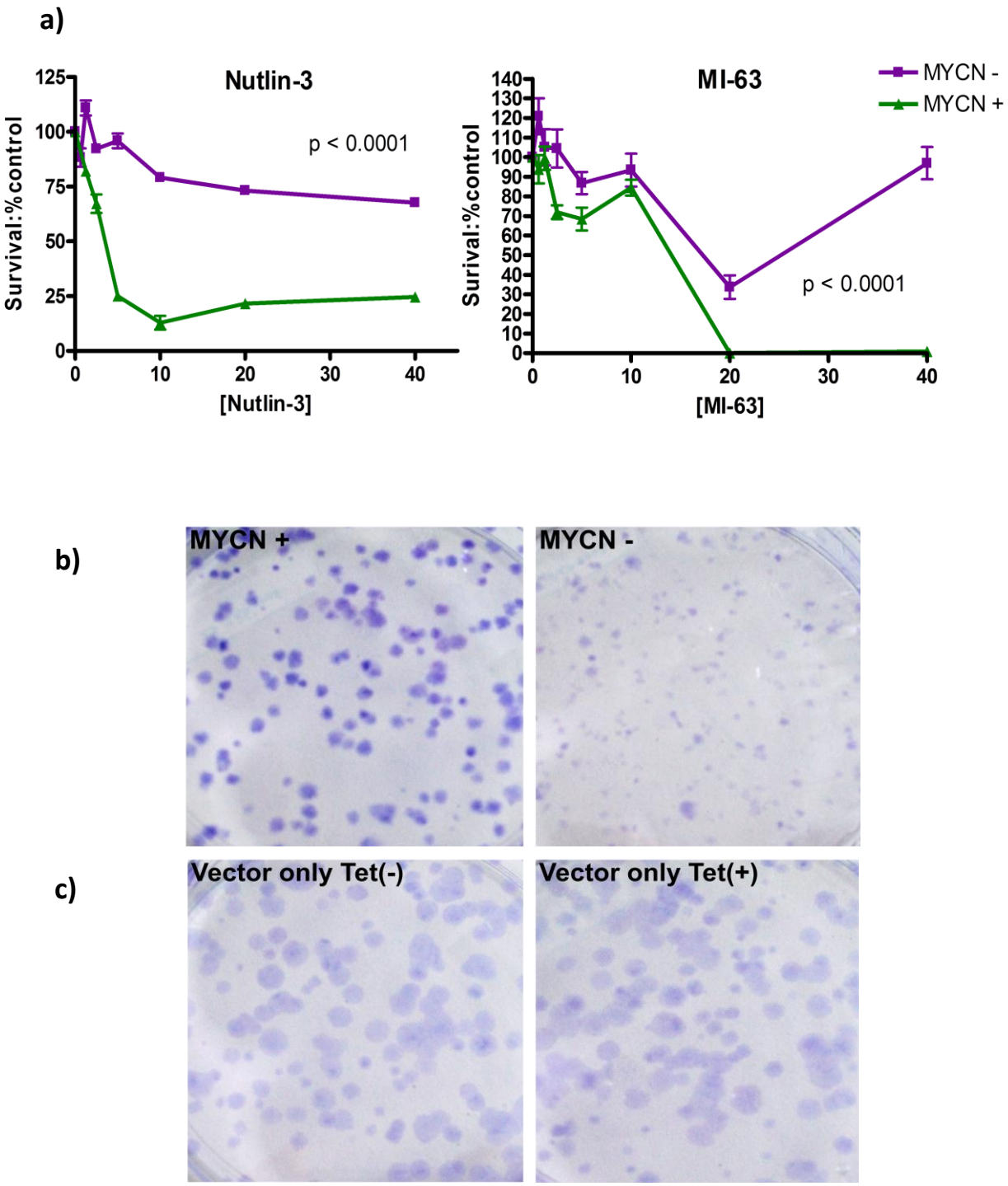


Figure 3.5. **MYCN(-) Tet21N cells have increased cell survival following MDM2-p53 antagonist treatment compared to MYCN(+) Tet21N cells. a)** Clonogenic survival curves (24 hour drug exposure followed by 2 week incubation) shows MYCN(+) cells are much more sensitive to Nutlin-3 and MI-63 long term survival compared to MYCN(-) cells ($P<0.0001$, 2-way ANOVA). **b)** In untreated controls, MYCN(+) cells have larger and denser colonies compared to MYCN(-) cells. **c)** In untreated control, vector only tetracycline(+) and tetracycline(-) cells have the same colony appearance suggesting no effect of tetracycline alone.

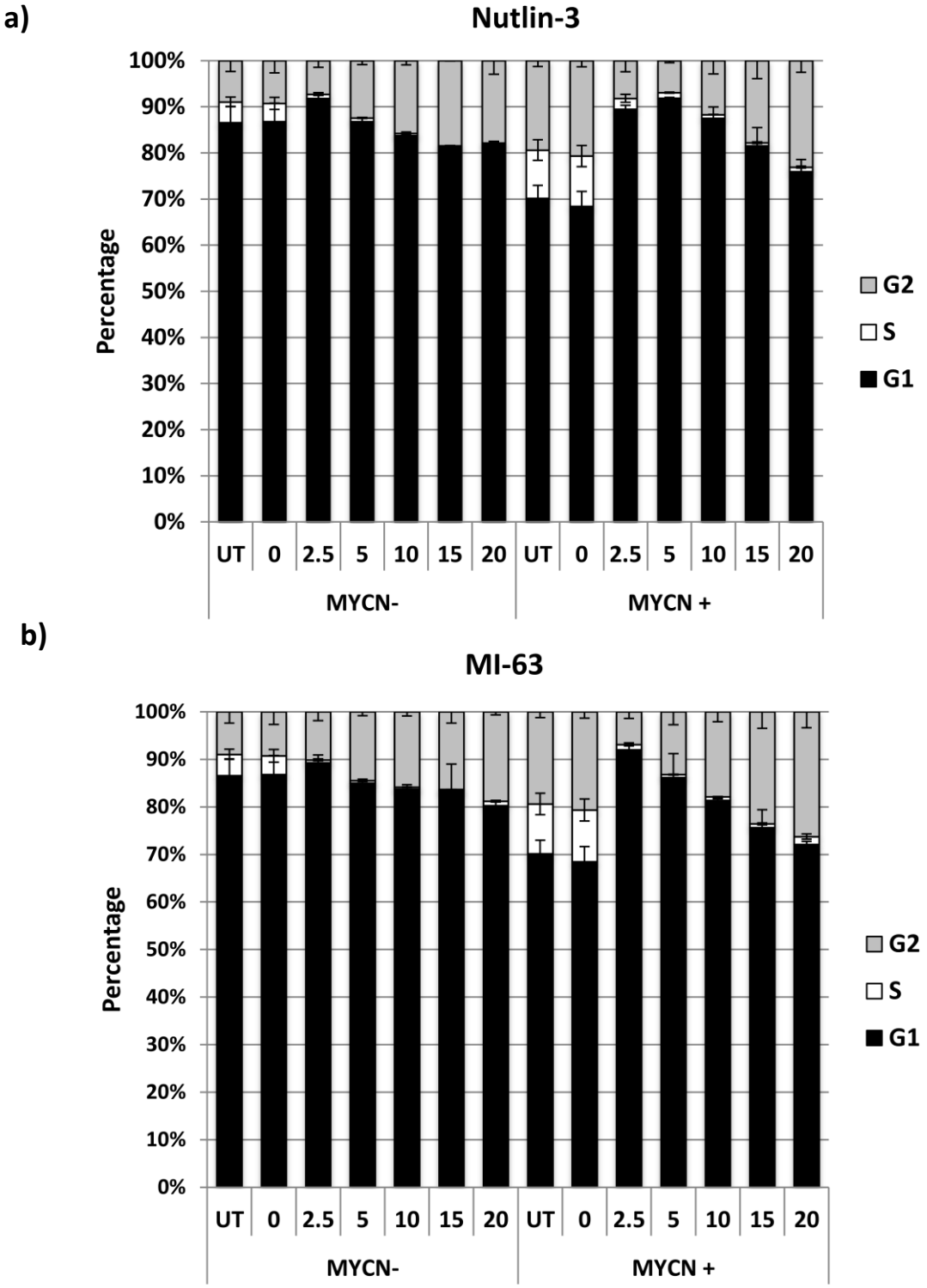
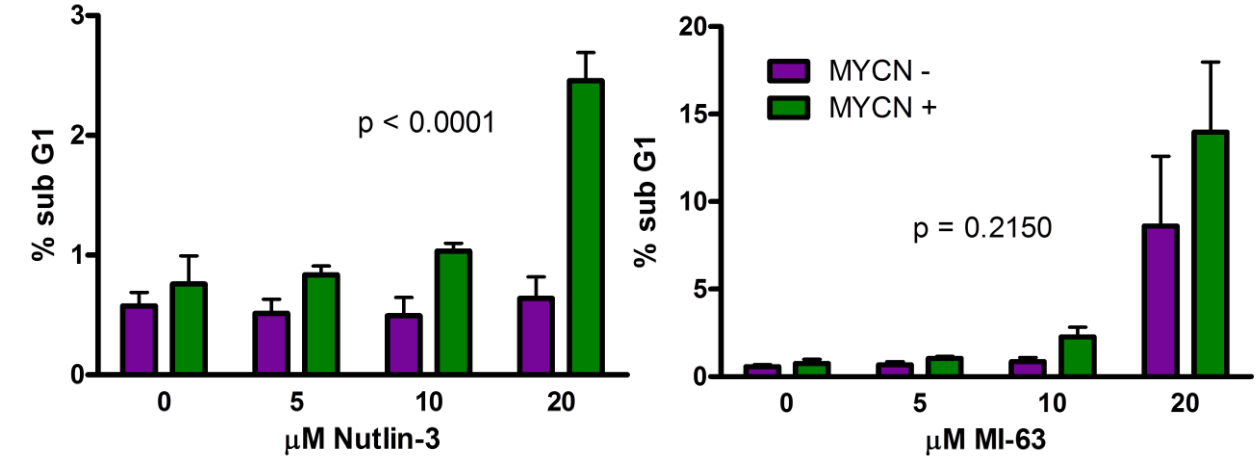
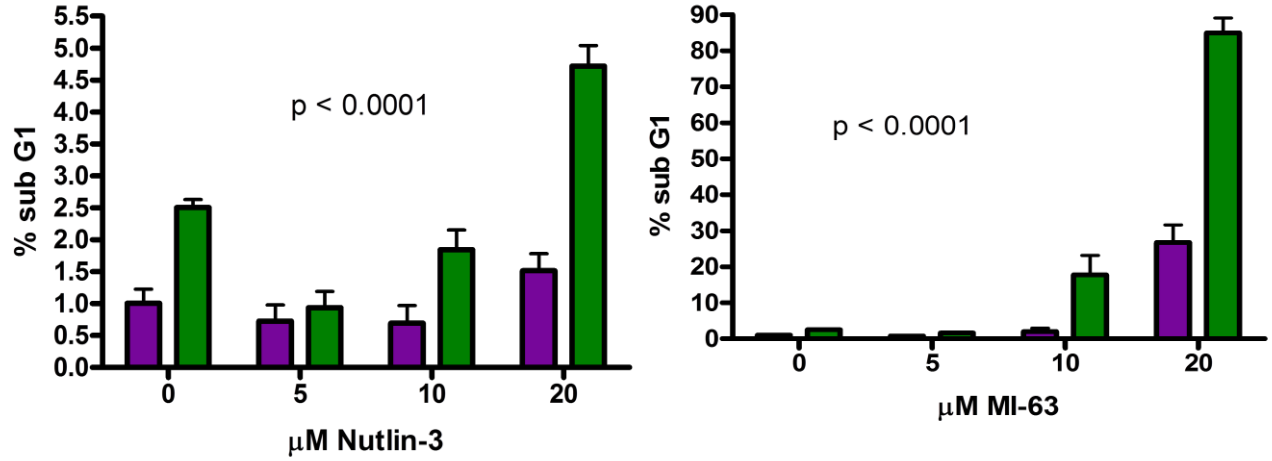


Figure 3.6. **Cell cycle analysis after 24 hour drug exposure shows that both MYCN(–) and MYCN(+) cells G_1 arrest in response to just 2.5µM a) Nutlin-3 or b) MI-63, and also shows that MYCN(–) cells have an increased proportion of cells in G_1 in control samples compared to MYCN(+) cells. At increasing concentrations of Nutlin-3 and MI-63, an increasing number of cells appear to accumulate in G_2.**

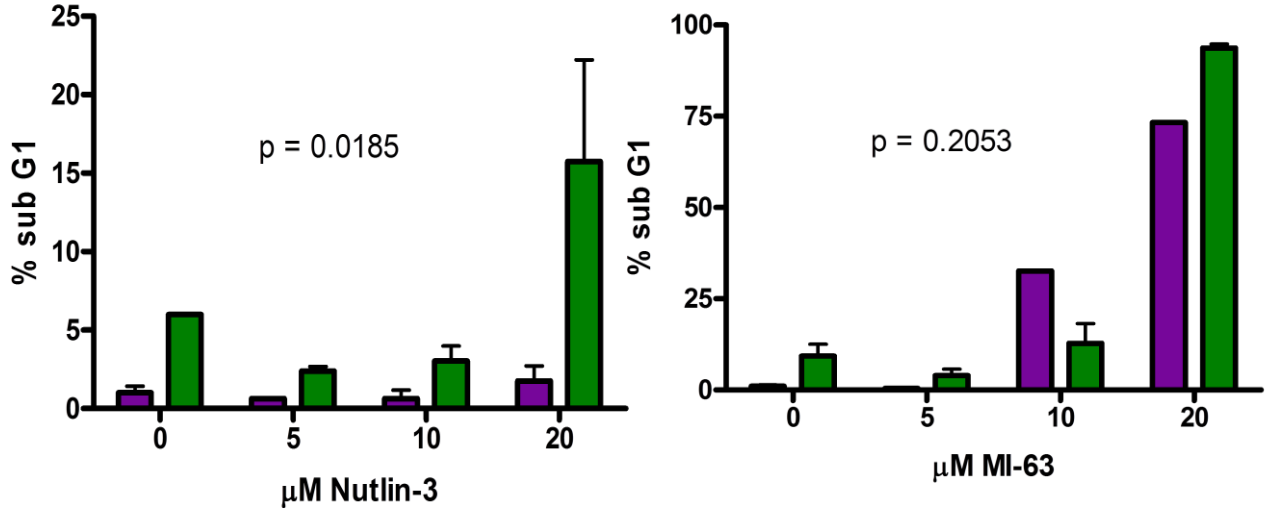
a) 24 hours



48 hours



72 hours



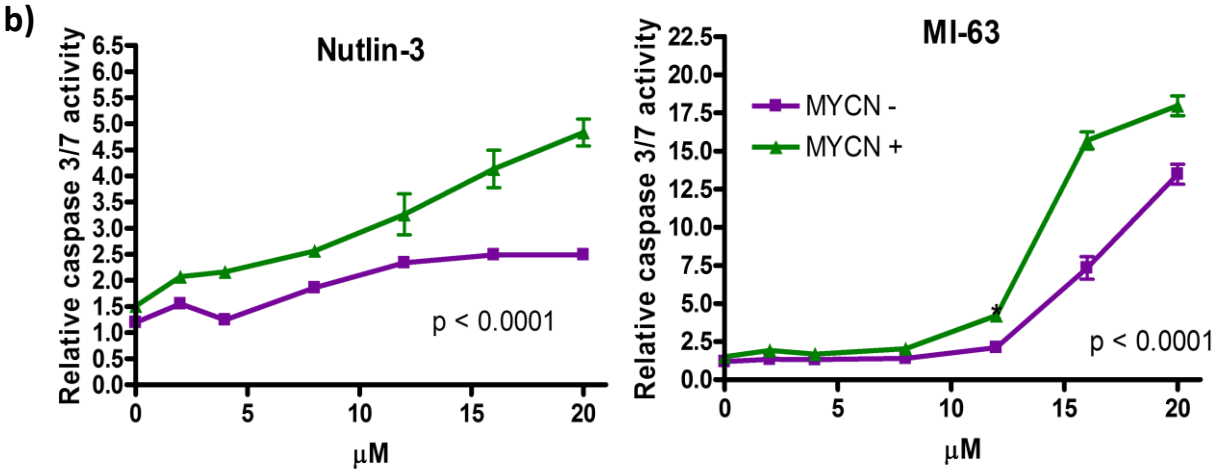


Figure 3.7. **MYCN(+) Tet21N cells are more sensitive to MDM2-p53 antagonist mediated apoptosis than MYCN(-) Tet21N cells. a)** The sub G_1 DNA fraction was measured in MYCN(-) and MYCN(+) Tet21N cells following Nutlin-3 and MI-63 treatment at 24, 48 and 72 hours (24 hours: Nutlin-3 p<0.0001, MI-63 p=0.2150l; 48 hours: Nutlin-3 p<0.0001, MI-63 p<0.0001; 72 hours: Nutlin-3 p=0.0185, MI-63 p=0.2053; 2-way ANOVA) . **b)** Caspase 3/7 activity was significantly reduced in MYCN(-) compared to MYCN(+) cells following Nutlin-3 and MI-63 treatment (p<0.0001 for both Nutlin-3, and MI-63, 2-way ANOVA).

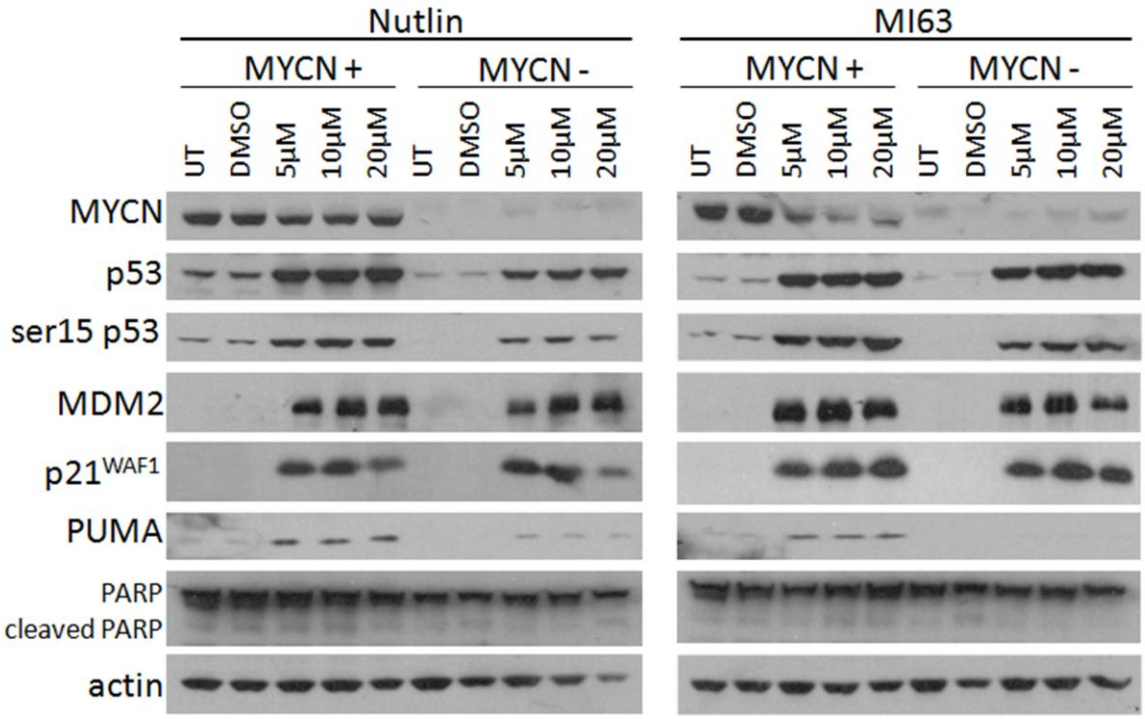


Figure 3.8. **Western blots showing no difference in induction of p53, MDM2 and p21^{WAF1} in MYCN(-) and MYCN(+) cells following 24 hours Nutlin-3 and MI-63 treatment but there were increased levels of phosphorylated p53 and PUMA in MYCN(+) compared to MYCN(-) cells. Actin was used as a loading control.**

3.4.5 Nutlin-3 and MI-63 induce a p53 response and apoptosis in NGP and LS cells

NGP cells (Figure 3.9a) were treated with 10µM Nutlin-3 and MI-63 for 4 and 24 hours. Whilst p53 was induced at 4 hours, $p21^{WAF1}$ and cleaved PARP were detected at 24 hours, and p53 levels remained the same as at 4 hours. 24 hours of Nutlin-3 or MI-63 treatment was therefore chosen as a suitable time-point to look for induction of p53, p53 responsive genes and apoptotic markers. NGP (Figure 3.9b) and LS (Figure 3.9c) cells were treated with 0, 5 and 10µM Nutlin-3 and MI-63 for 24 hours, and Western analysis used to analyse p53 and the p53 response. There was induction of p53, and the p53 transcriptional targets $p21^{WAF1}$, MDM2 and PUMA (pro-apoptotic protein), as well as increased levels of the apoptotic markers cleaved caspase 3 and cleaved PARP. Caspase 3/7 activity increased in a dose-dependent manner following Nutlin-3 and MI-63 treatment in both cell lines (Figure 3.9d), and for a given dose was stronger following MI-63 treatment compared to Nutlin-3 treatment, reflecting the higher potency of MI-63.

3.4.6 The cell cycle response to Nutlin-3 and MI-63 is cell line dependent

Following Nutlin-3 and MI-63 treatment, the cell cycle response was analysed at 4 and 24 hours in NGP cells (Figure 3.10a). A cell cycle arrest was induced by 24 hours following treatment with just 2.5µM Nutlin-3 or MI-63 in NGP cells. LS cells were also treated for 24 hours with Nutlin-3 but did not undergo a G_1 arrest (Figure 3.10b) suggesting that different neuroblastoma cell lines respond differently, in terms of cell cycle arrest, to these compounds.

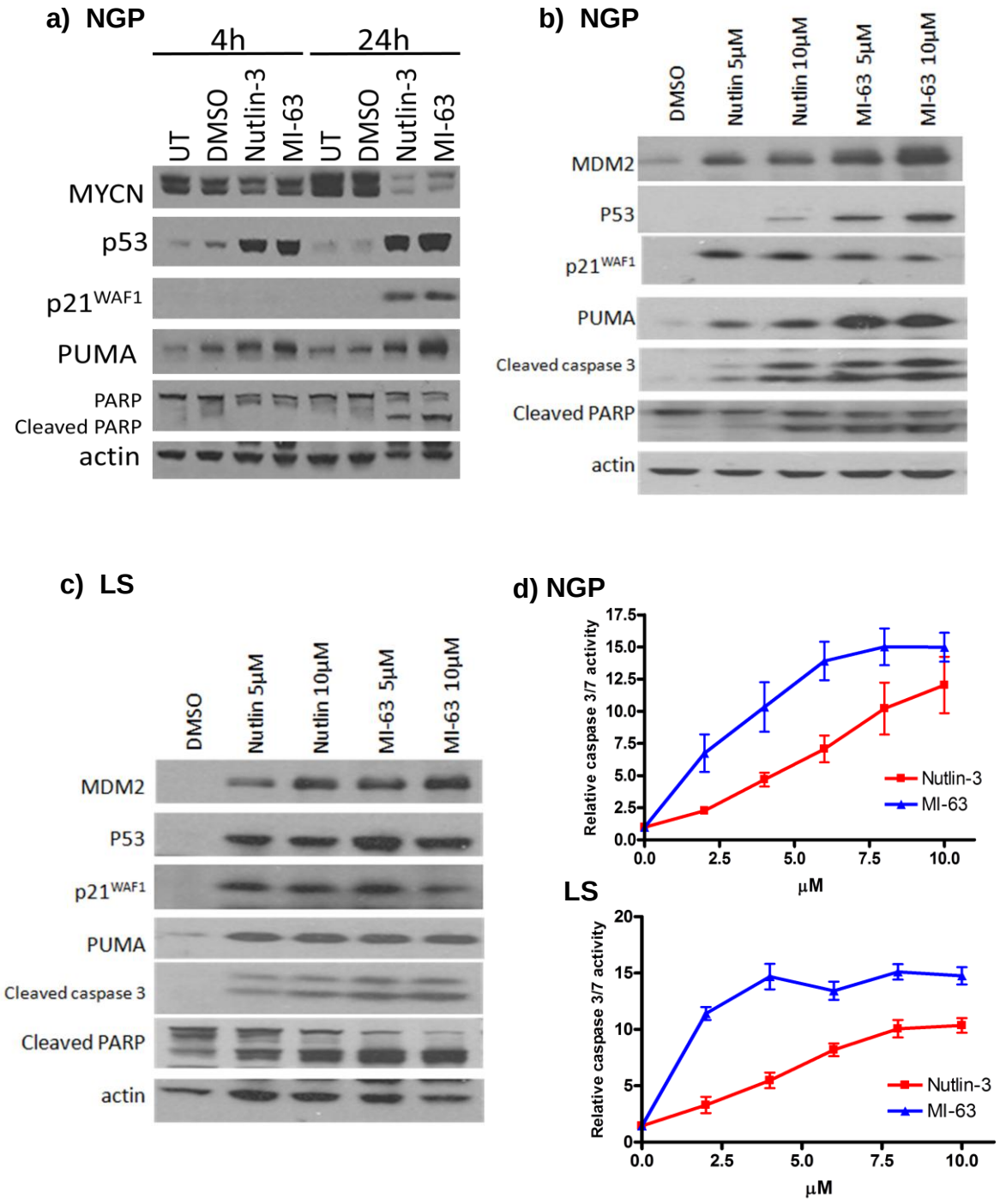


Figure 3.9. **MDM2-p53 antagonists induce a p53 response and apoptosis.** **a)** NGP cells were treated with 10μM Nutlin-3 for 4 and 24 hours. **b)** NGP cells and **c)** LS cells were treated with 5 and 10μM Nutlin-3 and MI-63 for 24 hours resulting in induction of p53 responsive genes and apoptotic markers, detected by western blot. Actin was used as a loading control**. c)** A dose-dependent increase in caspase 3/7 activity was observed for both compounds, with higher levels of caspase 3/7 activity following MI-63 treatment compared to Nutlin-3.

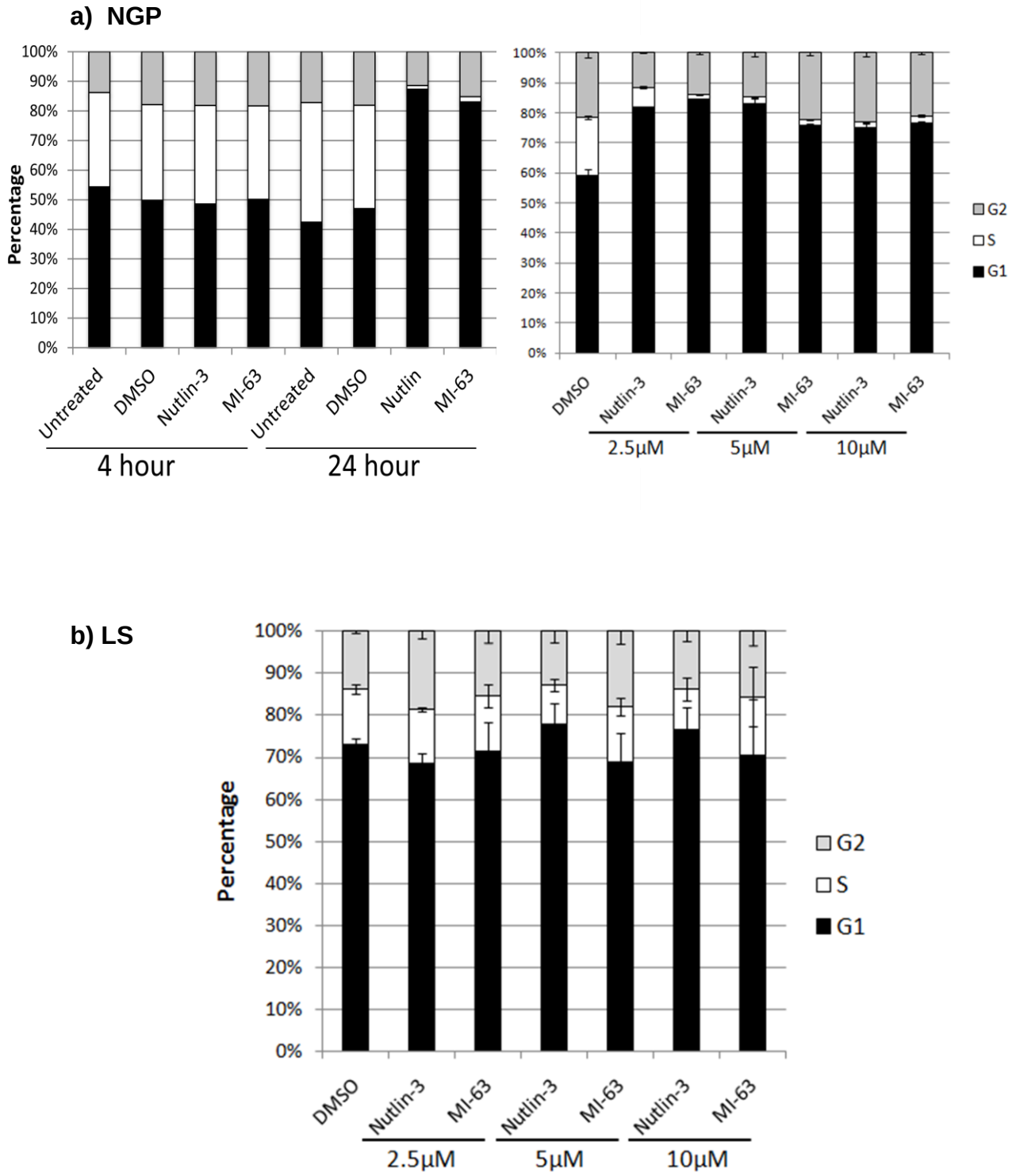


Figure 3.10. **Cell cycle analysis following Nutlin-3 and MI-63 treatment in NGP and LS cells. a)** NGP cells were treated with 10μM Nutlin-3 for 4 and 24 hours, and treated with 2.5, 5 and 10μM Nutlin-3 and MI-63 for 24 hours. They underwent a G_1 arrest at 24 hours with just 2.5μM of compound. **b)** LS cells were treated with 2.5, 5, and 10μM Nutlin-3 and MI-63 for 24 hours and did not undergo a G_1 arrest.

3.4.7 siRNA mediated knockdown of MYCN has little effect on p53 and p53 responsive genes

Interestingly, despite both MDM2 and p53 being reported as transcriptional targets of MYCN (Chen et al., 2010b; Slack et al., 2005a), MYCN knockdown (Figure 3.11) had differential effects on both p53 and MDM2. p53 decreased in TR14 cells, but remained unaltered in NGP, LS and NB1691 cells. MDM2 expression varied, increasing in NB1691 and NGP cells, but decreasing in LS and TR14 cells. There was little effect on $p21^{WAF1}$ protein expression levels following MYCN knockdown.

3.4.8 Knockdown of MYCN increases resistance of *MYCN*-amplified neuroblastoma cell lines to Nutlin-3 and MI-63 mediated induction of p53 and apoptosis detected by Western blot

To further investigate the role of MYCN on the sensitivity of neuroblastoma cell lines to MDM2-p53 antagonists, MYCN was knocked down by siRNA treatment in three cell lines co-amplified for *MYCN* and *MDM2*; NGP (40nmol/L), TR14 (30nmol/L) and LS (40nmol/L), and 2 *MYCN*-amplified but non-*MDM2* amplified cell lines; LAN5 (50nmol/L) and IMR-32 (40nmol/L). MYCN siRNA or SCR siRNA was added to cells for 24 hours then removed and replaced with 0, 5 or 10μM Nutlin-3 or MI-63 for a further 24 hours. Western analysis in all five cell lines, NGP (Figure 3.12a), LS (Figure 3.13a), LAN5 (Figure 3.14a), TR14 (Figure 3.15a) and IMR32 (Figure 3.16) showed that treatment with Nutlin-3 or MI-63, resulted in a clear increase in p53, p53 phosphorylation (serine 15), induction of p53 target genes ($p21^{WAF1}$, MDM2 and PUMA) and induction of the apoptotic markers PUMA, cleaved caspase 3 and cleaved PARP (lanes 4-8). In all cell lines, high levels of MYCN knockdown were achieved as shown by comparison of lanes 2 and 3 (at 24 hours after knockdown before treatment) and lanes 4 and 9 (DMSO controls with SCR and siMYCN at 48 hours after knockdown). MYCN knockdown alone had little effect on p53 or induction of p53 targets but in combination with MDM2-p53 antagonist treatment MYCN knockdown resulted in a decreased p53 and apoptotic response. p53 levels decreased in 4 of 5 cell lines, and levels of phosphorylated p53 decreased in all cell lines (Figure 3.12a-3.16a; lanes 10-13 compared to lanes 5-8) indicating reduced p53 activation. $p21^{WAF1}$ or MDM2 levels either decreased or did not change and in all cases, a decrease in at least 2 of the apoptotic markers PUMA, cleaved caspase 3 and cleaved PARP was observed. NGP cells were also investigated after 4 hours treatment with Nutlin-3 and MI-63

following MYCN knockdown (Figure 3.17) and whilst p53 was induced at this time point, no p53 targets or apoptotic markers were detected.

3.4.9 Knockdown of MYCN increases resistance of *MYCN*-amplified neuroblastoma cell lines to Nutlin-3 and MI-63 mediated induction of apoptosis

Apoptosis following MYCN knockdown and MDM2-p53 antagonist treatment was further investigated in NGP, LS, LAN5 and TR14 cells. Caspase 3/7 activity assays were performed in NGP (Figure 3.12b), LAN5 (figure 3.14b) and TR14 (Figure 3.15b) cells. Percentage sub G_1 DNA was determined in LS cells (Figure 3.13b) following 10μM Nutlin-3 or MI-63 treatment for 24 hours. After Nutlin-3 or MI-63 treatment alone, a dose-dependent increase in caspase 3/7 activity was seen in all cell lines with both compounds, and an increase in sub G_1 DNA % in LS cells (SCR control). Following MYCN knockdown alone, there was a reduction in caspase 3/7 activity in LAN5 and TR14 cells (at 0μM) (Figures 3.14b and 3.15b) compared to SCR control. A significant reduction in caspase activity was observed in NGP, LAN5 and TR14 cell lines following MYCN knockdown and Nutlin-3 or MI-63 treatment (NGP $p=0.013$ Nutlin-3 and $p=0.011$ MI-63; LAN5 $p=0.0026$ Nutlin-3 and $p=0.008$ MI-63; TR14 $p=0.0015$ Nutlin-3, $p=0.0001$ MI63). Levels of sub G_1 DNA in LS cells (Figure 3.13b) reduced after MYCN knockdown following Nutlin-3 ($p=0.08$) and MI-63 ($p<0.05$) treatment compared to SCR. The NGP cell line was also analysed for annexin-V staining following MYCN knockdown and MDM2-p53 antagonist treatment (Figure 3.12c). The proportion of annexin-V positive cells was increased following 10μM Nutlin-3 or MI-63 treatment compared to SCR control (Nutlin-3, $p=0.015$; MI-63, $p=0.011$). Photomicrographs of NGP cells treated with 10μM Nutlin-3 or MI-63 following MYCN knockdown shows that more cells are morphologically intact and are still adherent following 24 hours of 10μM Nutlin-3 or MI-63 treatment compared to SCR (Figure 3.12d). Interestingly, the TR14 cell line (Figure 3.15b) had increased levels of caspase 3/7 activity with SCR control compared to Nutlin-3 or MI-63 only, suggesting this cell line is particularly affected by the siRNA treatment and/or Lipofectamine. However, the data shows the same trend as the other 4 cell lines. These data are consistent with the reduction in levels of apoptotic markers shown by Western blot and show that reduction of MYCN by siRNA knockdown results in a decreased apoptotic response to MDM2-p53 antagonists.

3.4.10 Knockdown of MYCN does not alter the cell cycle response to MDM2-p53 antagonists

The effect of MDM2-p53 antagonists on the cell cycle was investigated in NGP, LS, LAN5 and TR14 cells. NGP cells underwent a G_1 arrest (Figure 3.12e) following MDM2-p53 antagonist treatment (SCR control), but not following MYCN knockdown alone (0μM). LS cells did not undergo a G_1 arrest following MDM2-p53 antagonist treatment but arrested after MYCN knockdown (Figure 3.13c). LAN5 cells did not G_1 arrest following neither MDM2-p53 antagonist treatment nor MYCN knockdown (Figure 3.14c) and TR14 cells underwent a slight G_1 arrest upon both Nutlin-3 or MI-63 treatment and MYCN knockdown (Figure 3.15c). This data suggests that upon MDM2-p53 antagonist treatment, or MYCN knockdown, neuroblastoma cell lines have a very varied cell cycle response. However, despite these varied responses, knockdown of MYCN did not influence the response to Nutlin-3 or MI-63, with each cell line responding in the same manner as with the SCR control.

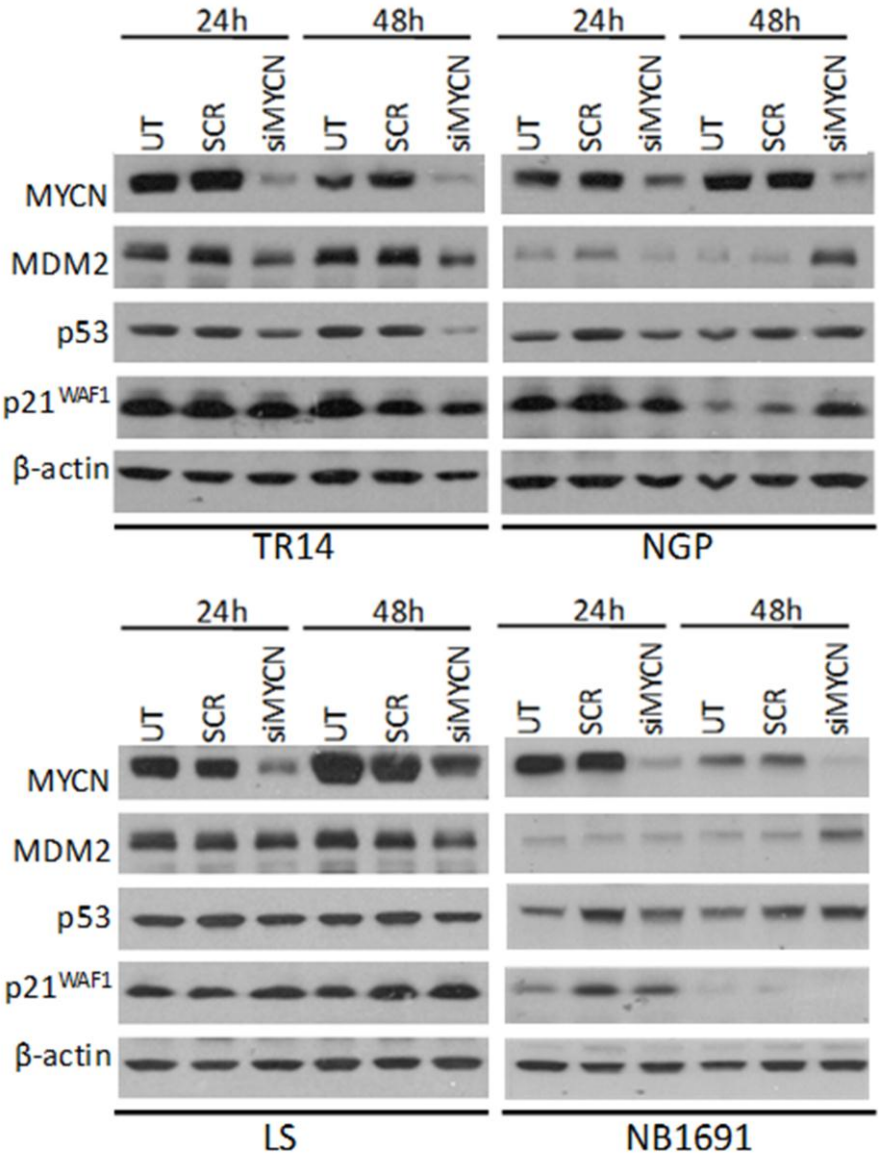
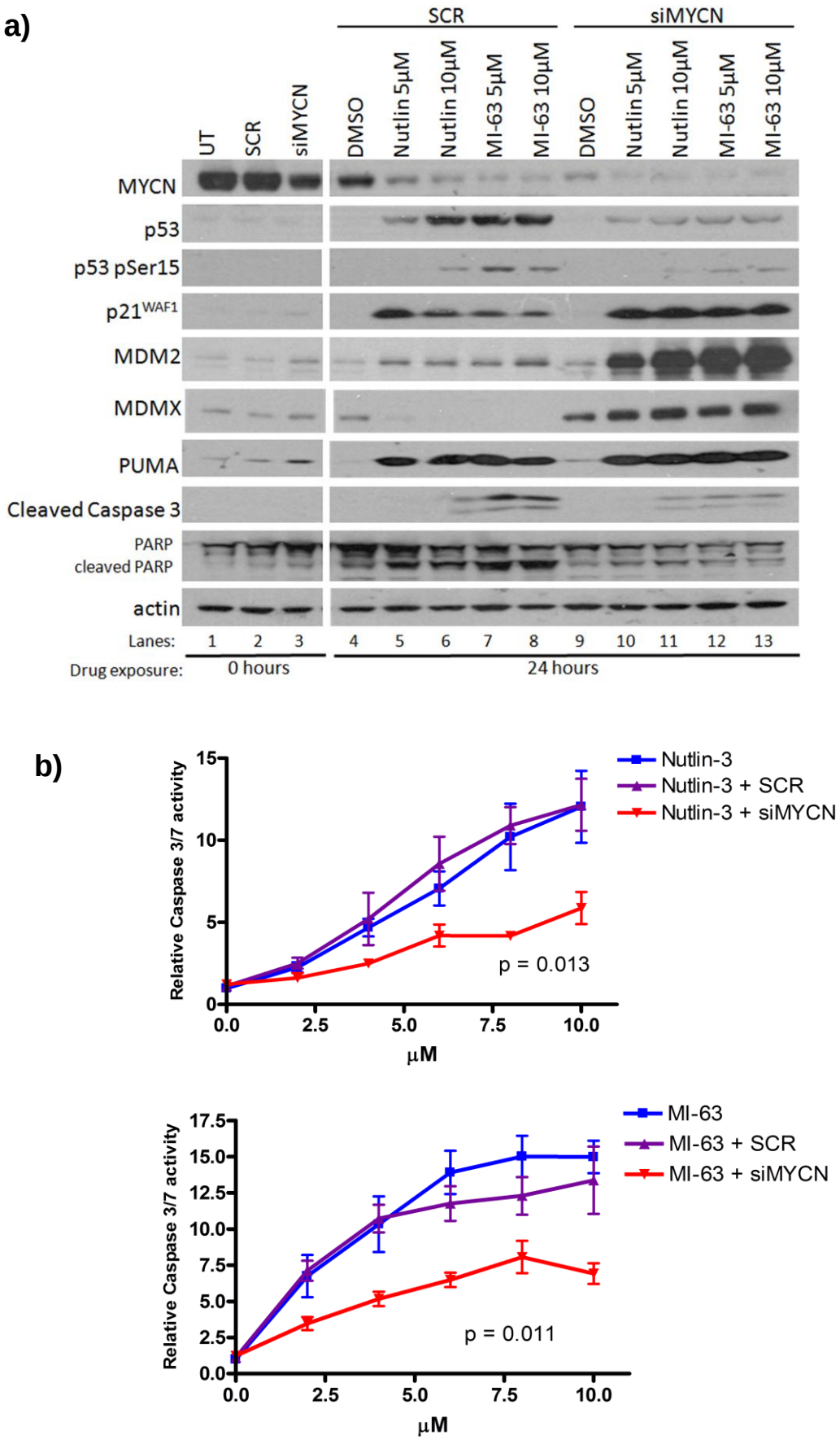
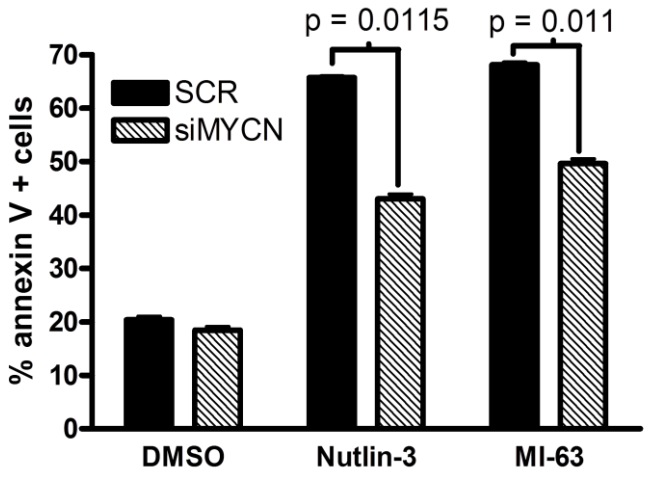


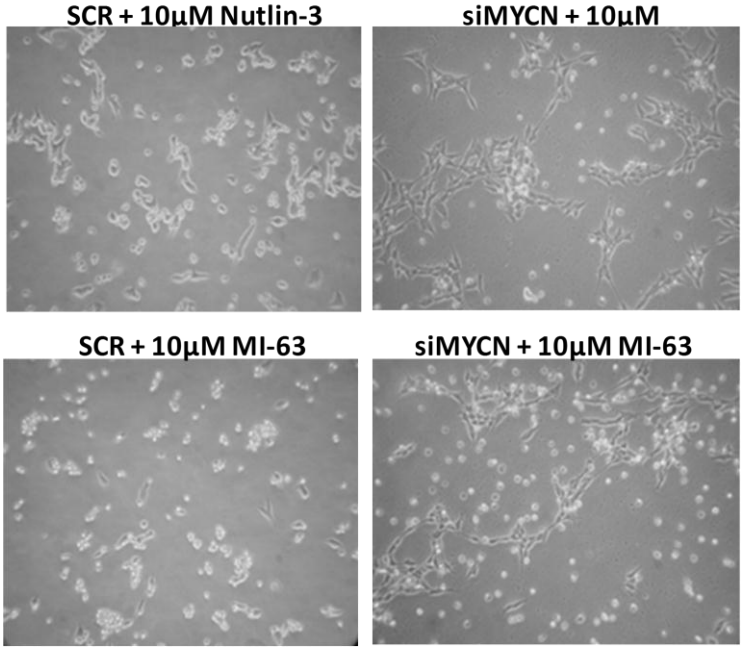
Figure 3.11. **siRNA mediated knockdown of MYCN has little effect on p53 and p53 responsive genes.** MYCN was knocked down in TR14, NGP, LS and NB1691 cells for 24 and 48 hours.


a)
SCR
siMYCN
UT
SCR
siMYCN
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
MYCN
p53
p53 pSer15
p21WAF1
MDM2
MDMX
PUMA
Cleaved Caspase 3
PARP
cleaved PARP
actin
Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13
Drug exposure: 0 hours 24 hours
b)
Relative Caspase 3/7 activity
μM
Nutlin-3
Nutlin-3 + SCR
Nutlin-3 + siMYCN
p = 0.013
MI-63
MI-63 + SCR
MI-63 + siMYCN
p = 0.011

c)


p = 0.0115
p = 0.011
SCR
siMYCN
% annexin V + cells
70
60
50
40
30
20
10
0
DMSO
Nutlin-3
MI-63

d)


SCR + 10µM Nutlin-3
siMYCN + 10µM
SCR + 10µM MI-63
siMYCN + 10µM MI-63

e)

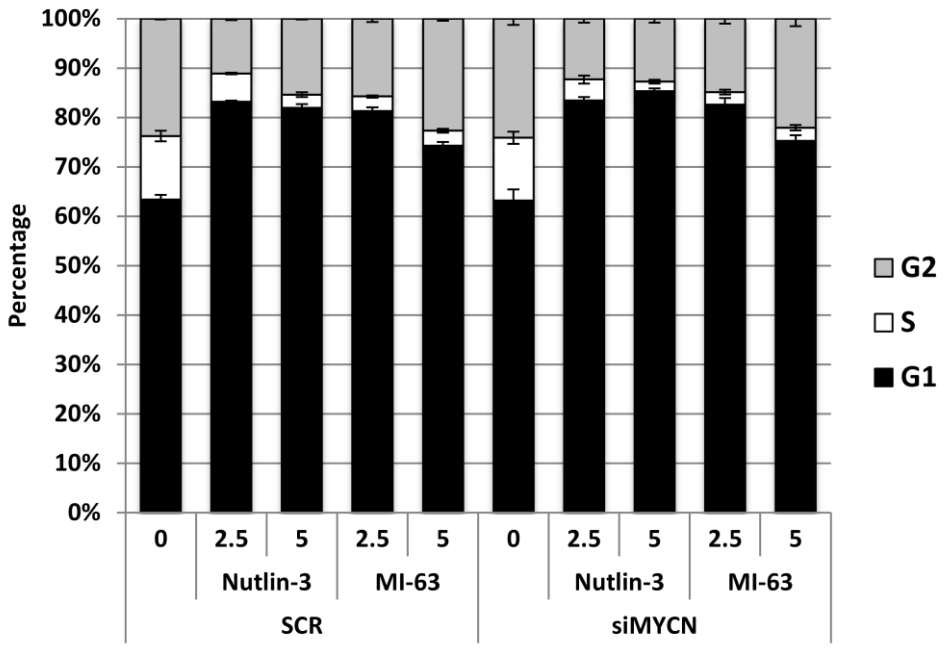
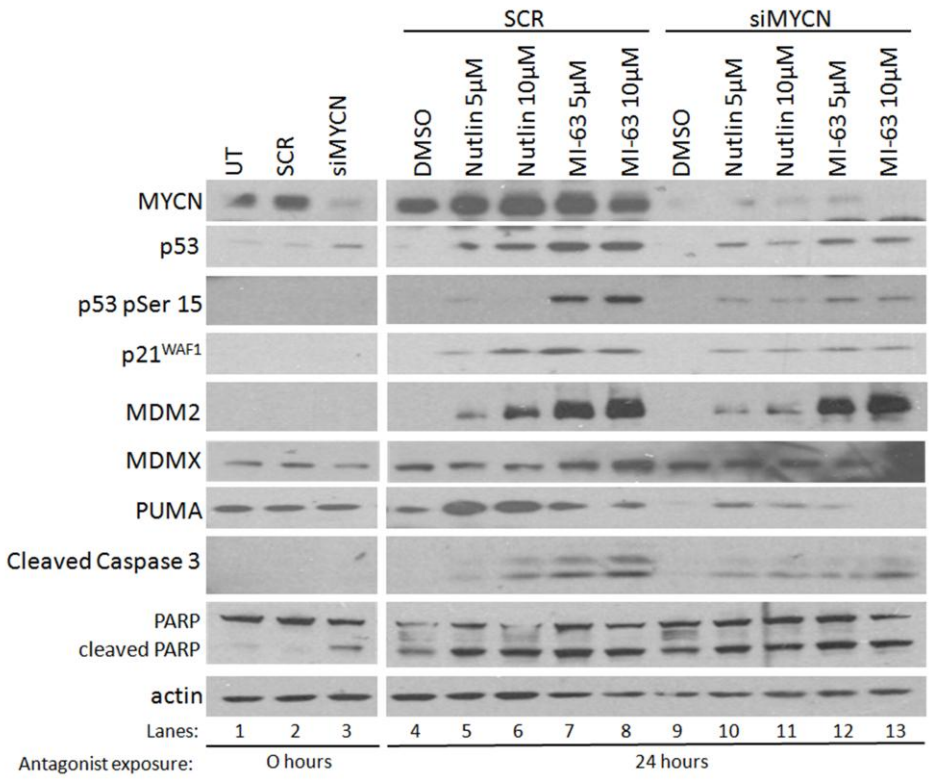
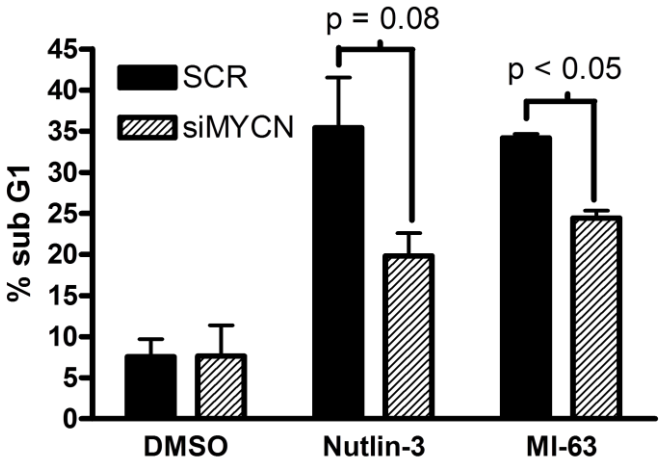

Percentage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
2.5
5
2.5
5
0
2.5
5
2.5
5
Nutlin-3
MI-63
Nutlin-3
MI-63
SCR
siMYCN
G2
S
G1

Figure 3.12. **MYCN knockdown using siRNA in the NGP *MYCN*-amplified neuroblastoma cell line. a)** Western blot showing induction of p53, p53 target genes and apoptotic markers following MYCN knockdown compared to SCR control at 0 hours (before Nutlin-3/MI-63 treatment) and 24 hours after Nutlin-3/MI-63 treatment. Actin was used as a loading control**. b)** Caspase 3/7 activity following MYCN knockdown in combination with Nutlin-3 or MI-63 treatment compared to SCR control and Nutlin-3 only (Nutlin-3, p=0.013; MI-63 p=0.011; 2-way ANOVA). **c)** Annexin V staining following 24 hours 10μM Nutlin-3 or MI-63 treatment after MYCN knockdown (Nutlin-3, p=0.015; MI-63, p=0.011; paired t-test). **d)** Photomicrographs of cells treated with 10μM Nutlin-3 or MI-63 following MYCN knockdown or SCR control**. e)** Cell cycle response following MYCN knockdown and 24 hour antagonist treatment.

a)


SCR
siMYCN
UT
SCR
siMYCN
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
MYCN
p53
p53 pSer 15
p21WAF1
MDM2
MDMX
PUMA
Cleaved Caspase 3
PARP
cleaved PARP
actin
Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13
Antagonist exposure:
O hours
24 hours

b)


p = 0.08
p < 0.05
SCR
siMYCN
% sub G1
45
40
35
30
25
20
15
10
5
0
DMSO
Nutlin-3
MI-63

c)

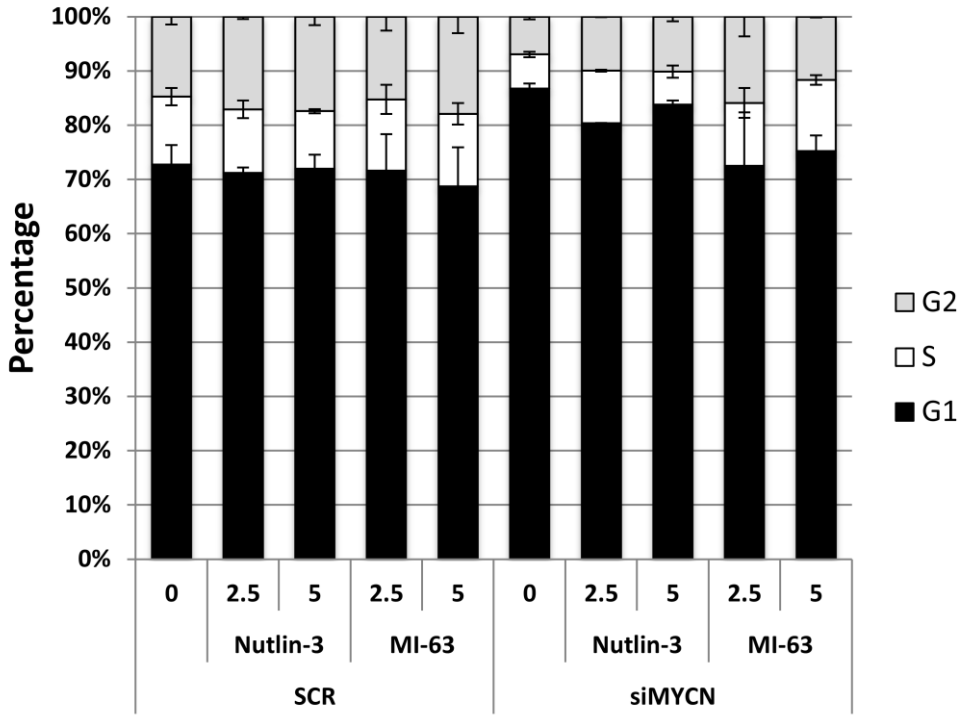
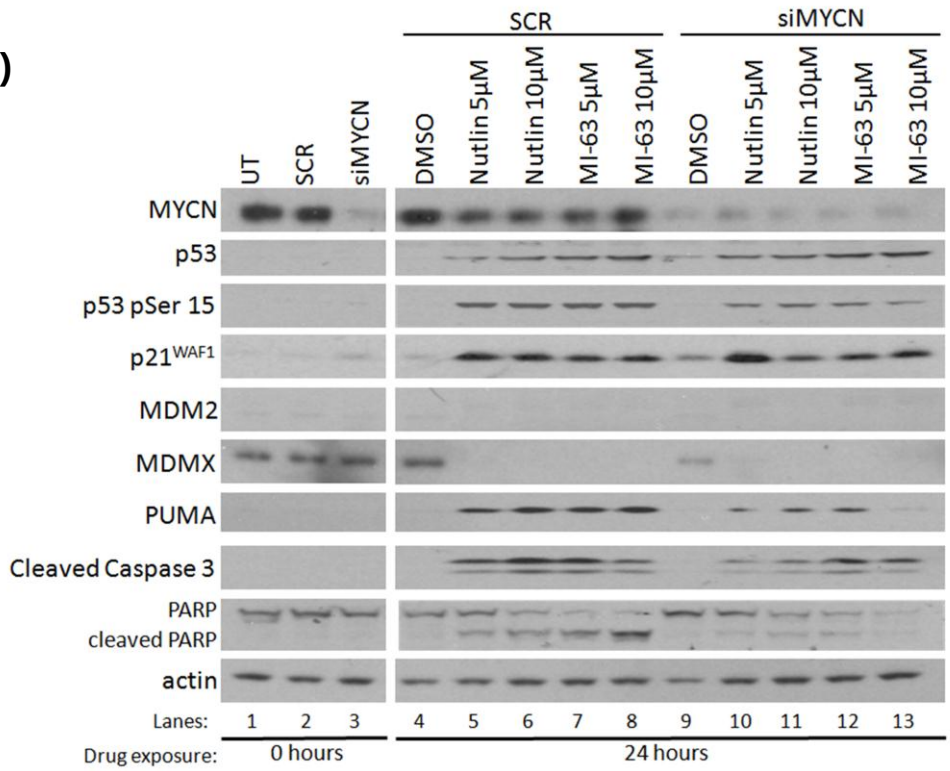
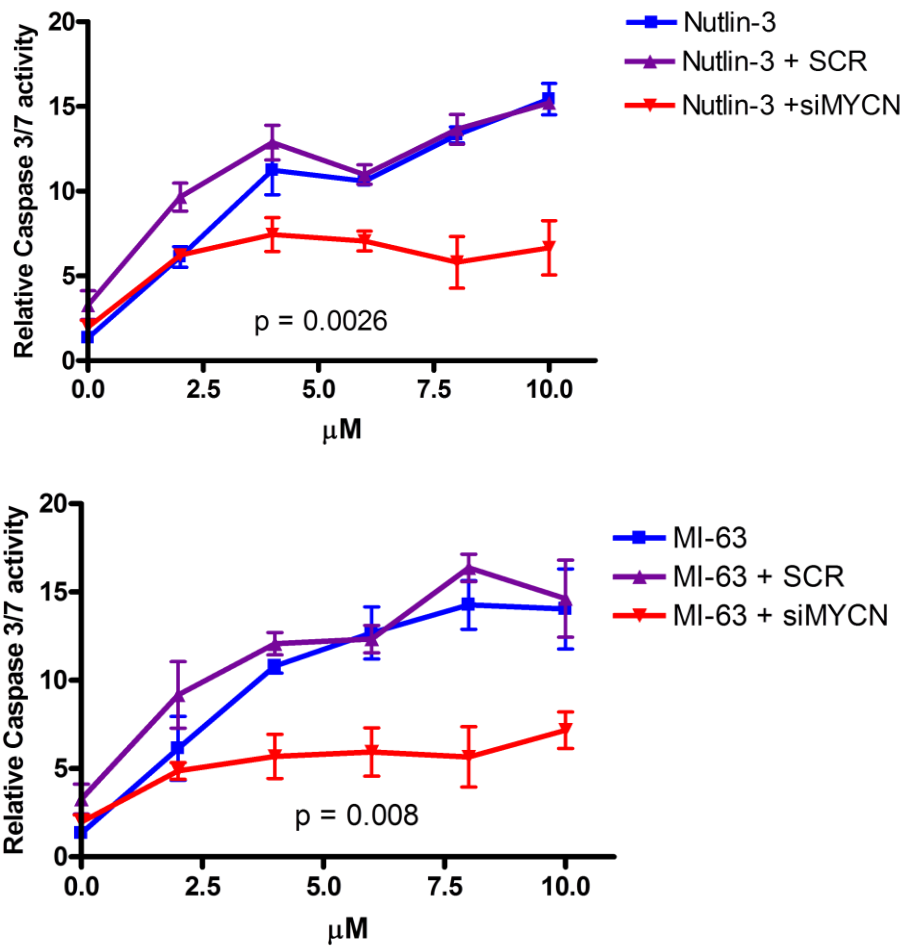


Figure 3.13. **MYCN knockdown using siRNA in the LS *MYCN*-amplified neuroblastoma cell line. a)** Western blot showing induction of p53, p53 target genes and apoptotic markers following MYCN knockdown compared to SCR control at 0 hours (before Nutlin-3/MI-63 treatment) and 24 hours after Nutlin-3/MI-63 treatment. Actin was used as a loading control. **b)** Percentage sub G_1 DNA as determined by FACs analysis following 10μM Nutlin-3 or MI-63 treatment for 24 hours (Nutlin-3, $p=0.08$; MI-63, $p<0.05$; two-tailed unpaired t-test). **c)** Cell cycle response following MYCN knockdown and 24 hour antagonist treatment.

a)


SCR
siMYCN
UT
SCR
siMYCN
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
MYCN
p53
p53 pSer 15
p21WAF1
MDM2
MDMX
PUMA
Cleaved Caspase 3
PARP
cleaved PARP
actin
Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13
Drug exposure: 0 hours 24 hours

b)


Relative Caspase 3/7 activity
20
15
10
5
0
0.0
2.5
5.0
7.5
10.0
μM
p = 0.0026
Nutlin-3
Nutlin-3 + SCR
Nutlin-3 +siMYCN
Relative Caspase 3/7 activity
20
15
10
5
0
0.0
2.5
5.0
7.5
10.0
μM
p = 0.008
MI-63
MI-63 + SCR
MI-63 + siMYCN

c)

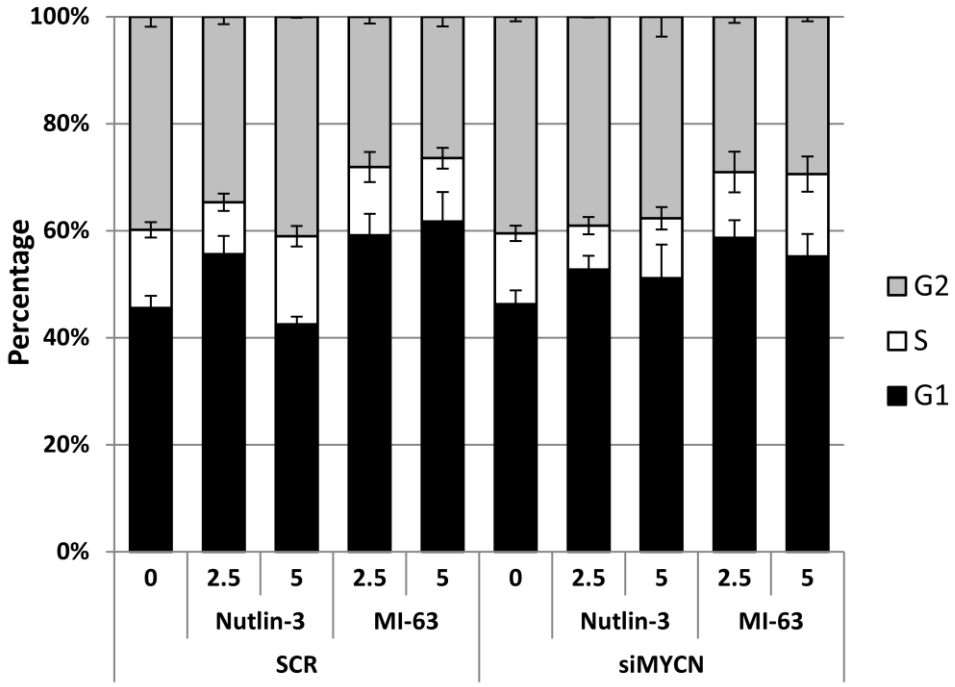
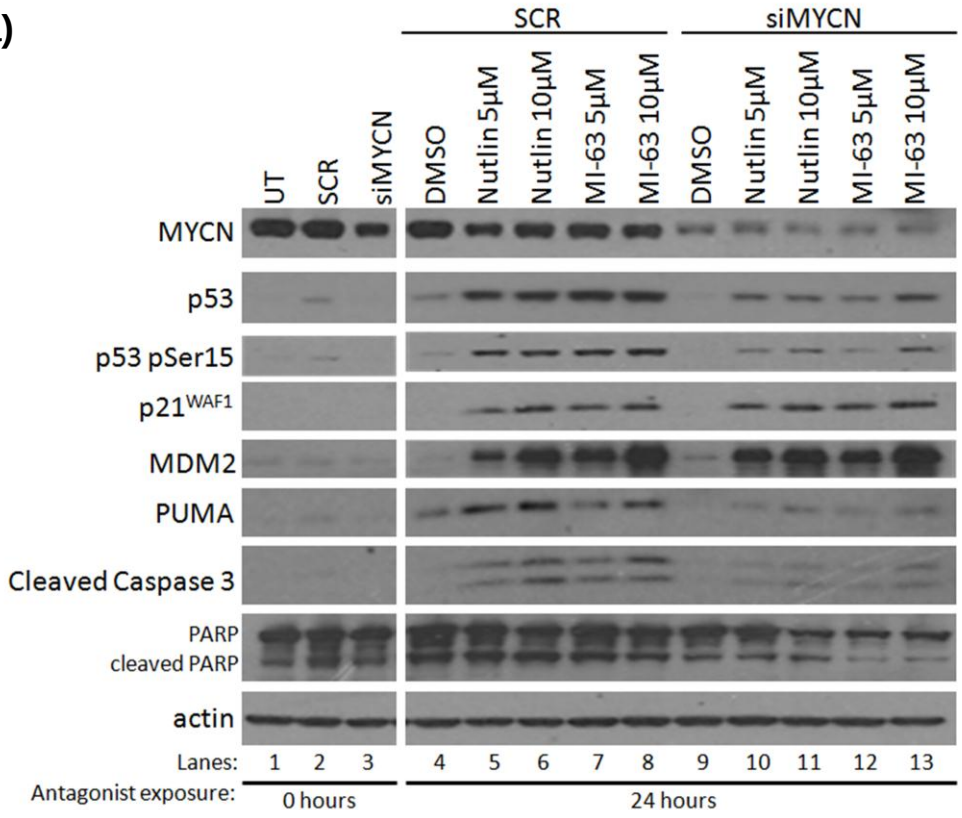
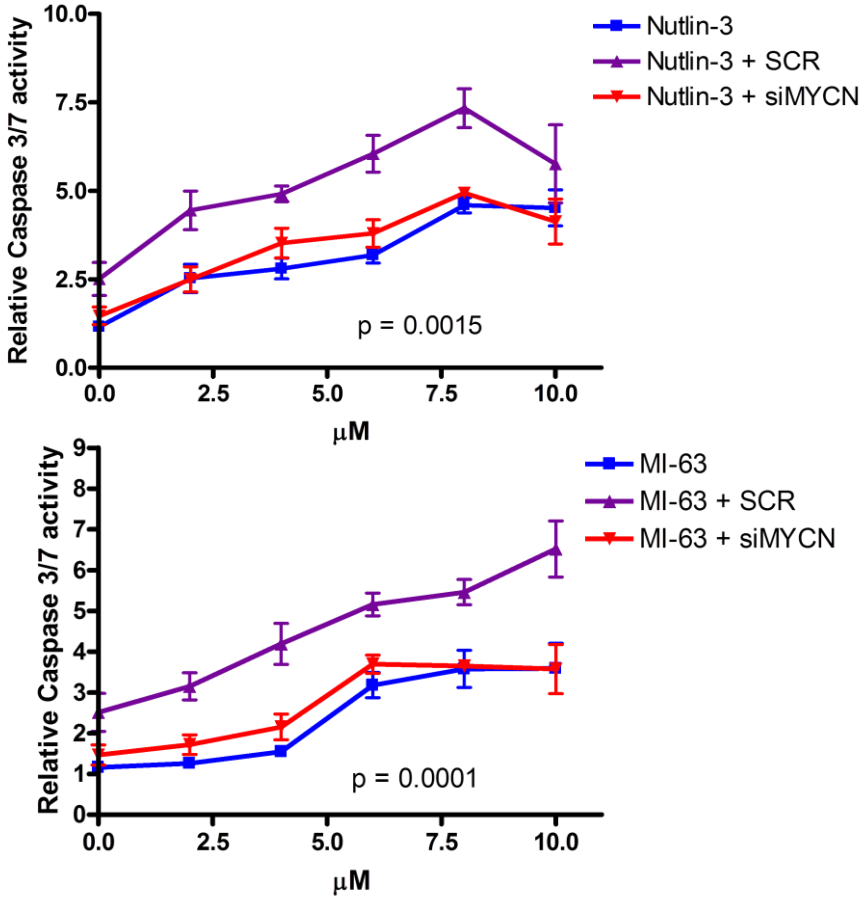


Figure 3.14. **MYCN knockdown using siRNA in the LAN5 *MYCN*-amplified neuroblastoma cell line. a)** Western blot showing induction of p53, p53 target genes and apoptotic markers following MYCN knockdown compared to SCR control at 0 hours (before Nutlin-3/MI-63 treatment) and 24 hours after Nutlin-3/MI-63 treatment. Actin was used as a loading control. **b)** Caspase 3/7 activity following MYCN knockdown in combination with Nutlin-3 or MI-63 treatment compared to SCR control and Nutlin-3 only (Nutlin-3, $p = 0.0026$; MI-63, $p = 0.008$; 2-way ANOVA). **c)** Cell cycle response following MYCN knockdown and 24 hour antagonist treatment.

a)


SCR
siMYCN
UT
SCR
siMYCN
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
DMSO
Nutlin 5μM
Nutlin 10μM
MI-63 5μM
MI-63 10μM
MYCN
p53
p53 pSer15
p21WAF1
MDM2
PUMA
Cleaved Caspase 3
PARP
cleaved PARP
actin
Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13
Antagonist exposure:
0 hours
24 hours

b)


Relative Caspase 3/7 activity
10.0
7.5
5.0
2.5
0.0
0.0
2.5
5.0
7.5
10.0
μM
Nutlin-3
Nutlin-3 + SCR
Nutlin-3 + siMYCN
p = 0.0015
Relative Caspase 3/7 activity
9
8
7
6
5
4
3
2
1
0
0.0
2.5
5.0
7.5
10.0
μM
MI-63
MI-63 + SCR
MI-63 + siMYCN
p = 0.0001

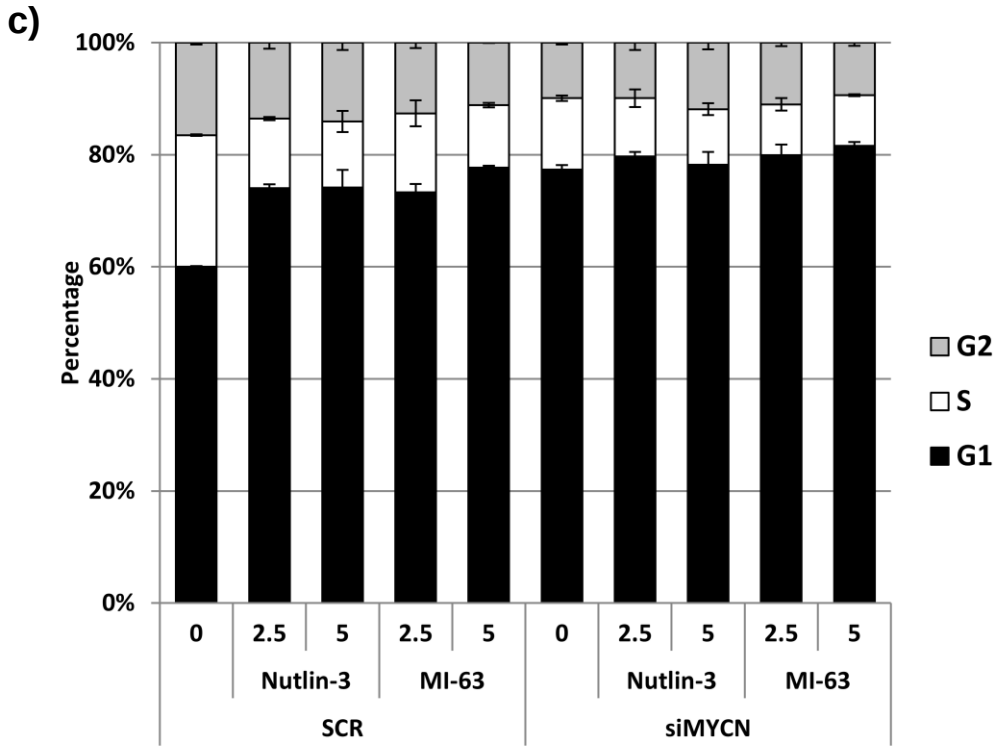


Figure 3.15. **MYCN knockdown using siRNA in the TR14 *MYCN*-amplified neuroblastoma cell line. a)** Western blot showing induction of p53, p53 target genes and apoptotic markers following MYCN knockdown compared to SCR control at 0 hours (before Nutlin-3/MI-63 treatment) and 24 hours after Nutlin-3/MI-63 treatment. Actin was used as a loading control**. b)** Caspase 3/7 activity following MYCN knockdown in combination with Nutlin-3 or MI-63 treatment compared to SCR control and Nutlin-3 only (p=0.0015 for Nutlin-3, p=0.0001 for MI-63, 2-way ANOVA). **c)** Cell cycle response following MYCN knockdown and 24 hour antagonist treatment.

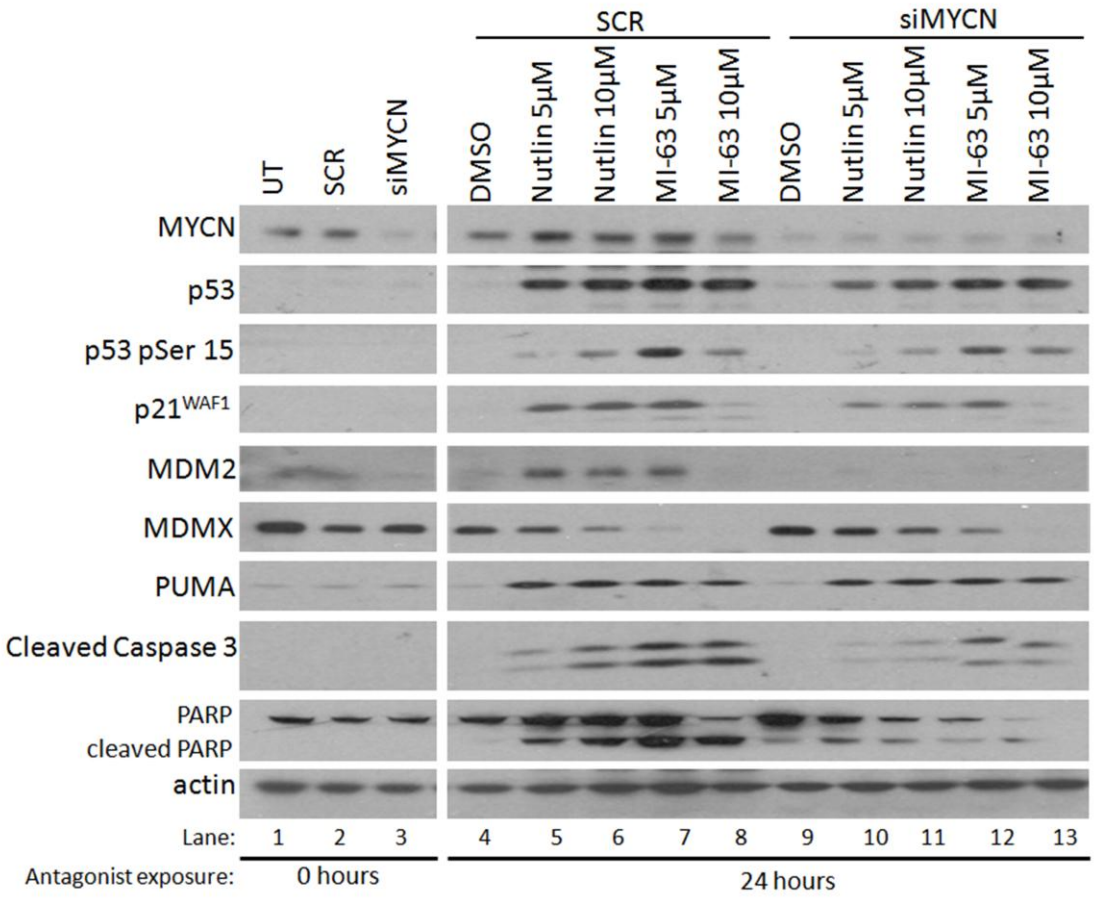


Figure 3.16. **MYCN knockdown using siRNA in the IMR32 *MYCN*-amplified neuroblastoma cell line.** Western blot showing induction of p53, p53 target genes and apoptotic markers following MYCN knockdown compared to SCR control at 0 hours (before Nutlin-3/MI-63 treatment) and 24 hours after Nutlin-3/MI-63 treatment. Actin was used as a loading control.

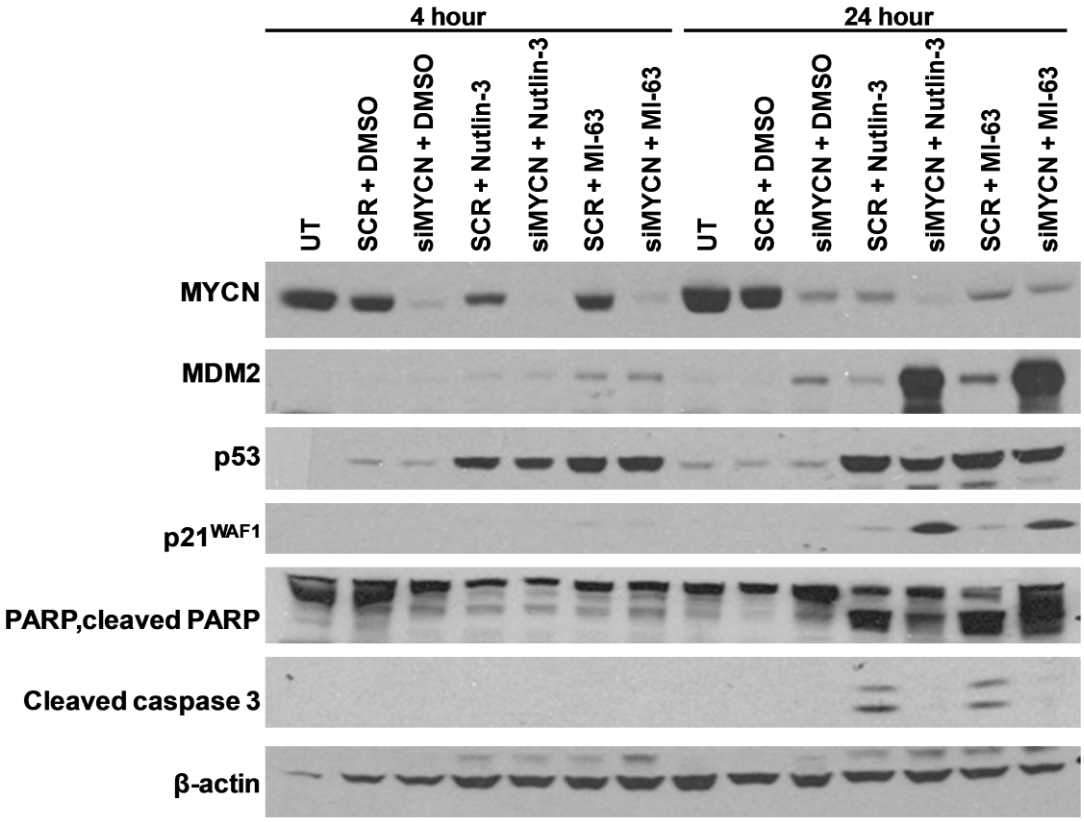


Figure 3.17. **Knockdown of MYCN at 4 and 24 hours followed by Nutlin-3 or MI-63 treatment in NGP cells.**

3.4.11 *MYCN* amplified neuroblastoma cell lines are more sensitive to MDM2-p53 antagonist mediated growth inhibition compared to non-*MYCN*-amplified neuroblastoma cell lines.

The SRB cell culture growth inhibition assay takes into account both cell cycle arrest and apoptosis, 2 important p53 responses. A panel of 12 *MYCN*-amplified (including 4 *MYCN* and *MDM2* co-amplified) and 8 non-*MYCN*-amplified neuroblastoma cell lines were investigated for their sensitivity to Nutlin-3 and MI-63 mediated growth inhibition. GI_{50} values for all p53 wildtype cell lines are shown in Table 3.2, and the growth curves they were determined from data presented in Figure 3.18.

In response to Nutlin-3 (Figure 3.19a) and MI-63 (Figure 3.20a) *MYCN*-amplified neuroblastoma cell lines underwent more growth inhibition compared to non-amplified cell lines with a significant difference in mean GI_{50}s as shown in the scatter plots (Figure 3.19b and 3.20b) ($p<0.001$ for Nutlin-3 and $p<0.05$ for MI-63). Overall a more varied response to the antagonists was seen in the non-amplified cell lines compared to *MYCN*-amplified cell lines. Interestingly, and despite being p53 wildtype the SKNRA cell line was most resistant to both Nutlin-3 and MI-63 (discussed in Chapter 5.4).

MYCN-only amplified cell lines were more sensitive to Nutlin-3 mediated growth inhibition compared to neuroblastoma cell lines co-amplified for *MYCN* and *MDM2* ($p=0.0095$) (Figure 3.19c) and although this difference did not reach statistical significance, the same trend was observed for MI-63 ($p = 0.0667$) (Figure 3.20c). A number of cell lines in this panel had impaired $p14^{ARF}$ function; PER-108 (methylated) and GIMEN (methylated), SHEP (homozygous deletion) and LAN-6 (homozygous deletion) (Carr et al., 2006). There appeared to be a varied response to MDM2-p53 antagonist mediated growth inhibition with no evidence that $p14^{ARF}$ status affects the response to these compounds (figure 3.19a and 3.20a).

3.4.12 p53 mutant cells lines are resistant to MDM2-p53 antagonists regardless of MYCN status

Two *MYCN*-amplified neuroblastoma cell lines, (SKNBe2C (Be2C) and IGRN91), a non-*MYCN*-amplified neuroblastoma cell line (SKNAS) and a non-*MYCN*-amplified PNET cell line (NB100), all mutant for p53, were investigated for their sensitivity to Nutlin-3 and MI-63. As shown in Figure 3.21, p53 mutant cell lines were highly resistant to these compounds regardless of *MYCN* status. 50% growth inhibition was not

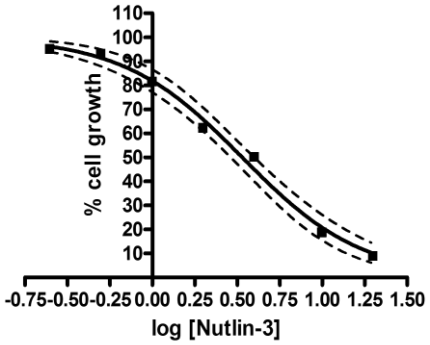
achieved in these cell lines with the highest concentrations of Nutlin-3 (20µM) and MI-63 (10µM) used to generate GI_{50} values in the other p53 wildtype cell lines tested.

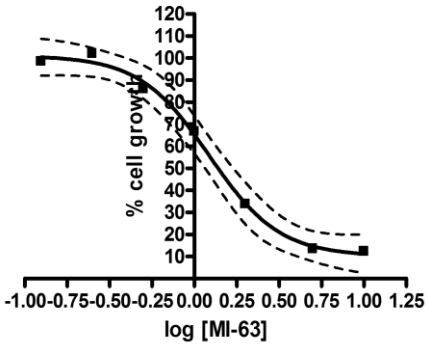
3.4.13 *MYCN*-amplified neuroblastoma cell lines are more sensitive to MDM2-p53 antagonist mediated apoptosis compared to non-*MYCN*-amplified neuroblastoma cell lines

As a marker of apoptosis, caspase 3/7 activity following Nutlin-3 or MI-63 treatment was determined in the same panel of neuroblastoma cell lines that were assessed for growth inhibition. In response to 5µM Nutlin-3 (Figure 3.22a) and 2.5µM MI-63 (Figure 3.23a), *MYCN*-amplified neuroblastoma cell lines showed higher mean caspase 3/7 activity compared to non-*MYCN*-amplified cell lines (Nutlin-3, p=0.0343; MI-63 p=0.0111) (Figure 3.22b and 3.23b). Interestingly, despite no obvious resistance to growth inhibition, $p14^{ARF}$ impaired cell lines, particularly those that are not *MYCN*-amplified, were especially resistant to MDM2-p53 antagonist mediated activation of caspase 3/7 activity (Nutlin-3, p = 0.0093; MI-63 p = 0.0078), which was comparable to that of p53 mutant cell lines (Figure 3.22a and 3.23a). The sub population of non-*MYCN* amplified cells in Figure 3.22b and 3.23b with very low caspase activity are infact 3 of the 4 $p14^{ARF}$ impaired cell lines; LAN6, SHEP and GIMEN, in addition to the p14ARF normal, p53 wildtype SKNRA cell line.

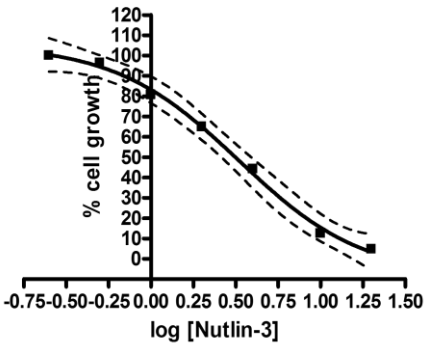
In addition the *MYCN* and *MDM2* co-amplified cell lines displayed increased caspase 3/7 activity compared to other cell lines, and this was significant for MI-63 (Nutlin-3, p = 0.0998; MI-63, p = 0.0169) (Figure 3.22d and 3.23d). They did not however, have significantly increased caspase activity compared to *MYCN*-amplified only cell lines (p = 0.48 for Nutlin-3, p=0.11 for MI-63, Mann-Whitney test). These *MYCN* and *MDM2* co-amplified cell lines have previously been reported to express high levels of $p14^{ARF}$ mRNA and protein (Carr et al., 2006).

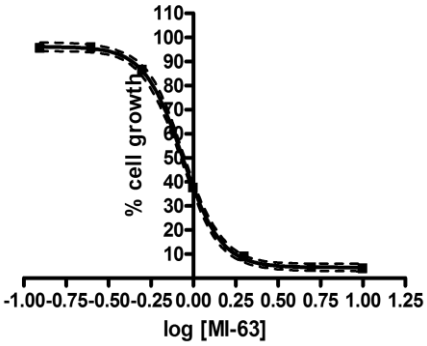
NGP





LS





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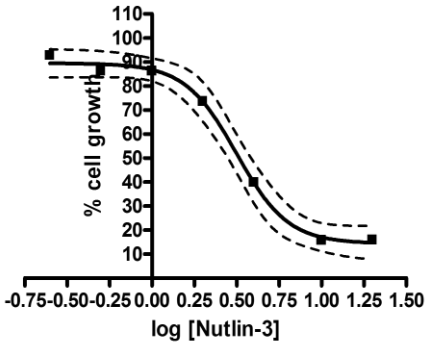

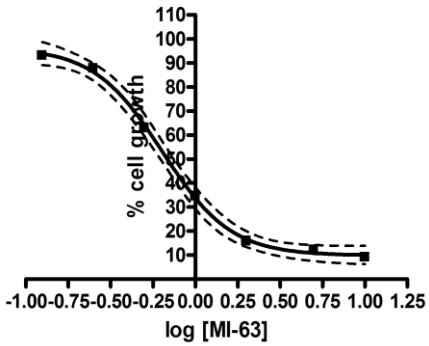

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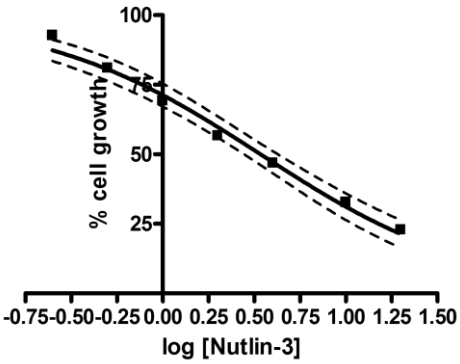

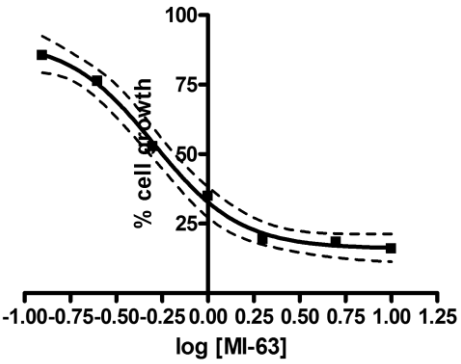

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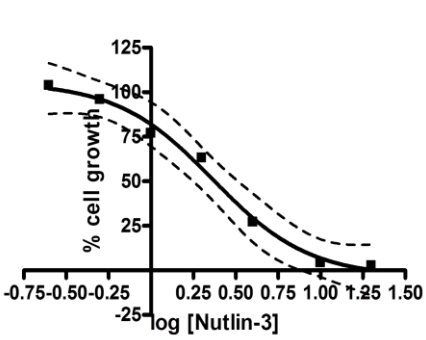

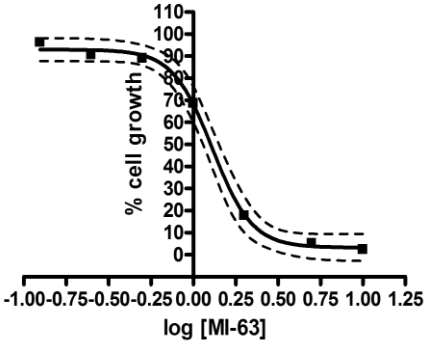

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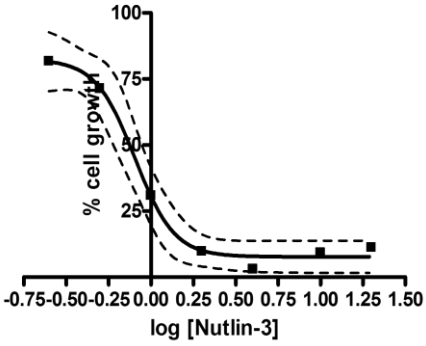

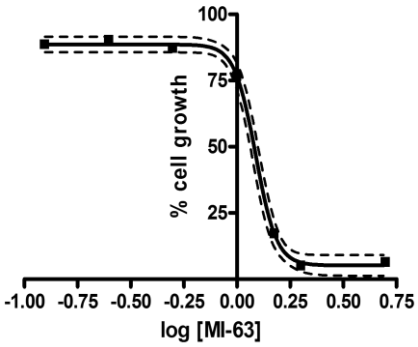

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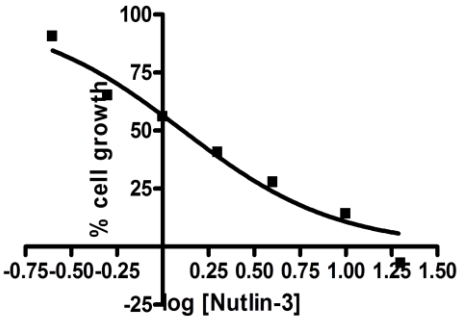

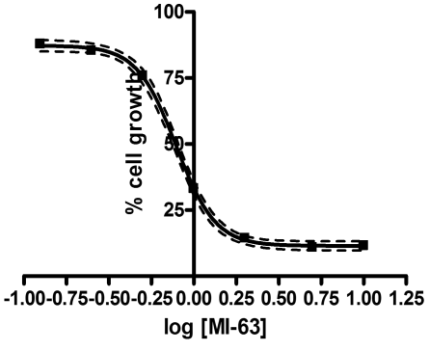

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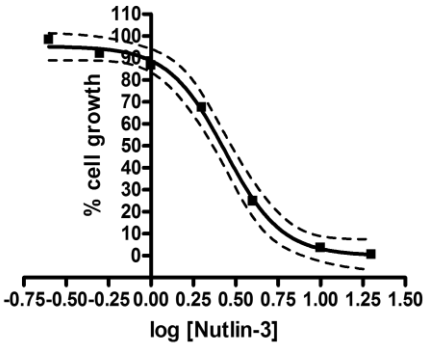

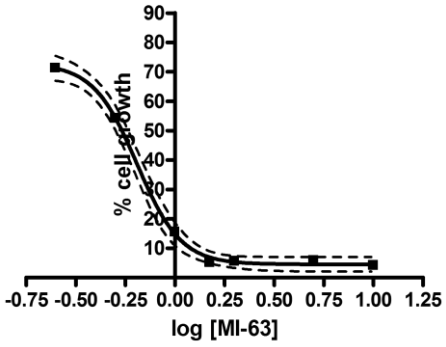

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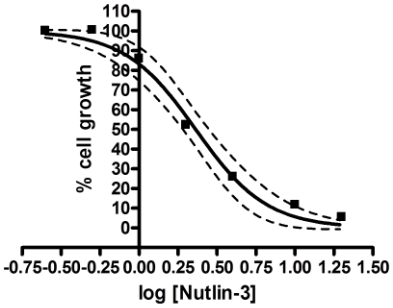

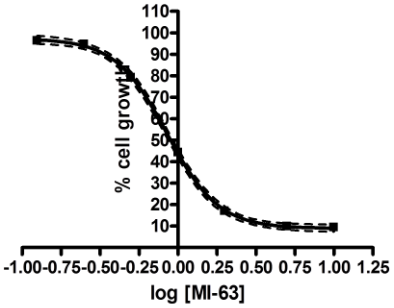

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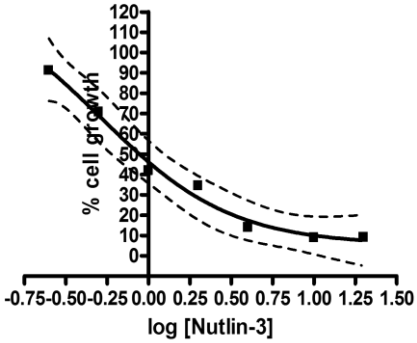

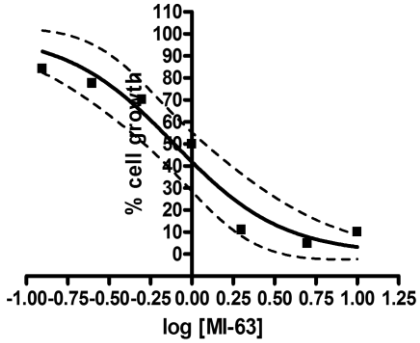

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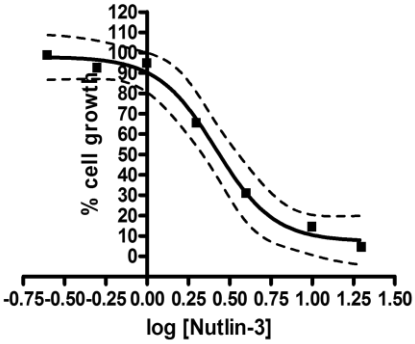

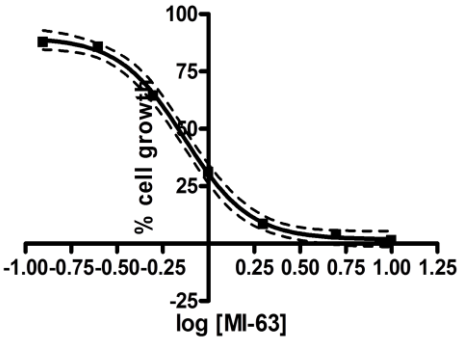

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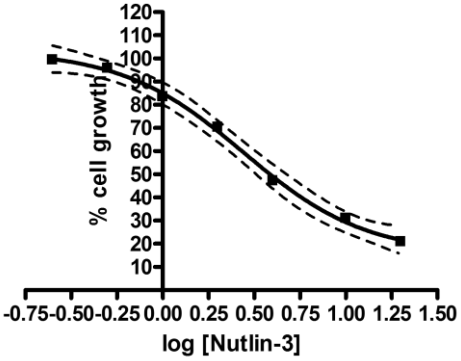

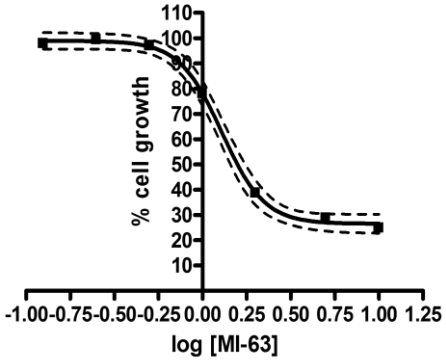

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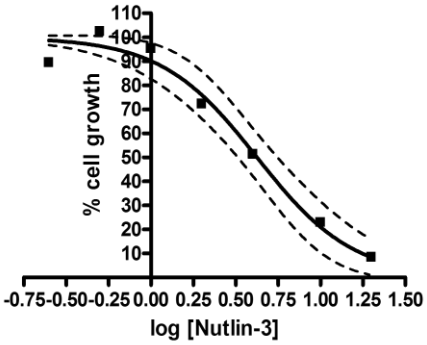

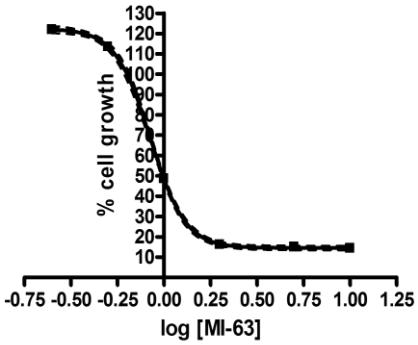

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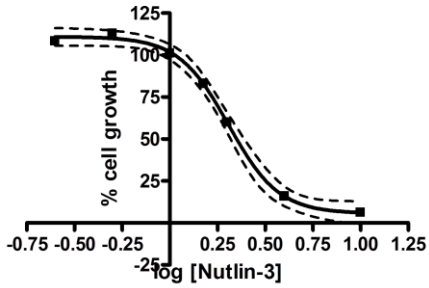

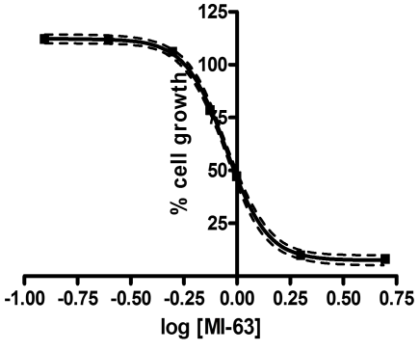

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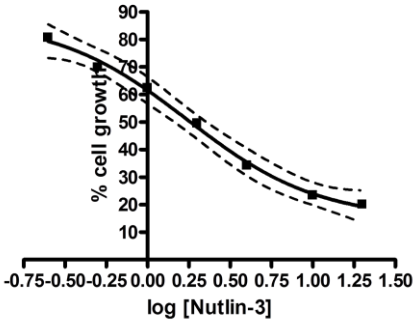

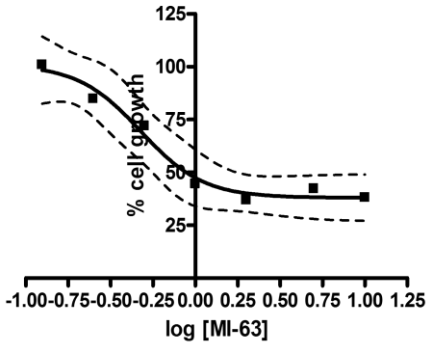

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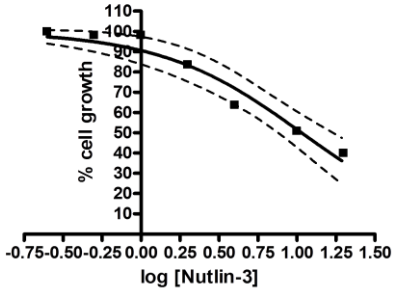

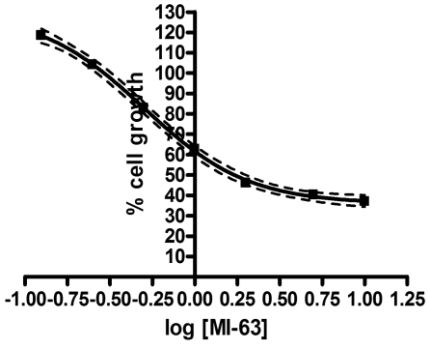

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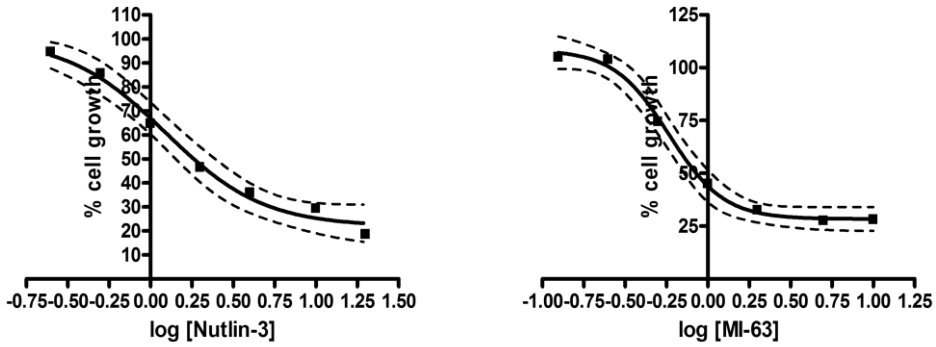

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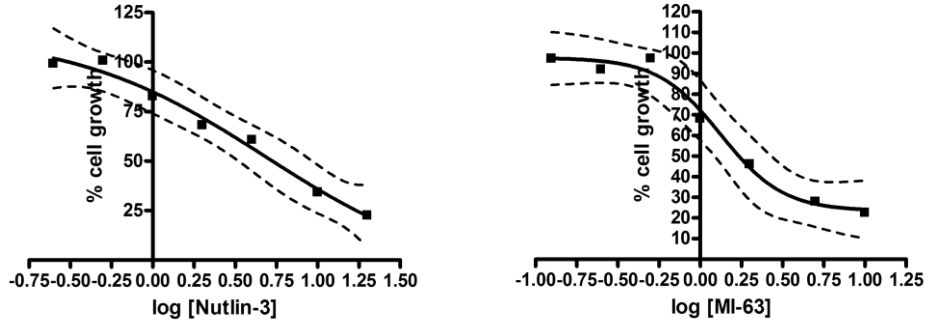


Figure 3.18. **Concentration dependent growth inhibition curves for the panel of *MYCN*-amplified and non-*MYCN*-amplified neuroblastoma cell lines used to generate GI_{50} values.**

Cell Line	*MYCN* status	*MDM2* status	GI_{50} (μM)	
			Nutlin-3	MI-63
NGP	amp	amp	2.53 ± 0.43	1.21 ± 0.04
LS	amp	amp	2.95 ± 0.12	0.98 ± 0.06
NB1691	amp	amp	2.80 ± 0.17	0.87 ± 0.22
TR14	amp	amp	2.91 ± 0.28	1.09 ± 0.25
IMR32	amp	non-amp	2.53 ± 0.20	1.00 ± 0.29
NBLW	amp	non-amp	0.74 ± 0.07	0.85 ± 0.21
SMSKCNR	amp	non-amp	1.18 ± 0.08	0.74 ± 0.05
LAN5	amp	non-amp	1.52 ± 0.21	0.90 ± 0.16
PER108	amp	non-amp	1.64 ± 0.29	0.85 ± 0.09
CHLA136	amp	non-amp	0.64 ± 0.12	0.83 ± 0.03
SHSY5Y	non-amp	non-amp	3.85 ± 0.98	2.01 ± 0.73
GIMEN	non-amp	non-amp	4.61 ± 0.99	1.81 ± 0.45
SJNB1	non-amp	non-amp	4.32 ± 0.10	1.27 ± 0.20
NB69	non-amp	non-amp	1.77 ± 0.12	0.72 ± 0.06
LAN6	non-amp	non-amp	2.97 ± 0.75	2.28 ± 0.62
SKNRA	non-amp	non-amp	9.85 ± 0.61	4.63 ± 0.79
SHEP	non-amp	non-amp	3.92 ± 0.61	0.86 ± 0.14
NBLS	non-amp	non-amp	3.08 ± 0.68	1.25 ± 0.24
MYCN + (Tet21N)		non-amp	3.33 ± 0.74	1.08 ± 0.14
MYCN – (Tet21N)		non-amp	13.76 ± 2.57	6.56 ± 1.18

Table 3.2. **Summary of GI_{50} values for Nutlin-3 and MI-63 in 18 p53 wild-type neuroblastoma cell lines of varying *MYCN* and *MDM2* amplification status, and the Tet21N conditional MYCN expression system.** amp - amplified, non-amp – non-amplified**.**

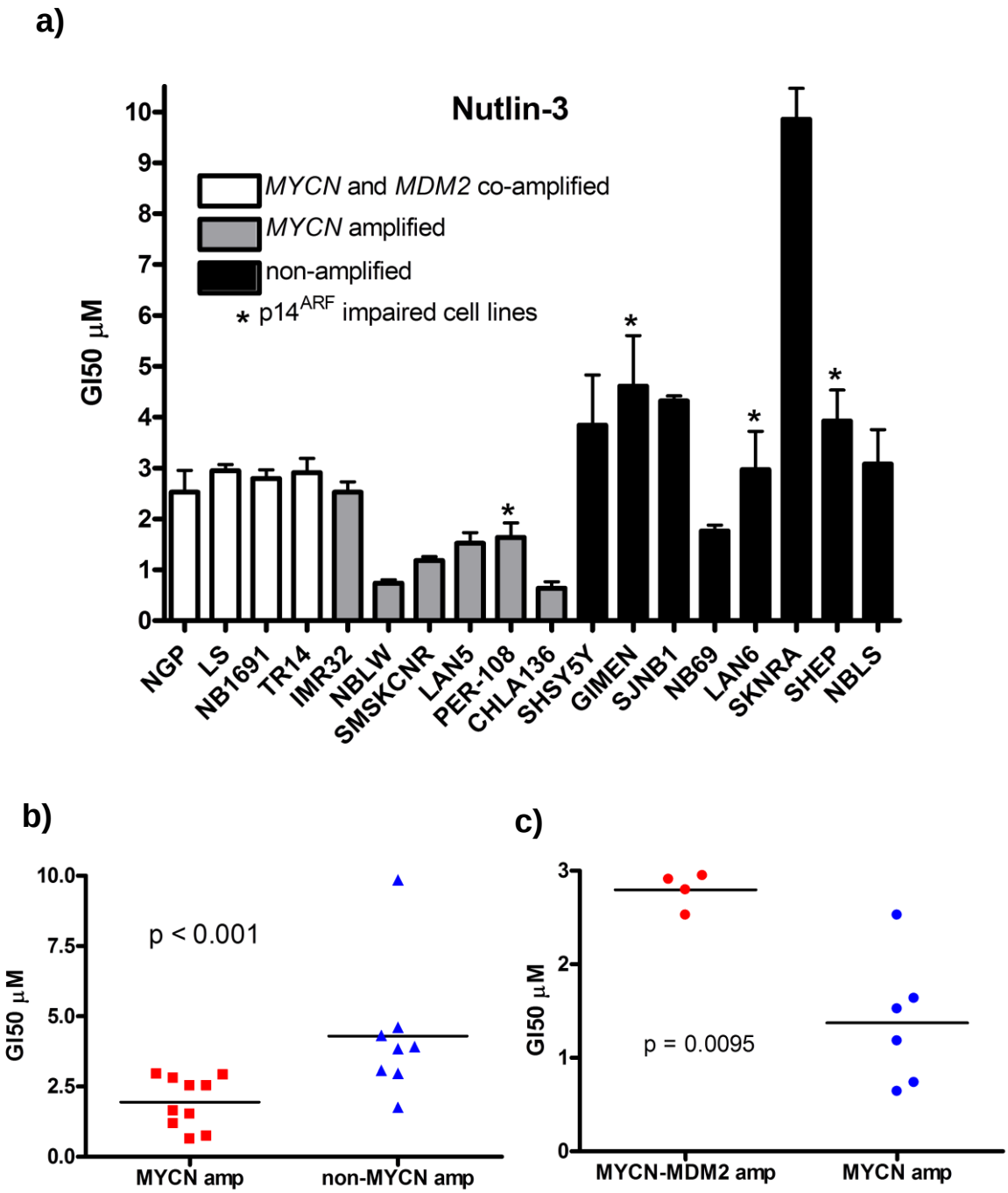


Figure 3.19. **Comparison of GI_{50} values in *MYCN*-amplified compared to non-*MYCN*-amplified cell lines following Nutlin-3 treatment. a)** GI_{50} values in a panel of p53 wildtype neuroblastoma cell lines with *MYCN*-amplification, *MDM2*-amplification and *p14*^{ARF} impairment**. b)** Scatter plot of *MYCN*-amplified vs. non-*MYCN*-amplified cell lines ($p<0.05$, Mann-Whitney test). **c)** Scatter plot of *MYCN* and *MDM2* co-amplified cell lines compared to *MYCN*-amplified cell lines ($p = 0.0095$, Mann-Whitney test).

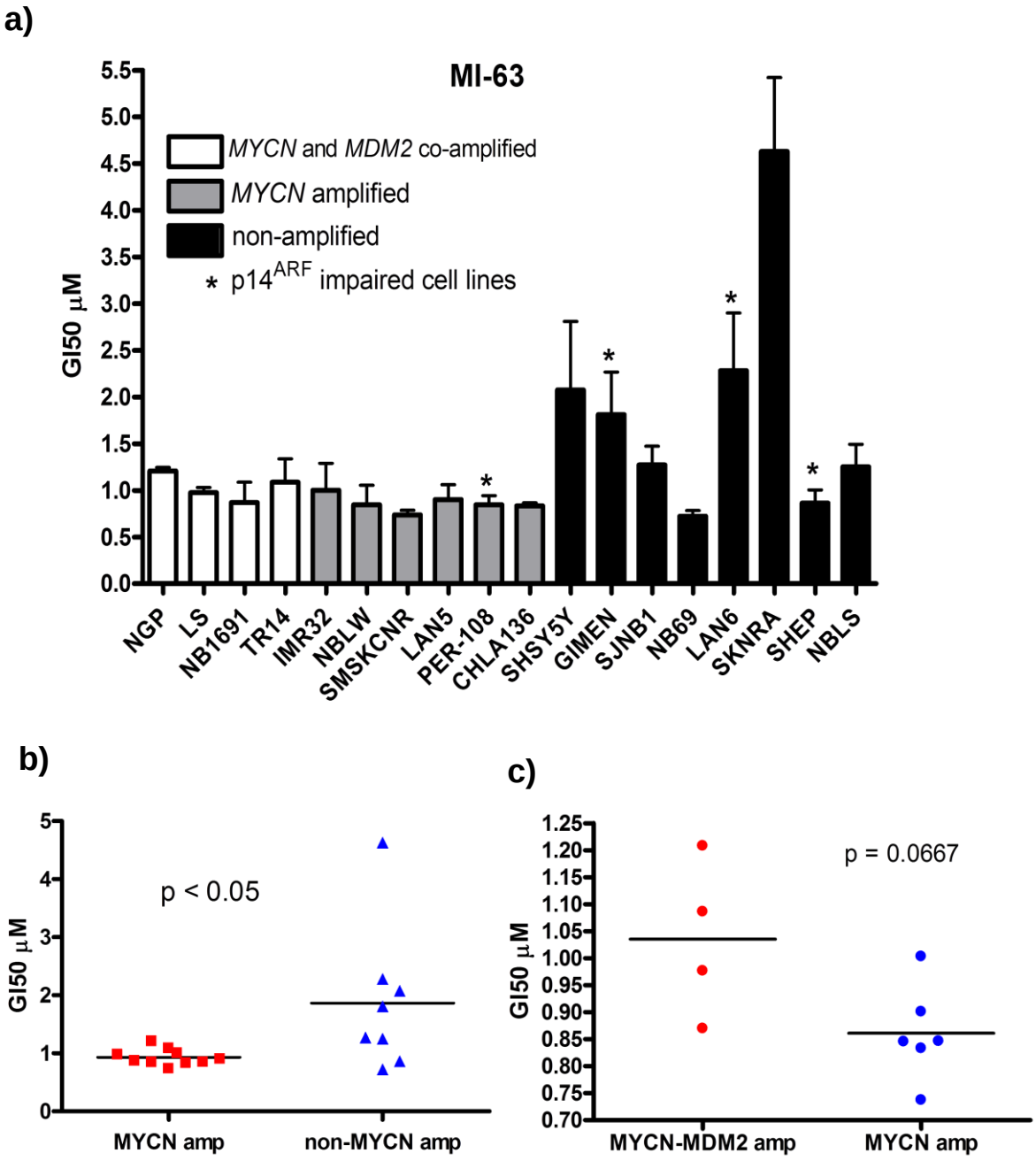


Figure 3.20. **Comparison of GI_{50} values in *MYCN*-amplified compared to non-*MYCN*-amplified cell lines following MI-63 treatment. a)** GI_{50} values in a panel of p53 wildtype neuroblastoma cell lines with *MYCN*-amplification, *MDM2*-amplification and *p14*^{ARF} impairment. **b)** Scatter plot of *MYCN*-amplified vs. non-*MYCN*-amplified cell lines ($p < 0.05$, Mann-Whitney test). **c)** Scatter plot of *MYCN* and *MDM2* co-amplified cell lines compared to *MYCN*-only amplified cell lines ($p = 0.0667$, Mann-Whitney test).

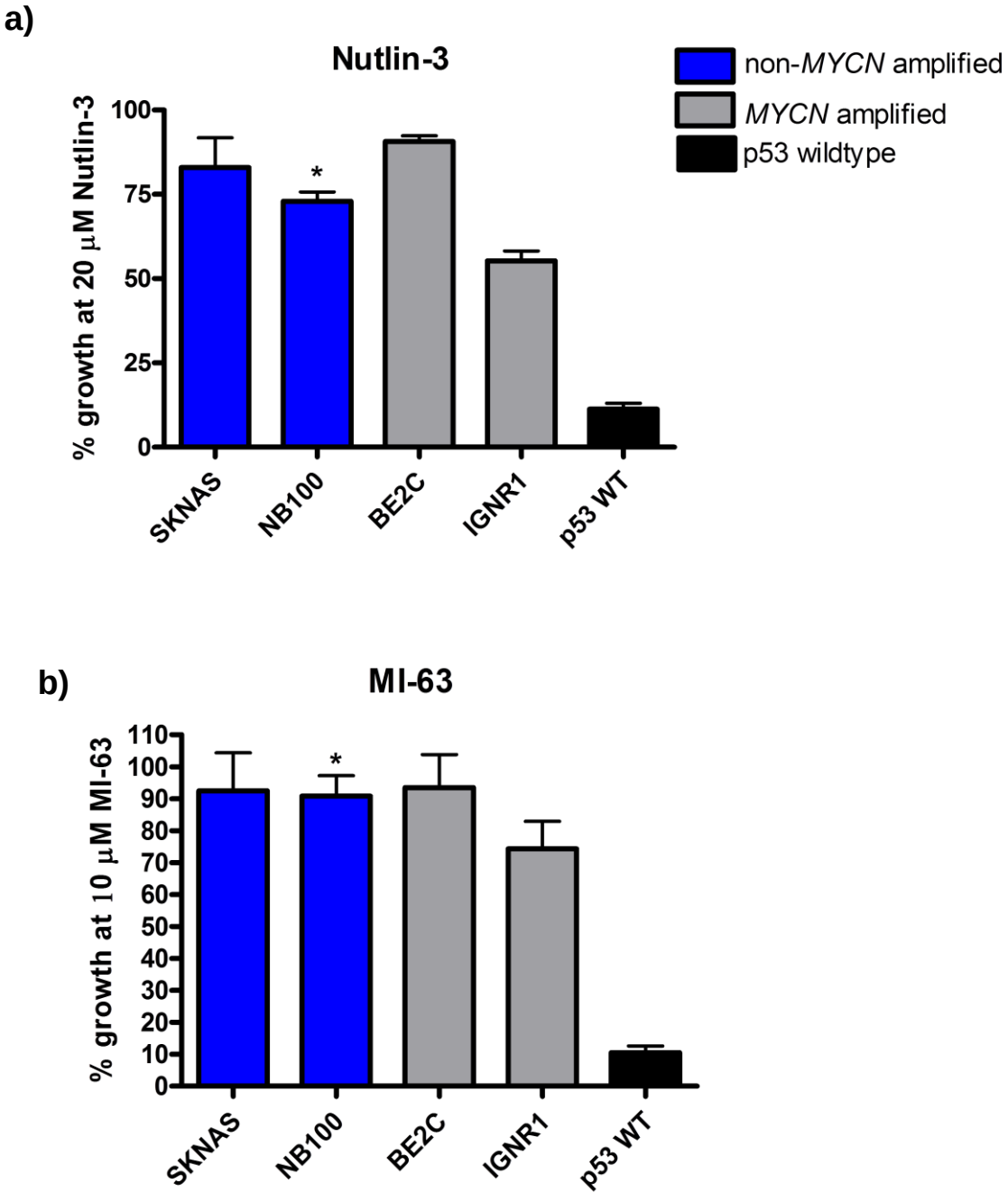
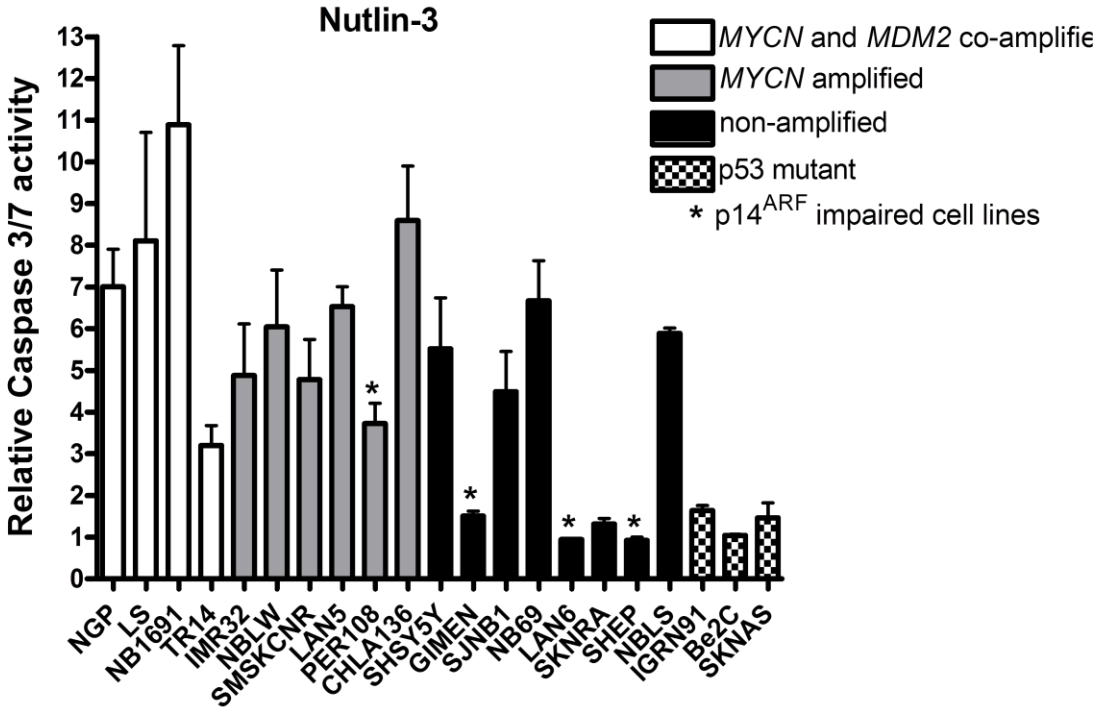
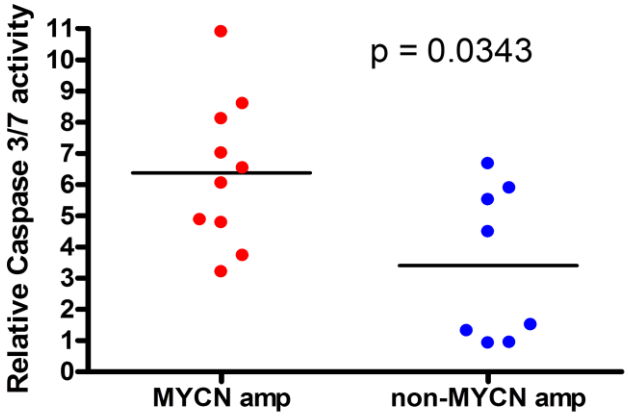


Figure 3.21. **p53 mutant cells lines are resistant to MDM2-p53 antagonists mediated growth inhibition regardless of *MYCN* status. p53 mutant cell lines were treated with a)** Nutlin-3 (up to 20μM) and **b)** MI-63 (up to 10μM) for 72 hours and did not reach 50% growth inhibition to obtain a GI_{50} value. *NB100 is a PNET cell line

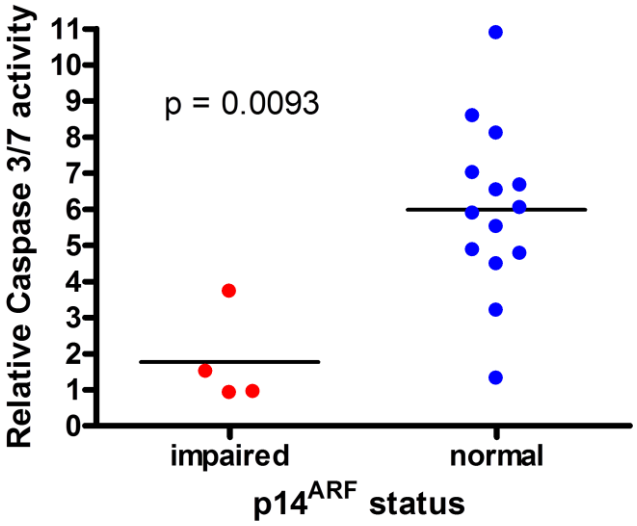
a)


Nutlin-3
MYCN and MDM2 co-amplifie
MYCN amplified
non-amplified
p53 mutant
* p14ARF impaired cell lines
Relative Caspase 3/7 activity
0
1
2
3
4
5
6
7
8
9
10
11
12
13
NGP
LS
NB1691
TR14
IMR32
NBLW
SMSKCNR
LAN5
PER108
CHLA136
SHSY5Y
GIMEN
SJNB1
NB69
LAN6
SKNRA
SHEP
NBLS
IGRN91
Be2C
SKNAS

b)


Relative Caspase 3/7 activity
0
1
2
3
4
5
6
7
8
9
10
11
p = 0.0343
MYCN amp
non-MYCN amp

c)



d)

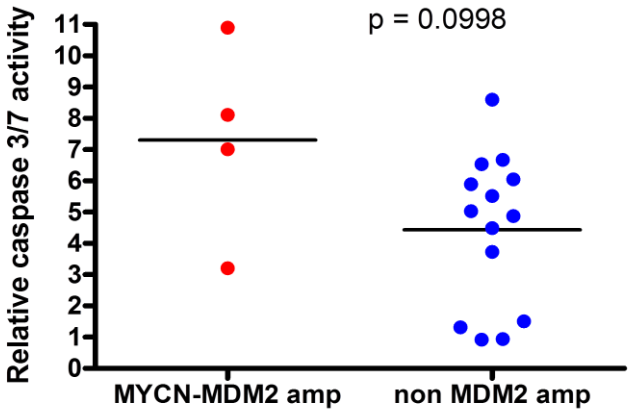
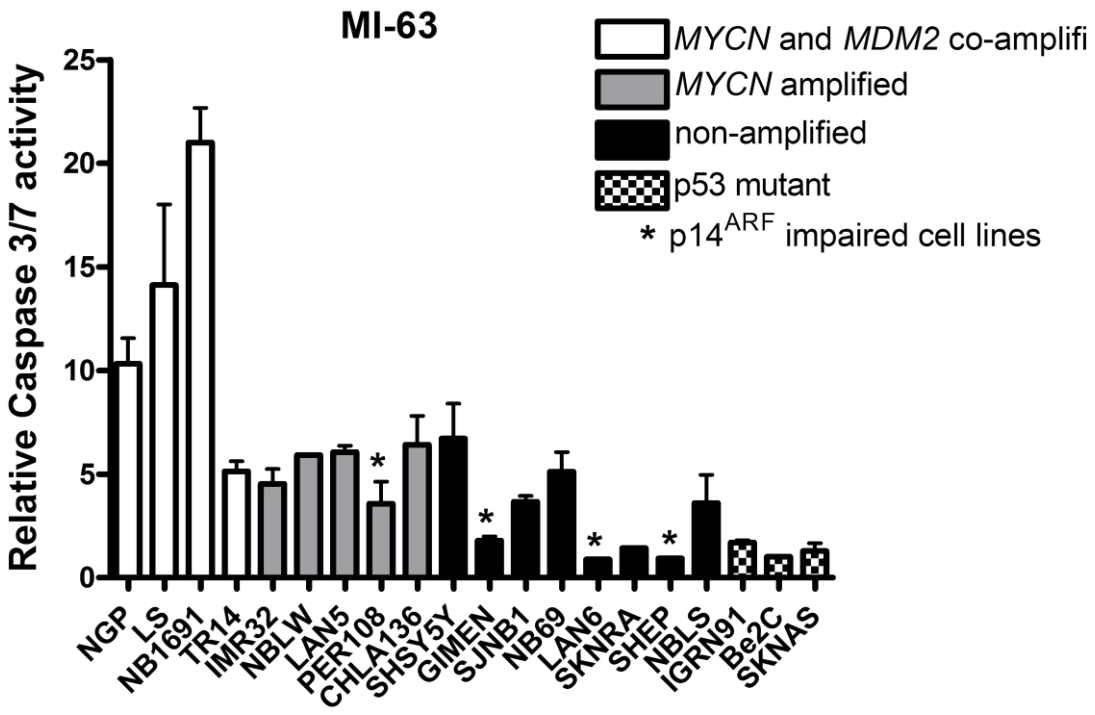
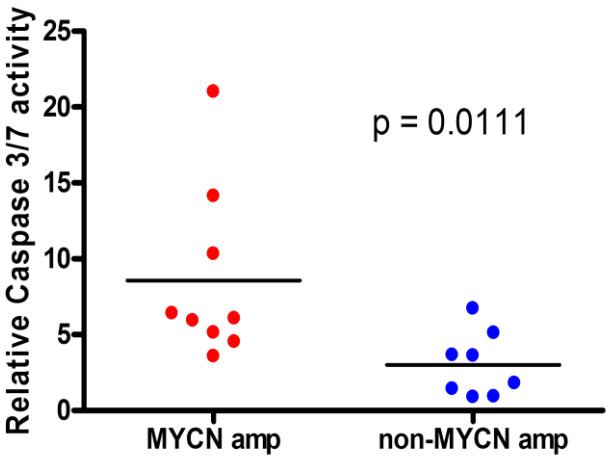


Figure 3.22. **Caspase 3/7 activity in a panel of neuroblastoma cell lines following Nutlin-3 treatment. a)** Caspase activity in panel of cell lines. **b)** scatter plot of *MYCN*-amplified vs. non-*MYCN*-amplified (p = 0.0343, Mann-Whitney test). **c)** scatter plot of $p14^{ARF}$ impaired vs. $p14^{ARF}$ normal (p = 0.0093, Mann-Whitney test). **d)** scatter plot of *MYCN* and *MDM2* co-amplified vs. p53 wt cell lines (p = 0.0998, Mann-Whitney test).

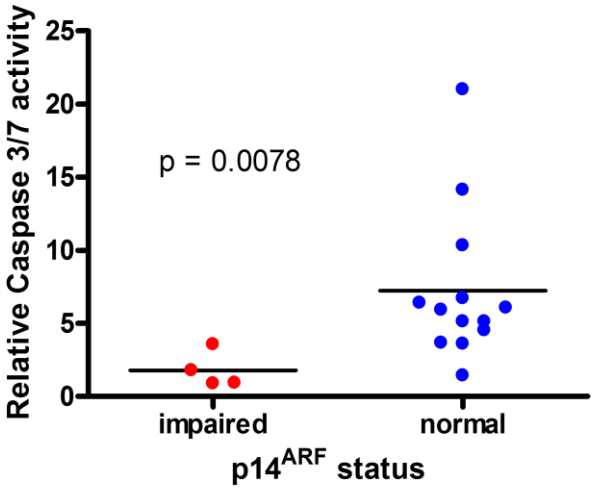
a)


MI-63
MYCN and MDM2 co-amplifi
MYCN amplified
non-amplified
p53 mutant
* p14ARF impaired cell lines
Relative Caspase 3/7 activity
25
20
15
10
5
0
NGP
LS
NB1691
TR14
IMR32
NBLW
LAN5
PER108
CHLA136
SHSY5Y
GIMEN
SJNB1
NB69
LAN6
SKNRA
SHEP
NBLS
IGRN91
Be2C
SKNAS

b)


Relative Caspase 3/7 activity
25
20
15
10
5
0
p = 0.0111
MYCN amp
non-MYCN amp

c)



d)

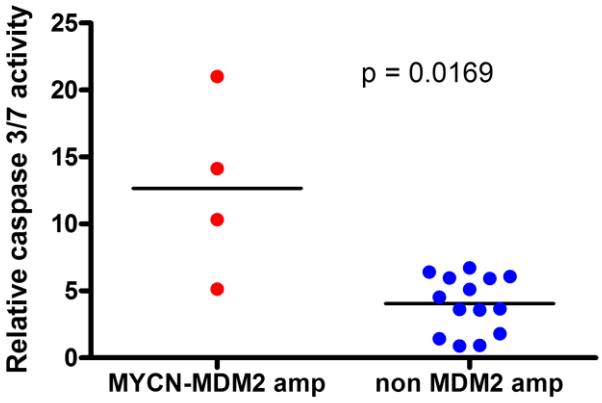


Figure 3.23. **Caspase 3/7 activity in a panel of neuroblastoma cell lines following MI-63 treatment. a)** Caspase activity in panel of cell lines **b)** scatter plot of *MYCN*-amplified vs. non-*MYCN*-amplified (p = 0.0111, Mann-Whitney test). **c)** scatter plot of $p14^{ARF}$ impaired vs. $p14^{ARF}$ normal (p = 0.0078, Mann-Whitney test) **d)** scatter plot of *MYCN* and *MDM2* co-amplified vs. p53 wt cell lines (p = 0.0168, Mann-Whitney test).

3.4.14 Investigating the relationship between cell type, growth inhibition and caspase activity

As shown in Figure 3.24, N-type neuroblastoma cell lines had increased caspase 3/7 activity and GI_{50} values compared to S-type neuroblastoma cell lines. The outliers in the N-type cells that had low caspase activity were the $p14^{ARF}$ impaired cell lines: LAN6 and PER108 (discussed in Chapter 5), and the S-type cell that underwent high levels of apoptosis was the NBLS cell line. There was a clear divide in the mixed N/S type cells where the cell lines that were predominantly N-type (NB69 and NBLW) underwent high levels of apoptosis and growth inhibition compared to cell lines that were predominantly S-type (GIMEN and SJNB1).

3.4.15 Irradiation induced apoptosis in *MYCN*-amplified cells is dependent on p53 expression

To determine whether higher levels of apoptosis in *MYCN*-amplified neuroblastoma cell lines is dependent on higher levels of p53 in the presence of MYCN, *MYCN*-amplified NGP cells were treated with MYCN and/or p53 siRNA or SCR siRNA prior to irradiation induced DNA damage. It has been previously shown that NGP cells undergo high levels of apoptosis following irradiation (Tweddle et al., 2001b). Apoptosis was determined by analysis of expression of the apoptosis mediator PUMA and cleavage of caspase-3 and PARP (Figure 3.25a) together with quantification of caspase 3/7 activity (Figure 3.25b). Twenty-four hours after irradiation, inhibition of MYCN or p53 led to decreased caspase 3/7 activity compared with SCR siRNA. Furthermore dual inhibition of MYCN and p53 led to a slightly greater reduction in caspase 3/7 activity than MYCN or p53 alone and also resulted in a greater reduction in cleavage of caspase-3 and decreased expression of PUMA. This suggests that increased levels of apoptosis are observed when both p53 and MYCN are present, and that irradiation induced apoptosis in *MYCN*-amplified cell lines is at least partly dependent on p53 expression.

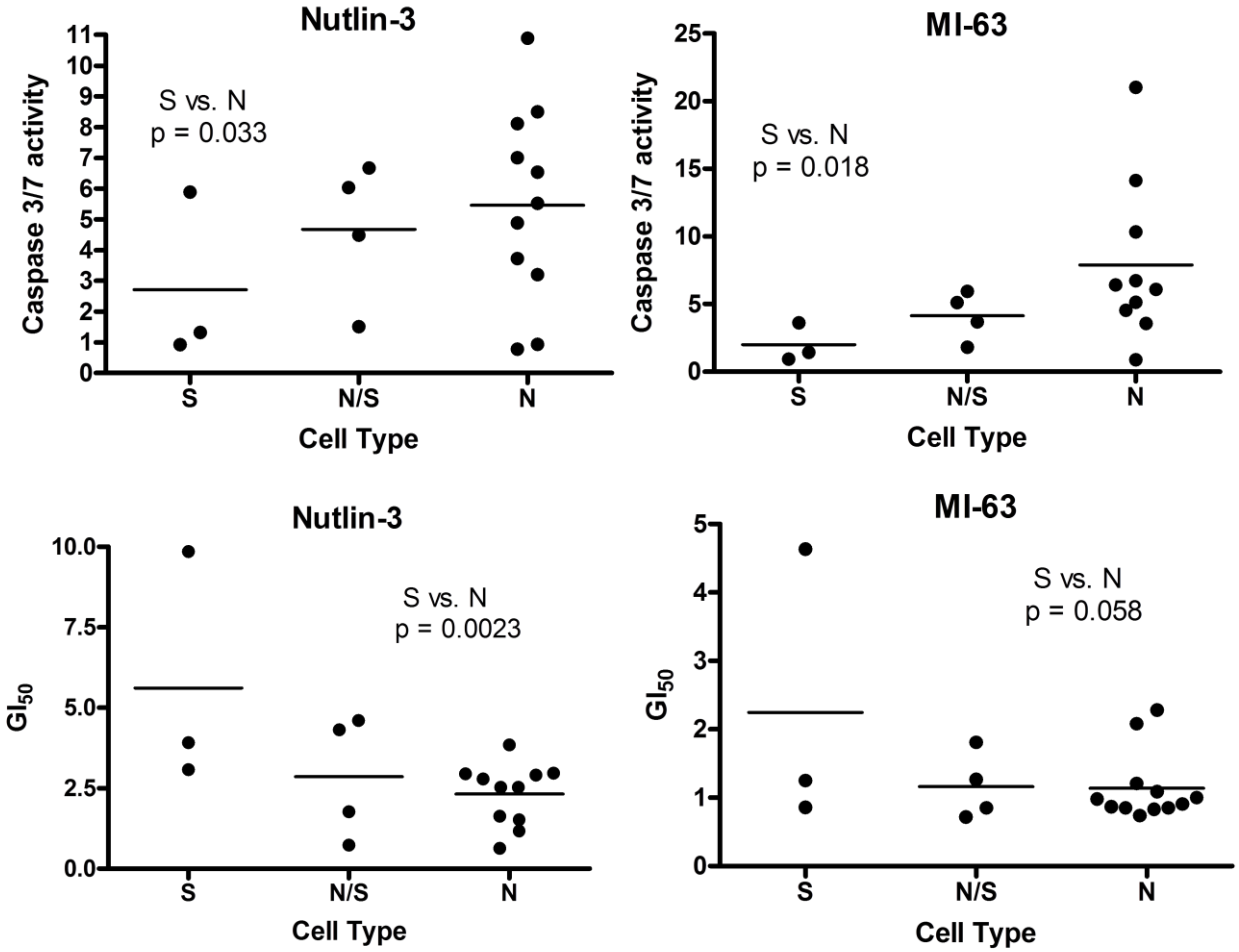


Figure 3.24. **N-type neuroblastoma cells have increased caspase 3/7 activity and decreased growth inhibition following Nutlin-3 and MI-63 treatment compared to S-type neuroblastoma cells.** Caspase activity values and GI_{50} values are those as shown in Figures 19-23. For statistical analysis, the N/S type cells were grouped into N and S depending on which was their dominant cell type (NB69, N>S, SJNB1 S>N, GIMEN S>N, NBLW N>S). p values were calculated using Mann-Whitney tests.

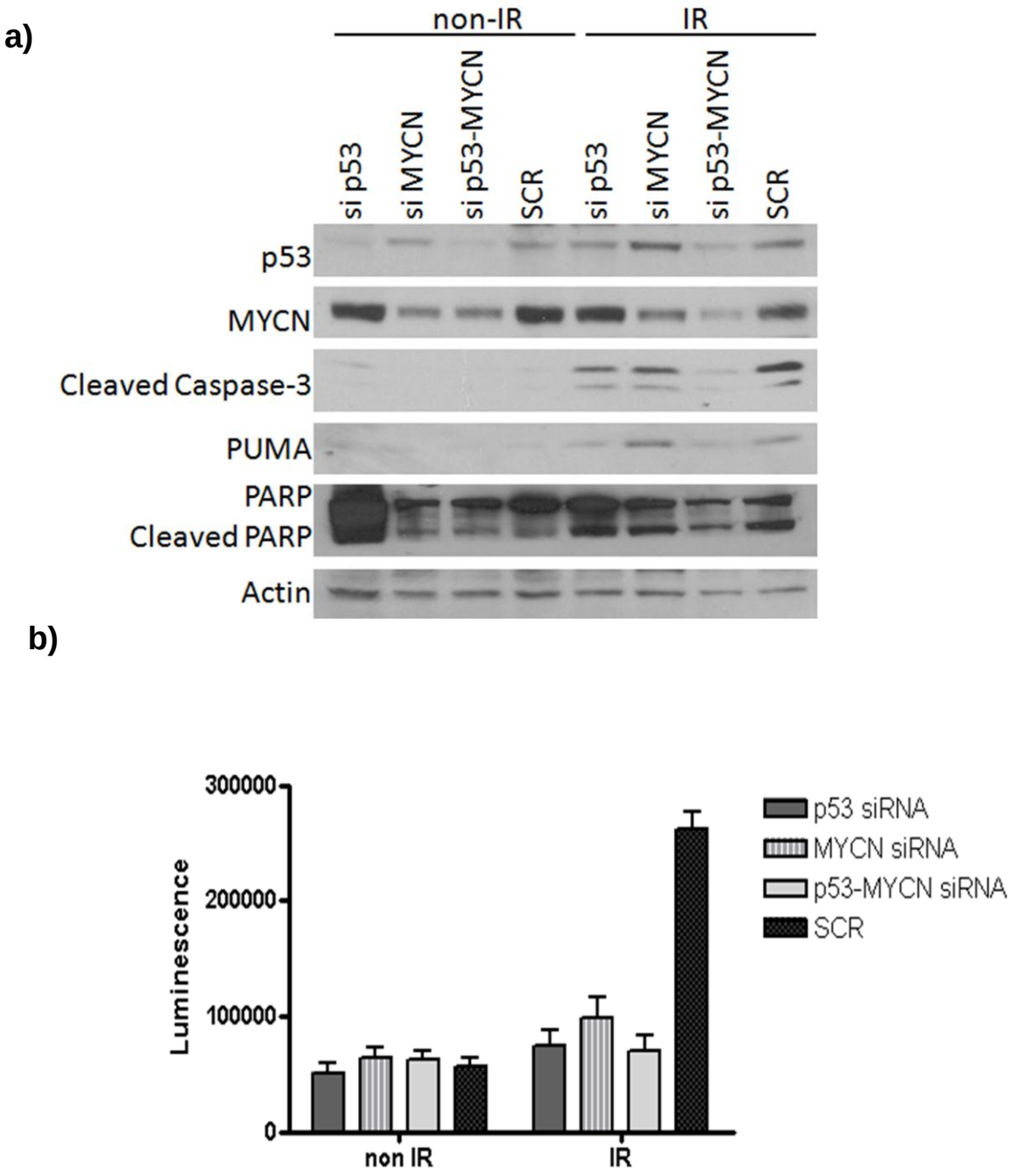


Figure 3.25. **Irradiation induced apoptosis in *MYCN*-amplified cell lines is dependent on p53.** NGP cells were treated with MYCN, p53, MYCN and p53 or SCR siRNA for 48 hours prior to irradiation-induced DNA damage and harvested 24 hours later. Expression of apoptotic markers was determined by **a)** Western blot and **b)** caspase 3/7 activity.

3.5 Discussion

Neuroblastoma accounts for 15% of childhood cancer related deaths (Maris and Matthay, 1999). *MYCN*-amplification is a powerful and reliable biomarker of poor prognosis in neuroblastoma and is used to stratify patients into a high risk group requiring intensive treatment. However, current therapies are insufficient for these patients, resulting in high mortality rates, a high incidence of relapse and treatment related toxicity (Matthay et al., 1999).

3.5.1 MYCN and the p53/MDM2/p14^{ARF} network

MYCN plays roles in the contradictory pathways of promoting both cell survival (Lutz et al., 1996) and sensitizing cells to apoptosis (van Noesel et al., 2003; Fulda et al., 2000), but in neuroblastoma abnormalities within the apoptotic pathways can occur in association with *MYCN*-amplification (Hogarty, 2003). p53, the major tumour suppressor in the cell, is generally wild-type and active at diagnosis and patients respond well to initial therapy. However, high risk patients often relapse and although p53 mutations are still relatively rare (~15% of cases), p53 function is often inactivated through disruption of the p53/MDM2/p14^{ARF} network, of which MYCN is a central modulator (Figure 3.26), resulting in chemoresistant disease (Carr-Wilkinson et al., 2010). A number of studies have shown that pathways downstream of p53 are intact in neuroblastoma and that p53 can induce apoptotic responses (Van Maerken et al., 2011; Hogarty, 2003; Tweddle et al., 2003; Hosoi et al., 1994; Vogan et al., 1993). It has been previously reported that the negative regulator of p53, MDM2, is the critical oncogene product by which *MYCN*-amplified neuroblastomas acquire a more aggressive behaviour (Slack and Shohet, 2005), and that MYCN and MDM2 work together to inhibit apoptosis (Wang et al., 2006; Alt et al., 2003). There are a number of mechanisms by which MDM2 might contribute to the aggressive phenotype of *MYCN*-amplified neuroblastoma. MDM2 has been reported to be a direct transcriptional target of MYCN and therefore MYCN-driven expression of MDM2 may contribute to p53 inactivation in neuroblastoma (Slack et al., 2005a). *MDM2* is co-amplified with *MYCN* in about 6% of neuroblastomas but more frequently the negative regulator of MDM2, p14^{ARF} is inactivated through methylation or deletion, and has been reported to occur in 29% of tumours, resulting in hyperactive MDM2 (Carr-Wilkinson et al., 2010; Corvi et al., 1995b). In addition, we have recently reported that p53 is a direct transcriptional target of MYCN, and that p53 induction by MYCN may be an important contributory mechanism by which MYCN sensitizes cells to apoptosis (Chen et al., 2010b).

In this chapter, the effect of MYCN on the response to the MDM2-p53 antagonists Nutlin-3 and the more potent MI-63 was investigated. MI-63 has not been previously investigated in neuroblastoma. Three independent methods show that *MYCN*-amplification and expression sensitizes neuroblastoma cell lines to MDM2-p53 antagonists.

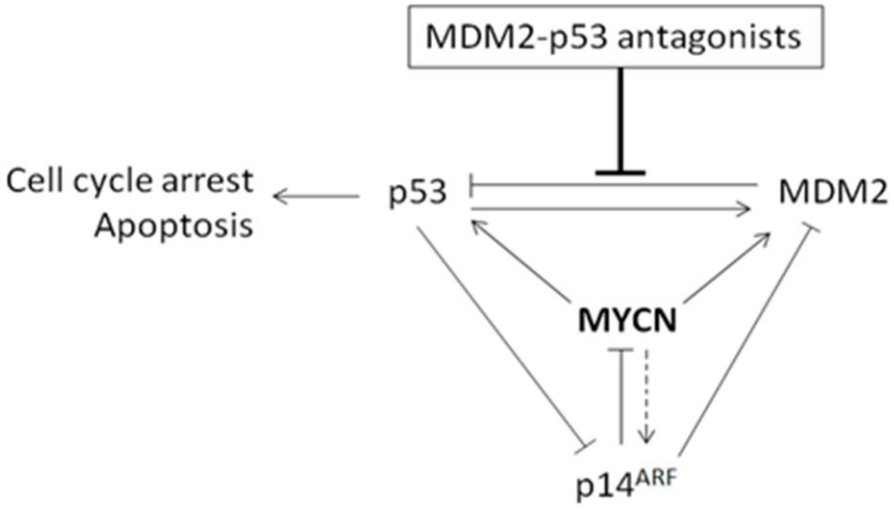


Figure 3.26. **MYCN is a central modulator in the p53/MDM2/p14^{ARF} network.**

3.5.2 The Tet21N cell line and response to MDM2-p53 antagonists

SHEP cells (described in Chapter 2.2) were used to generate a synthetic inducible system on the basis of the tetracycline repressor of E coli, to reversibly express MYCN in SHEP cells, which have barely detectable endogenous MYCN. This cell line allows the role of the MYCN protein in the biology of neuroblastoma to be investigated and has previously been used in many studies. MYCN induction has been shown to increase DNA synthesis and the proliferation rate (Lutz et al., 1996), MYCN(+) Tet21N cells are more sensitive to cytotoxic drugs (Fulda et al., 2000), and MYCN(+) cells were previously shown to have lower IC_{50} values following Nutlin-3 treatment compared to MYCN(-) cells (Barbieri et al., 2006).

SHEP Tet21N MYCN regulatable cells were used to assess the effect of manipulation of MYCN where tetracycline removal results in induction of MYCN expression. Data in this chapter has shown that both Nutlin-3 and MI-63 are more effective at inducing growth inhibition and apoptosis in MYCN(+) compared to MYCN(-) cells, as shown by

growth inhibition assays, clonogenic survival curves, caspase 3/7 activity, sub G_1 DNA content and induction of the p53 transcriptional target and apoptotic marker PUMA. The increase in baseline p53 on MYCN induction is consistent with previous studies in our group (Chen et al., 2010b; Bell et al., 2006).

3.5.2.1 SHEP cells do not undergo high levels of apoptosis following MDM2-p53 antagonist treatment

The Tet21N SHEP cell line has previously been reported to be very chemoresistant compared to SHSY5Y, both of which were derived from SKNSH cells (Rodriguez-Lopez et al., 2001; Tweddle et al., 2001b; Jasty et al., 1998). The Tet21N SHEP cell line was quite resistant to the apoptotic effects of MDM2-p53 antagonists, resulting in little caspase 3/7 activation at MI-63 concentrations less than 10µM and up to 20µM Nutlin-3. In other responsive neuroblastoma cell lines, 10µM Nutlin-3 or MI-63 was a maximum concentration used and was sufficient to detect high levels of apoptosis. In the panel of cell lines, just 2.5µM MI-63 and 5µM Nutlin-3 was sufficient to induce caspase 3/7 activity and to observe a difference in caspase activities between *MYCN*-amplified and non-*MYCN*-amplified cell lines. In SHEP cells, sub G_1 DNA was looked at and again a concentration of 20µM had to be used to see sufficient induction of apoptosis at 24, 48 and 72 hours. Surprisingly, just 2.5µM of Nutlin-3 or MI-63 was sufficient to induce cell cycle arrest in both MYCN(+) and MYCN(-) cells. This suggests that these cells have an intact cell cycle control mechanism, but since they undergo low levels of apoptosis upon MDM2-p53 antagonist treatment, suggests that they have defective apoptotic pathways in response to MDM2-p53 antagonists. Like SHEP Tet21N cells, the original SHEP cells were also resistant to MDM2-p53 antagonists. Previous studies, however, have shown that SHEP cells are suitable for investigating drug induced apoptosis following Paclitaxel treatment (Janssen et al., 2007), Betulinic acid and doxorubicin treatment (Fulda and Debatin, 2005), mannitol treatment (Kim and Feldman, 2002), and treatment with resveratrol (Fulda and Debatin, 2004), suggesting that the cells have intact apoptotic pathways but have a poor apoptotic response to MDM2-p53 antagonists. In support of this data, Van Maerken *et al* reported that despite an increase in caspase activity and sub G_1 DNA following Nutlin-3 treatment in the related SKNSH and SHSY5Y cells, no increase was observed in SHEP cells, but the cells underwent a pronounced cell cycle arrest, as has been observed in this study in Tet21N cells (Van Maerken et al., 2011). Despite SKNSH, the original cells SHEP cells were derived from, being caspase 8 deficient (Rebbaa et al.,

2001), a major pathway by which cytotoxic drugs act, SHEP cells were used as a positive control for intact caspase 8 in another study (Braun et al., 2010), suggesting SHEP cells have intact caspase 8 and therefore this does not contribute to the resistance to induction of apoptosis. Another feature of SHEP cells are that they are homozygously deleted for $p14^{ARF}$ and this may be a mechanism by which SHEP cells are resistant to apoptosis, particularly as LAN6 and GIMEN non-*MYCN*-amplified cells are $p14^{ARF}$ impaired and are also particularly resistant to Nutlin-3 and MI-63 mediated caspase activation. Like Tet21N cells and SHEP cells, LAN6 and GIMEN cells were not resistant to MDM2-p53 antagonist mediated growth inhibition. The effects of $p14^{ARF}$ on the response to MDM2-p53 antagonists in neuroblastoma cell lines is investigated in more depth in Chapter 5.

3.5.2.2 Differences in colony formation between MYCN(+) and MYCN(-) Tet21N cells

The appearance of the colonies formed from MYCN(+) cells in both untreated controls and following 24 hours treatment with Nutlin-3 or MI-63, followed by a further 14 days incubation, differed from those formed by MYCN(-) cells. The MYCN(+) colonies were larger and stained darker, suggesting increased cell density in each colony compared to MYCN(-) cells. This agrees with published data within our group suggesting that MYCN(+) cells have a faster doubling time compared to MYCN(-) cells (Bell et al., 2006). Since the appearance of colonies formed from DMSO only treated cells had an appearance comparable to colonies formed from treated cells, this also suggests that surviving cells either initially underwent a reversible growth arrest, or that the surviving population of cells did not growth arrest, despite seeing a growth arrest by FACs analysis following 24 hours MDM2-p53 antagonist treatment, and $p21^{WAF1}$ induction at 24 hours post treatment.

3.5.2.3 Tet21N cells and control Tet21 cells

Tet21 Vector only cells were used to control for tetracycline. To eliminate tetracycline as a possible reason for the differences observed in colony appearance in Tet21N cells clonogenic assays were performed with Tet21 cells. Surprisingly, compared to Tet21N cells (MYCN+ or MYCN-), Tet21 cells formed much larger flatter colonies. Also the GI_{50} value generated for Tet21 cells (MYCN-) was similar to the GI_{50} value for Tet21N (MYCN+) cells, where a GI_{50} value similar to MYCN(-) Tet21N cells would have been expected. This data suggests that there is something fundamentally different between these two cell lines, or that the addition of the transfected plasmids and MYCN

promoter within Tet21N cells has a greater effect than expected on the cell, and results with the Tet21 control should be used with caution for clonogenic assays.

3.5.3 MYCN knockdown and response to MDM2-p53 antagonists

RNA interference allows the effect of manipulating expression of a single gene to be investigated. siRNA was used to knockdown MYCN in 5 *MYCN*-amplified neuroblastoma cell lines, and the effect of MDM2-p53 antagonists on the p53 response, apoptosis and the cell cycle investigated. All cell lines that were used had a strong p53 and apoptotic response to MDM2-p53 antagonists.

3.5.3.1 MYCN knockdown alone

MYCN knockdown alone resulted in varying effects on MDM2 and $p21^{WAF1}$, and either a decrease or no change in p53 despite both p53 and MDM2 having been reported as a direct transcriptional target of MYCN (Chen et al., 2010b; Slack et al., 2005a). Previous data shows that knockdown of MYCN results in decreased p53 at transcriptional and protein levels, as well as p53 transcriptional targets MDM2 and PUMA (Chen et al., 2010b). In this study, although similar effects have been found, it is highly variable. However, p53 as a direct transcriptional target of MYCN supports the hypothesis that MYCN knockdown may decrease the p53 response to MDM2-p53 antagonists due to decreased transcriptional activity.

3.5.3.2 MYCN knockdown and MDM2-p53 antagonist treatment

Following siRNA-mediated knockdown of MYCN in two *MYCN*-amplified and three *MYCN* and *MDM2* co-amplified neuroblastoma cell lines, Nutlin-3 and MI-63 treatment resulted in a decreased p53 response and reduction in the levels of apoptosis in all cases. Western analysis was performed in all cell lines and the apoptotic markers PUMA, cleaved caspase 3 and cleaved PARP detected as markers of apoptosis. Interestingly, in NGP cells levels of PUMA did not alter following MYCN knockdown, but caspase 3 and PARP cleavage decreased, and similar effects were seen in IMR32 cells. This may suggest aberrant p53-PUMA signalling in these cell lines, but other pathways by which Nutlin-3 and MI-63 induce apoptosis are intact.

3.5.4 Response to MDM2-p53 antagonists in a panel of neuroblastoma cell lines

Finally, growth inhibition assays and caspase 3/7 activity assays were performed in a panel of 18 p53 wildtype and 3 p53 mutant neuroblastoma cell lines, to assess the

effects of Nutlin-3 and MI-63 in *MYCN*-amplified compared to non-*MYCN*-amplified cell lines. Both Nutlin-3 and MI-63 were more effective at inducing growth inhibition and apoptosis in *MYCN*-amplified cells, and the effect was dependent on wildtype p53, consistent with a number of reports showing that these compounds require wildtype p53, as expected if their action is target-specific (Van Maerken et al., 2006; Vassilev et al., 2004). Whereas all wild-type p53 *MYCN*-amplified cell lines investigated were responsive to MDM2-p53 antagonists, the response of non-amplified cell lines was more variable. This is unsurprising as *MYCN*-amplification is directly responsible for transformation; non-amplified cases are likely to have a variety of other genetic abnormalities or defects in apoptotic pathways that may not be present in *MYCN*-amplified tumours.

3.5.5 Comparison to published data

Our data differs from previous findings that report no correlation between *MYCN* status and response to Nutlin-3 (Barbieri et al., 2006; Van Maerken et al., 2006). Van Maerken *et al*. tested a limited panel of 7 p53 wildtype cell lines (3 *MYCN*-amplified), and found no significant difference in the cell viability response or apoptotic response to Nutlin-3 in *MYCN*-amplified compared to non-*MYCN* amplified cell lines (Van Maerken et al., 2006). In another study IC_{50} values for Nutlin-3 in 2 MYCN regulatable cell lines were determined and whilst in agreement with our extensive observations that there was increased sensitivity in MYCN(+) compared to MYCN(-) Tet21N cells, it was reported that there was no difference in IC_{50}s for MYCN(+/-) MYCN3 cells, although when used in conjunction with cisplatin the IC_{50} for Nutlin-3 was lower in MYCN+ MYCN3 cells (Barbieri et al., 2006). To test the effect of MYCN on sensitivity to MDM2-p53 antagonists in this study, several methods were used and a total of 22 cell lines to investigate the influence on cell growth and cell death, and similar results were found using two structurally unrelated MDM2-p53 antagonists. Furthermore, previous studies with Tet21N cells have reported that MYCN(+) cells were more sensitive to apoptosis from cytotoxic drugs than MYCN(-) cells (Paffhausen et al., 2007; Fulda et al., 2000), and a recent study reports that MYCN sensitizes neuroblastoma cell lines to the apoptotic effects of bleomycin (Petroni et al., 2011). These studies are all in agreement with the data presented in this chapter showing that MYCN sensitises cells to apoptosis.

3.5.6 $p14^{ARF}$ and the response to MDM2-p53 antagonists

$p14^{ARF}$ is often inactivated in neuroblastoma (Carr-Wilkinson et al., 2010), is expressed at higher levels in *MYCN* and *MDM2* co-amplified cell lines compared to *MYCN*-amplified non-*MDM2* amplified cell lines (Carr et al., 2006), and may inhibit *MYCN* transcriptional activity (Amente et al., 2007). There is also evidence that $p14^{ARF}$ is induced by MYCC, and therefore MYCN may function in a similar way (Zindy et al., 1998). Interestingly the 3 non-*MYCN*-amplified $p14^{ARF}$ impaired cell lines were especially resistant to Nutlin-3 and MI-63 mediated apoptosis, supporting recent findings showing that silencing of $p14^{ARF}$ results in decreased susceptibility to undergo apoptosis and overexpression of $p14^{ARF}$ results in a stronger caspase 3/7 response (Van Maerken et al., 2011). The effect of $p14^{ARF}$ is investigated further and discussed in more detail in Chapter 5.

3.5.7 *MDM2*-amplification and the response to MDM2-p53 antagonists

In this study, *MYCN* and *MDM2* co-amplified neuroblastoma cell lines had reduced sensitivity to Nutlin-3 mediated growth inhibition, and a similar trend with MI-63, compared to *MYCN*-only amplified cell lines. However, there was increased levels of caspase 3/7 activity in these cell lines compared to *MYCN*-amplified and non-amplified cell lines, consistent with previous reports that increased levels or amplification of *MDM2* increases Nutlin-3 induced apoptosis as shown in liposarcomas and AML (Müller CR, 2007; Kojima et al., 2005). However, *MYCN* and *MDM2* co-amplified cell lines have previously been reported to have increased $p14^{ARF}$ mRNA and protein expression (Carr et al., 2006), and it may be this increase in $p14^{ARF}$ that is sensitizing these cell lines to increased caspase activity. The data presented in this chapter indicates that *MDM2* co-amplification may increase the resistance of *MYCN*-amplified neuroblastomas to Nutlin-3 mediated growth inhibition but may sensitise to apoptosis. The effect of MDM2 on the response to MDM2-p53 antagonists is explored further in Chapter 4.

3.5.8 Variation in response to MDM2-p53 antagonists in panel of cell lines

3.5.9 The effect of MDM2-p53 antagonists on MYCN expression

Interestingly, MYCN levels decreased dramatically in MYCN(+) Tet21N cells when treated with MI-63 (and to a lesser extent with Nutlin-3), and in NGP cells when treated

with both Nutlin-3 and MI-63. A previous report has shown a decrease in MYCN in Tet21N MYCN(+) cells following 2μM Nutlin-3 treatment (Peirce and Findley, 2009a). MYCN levels remained unchanged in LS cells, and decreased slightly in LAN5 and TR14 cells. Interestingly, NGP and Tet21N cells were the only cell lines to G_1 arrest following Nutlin-3 and MI-63 treatment, and Bell et al. previously found that MYCN expression was linked with a failure of neuroblastoma cell lines to G_1 arrest in response to DNA damage. Our results suggests that this may also be true of MDM2-p53 antagonists, whereby cell lines that have reduced MYCN expression following Nutlin-3 treatment G_1 arrest, and those that still have high MYCN protein levels do not. However, knockdown of MYCN in other *MYCN*-amplified cell lines such as IMR32 does not lead to a G_1 arrest following irradiation (Bell et al., 2006). However, the mechanism for Nutlin-3 reducing MYCN expression in these cell lines is unknown.

3.5.10 Irradiation induced apoptosis in *MYCN*-amplified cell lines is dependent on p53

The mechanisms by which members of the MYC family induce apoptosis are not fully understood (reviewed in (Adhikary and Eilers, 2005)). There is evidence that *MYCN*-amplified tumours may circumvent MYCN-driven apoptosis by selecting for cells with aberrations within the p53/MDM2/$p14^{ARF}$ pathway and analysis of neuroblastoma cell lines reported to date with aberrations in the p53/MDM2/$p14^{ARF}$ pathway show that 25 of 34 (74%) cell lines are *MYCN*-amplified and predominantly established following previous therapy at relapse (Chen et al., 2010b; Carr et al., 2006). Possible mechanisms of MYC driven apoptosis is through $p14^{ARF}$-mediated expression, stabilisation and activity of p53 through inhibition of MDM2, and also p53 is a direct transcriptional target of MYCN (Chen et al., 2010b). In this study, it has been found that dual knockdown of MYCN and p53 results in decreased levels of caspase 3 and PARP cleavage, decreased levels of the p53-dependent apoptotic mediator PUMA, and decreased levels of caspase 3 activity. This suggests that increased levels of apoptosis are observed when both p53 and MYCN are present, and that irradiation induced apoptosis in *MYCN*-amplified cell lines may be dependent on p53. Levels of apoptosis were also significantly reduced following knockdown of either MYCN alone or p53 alone, suggesting that individually, these proteins also have a significant impact on the ability for the cells to undergo apoptosis following DNA damage. It would be interesting to see if MDM2-p53 antagonists produced similar results.

3.5.11 p53 and mTOR

Interestingly, p53 has been shown to inhibit the mTOR pathway which is involved in senescence (Demidenko et al., 2010), and there is evidence that p53 induced cell cycle arrest is reversible as long as mTOR is also inhibited, suggesting that induction of senescence, reversible cell cycle arrest and cell death in response to Nutlin-3 may be determined by the status of mTOR (Korotchkina et al., 2010). The activation status of mTOR may therefore be important in determining the response to MDM2-p53 antagonists in neuroblastoma.

3.5.12 Conclusions and Future Work

The observations in this study support reports that both MDM2 and p53 are induced by MYCN, that MDM2 is a critical oncogene product by which *MYCN*-amplified neuroblastomas acquire a more aggressive phenotype and that MYCN sensitizes cells to p53-mediated apoptosis. Under normal circumstances, both p53 and MYCN induce MDM2, but upon MDM2 inhibition MYCN-mediated transcription of p53 allows p53 to accumulate and increases activity. In addition there is evidence that both p53 and MYCC inhibit anti-apoptotic factors such as the Bcl-2 and Bcl-x, another mechanism by which amplification of *MYCN* may promote apoptosis together with p53 if, as is likely, this is also true of MYCN (Chipuk and Green, 2006; van Noesel and Versteeg, 2004).

In conclusion, these studies present several lines of evidence that MYCN sensitizes neuroblastoma cell lines to MDM2-p53 antagonists through p53-dependent growth inhibition and apoptosis, and may provide a promising therapeutic approach for patients with high-risk *MYCN*-amplified neuroblastoma with wild-type p53. Previous studies show that *MYCN*-amplification sensitizes cells to chemotherapeutic drugs and that Nutlin-3 induces senescence in normal cells that might actually protect the cell against cytotoxic drugs (Efeyan et al., 2007; Fulda et al., 2000). Furthermore Nutlin-3 synergises with chemotherapy in neuroblastoma cells (Barbieri et al., 2006) suggesting that patients with *MYCN*-amplified tumours may be particularly responsive to MDM2-p53 antagonists in combination with chemotherapeutic drugs. To confirm the sensitivity *MYCN*-amplification confers to MDM2-p53 antagonists in neuroblastoma, further studies should be carried out in mouse models, such as the MYCN transgenic mouse model or xenograft studies with *MYCN*-amplified and non-*MYCN*-amplified cell lines.

Chapter 4. The effect of MDM2 and MDMX in neuroblastoma cell lines on the response to MDM2-p53 antagonists

4.1 Introduction

The major negative regulator of p53 is MDM2, an E3 ubiquitin ligase that promotes ubiquitination and relocalisation of p53 to the cytoplasm where it is degraded by the proteosome, and also inhibits p53 transcriptional activity by binding the transactivation domain within the N-terminal region of p53 (Zhang and Xiong, 2001). MDM2 is also a direct transcriptional target of p53, forming an autoregulatory negative-feedback loop. MDMX, another negative regulator of p53, shares high amino acid sequence and structural homology to MDM2 (Shvarts et al., 1997; Shvarts et al., 1996). However, whereas MDM2 targets p53 for proteosomal degradation, MDMX has neither E3 ubiquitin ligase activity nor a nuclear localisation signal despite possessing a RING domain (Stad et al., 2001; Shvarts et al., 1996). Despite their similarities, neither protein can substitute for the loss of the other; MDMX null mice die *in utero* in a p53-dependent manner, but can be rescued upon p53 knockout (Parant et al., 2001), and MDM2 knockout is lethal during early embryogenesis in mice as a result of hyperactive p53, but can also be rescued by p53 knockout (Montes de Oca Luna et al., 1995).

4.1.1 MDMX and the p53/MDM2 pathway

p53 regulation by MDMX is not fully understood but there is growing evidence that it is complex, and that there is complicated interplay between MDM2 and MDMX. MDMX binds to the p53 transactivation domain, repressing transcriptional activity (Toledo and Wahl, 2006), and can act alone to repress p53 transcription (Marine et al., 2007) or as a heterodimer with MDM2 through the C-terminal RING domain to enhance ubiquitination of p53 (Stad et al., 2001; Sharp et al., 1999). MDM2 and MDMX were found to exist in cells mainly as a heterocomplex (Kawai et al., 2007). There is evidence that MDM2 is relatively ineffective as an E3 ubiquitin ligase when not in complex with MDMX (Kawai et al., 2007; Poyurovsky et al., 2007; Uldrijan et al., 2007), and that the MDM2-MDMX heterodimer is a more effective E3 ubiquitin ligase for p53 than MDM2 alone, promoting MDM2-mediated degradation of p53 (Linares et al., 2003; Gu et al., 2002). An MDM2-MDMX complex is required for control of p53 activity *in vivo* (Huang et al., 2011). This data is in line with neither being able to substitute for the other, nor both embryonic lethal phenotypes being rescued by p53 knockout.

In addition, MDMX is relatively stable compared to MDM2 and there is evidence that MDMX promotes MDM2 stabilisation (Pereg et al., 2005).

Following cellular stress, MDM2 undergoes autodegradation, and promotes ubiquitination and subsequent degradation of MDMX (de Graaf et al., 2003; Kawai et al., 2003a; Pan and Chen, 2003; Fang et al., 2000). As activated p53 transactivates MDM2, increased MDM2 degrades MDMX more efficiently allowing full p53 activation. Overexpression of MDMX may prevent MDM2 from degrading p53 due to competition for p53 binding.

4.1.2 MDMX and response to MDM2-p53 antagonists

The MDM2-p53 antagonists used in this study, Nutlin-3 and MI-63, do not disrupt the MDMX-p53 interaction (Popowicz et al., 2010). Previously, MDMX has been shown to affect the efficiency of MDM2 inhibitors. The cellular activity of MDM2 inhibitors is decreased by MDMX, and Nutlin-3 does not induce apoptosis in cancer cells that express high levels of MDMX (Hu et al., 2006; Patton et al., 2006; Wade et al., 2006). MDMX siRNA or a peptide that disrupts the interaction of p53 with MDM2 or MDMX (pDI) increases p53 activation, inhibits tumour growth and sensitises MCF-7 cells to apoptosis (Hu et al., 2007a; Wade et al., 2006). Furthermore, a number of studies have shown that inhibition of MDMX enhances the response to MDM2-p53 antagonists. A new compound, a benzofluroxan derivative, repressed the MDMX promoter in breast cancer cells, activating p53 and inducing apoptosis, and acted additively with Nutlin-3 (Wang et al., 2011). This effect was less efficient in cells with low MDMX expression. The Hsp90 inhibitor 17AAG destabilises MDMX, but in addition to a range of signalling proteins including Bcr-Abl, Her2, Akt, and Raf-1, and induces apoptosis (Vaseva et al., 2011), and MDMX overexpression in CLL resulted in a poor response to Nutlin-3 (Bo et al., 2010).

4.1.3 MDMX expression in neuroblastoma and other cancer types

MDMX overexpression in cancer is mainly caused by aberrant transcription (Gilkes et al., 2008) and MDM2 and MDMX expression has an inverse relationship in cancer cells (Danovi et al., 2004). MDMX is overexpressed in many cancers, including 18-19% of lung and colon cancers (Danovi et al., 2004), 50% of head and neck squamous carcinomas (Valentin-Vega et al., 2007) and 65% of retinoblastomas (Laurie et al., 2006), and many human cancer cell lines have been shown to overexpress MDMX

(Ramos et al., 2001). Inactivation of p53 by ectopically overexpressed MDMX has been found to be oncogenic (Marine et al., 2006).

MDMX expression has not been explored previously in neuroblastoma and as previous studies have indicated that MDMX removal may be necessary to fully activate the p53 response in other cancer types, the effect of MDMX expression in neuroblastoma on the response to two MDM2-p53 antagonists, Nutlin-3 and MI-63 is investigated in this study.

4.1.4 *MDM2*-amplification in neuroblastoma and other cancer types, and response to MDM2-p53 antagonists

Amplification of *MDM2* is reported in over 10% of 8000 human cancers from various sites (reviewed in (Toledo and Wahl, 2006)), and is amplified in a high proportion of sarcomas (20%) with wildtype p53 (Momand, 1998; Oliner et al., 1992). In neuroblastoma, a study by Carr-Wilkinson *et al* found *MDM2*-amplification in 17% of cell lines all established at relapse and with co-amplification of *MYCN*, and 13% of tumours both at diagnosis and relapse, with and without *MYCN*-co-amplification (Carr-Wilkinson et al., 2010; Carr et al., 2006). Corvi *et al.* reported *MDM2*-amplification only in the presence of *MYCN*-amplification in 15% of cell lines established at relapse, and just 1 of 25 *MYCN*-amplified tumours, from which the LS cell line was derived (Corvi et al., 1995b). There was a lack of *MDM2*-amplification in 11 tumours in one study, and 15 neuroblastomas in another (Moll et al., 1995; Waber et al., 1993). Previous reports suggest that in various cancer types amplification or overexpression of MDM2 sensitizes cells to MDM2-p53 antagonists (Gu et al., 2008b; Tovar et al., 2006; Kojima et al., 2005) whilst other groups report no effect (Liu et al., 2009; Kojima et al., 2006; Van Maerken et al., 2006). Early studies suggest that MDM2-p53 antagonists may be particularly effective in sarcomas because *MDM2* is frequently amplified in these tumours (Vassilev, 2007; Freedman et al., 1999; Momand, 1998; Florenes et al., 1994). In a panel of 18 neuroblastoma cell lines, *MYCN*-amplified neuroblastomas were found to be more sensitive to MDM2-p53 binding antagonists than non-amplified cell lines, but within the *MYCN*-amplified set, the cell lines co-amplified for *MYCN* and *MDM2* had a higher average GI_{50} value for Nutlin-3 and MI-63 than the subset with *MYCN*-amplification alone (Chapter 3, and (Gamble et al., 2011a)). In addition, there was increased caspase 3/7 activity in *MDM2*-amplified neuroblastoma cell lines following Nutlin-3 and MI-63 treatment compared to other neuroblastoma cell lines.

In this chapter, the effect of MDM2-p53 antagonists and the effect of *MDM2*-amplification in neuroblastoma is investigated further, and also the effects of MDM2-p53 antagonists in *MDM2*-amplified neuroblastoma cell lines is compared to that in *MDM2*-amplified sarcoma cell lines, since *MDM2*-amplification occurs so frequently in sarcomas, and *MDM2*-amplified sarcoma cell lines were available for comparison.

4.2 Aims and Hypotheses

Hypotheses:

- MDM2 knockdown does not alter the apoptotic response to MDM2-p53 antagonists in neuroblastoma
- High levels of MDMX expression impairs the ability of MDM2-p53 antagonists to fully activate the p53 response in neuroblastoma

Aims:

1. To examine the effect of MDM2 knockdown on the apoptotic response to MDM2-p53 antagonists in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines.
2. To examine MDMX expression in a panel of neuroblastoma cell lines and to investigate the relationship between MDMX expression, and levels of caspase activation and growth inhibition following MDM2-p53 antagonist treatment.
3. To assess the effects of MDMX knockdown in 3 neuroblastoma cell lines on induction of the p53 response, and apoptosis.
4. To investigate the relationship between MYCN and MDMX.
5. To karyotype and characterise *MDM2*-amplified non-neuroblastoma cell lines.
6. To assess differences in growth inhibition in *MDM2*-amplified neuroblastoma cell lines compared to *MDM2*-amplified sarcoma cell lines.

4.3 Specific Materials and Methods

4.3.1 siRNA mediated knockdown of MDM2

4.3.1.1 Optimisation of MDM2 knockdown

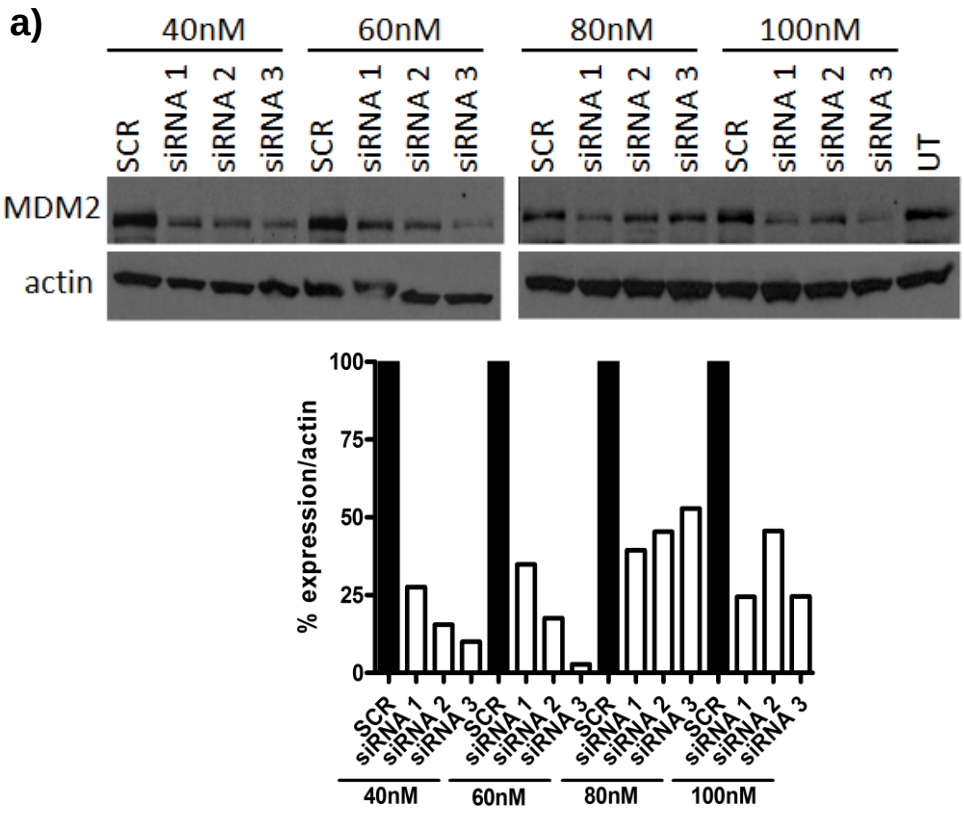
siRNA transfection was performed as described in Chapter 2.3. Initially, MDM2 was knocked down in NGP cells to assess the effectiveness of 3 siRNAs generated by Eurogentec. The sequences of the 3 siRNA are displayed in Table 2.2. Four concentrations of siRNA were chosen to test; 40, 60, 80 and 100nM. As shown in Figure 4.1a, maximum levels of knockdown were achieved using siRNA 3 at concentrations of 40nM and 60nM. To further optimise knockdown a time course was carried out at 24, 48 and 72 hour using 40nM and 60nM siRNA 3 (Figure 4.1b). The optimal levels of knockdown were achieved at either 24 hours with 40nM siRNA (90%) or 48 hours with 60nM siRNA (89%). 40nM at 24 hour was chosen so that a lower concentration of Lipofectamine® Reagent (Invitrogen) could be used.

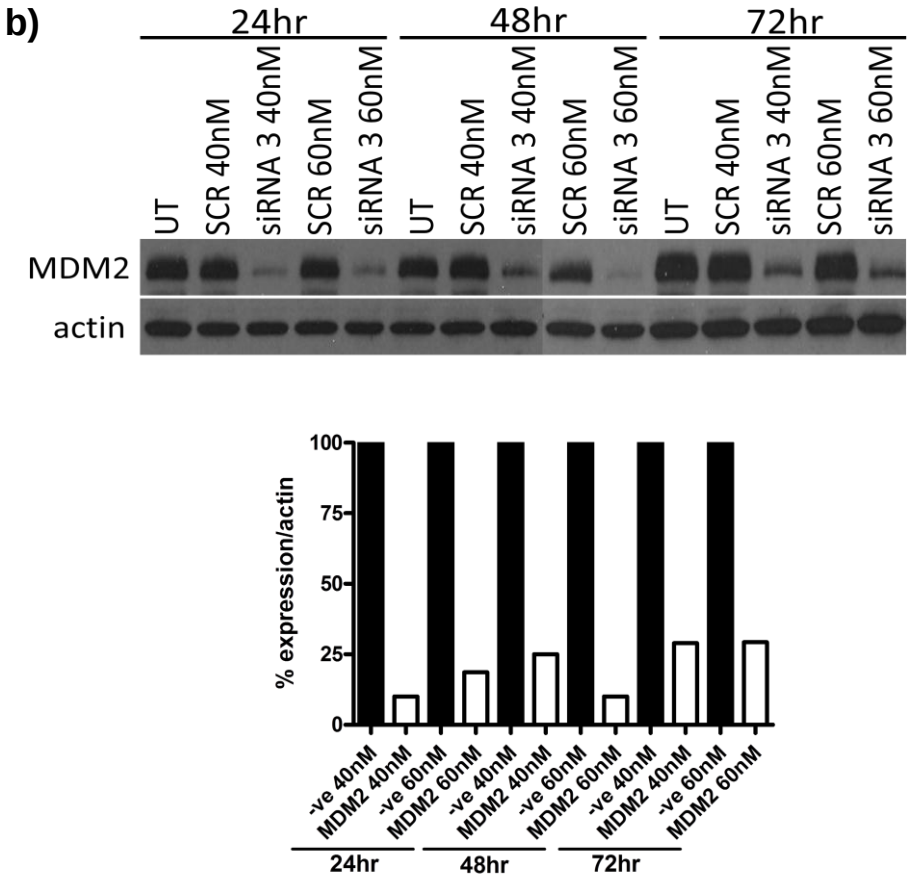
siRNA was then tested in the LS cell line, where again the optimal knockdown was achieved at 24 hour with 40nM siRNA (76%) (Figure 4.1c).

4.3.1.2 MDM2 knockdown for Western blot and caspase assay

$4x10^5$ cells were seeded in 6-well plates for Western blot analysis and left for 24 hours to adhere. Cells were treated with siRNA, and either harvested at 24 hours, media replaced with RPMI 1640 and harvested after 48 hours. For MDM2-p53 antagonist treatment studies, cells were treated with 0, 5 or 10µM Nutlin-3 or MI-63 in 2ml of media, following 24 hour knockdown.

For caspase 3/7 activity assays, cells were seeded in 96-well plates at a density of 5000 cells/well, and then treated as for Western analysis. Instead of harvesting cells, caspase assays were performed as described in Chapter 2.7.


a)
40nM
60nM
80nM
100nM
SCR
siRNA 1
siRNA 2
siRNA 3
UT
MDM2
actin
% expression/actin
100
75
50
25
0
40nM
60nM
80nM
100nM


b)
24hr
48hr
72hr
UT
SCR 40nM
siRNA 3 40nM
SCR 60nM
siRNA 3 60nM
MDM2
actin
% expression/actin
100
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50
25
0
-ve 40nM
MDM2 40nM
-ve 60nM
MDM2 60nM
24hr
48hr
72hr

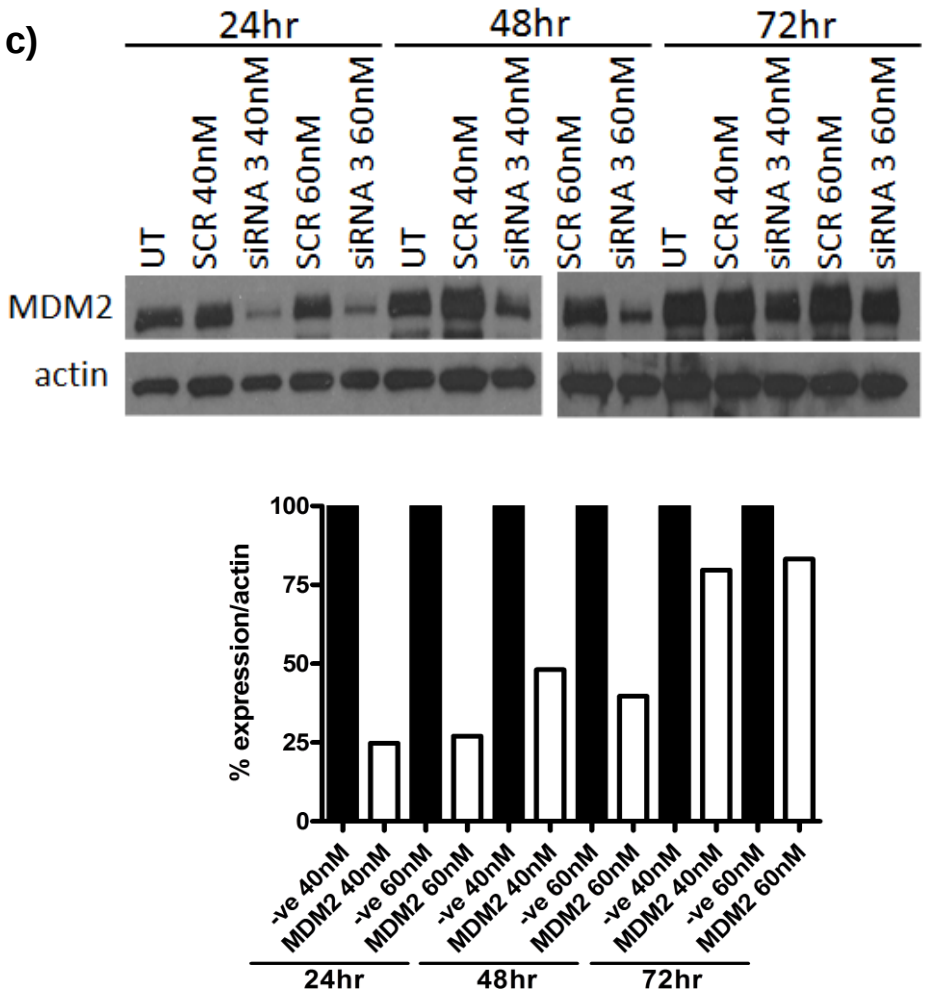


Figure 4.1. **Optimisation of MDM2 knockdown in NGP and LS cells. a)** 3 siRNAs were tested at 24 hours in NGP cells. **b)** The optimal concentrations of siRNA were tested at 24 and 48 hours in NGP cells. **c)** 40nM and 60nM siRNA 3 was tested in LS cells. The percentage expression is relative to actin, and was estimated by densitometry. siRNA sequences are shown in Table 2.2.

4.3.2 siRNA-mediated knockdown of MDMX

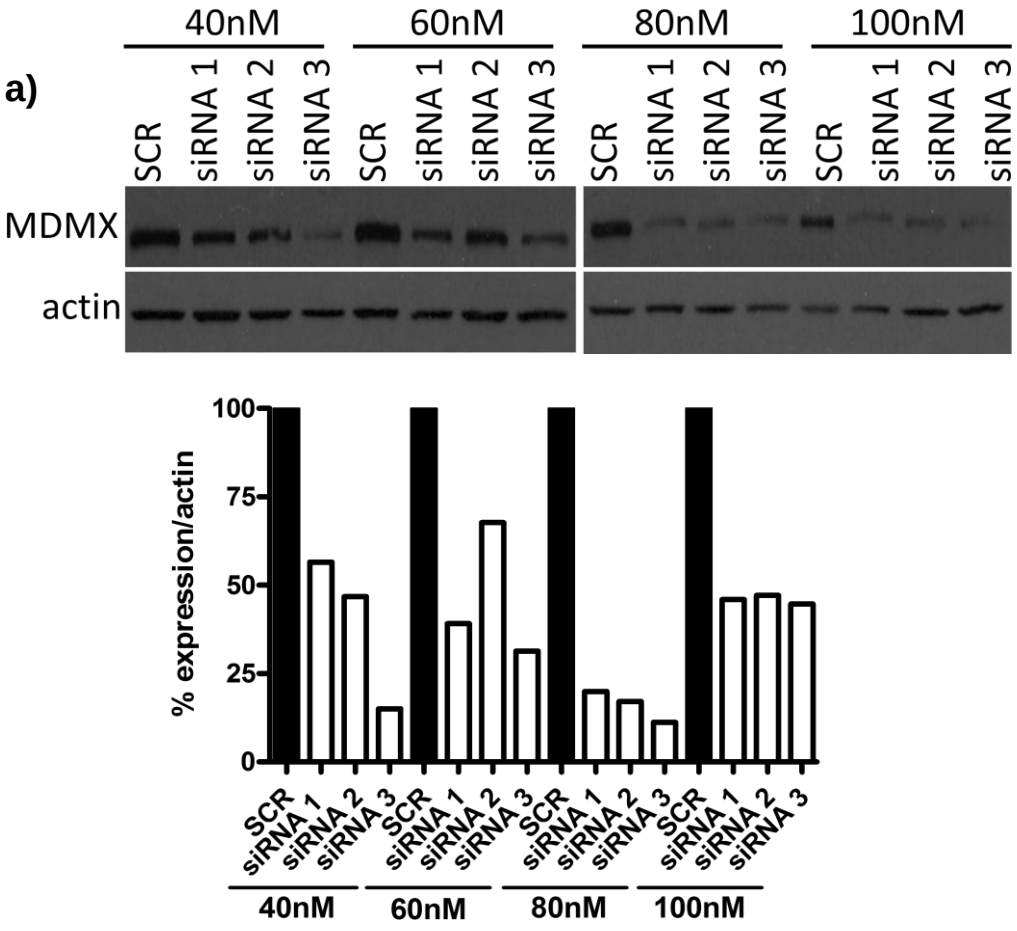
4.3.2.1 Optimisation of MDMX knockdown

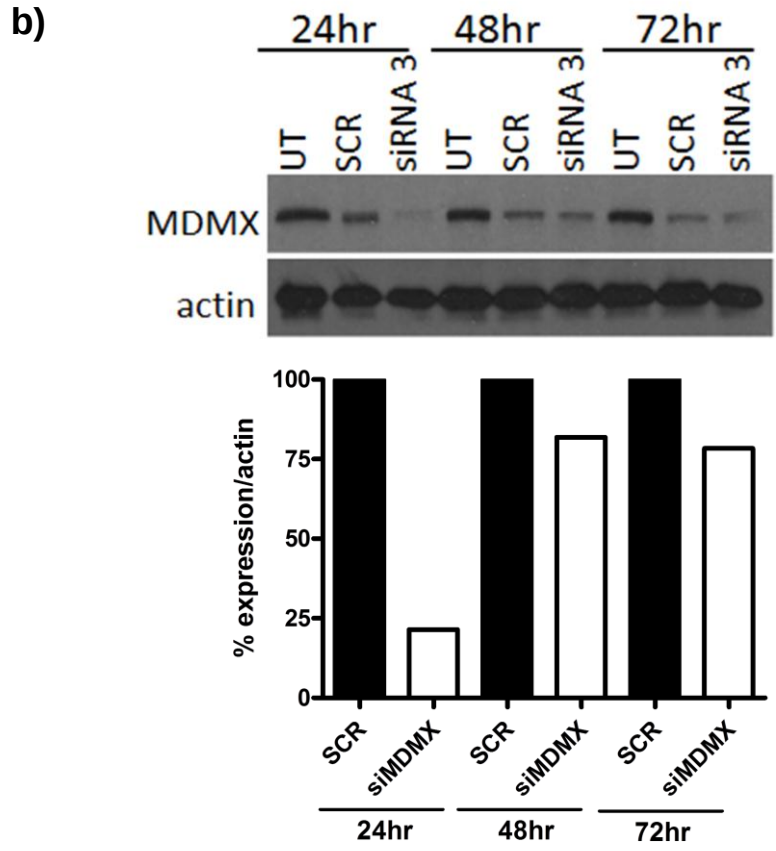
MDMX knockdown was optimised in NGP and LS cell lines. MDMX siRNA sequences are shown in Table 2.2. As shown in Figure 4.2a siRNA 3 generated the highest levels of knockdown in NGP cells. siRNA 3 was further optimised in NGP and LS cells at 24, 48 and 72 hours (Figure 4.2b and 4.2c). Greatest levels of knockdown were achieved using 40nM siRNA at 24 hours in NGP (79.58%) and LS (88.52%) cells. MDMX was knocked down in LAN5 cells, and an initial test of 40nM for 24 hours achieved high levels of knockdown.

4.3.2.2 MDMX knockdown for Western blot and caspase assay

$4x10^5$ cells were seeded in 6-well plates for Western blot analysis (2-wells per treatment) or for FACs analysis (1 well per treatment) and left for 24 hours to adhere. Cells were treated with siRNA, and either harvested at 24 hours or media replaced with RPMI 1640 and harvested after 48 hours. For MDM2-p53 antagonist studies, cells were treated with 0, 5 or 10μM Nutlin-3 or MI-63 in 2ml of media following MDMX knockdown at the optimal time point (24 hours).

Cells were seeded at a density of 5000 cells/well for caspase 3/7 activity assays and then treated as for Western analysis. Instead of harvesting cells, caspase assays were carried out as described in Chapter 2.7.


a)
40nM
60nM
80nM
100nM
SCR
siRNA 1
siRNA 2
siRNA 3
MDMX
actin
% expression/actin
100
75
50
25
0
40nM
60nM
80nM
100nM


b)
24hr
48hr
72hr
UT
SCR
siRNA 3
MDMX
actin
% expression/actin
100
75
50
25
0
SCR
siMDMX
24hr
48hr
72hr

c)

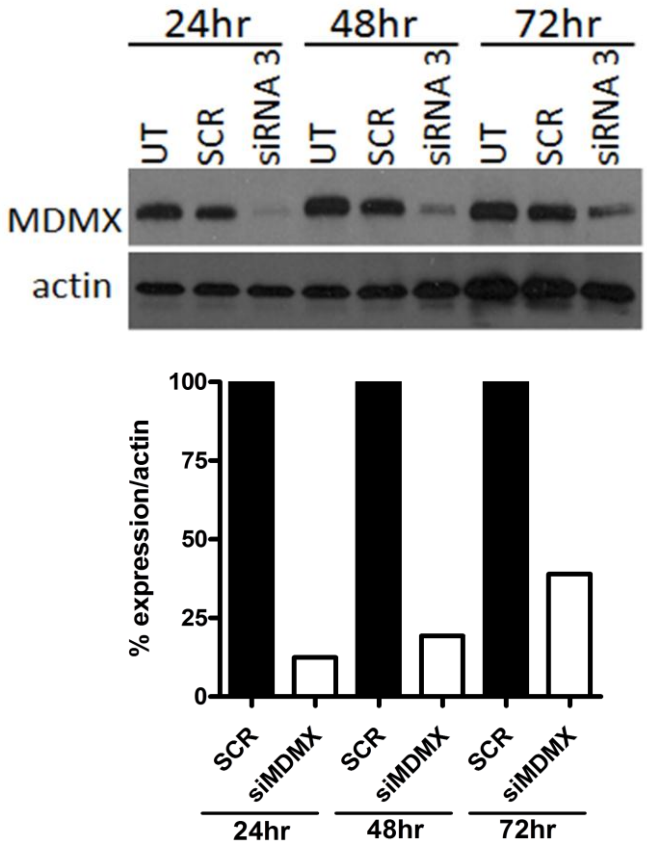


Figure 4.2. **Optimisation of MDMX knockdown in NGP and LS cells. a)** siRNAs 1-3 were tested at 24 hours with 40, 60, 80 and 100nM siRNA in NGP cells, and levels of knockdown determined by Western blot analysis and densitometry. The optimal concentration of 40nM siRNA 3 was tested at 24, 48 and 72 hours in **b)** NGP cells, and **c)** LS cells. siRNA sequences are shown in Table 2.2.

4.3.3 Tet21N time-course for MYCN and MDMX protein and mRNA expression

The relationship between MYCN and MDMX was investigated in the Tet21N cells. A time-course was performed to look at the effect on MDMX mRNA and protein following MYCN induction or MYCN removal. Protein was harvested at 0, 2, 6, 8, 24, 48 and 72 hours for MYCN(+) to MYCN(-) (tetracycline addition), and at 0, 24. 48, 72, 96 and 120 hours for MYCN(-) to MYCN(+) (tetracycline removal). mRNA was collected at early time points for both tetracycline addition and removal as if MDMX was a transcriptional target of MYCN, the effect on MYCN mRNA would occur before the effect on protein could be observed. mRNA samples were collected at 0, 2, 4, 12, 24, 48 and 72 hours for both MYCN(+) to MYCN(-) and MYCN(-) to MYCN(+) time courses.

4.3.3.1 Seeding of Tet21N cells for RNA and protein extraction

Cells were seeded for protein and RNA extraction simultaneously. Tet21N cells were grown in 6-well plates (2 wells per time point for protein, and 1 well per time point for mRNA) at a density of $2x10^4$cells/well until 48 hours, then $1x10^4$cells/well 72 hour onwards. 24 hours after seeding, tetracycline was either added (to remove MYCN expression) or removed from cells (to induce MYCN expression) and protein or RNA extracted at the specified time-point. Protein was extracted as previously described (Chapter 2.4.2). For RNA extraction, 300µl of RLT buffer was added to the well to lyse the cells, and cells gently scraped, placed in a microfuge tube (Eppendorf) and stored at -80°C until use.

4.3.3.2 Western analysis of Tet21N cells

Western blotting was performed as previously described (Chapter 2.4), and membranes probed for MYCN and MDMX. Vector only Tet21 cells were used as controls, and samples harvested at 48 hours run alongside time-course samples.

4.3.3.3 RNA extraction and determination of concentration

Lysed cells were defrosted on ice, and the RNeasy® Mini kit (Qiagen, Crawley, UK) was used to extract total cellular RNA, according to the manufacturer's protocol for 'Purification of total RNA from Animal Cells using Spin Technology'. Samples were homogenised by passing through a 23-gauge syringe 7 times, and RNA was eluted in 50µl of RNase-free water. The NanoDrop™ ND-1000 Spectrophotometer (NanoDrop

Technologies, Inc., Wilmington, DE, USA) was used to determine concentration and to assess the quality of the RNA. 1.2µl of sample was applied to the NanoDrop and absorbance measured at 260 and 280nm. The purity of the RNA was determined by the 260nm:280nm ratio, which should be between 1.9 and 2.1 for good quality RNA.

4.3.3.4 Reverse transcription to generate cDNA

cDNA was generated from the RNA samples using the TaqMan® Reverse Transcription Kit (Applied Biosystems). 1µg of RNA (made up in 19.25µl RNase-free water) was reverse transcribed for each sample in a total volume of 50µl (30.75µl reaction mix shown in Table 4.1). Thermal reaction conditions using a programmable heating block were as follows:

25°C for 10 minutes

48°C for 30 minutes

95°C for 5 minutes

4°C hold (short-term storage)

cDNA was then stored at -20°C up to 1 month.

Reagent	Volume per reaction (µl)
10x RT buffer	5
$MgCl_2$	11
dNTPs	10
Random Hexamers	2.5
RNAase inhibitors	1
RT Multiscribe	1.25

Table 4.1. **Reverse transcription PCR reagents.**

4.3.3.5 Quantitative Reverse-Transcription Polymerase Chain Reaction

4.3.3.5.1 Principles of quantitative PCR

PCR is a technique used to amplify a DNA target sequence, and with real-time technology, the levels of the DNA target sequence can be quantified. With standard PCR, the reaction product is measured at the end, but real-time quantitative PCR allows for the detection of PCR amplification during the early phases of the reaction and the product is measured as the reaction progresses and the product accumulates. The higher

the starting level of the target sequence, the fewer cycles at which a significant level is reached.

There are 3 steps to a PCR reaction;

1. Denaturation at 95°C. Double stranded DNA is melted to single strands for replication.
2. Primer annealing. Primers complementary to the two strands at specific sequences flanking the target DNA anneal to the target DNA when the reaction is cooled to 60°C. Primers are in large excess preventing the two original strands of DNA reannealing.
3. Elongation. A thermostable DNA polymerase elongates the primer, producing a complementary strand of DNA. This process is repeated again and again, amplifying target DNA.

The Taqman™ (Applied Biosystems) system was used, which involves a fluorogenic labelled probe that binds to the target sequence downstream of the primer, which is designed with a high energy (has a high energy transfer efficiency when excited) reporter dye (fluorescein) at the 5' end, and a low energy quencher molecule (TAMRA) at the 3' end. When the probe is intact and excited by a light source, the emission from the reporter dye is suppressed by the quencher dye due to the close proximity of the two, and energy is transferred from high to low through fluorescence resonance energy transfer (FRET), reducing the level of fluorescence from the reporter dye. AmpliTaq Gold® DNA polymerase has 5'exo-nuclease activity. As the DNA polymerase extends the primer, it encounters and digests the probe, releasing the reporter dye from the quencher and increasing the distance between the two, causing the transfer of energy to stop. The fluorescent emission of the reporter dye increases, and this increase is captured by the detection instrument within the 7900 HT Real-Time PCR system (Applied Biosystems), which detects fluorescence emissions at 500-600nm.

When the fluorescence signal reporter increases to a detectable level, it is captured and displayed as an amplification plot and the threshold line is the level of detection at which a reaction reaches fluorescence intensity above background. The threshold line is set in the exponential phase of amplification for the most accurate reading and the cycle at which the sample reaches this level is called the cycle Threshold (Ct).

4.3.3.5.2 Real-time PCR protocol

The primers and probes used were inventoried TaqMan® Gene Expression Assays (Applied Biosystems); MDMX, Hs00159092.m1; MYCN, Hs00232074_m1; 18S

ribosomal RNA, Hs0.928985_g1. All results were normalised to 18S ribosomal RNA control. Relative quantification using the standard curve method was used to determine expression levels of target genes within samples. A standard curve was included on all plates, generated from a sample with high levels of the target gene (in this case tetracycline(-) Tet21N cells at 0 hours, with high levels of MYCN) and prepared as follows:

Standard 1 – neat
Standard 2 – 1/10 dilution
Standard 3 – 1/100 dilution
Standard 4 – 1/1000 dilution
Standard 5 – 1/10000 dilution

MasterMixes were prepared as shown in Table 4.2, for use in 384 well plates with a final volume of 10µl per well and each sample set up in triplicate (2.5µl cDNA, and 7.5µl MasterMix per well). A total of 22 samples were set up for each set of primers and probes (6 standards, 7 time points for two experiments (MYCN(+) to MYCN(-), and MYCN(-) to MYCN(+) at 0, 2, 4, 12, 24, 48, and 72 hours), and no template control (NTC)).

Reagent	Volume per reaction (µl)	Volume for 22 samples (µl)	Volume for triplicate reactions (µl)	Volume + 12% (µl)
MasterMix (x2)	5	110	330	369.6
Primers and Probes (x20)	0.5	11	33	37.0
Nuclease-free water	2	44	132	147.8

Table 4.2. **Quantitative real-time PCR reaction reagents per primer/probe set.**

Once plates were set up, they were spun at 1500rpm for 1 minute in a 4K15 centrifuge (Sigma) to remove any air bubbles, and run on a 7900 HT RT PCR system (Applied Biosystems), with SDS software (Applied Biosystems). Thermal cycling conditions are as shown in Figure 4.3, and fluorescence emissions at 500-600nM wavelength were detected.

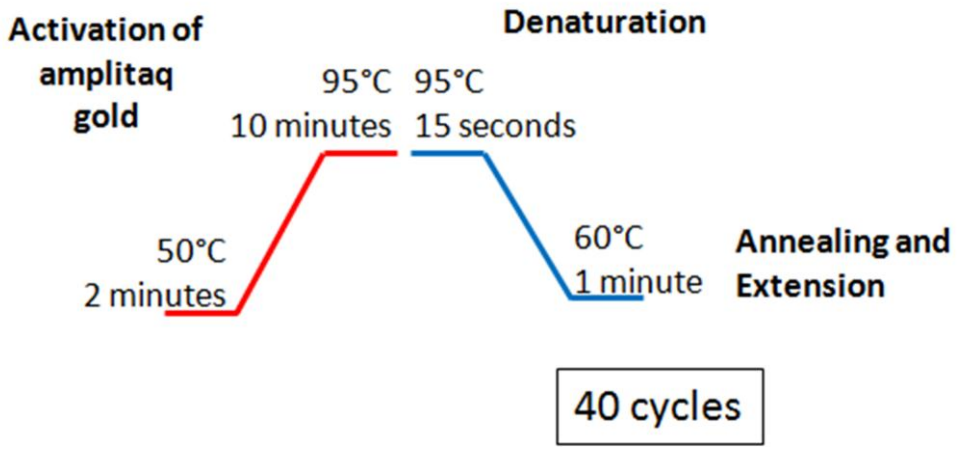
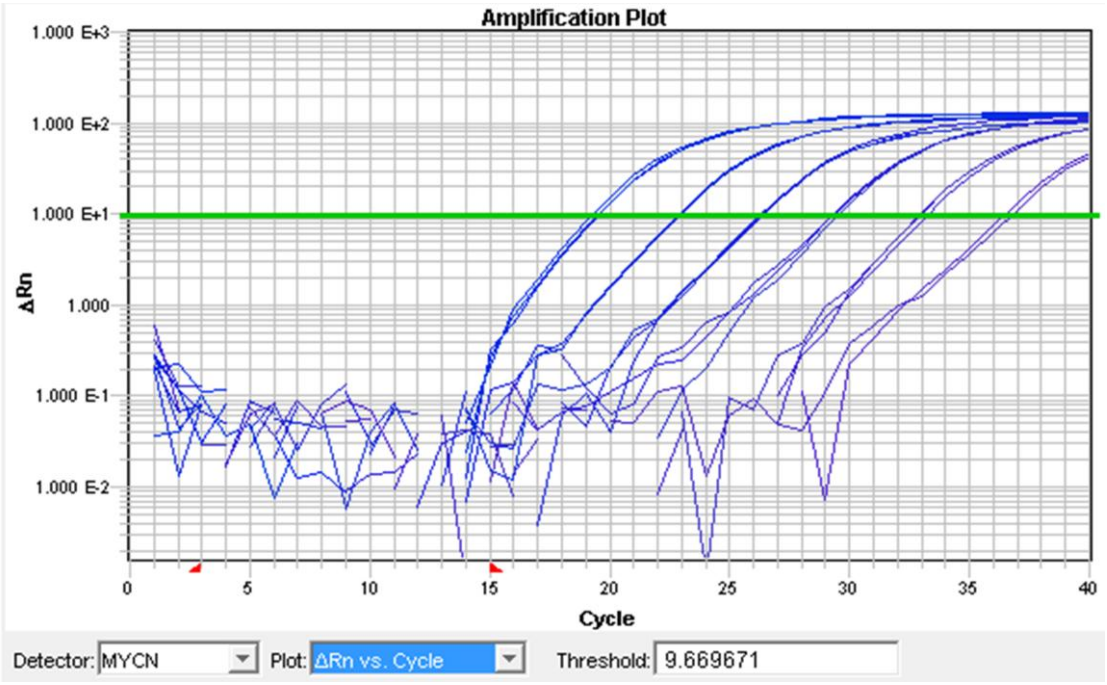


Figure 4.3. **Thermal cycling programme for quantitative real-time PCR.**

4.3.3.5.3 Analysis of real-time PCR results

SDS 2.2 software (Applied Biosystems) was used to analyse real-time PCR results. Amplification plots were generated for each well (magnitude of signal, ΔRn (the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye), against cycle number) and a standard curve generated as shown in Figure 4.4. Unknown values were calculated relative to the standard curve. Ct values are the number of cycles required to meet the threshold level of expression. The mean quantities were then normalised to mean 18S ribosomal RNA quantities for each time point, and graphs plotted using GraphPad Prism software.

a)



b)

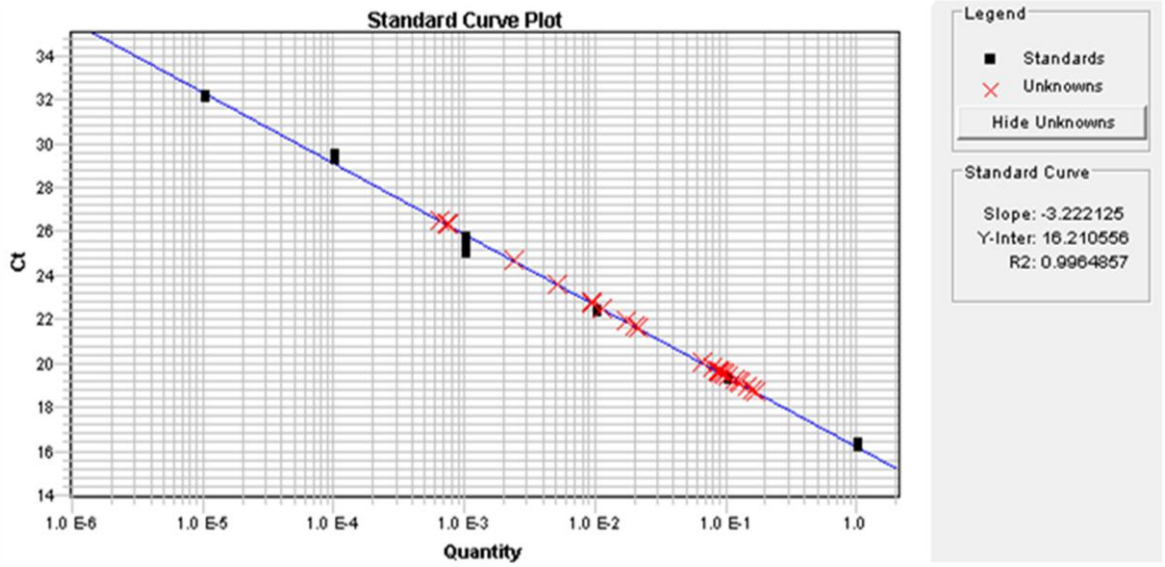


Figure 4.4. **a)** amplification plots for MYCN standards, generated by SDS software. Threshold level is indicated in green. **b)** Standard curve for MYCN.

4.3.4 Growth inhibition assays

The cell density was determined as previously described in Section 2.6.1. Growth inhibition curves, SRB assays and GI_{50} values were calculated as described in Section 2.6. All neuroblastoma cell lines were seeded at the densities specified in Table 3.1, and sarcoma cell lines at the following number of cells per well in 96-well plates: SJSA-1 - 2500, RH18 - 4000, MHM - 3000, T449 - 5000, T778 - 3000.

4.3.5 Fluorescence in situ hybridisation (FISH)

4.3.5.1 Principles of FISH

FISH is a cytogenetic technique used to detect the presence of specific nucleic acid sequences on chromosomes by hybridisation using fluorescent labelled DNA probes. The probes bind to parts of the chromosome with high sequence similarity, and hybridisation results in duplex formation of sequences present in the test material and the specific gene probe. Fluorescence microscopy is then used to visualise the location and copy number per cell of the probes.

4.3.5.2 ZytoLight® System

The ZytoLight SPEC MDM2/CEN 12 Dual Color Probe Kit (ZytoVision Gmbh, Bremerhaven, Germany) is designed for the detection of MDM2, and chromosome 12 centromeric alpha-satellite sequences by FISH. The probe is a mixture of an orange fluorochrome direct labelled CEN 12 probe, specific for the alpha satellite centromeric region of chromosome 12 (D12Z3) (ZyOrange: excitation at 547nm and emission at 572nm), and a green fluorochrome direct labelled SPEC MDM2 probe, hybridising distal and proximal to the human MDM2 gene in chromosomal region 12q14.3-12q15 (ZyGreen: excitation at 503nm and emission at 528nm). Duplex formation of the fluorescence-labelled probes can be visualised using fluorescence microscopy. In a normal diploid cell interphase nucleus, two orange and two green signals are expected. In a cell with amplification of the *MDM2* gene locus as double minutes (DMs) or homogeneously staining regions (HSRs), multiple copies of the green signal or green signal clusters will be observed as shown in Figure 4.5.

4.3.5.3 Protocol

Cytospins of the cell lines were created, and the methods carried out according to the ZytoLight SPEC MDM2/CEN 12 Dual Color Probe Kit protocol. The pre-treatment

step was not required as cells were not paraffin embedded. For the denaturation and hybridisation step, 10µl of probe was pipetted onto the slide, and cells gently covered with a coverslip ensuring no air bubbles. The coverslip was then sealed with rubber cement, and placed on a 75°C hotplate for 10 minutes to allow denaturation. Slides were then transferred to a humidity chamber and probes allowed to hybridise overnight at 37°C. The coverslip was removed by submerging in 1x wash buffer A at 37°C for 3 minutes, and then washed in the same buffer for 2x 5 minutes at 37°C. Slides were incubated in 70%, 90% and 100% ethanol for 1 minute each (protected from light), and 30µl of DAPI/Antifade-Solution added, and cells covered again with a coverslip. After 15 minutes, excess DAPI/Antifade-Solution was removed and slides stored in the dark. A fluorescence detection microscope (Olympus BX61) and Cytovision software version 7.1 (Molecular Devices) was then used to visualise copies of MDM2 and the centromeric region of chromosome 12.

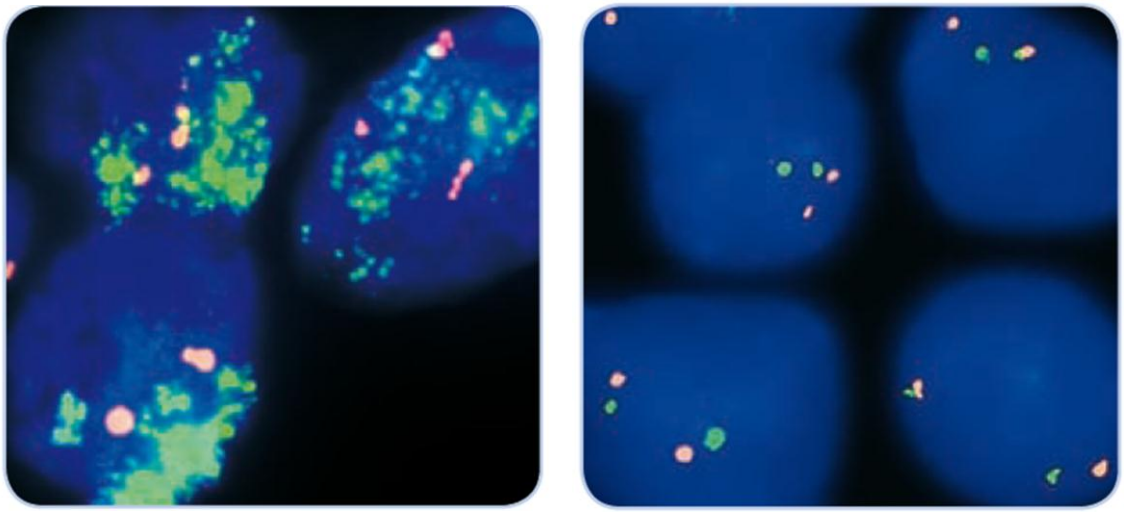


Figure 4.5. **a)** FISH showing amplification of MDM2 gene (green) and CEN 12 (orange). **b)** A normal interphase cell showing 2 copies of MDM2 (green) and 2 copies CEN 12 (orange). (http://www.zytovision.com/Manuals/zytolightmanuals.html).

4.4 Results

4.4.1 siRNA-mediated knockdown of MDM2 results in induction of p53 and p21^{WAF1} protein expression, and increased caspase 3/7 activity

Two *MYCN* and *MDM2* co-amplified neuroblastoma cell lines (NGP and LS) were used to assess the effect of knocking down MDM2 expression on the p53 response. As shown in Figure 4.6a, knockdown of MDM2 alone resulted in induction of p53 and the p53 transcriptional target p21^{WAF1} at both 24 and 48 hours. Caspase 3/7 activity also increased 24 hours following MDM2 knockdown (Figure 4.6b). This increase in p53, p21^{WAF1} and caspase activity following MDM2 knockdown supports a role for MDM2 inhibitors as a therapeutic approach in neuroblastoma.

4.4.2 MDM2 knockdown does not affect the p53 or apoptotic response to Nutlin-3 and MI-63

There are conflicting reports about the effect of MDM2 overexpression or amplification on the response to MDM2-p53 antagonists in different cancer cell types. To test the effect of *MDM2*-amplification in neuroblastoma on the response to Nutlin-3 and MI-63, MDM2 was knocked down in NGP and LS cells, followed by treatment with Nutlin-3 and MI-63. As shown in Figure 4.7, 5μM and 10μM Nutlin-3 or MI-63 treatment results in an induction of p53 and p53 phosphorylation at serine 15, and p21^{WAF1} and induction of the p53 target gene and apoptotic marker PUMA, and caspase 3 and PARP cleavage (SCR siRNA control). Induction of apoptosis is also observed with a dose-dependent increase in caspase 3/7 activity following Nutlin-3 and MI-63 treatment as shown in Figure 4.8 (SCR siRNA control).

Following MDM2 knockdown, despite a clear suppression of the induction of MDM2 protein levels by siMDM2, there is no evidence of an altered response to MDM2-p53 in terms of p53 induction, and apoptotic markers as shown in Figure 4.7 for both NGP and LS cell lines, and there is no significant difference in the levels of caspase 3/7 activity compared to SCR control (Figure 4.8). Interestingly, in NGP cells p21^{WAF1} and PUMA protein levels decreased after Nutlin-3 and MI-63 treatment following MDM2 knockdown, but increased in LS cells (Figure 4.7). The only consistent change is increased phosphorylated p53 at serine 15 following MDM2 knockdown and 5μM Nutlin-3 treatment compared to SCR control in both cell lines.

These results suggest that the initial MDM2 protein levels at the start of treatment are not important in determining the growth inhibitory or apoptotic signalling response to MDM2-p53 antagonists. Increased caspase 3/7 activity previously detected in *MDM2*-amplified cell lines following treatment with MDM2-p53 antagonists is likely to be due to increased $p14^{ARF}$ expression (Chapter 3.4.13), as $p14^{ARF}$ impairment or knockdown results in a desensitisation to MDM2-p53 antagonist mediated apoptosis as shown in Chapter 5. Results are also consistent with no difference in sensitivity to MDM2-p53 antagonists between *MYCN*/*MDM2* co-amplified cell lines compared with *MYCN*-amplified cell lines. Therefore there are two lines of evidence showing that MDM2 levels do not affect response to MDM2-p53 antagonists in neuroblastoma.

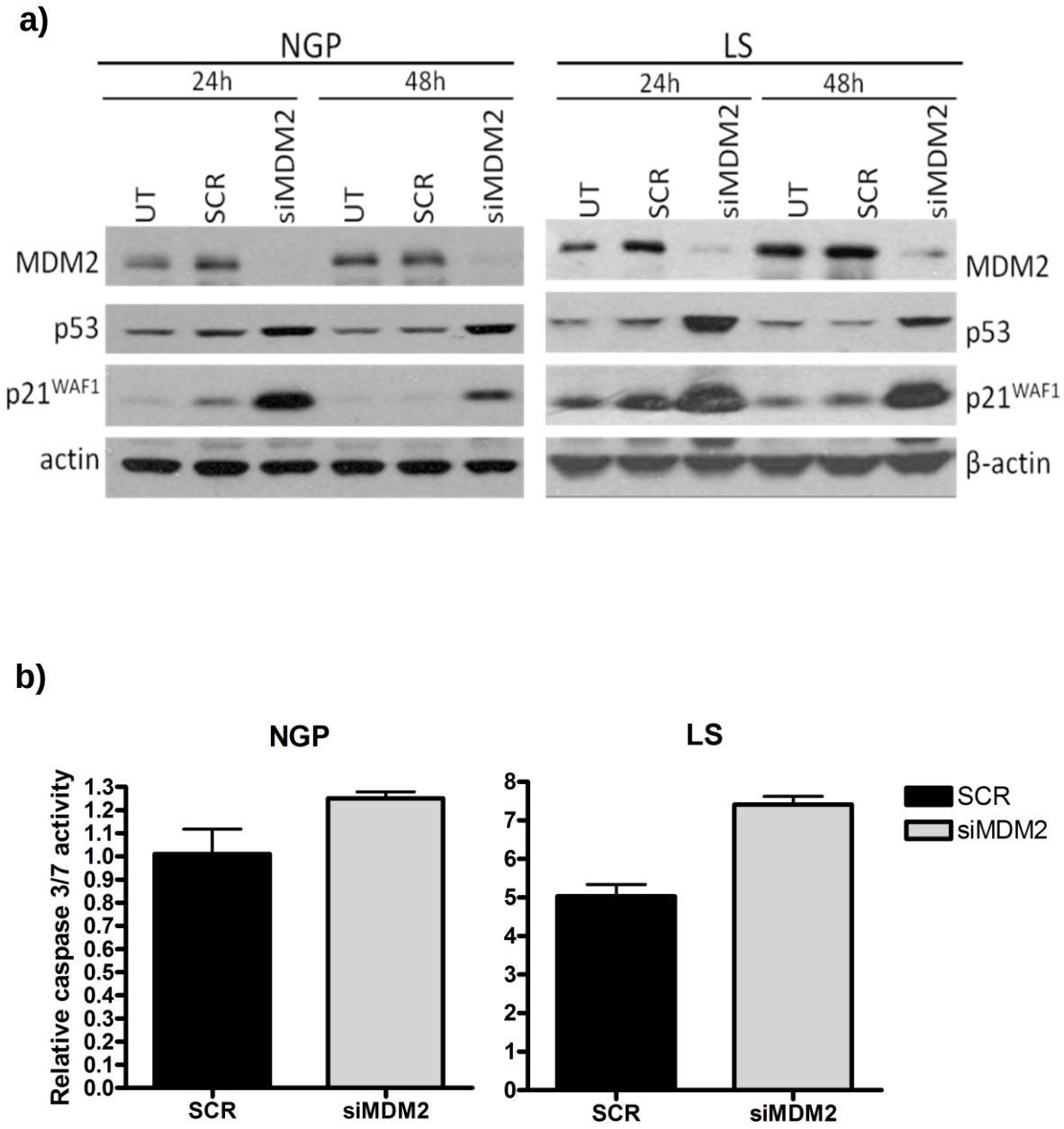


Figure 4.6. **MDM2 knockdown in 2 *MYCN* and *MDM2* co-amplified neuroblastoma cell lines (NGP and LS). a)** MDM2 knockdown induces p53 and $p21^{WAF1}$ at 24 and 48 hours. Actin was used as a loading control. **b)** caspase 3/7 activity increases 24 hours after MDM2 knockdown.

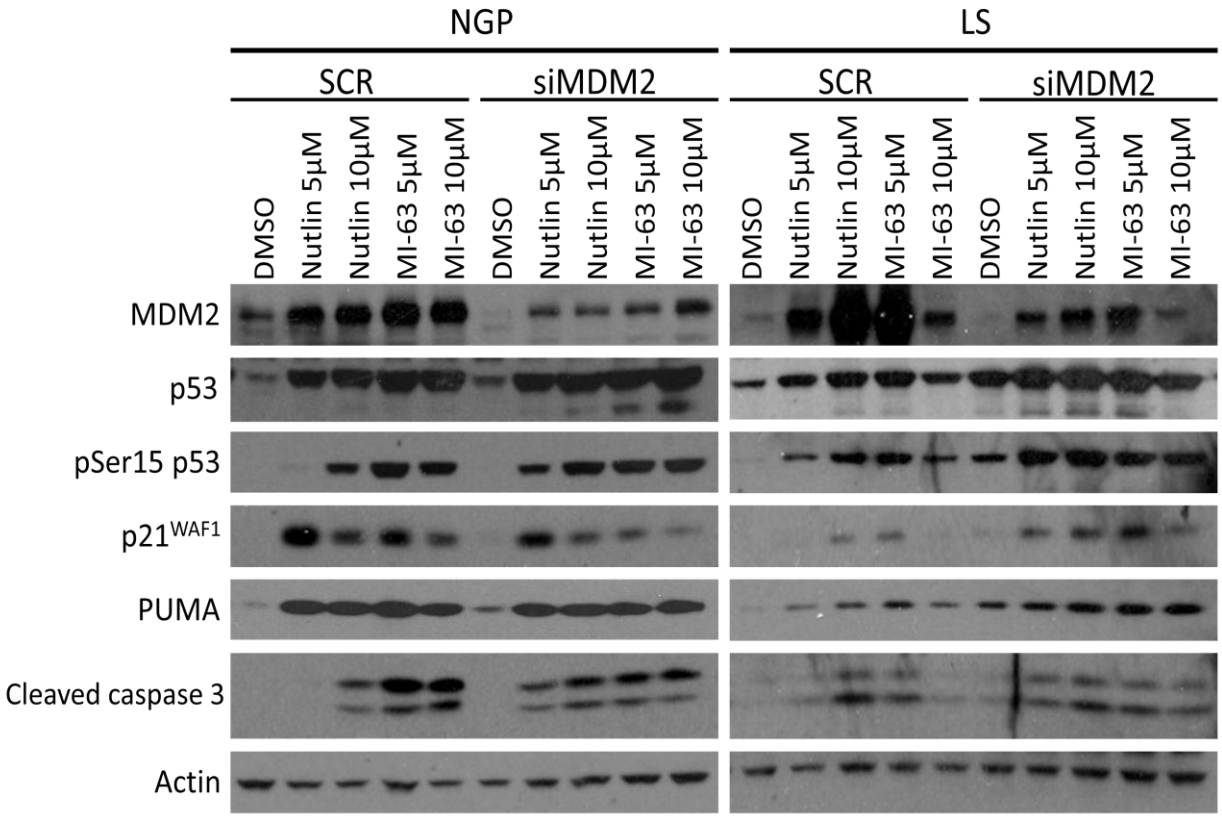


Figure 4.7. **MDM2 knockdown in NGP and LS cells followed by Nutlin-3 or MI-63 treatment results in no obvious change in the induction of p53 and p53 transcriptional targets or levels of apoptotic markers compared to control.** Actin was used as a loading control. (n=2 NGP, n=1 LS)

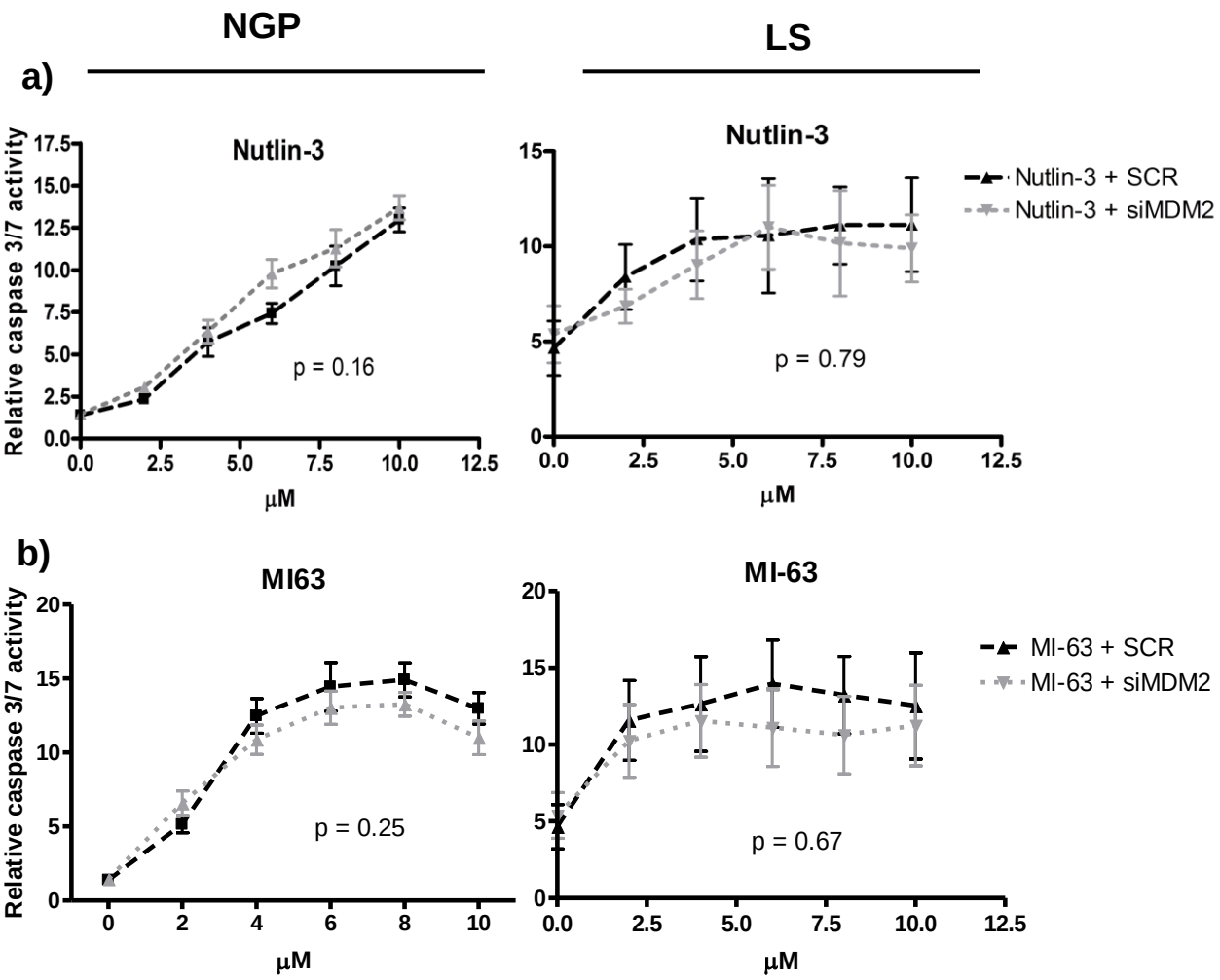


Figure 4.8. **No difference in caspase 3/7 activity was observed following MDM2 knockdown compared to SCR control following a) Nutlin-3 or b) MI-63 treatment** (NGP; Nutlin-3 p = 0.16, MI-63 p = 0.25. LS; Nutlin-3 p = 0.79, MI-63 p = 0.67, 2-way ANOVA).

4.4.3 MDMX expression varies across neuroblastoma cell lines but correlates with the extent of caspase 3/7 activity following MDM2-p53 antagonist treatment

MDMX protein expression levels were determined in a panel of 21 neuroblastoma cell lines (including MYCN(+) and MYCN(-) Tet21N cells). As shown in Figure 4.9a, MDMX protein expression is variable across 21 neuroblastoma cell lines and does not appear to have any relationship with *MYCN*-amplification status. *MDM2*-amplified cell lines have low MDMX expression levels compared to *MYCN*-amplified cell lines, supporting a role for MDM2 in the ubiquitination and subsequent degradation of MDMX. The caspase 3/7 activity and GI_{50} values following MDM2-p53 antagonist treatment in the panel of 21 cell lines previously determined in Chapter 3.4.11-13, was examined for any correlation with MDMX protein expression (quantitative values determined using densitometry). As shown in Figure 4.9b, apart from three cell lines which fell into an obvious separate group (NBLW, NBLS and NB69), and not including the low MDMX expressing *MDM2*-amplified cell lines or p53 mutant cell lines, the remaining 12 of 15 cell lines, including Tet21N, have a highly significant positive correlation between caspase 3/7 activity and MDMX protein expression for both Nutlin-3 ($p = 0.0001$, $r^2 = 0.78$) and MI-63 ($p < 0.0001$, $r^2 = 0.88$). Interestingly, cell lines with high MDMX expression show high induction of caspase 3/7 activity, but not growth inhibition in the SRB assay (as shown in Figure 4.9c). This implies that these cells did not show a cell cycle arrest response and suggests that a high level of MDMX expression is associated with defective cell cycle arrest. However, there was an inverse correlation between caspase 3/7 activity and GI_{50} values, which was significant for Nutlin-3 ($p=0.015$), but not MI-63 ($p = 0.076$). A lot of weight cannot be attributed to this since both had low r^2 values, a measure of how well the regression line approximates the real data points ('goodness of fit') (Figure 4.9d).

4.4.4 N-type neuroblastoma cells have increased MDMX protein expression compared to S-type cells

In Figure 4.9a, SHSY5Y cells have high levels of MDMX protein but interestingly, SHEP cells do not, despite both being derived from the SKNSH cell line. SHSY5Y is an N-type clone of SKNSH, whereas SHEP is an S-type clone. As shown in Figure 4.10, grouping the neuroblastoma cell lines into N- and S-type reveals that S-type neuroblastoma cells have lower overall MDMX expression (determined from

densitometric analysis of MDMX in Figure 4.9a) compared to N-type cells, with N/S mixed cell lines falling in the middle. (p=0.0047, S- and N/S-type vs. N type). This suggests that MDMX expression may differ according to differentiation status in neuroblastoma. Again *MDM2*-amplified cell lines were excluded from the analysis as they have low MDMX expression as a result of *MDM2*-mediated degradation.

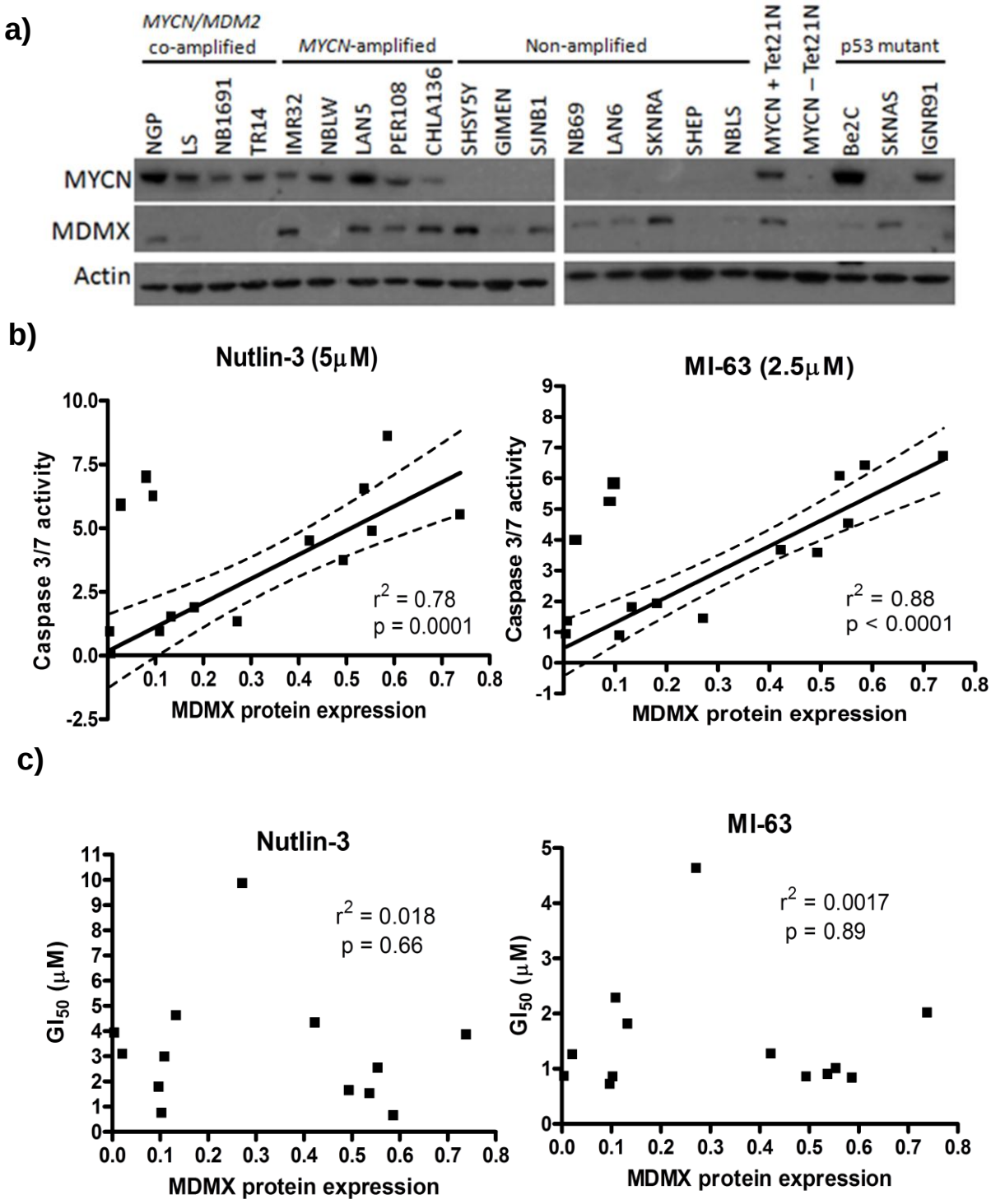
4.4.5 Knockdown of MDMX results in induction of p53 and $p21^{WAF1}$ and increased caspase 3/7 activity

The MDM2-p53 antagonists Nutlin-3 and MI-63 are not active against MDMX despite strong sequence and structural homology between MDM2 and MDMX. Knockdown of MDMX in two *MYCN* and *MDM2* co-amplified cell lines (LS and NGP), and one *MYCN*-amplified cell line (LAN5) resulted in a strong activation of the p53 response, resulting in induction of p53, p53 phosphorylation at serine 15, and induction of the p53 target gene $p21^{WAF1}$ (Figure 4.11a). MDM2 levels decreased slightly, supporting a role for MDMX in the stabilisation of MDM2, as previously published (Pereg et al., 2005). An increase in caspase 3/7 activity was also observed following MDMX knockdown (Figure 4.11b), and to a greater extent than that seen upon MDM2 knockdown at 24 hours (Figure 4.6b). Since MDMX is also a negative regulator of p53, this data suggests that MDMX removal may be necessary to fully activate p53 in response to MDM2-p53 antagonists in neuroblastoma.

4.4.6 siRNA-mediated knockdown of MDMX results in decreased MDM2-p53 antagonist-mediated apoptosis

Interestingly, despite induction of p53 and caspase 3/7 activity following MDMX knockdown alone, knockdown of MDMX followed by MDM2-p53 antagonist treatment resulted in decreased apoptosis. MDMX was knocked down for 24 hours in NGP, LS and LAN5 cells followed by treatment with 5μM or 10μM Nutlin-3 or MI-63. In NGP and LS cells, no effect on p53 and $p21^{WAF1}$ protein levels or p53 phosphorylation was observed compared to the SCR control (Figure 4.12). As shown in Figure 4.13, no alteration in cell cycle distribution was observed following MDMX knockdown compared to SCR control after Nutlin-3 or MI-63 treatment in NGP and LS cells with NGP cells G_1 arresting, and LS cells not (similar to the effects seen upon MYCN knockdown in Chapter 3.4). This is consistent with the unchanged $p21^{WAF1}$ levels. However, LAN5 cells, which are *MYCN*-amplified but not *MDM2*-amplified and have the highest MDMX protein levels (Figure 4.9a), had a reduced p53 response following

MDMX knockdown and MDM2-p53 antagonist treatment (Figure 4.12), suggesting that MDMX may be more important in non-*MDM2*-amplified cell lines. In all cases PUMA and cleaved caspase 3 levels decreased whether an effect on p53 was observed or not (Figure 4.12), and in support of these results, caspase 3/7 activity assays showed a significant and pronounced reduction in caspase 3/7 activity following 24 hours Nutlin-3 or MI-63 treatment (Figure 4.14) (LS; Nutlin-3 $p = 0.0024$, MI-63 $p = < 0.001$, NGP; Nutlin-3 $p < 0.001$, MI-63 $p < 0.001$, LAN5; Nutlin-3 $p = 0.0004$, MI-63 $p = 0.0024$). Since these results contradict published reports in other cancer cell types, caspase activity was checked at 12 hours and no difference in caspase activity was seen between SCR and siMDMX treated cells (Figure 4.15). Again, in all 3 cell lines, MDM2 levels decreased following MDMX knockdown (Figure 4.12). Since it had previously been observed that MDM2 levels do not affect the response to MDM2-p53 antagonists (refer to Section 4.4.2), it cannot be the destabilisation of MDM2 that is causing the unexpected resistance to these compounds following MDMX knockdown.


a)
MYCN/MDM2 co-amplified
MYCN-amplified
Non-amplified
MYCN + Tet21N
MYCN – Tet21N
p53 mutant
NGP
LS
NB1691
TR14
IMR32
NBLW
LAN5
PER108
CHLA136
SHSY5Y
GIMEN
SJNB1
NB69
LAN6
SKNRA
SHEP
NBLS
Be2C
SKNAS
IGNR91
MYCN
MDMX
Actin
b)
Nutlin-3 (5μM)
Caspase 3/7 activity
MDMX protein expression
r2 = 0.78
p = 0.0001
MI-63 (2.5μM)
r2 = 0.88
p < 0.0001
c)
Nutlin-3
GI50 (μM)
r2 = 0.018
p = 0.66
MI-63
r2 = 0.0017
p = 0.89

d)

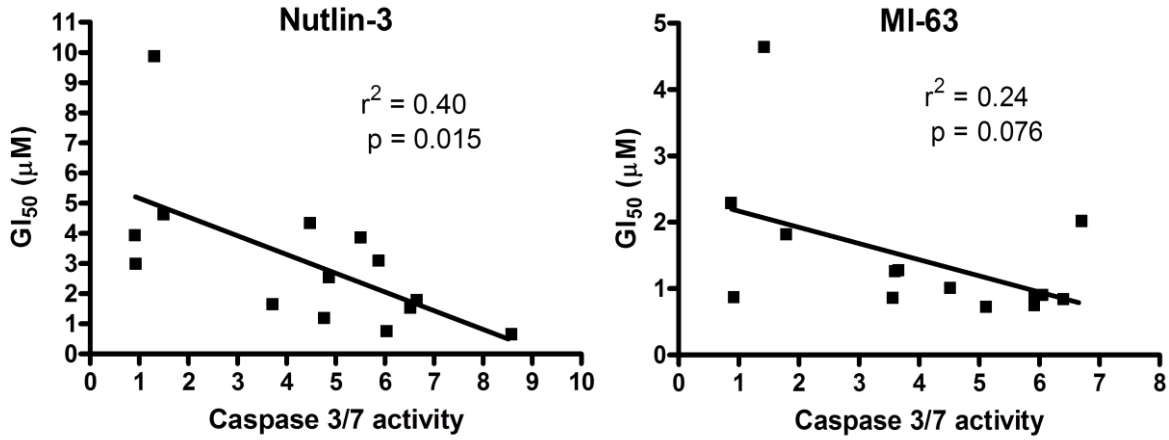


Figure 4.9. **Neuroblastoma cell lines have variable MDMX protein expression which correlates with caspase 3/7 activity but not growth inhibition. a)** MDMX expression in a panel of neuroblastoma cell lines alongside their *MYCN*, *MDM2* and p53 status. Be2C and IGNR91 p53 mutant cell lines are *MYCN*-amplified. Actin was used as a loading control. **b)** Caspase 3/7 activity following 24 hours Nutlin-3 (5μM) or MI-63 (2.5μM) treatment correlates with MDMX protein expression, with the exception of 3 cell lines; NBLW, NB69 and NBLS. 95% confidence intervals are displayed. (Nutlin-3, $p = 0.0001$, $r^2 = 0.78$; MI-63; $p < 0.0001$, $r^2 = 0.88$). **c)** GI_{50} values were calculated following 72 hour treatment, and do not correlate with MDMX protein expression (Nutlin-3, $p = 0.75$, $r^2 = 0.0085$; MI-63; $p = 0.971$, $r^2 = 0.0001$). **d)** There is a weak inverse correlation between Nutlin-3 induced caspase 3/7 activity and GI_{50} ($p=0.015$, Pearson correlation), and whilst not significant ($p=0.076$, Pearson correlation), there is also a trend towards a correlation with MI-63.

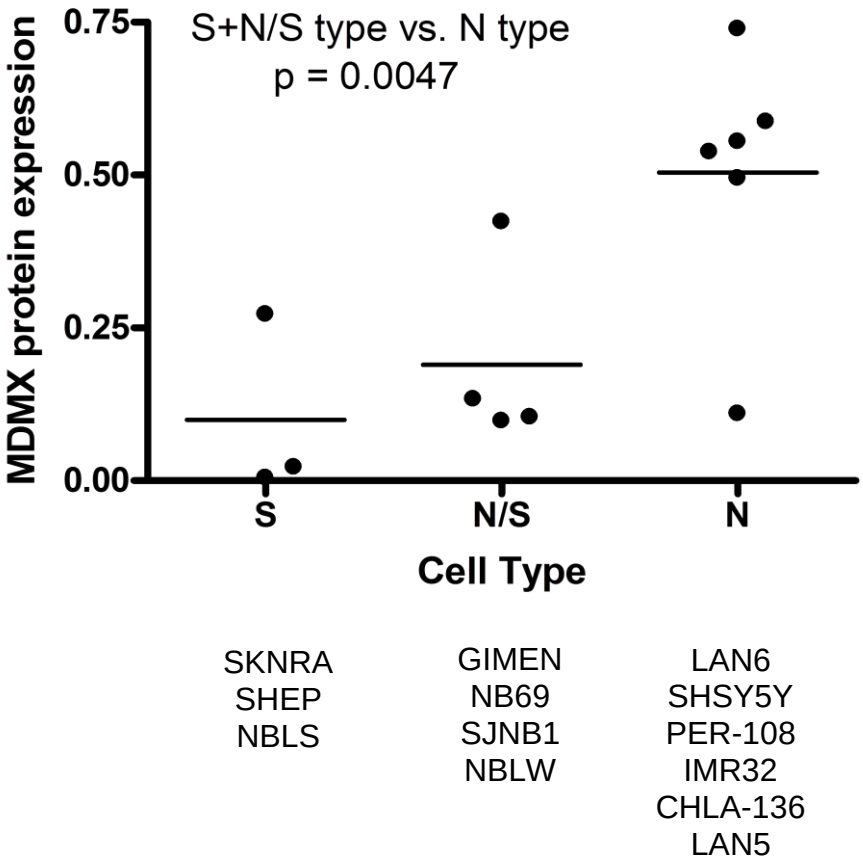
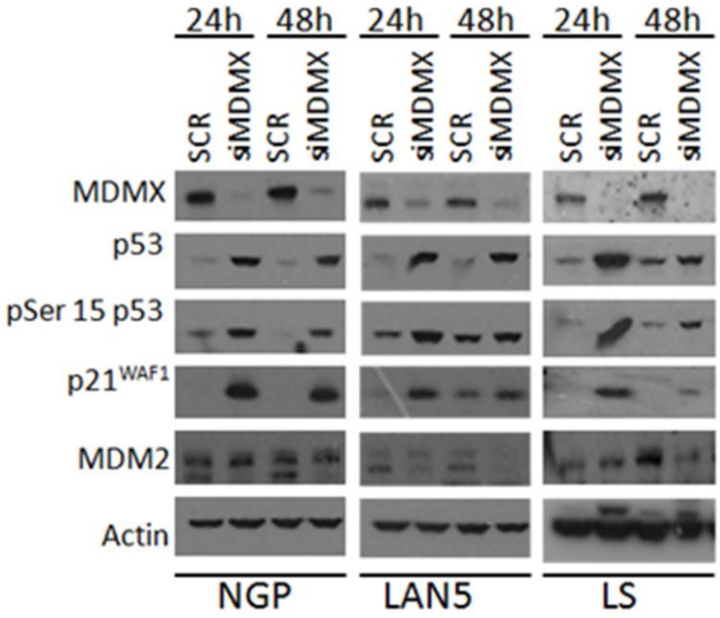


Figure 4.10. **MDMX protein expression levels may be influenced by neuroblastoma cell type. (S – substrate-adherent, N – neurite-bearing, N/S – mixture of N and S type). p = 0.0047 (unpaired t-test). *MDM2*-amplified and p53 mutant cell lines are excluded from analysis.**

a)



b)

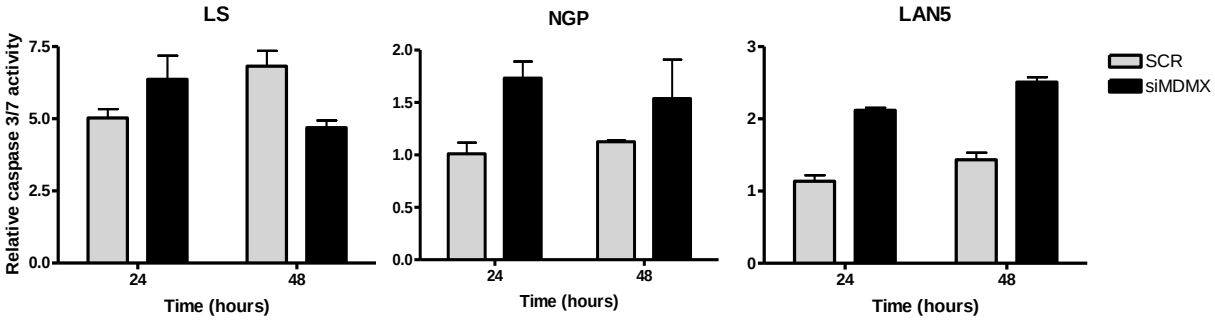


Figure 4.11. **MDMX knockdown induces a p53 response and apoptosis in 3 neuroblastoma cell lines; LS, NGP (*MYCN* and *MDM2* co-amplified) and LAN5 (*MYCN*-amplified). a)** Knockdown of MDMX results in induction of p53, p53 phosphorylation and induction of the p53 transcriptional target $p21^{WAF1}$. **b)** Caspase 3/7 activity is increased after MDMX knockdown compared to SCR control at 24 and 48 hours in all cell lines, except LS where an increase is seen at 24 hours only.

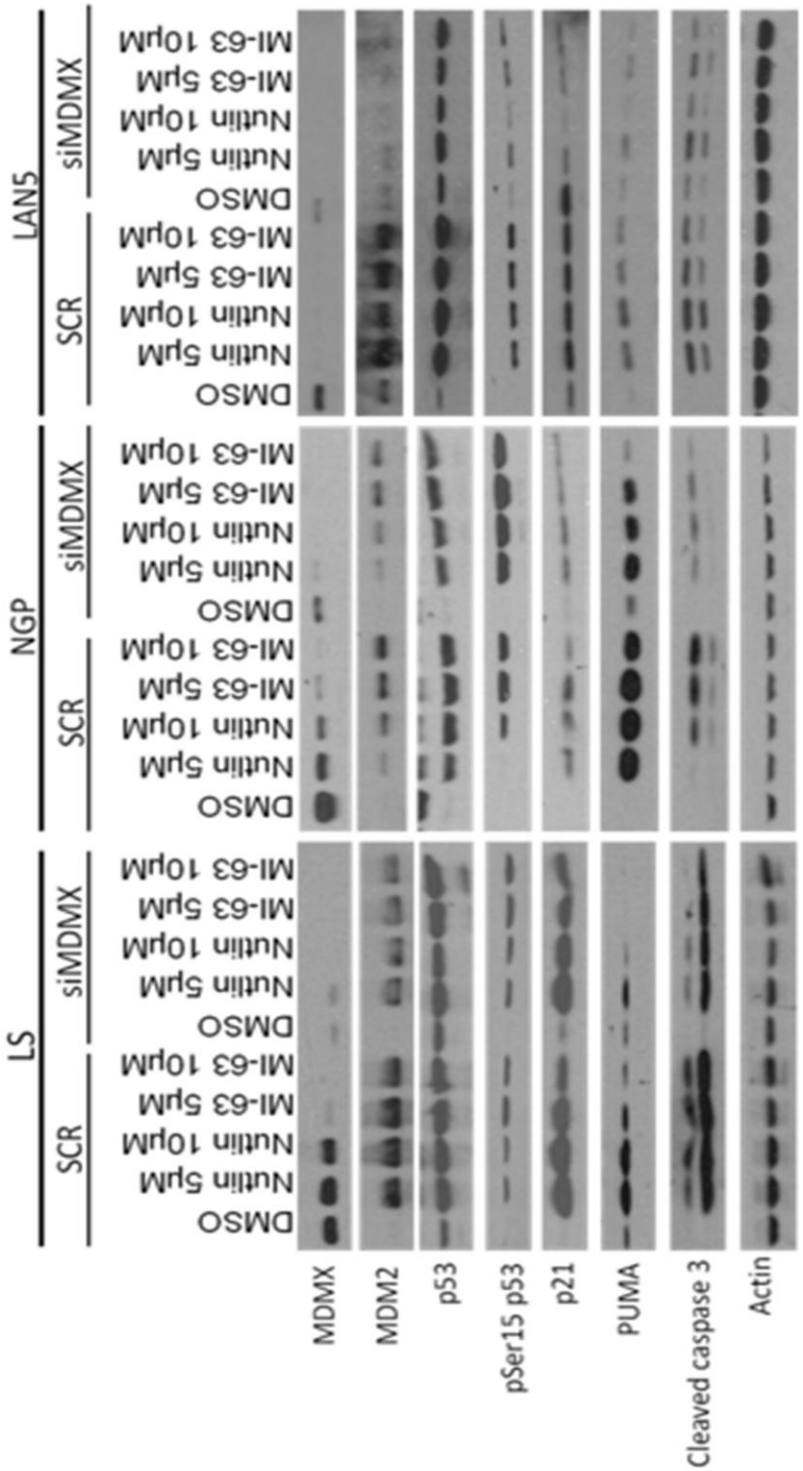


Figure 4.12. **LS, NGP and LAN5 cells were treated with Nutlin-3 or MI-63 following MDMX knockdown (or SCR control).** A decrease in apoptotic markers PUMA and cleaved caspase 3 was observed in all 3 cell lines following MDMX knockdown compared to SCR control. p53 responsive proteins were not affected in *MDM2*-amplified cell lines but were in the non-*MDM2* amplified high MDMX expressing LAN5 cell line. Actin was used as a loading control.

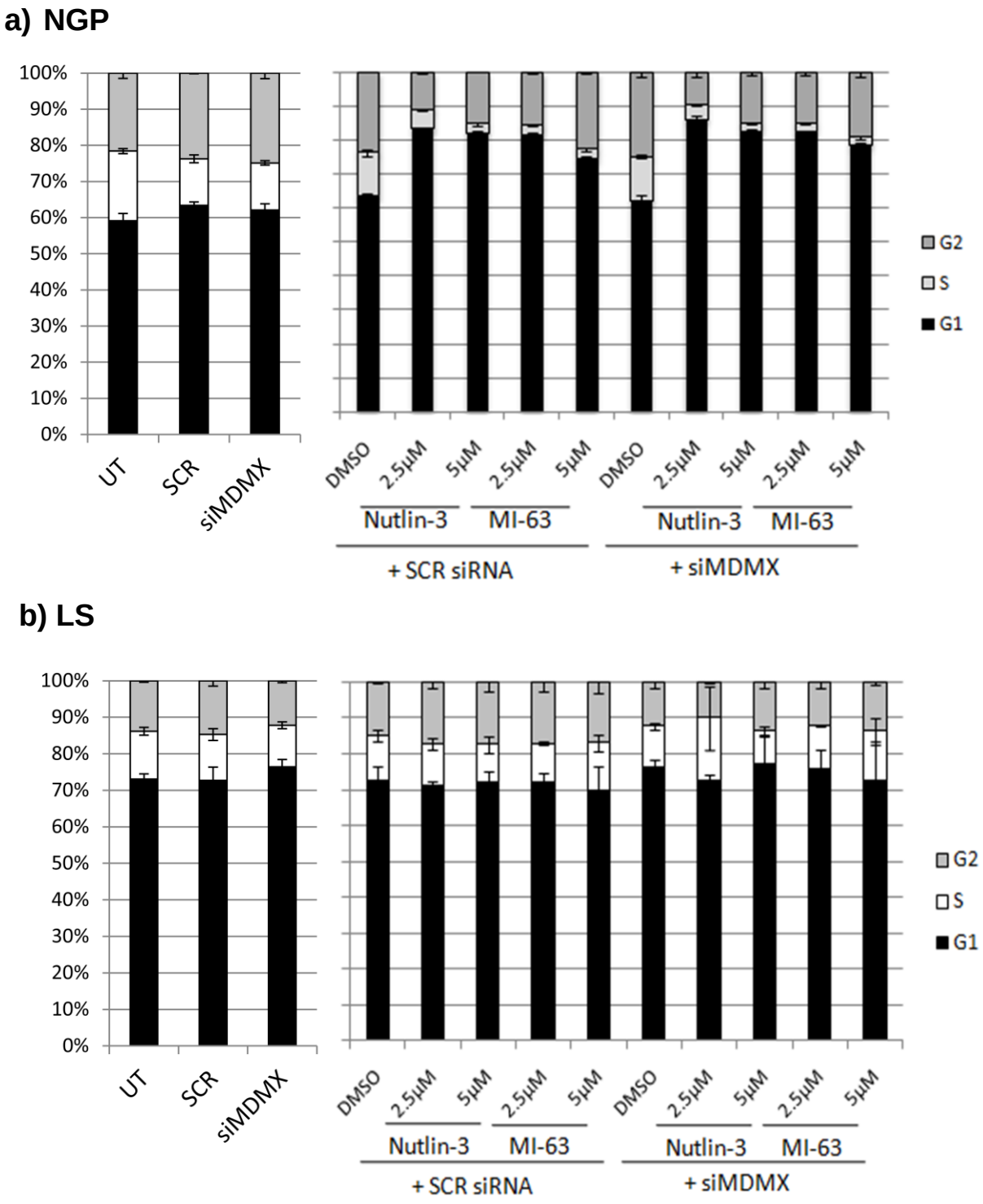


Figure 4.13. **MDMX knockdown in a) NGP and b) LS cells resulted in no change to the proportion of cells in the various phases of the cell cycle compared to SCR control consistent with unaltered $p21^{WAF1}$ protein levels. NGP cells continued to G_1 arrest, whereas LS cells did not G_1 arrest.**

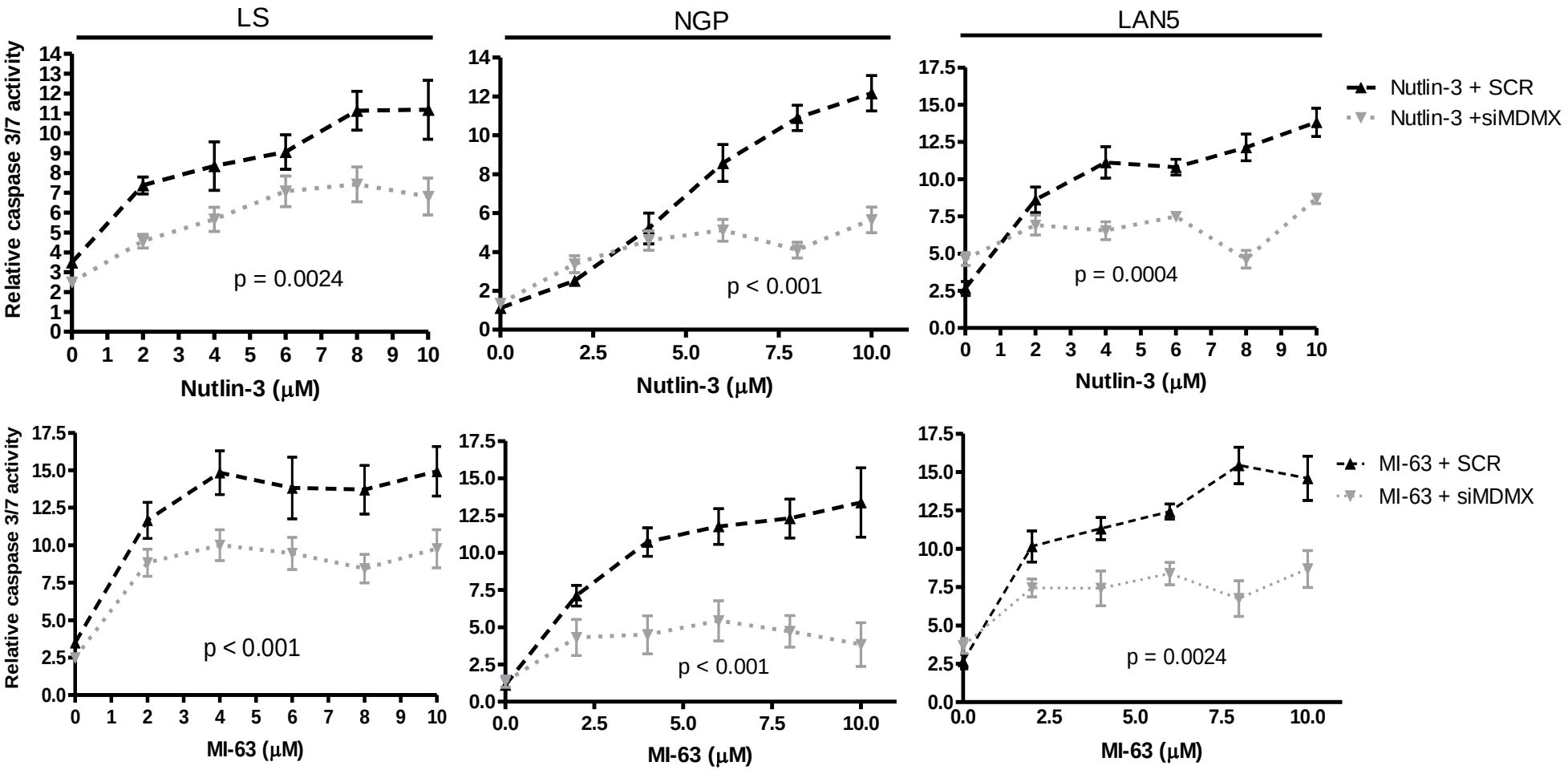


Figure 4.14. **Caspase 3/7 activity decreased after MDMX knockdown compared to SCR control at 24 hours in all 3 cell lines following Nutlin-3 or MI-63 treatment (LS, Nutlin-3 p = 0.0024, MI-63 p < 0.001; NGP, Nutlin-3 p < 0.001, MI-63 p < 0.001; LAN5, Nutlin-3 p = 0.0004, MI-63 p = 0.0024. 2-way ANOVA).**

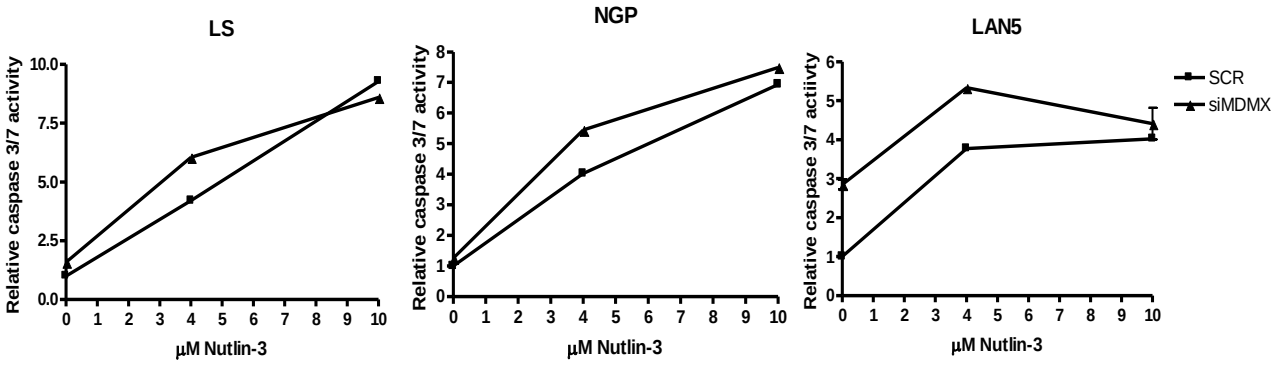


Figure 4.15. **There was no effect on caspase 3/7 activity following MDMX knockdown after 12 hours Nutlin-3 treatment compared to SCR control.**

4.4.7 MDMX expression may be indirectly regulated by MYCN

Using the tetracycline regulatable cell line Tet21N, there was a clear causal link between MYCN and MDMX protein expression (Figure 4.16a), with a decrease in MYCN occurring at >2 hours following tetracycline addition, and a reduction in MDMX >24 hours. MYCN expression returned to maximal levels following tetracycline removal between 24 and 48 hours, whereas MDMX protein levels returned to maximal levels between 72 and 96 hours. Vector only cells were used as a control, and interestingly, whilst they did not express either MYCN in the presence or absence of tetracycline, they did express MDMX. Under normal conditions, MDMX has a relatively long half-life, but is dramatically reduced in the presence of MDM2. The half-life of MDMX in Tet21N cells is not known, so mRNA levels were looked at to determine if the effect was at the transcriptional or post-transcriptional level. Following tetracycline addition, MYCN mRNA levels were very low by 8 hours, whereas MDMX levels decreased by 12 hours but not dramatically. Following tetracycline removal, MYCN levels were at a maximum at 24 hours, but MDMX mRNA levels did not alter (Figure 4.16b). This suggests that MYCN is not a direct transcriptional regulator of the *MDMX* gene.

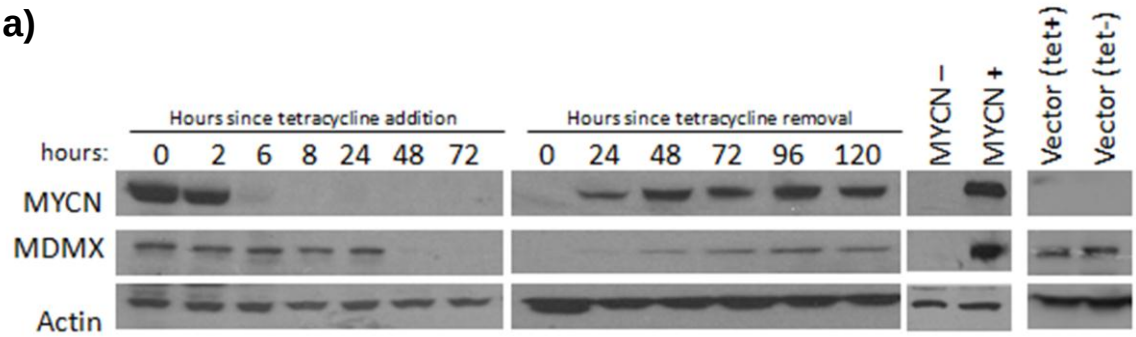
Despite a relationship between MYCN and MDMX in the Tet21N cell line, there was no correlation between MYCN and MDMX protein expression levels across a panel of 21 cell lines (Figure 4.16c), and in the 4 cell lines tested, no decrease was seen in MDMX expression up to 72 hours after MYCN knockdown by siRNA (Figure 4.16d). In fact, MDMX levels increased following 72 hours MYCN knockdown in NGP and NB1691 cells.

4.4.8 Knockdown of MDMX in MYCN-regulatable Tet21N cells

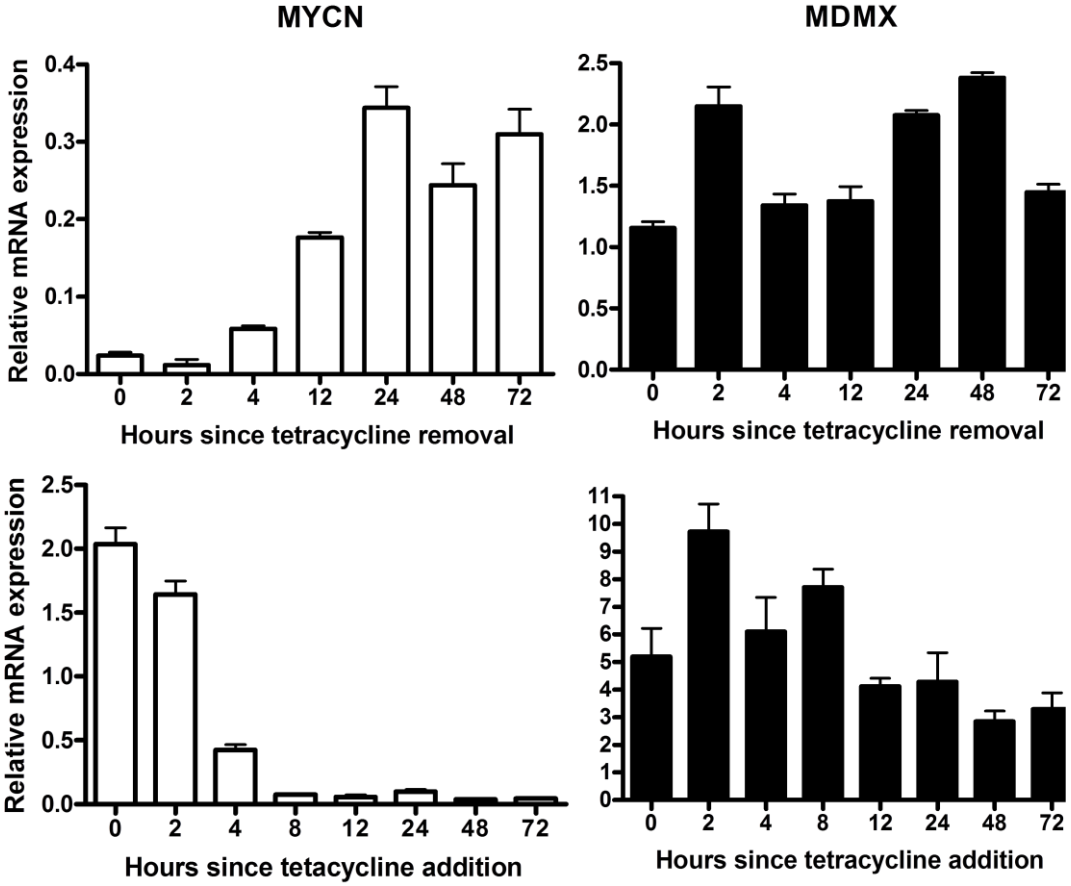
To further investigate the relationship between MYCN and MDMX in Tet21N cells, in which a correlation between MYCN and MDMX protein expression levels was seen, MDMX was knocked down in this cell line. MDMX knockdown, as shown in Figure 4.17a, resulted in increased p53 in both MYCN(+) and MYCN(-) cells, and little effect on induction of apoptotic markers or phosphorylated p53. However, this cell line does not undergo high levels of apoptosis (Chapter 3.1.4). MYCN(+) cells G_1 arrested following MDMX knockdown but since MYCN(-) cells are already G_1 arrested, an effect due to MDMX knockdown was difficult to determine (Figure 17b). This data

suggests that knockdown of MDMX has similar effects in MYCN(+) and MYCN(-) cells on induction of p53 and the p53 response.

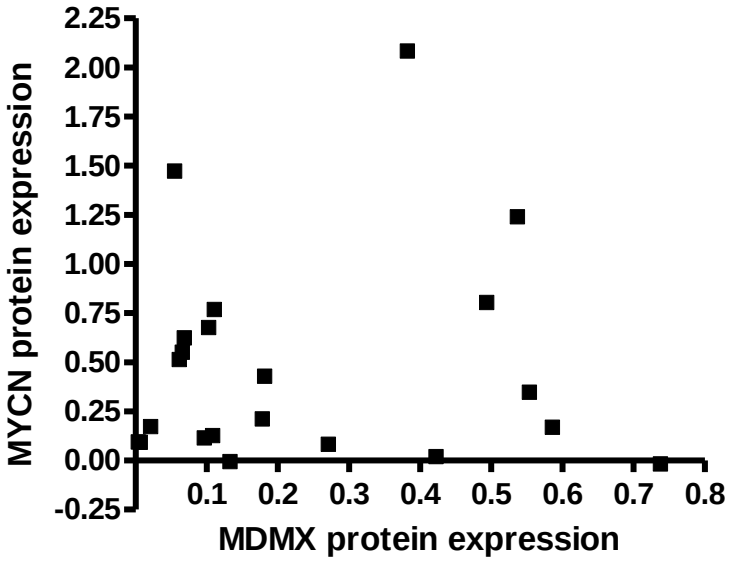
a)


Hours since tetracycline addition
Hours since tetracycline removal
MYCN –
MYCN +
Vector (tet+)
Vector (tet-)
hours:
0 2 6 8 24 48 72
0 24 48 72 96 120
MYCN
MDMX
Actin

b)


MYCN
MDMX
Relative mRNA expression
Hours since tetracycline removal
Hours since tetracycline removal
Relative mRNA expression
Hours since tetacycline addition
Hours since tetracycline addition

c)


MYCN protein expression
MDMX protein expression

d)

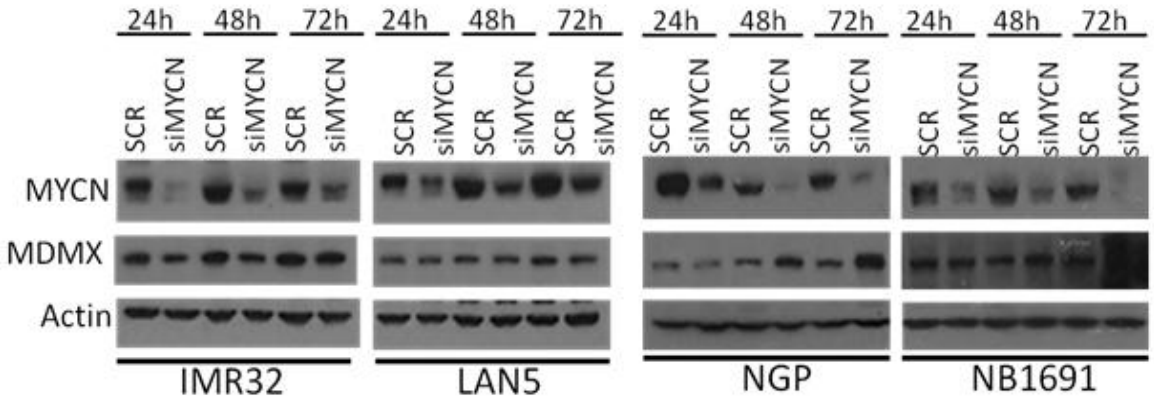


Figure 4.16. **The relationship between MDMX and MYCN expression in neuroblastoma.** The effect of MYCN induction (tetracycline removal) or MYCN removal (tetracycline addition) on **a)** MDMX protein expression (alongside vector only control), and **b)** MDMX mRNA levels. **c)** Comparison between MYCN and MDMX protein expression in a panel of 21 neuroblastoma cell lines (p=0.75, Pearson correlation). **d)** siRNA mediated knockdown of MYCN in 4 neuroblastoma cell lines (IMR32, LAN5, NGP and NB1691) did not affect MDMX protein expression.

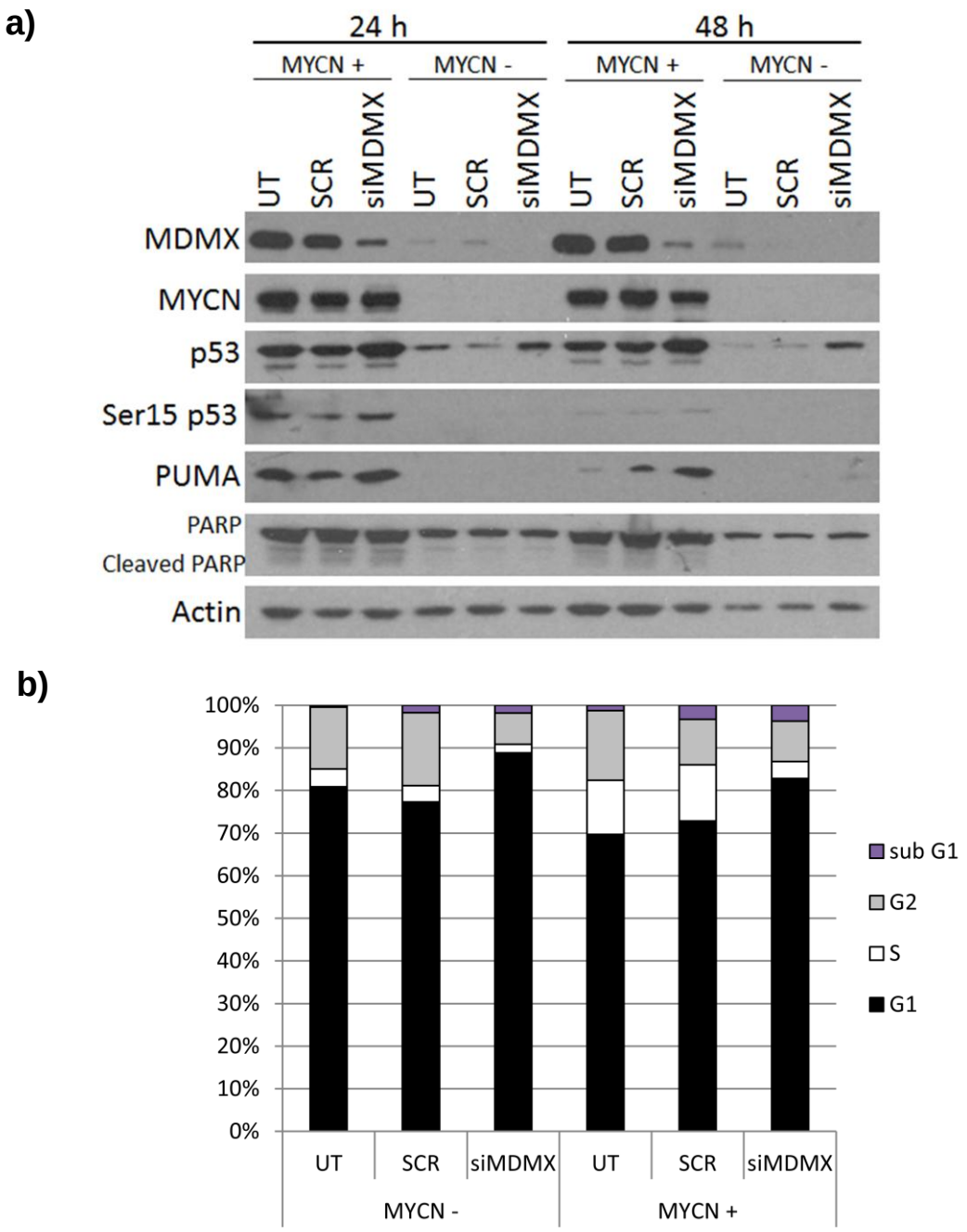


Figure 4.17. **MDMX knockdown in Tet21N cells. a)** MDMX knockdown in MYCN regulatable Tet21N cells did not show a difference in induction of p53 or apoptosis related markers between MYCN(+) and MYCN(-) cells. **b)** MDMX knockdown in MYCN(+) cells induced a G_1 arrest, and an increase in the proportion of cells in G1 was also observed in MYCN(-) cells.

4.4.9 Karyotyping of 4 *MDM2*-amplified sarcoma cell lines

A number of non-neuroblastoma cell lines were obtained to assess the effect of *MDM2*-amplification on the response to MDM2-p53 antagonists in neuroblastoma compared to other cell lines. All the cell lines obtained were derived from sarcomas. FISH analysis was used to confirm *MDM2*-amplification in RH18, MHM, T449 and T778 sarcoma cell lines (Figure 4.18). *MDM2*-amplified LS neuroblastoma cells were used as a positive control, and non-*MDM2*-amplified LAN5 cells used as a negative control. As seen with the LAN5 cells, in non-*MDM2*-amplified cells, equal copies of MDM2 (green) and chromosome 12 (red) signals are detected. G-banding was used to karyotype the cell lines, and the karyotypes and details of these cell lines are shown in Table 4.3. Karyotyping and FISH of metaphase spreads was performed by Dr Nick Bown, Institute of Human Genetics, Newcastle University. Figures 19-22 show karyograms for each cell line, alongside FISH analysis of metaphase spreads in which amplification by DMs is shown by a dispersed pattern, and HSRs is shown by clustering.

4.4.9.1 RH18

RH18 is a rhabdomyosarcoma cell line established from a patient at diagnosis before any treatment (Houghton et al., 1982). Analysis shows a complex near-triploid karyotype with multiple structural rearrangements including many marker chromosomes (a structurally abnormal chromosome in which no part can be identified) of unclear origin. Three metaphases were analysed (one of which is shown in Figure 4.19a), and showed variations amongst cells, with several clonal rearrangements. A composite karyotype is shown in Table 4.3. FISH analysis shows *MDM2*-amplification (Figure 19b), but unusually both DMs and HSRs were seen in metaphase spreads, and DMs in interphase spreads. DMs are visible in a significant minority of metaphases and these also hybridise MDM2. Most metaphases show a very small HSR on 12p, but a minority show a large MDM2 HSR on an unknown chromosome. Three chromosome 12 centromeres were detected in all FISH analyses (Figure 4.18 and 19b), and multiple copies of MDM2.

4.4.9.2 MHM

MHM is an osteosarcoma cell line derived from a patient at relapse following chemotherapy (Müller CR, 2007). Analysis shows a complex hypertriploid karyotype, with multiple structural rearrangements and many marker chromosomes of unclear origin. Three metaphases were analysed (one of which is shown in Figure 4.20a) and showed variations amongst cells, along with several clonal rearrangements. The

karyotype given in Table 4.3 is a composite karyotype. DMs were present in some metaphases. FISH analysis, shown in Figure 20b showed *MDM2*-amplification was localised to a HSR, but the chromosomal site could not be identified. Normal chromosome 12 with native MDM2 signals was also present. Interestingly, the MDM2 probe did not hybridise to the DM, suggesting a second gene may be amplified in this cell line. In Figure 4.18, FISH analysis shows many copies of MDM2 compared to the copies of chromosome 12, which varied amongst cells.

4.4.9.3 T449

T449 is derived from a primary well differentiated liposarcoma (Pedeutour et al., 1999). Analysis shows a complex near-diploid karyotype with multiple structural rearrangements including many marker chromosomes of unclear origin, and several large HSRs (Figure 21a). Three metaphases were analysed, and in two metaphases, one of the HSRs appeared to be incorporated into chromosome 17. The metaphases showed variations between cells and several clonal rearrangements. A composite karyotype is shown in Table 4.3 FISH analysis showed *MDM2*-amplification localised to HSRs, and normal chromosomes 12 with native MDM2 signal were present (Figure 21b). In Figure 4.18, FISH analysis showed 4 or 2 copies of chromosome 12, and many copies of MDM2.

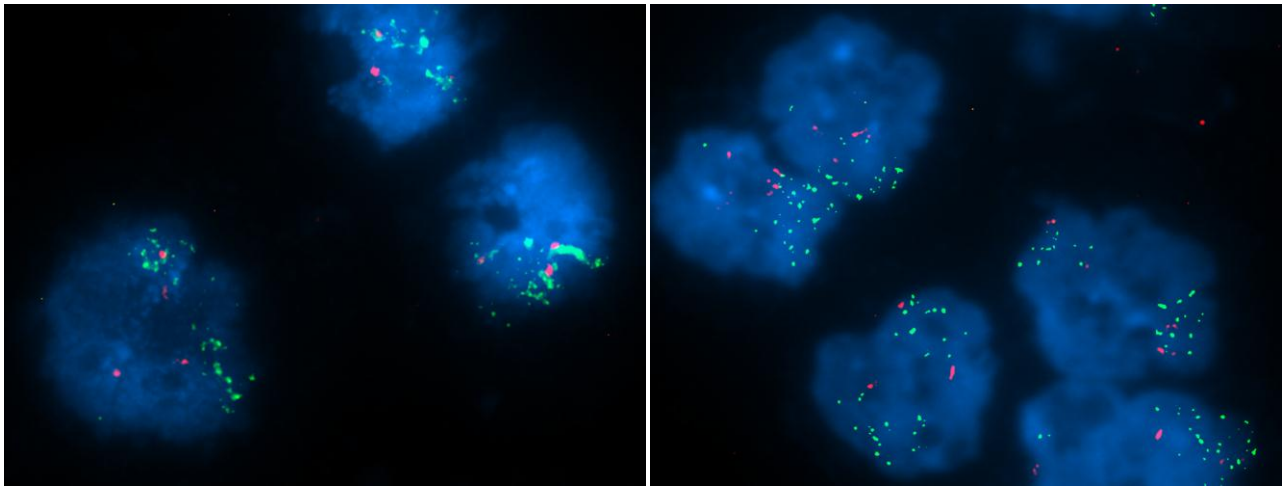
4.4.9.4 T778

T778 is paired with T449, and is from the well differentiated liposarcoma at relapse. At the time of writing, it was unknown whether this cell line is following chemotherapy or surgery only. Analysis shows a complex hypertriploid karyotype with multiple structural rearrangements with many marker chromosomes of unclear origin. Three metaphases were analysed and a composite karyotype shown in Table 4.3. These metaphases showed variation between cells and several clonal rearrangements (Figure 22a). T778 cells have a hypertriploid karyotype with multiple structural rearrangements (some of which cannot be characterised by G-banded analysis). There was no visible evidence of translocation t(12;16) with FUS-CHOP gene fusion, which is a recurrent rearrangement in liposarcoma. There is a fairly large metacentric chromosome (centromere of unknown origin) with a likely HSR on both sides, present in two copies in one of the cells, and there is likely to be a further HSR in a large submetacentric chromosome. FISH analysis shows 4 centromeres for chromosome 12 and 5 MDM2 HSRs (Figure 22b). In Figure 4.18, FISH analysis showed 3 copies of chromosome 12, and multiple copies of MDM2.

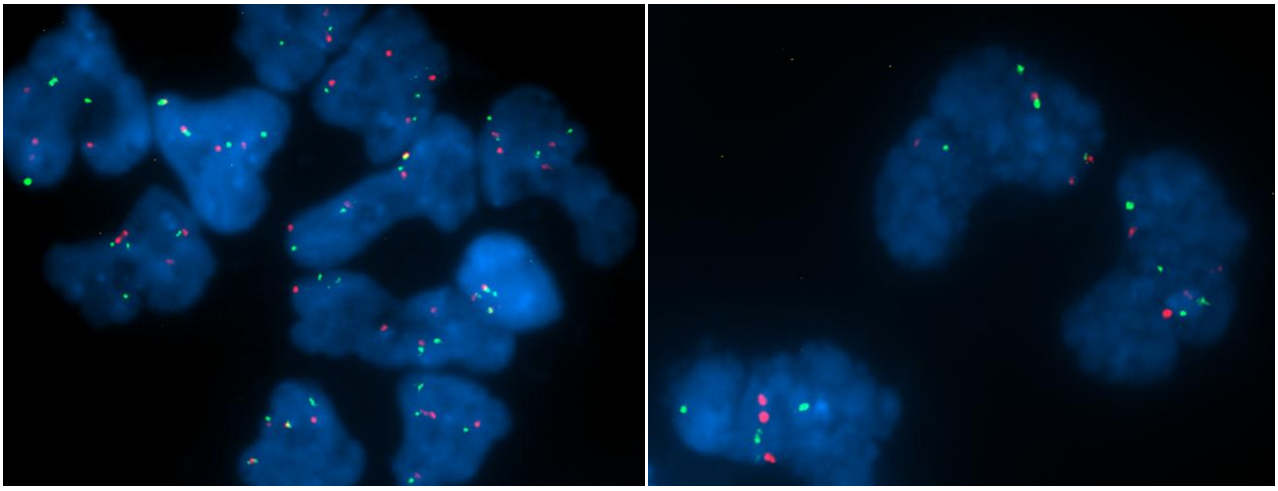
Cell Line	Cancer Type	Karyotype	*MDM2* amplification
RH18 (Houghton et al., 1982)	Rhabdomyosarcoma	61,XX,-1,+2,-3,add(3)(q21),add(6)(q21),-6,+7,+7,-10,-10,-11,add(11)(p15),-12,-12,hsr(12p),-13,-13,-17,-18,-19,-19,+20,add(20)(q13),21,+4mar	Large and small MDM2 HSRs DM pattern in interphase nucleus (Figure 4.18)
MHM (Müller CR, 2007)	Osteosarcoma	74<3n>X,-1,add(1)(q1?)x2,-2,-3,-4,add(4)(q1?),del(4)(q2?),-5,add(5)(p1?),-6,-7,-8,-9,add(9)(p1?),-10,add(11)(q14-21)x3,-12,hsr(12),-13,-15,-15,-16,+17,-18,-19,-21,-21,add(21)(p10),+23mar	1x MDM2 HSR DM not MDM2 (Figure 4.19)
T449 (paired) (Pedeutour et al., 1999)	Liposarcoma	47~51,X,hsr(17)(?p10),-11,-19,+10-15mar	HSR (Figure 4.20)
T778 (paired) (Pedeutour et al., 1999)	Liposarcoma	76~79<3n>,add(X)(p2?),add(1)(p1?),add(1)(q1?),-7,+10,+11,add(11)(p1?),-13,-14,+19,+19,-22,+11-13mar Chromosome with HSR of unclear origin	HSR (Figure 4.21)

Table 4.3. **Karyotypes of 4 sarcoma cell lines, and the mechanism of *MDM2*-amplification (double minutes (DMs) or homogeneously staining regions (HSRs)).** T449 and T778 are paired cell lines, with T449 established at diagnosis, and T778 at relapse (unknown at the time of writing whether T778 was after chemotherapy or surgery).

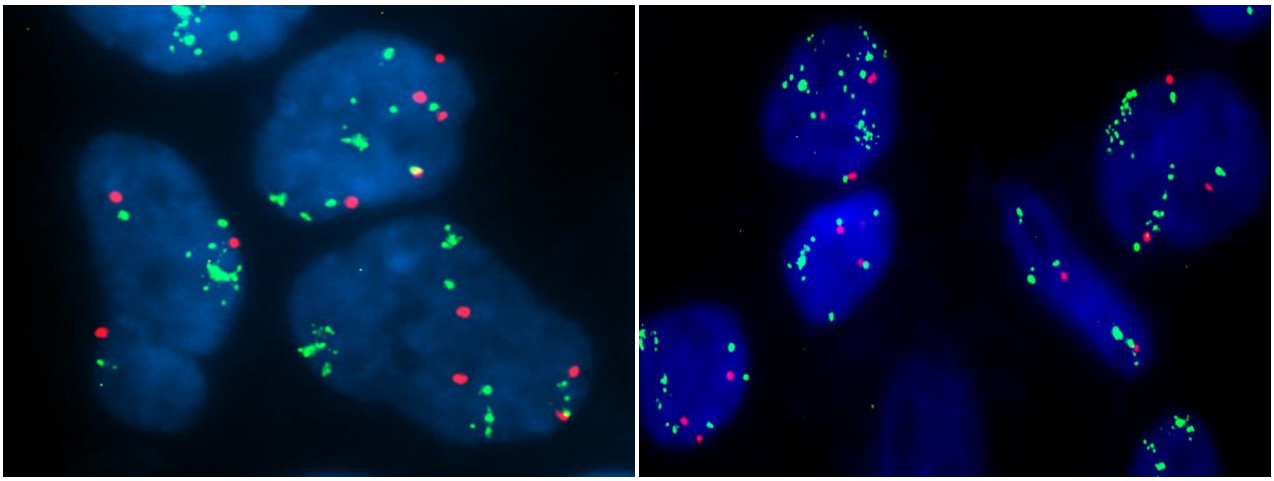
LS



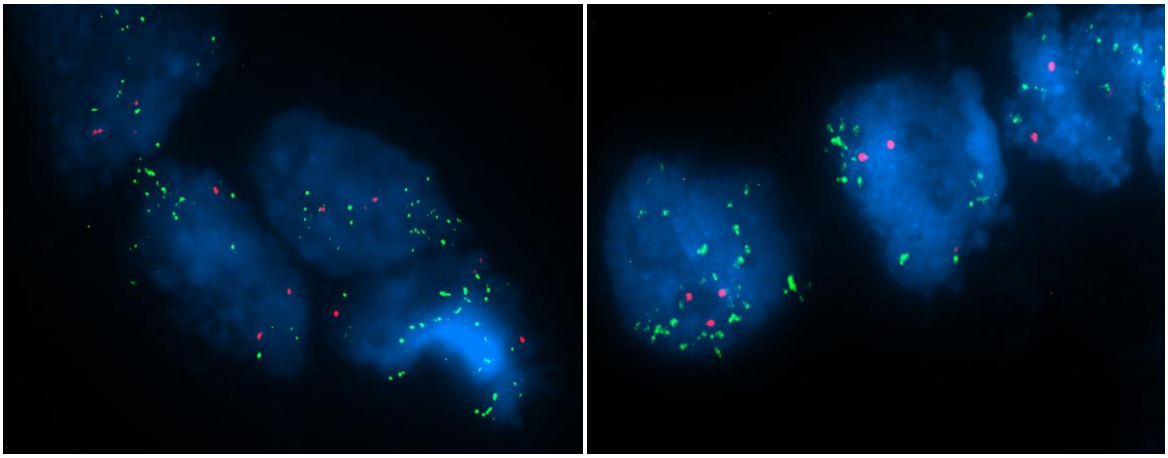
LAN5



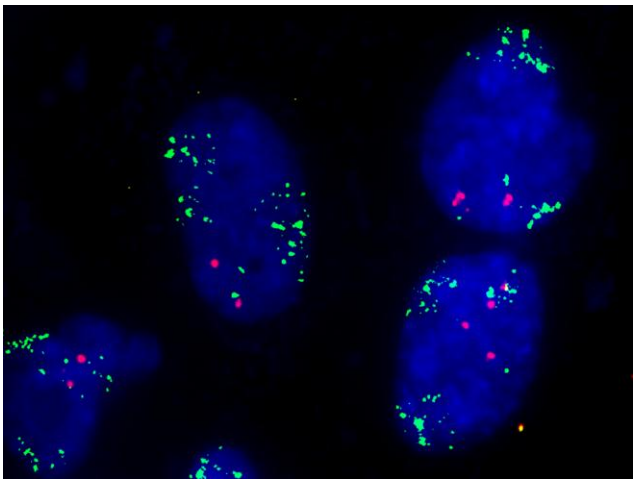
MHM



RH18



T449



T778

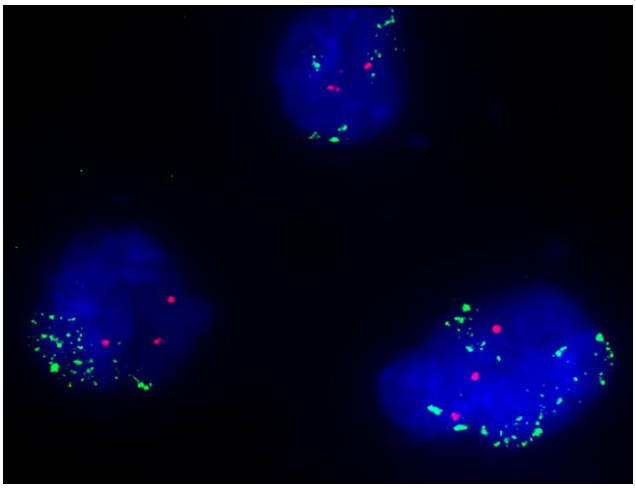


Figure 4.18. **FISH of sarcoma cell lines.** LAN5 was used as a non-*MDM2*-amplified control, and LS as a *MDM2*-amplified control.

a)


1
2
3
4
5
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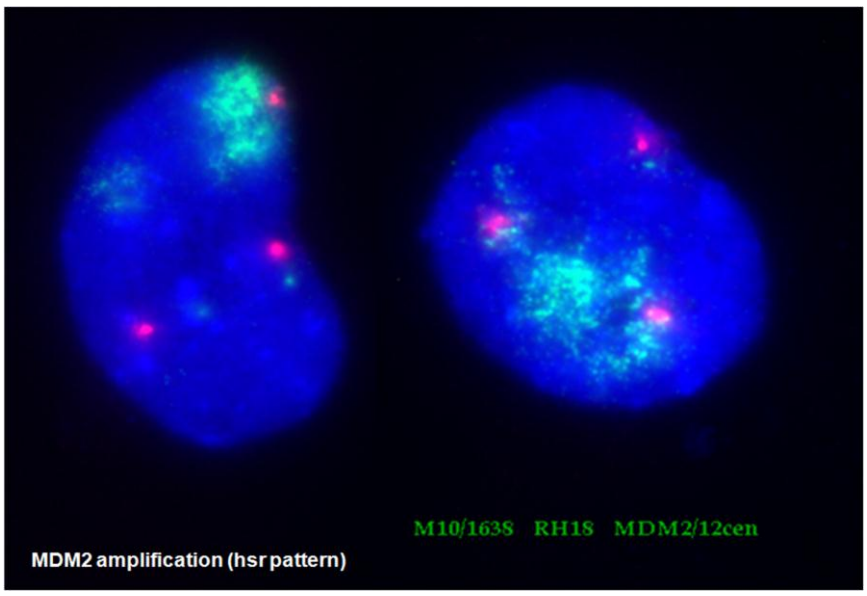
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

b)


M10/163S RH1S MDM2/12cen
MDM2 amplification (hsr pattern)

c)

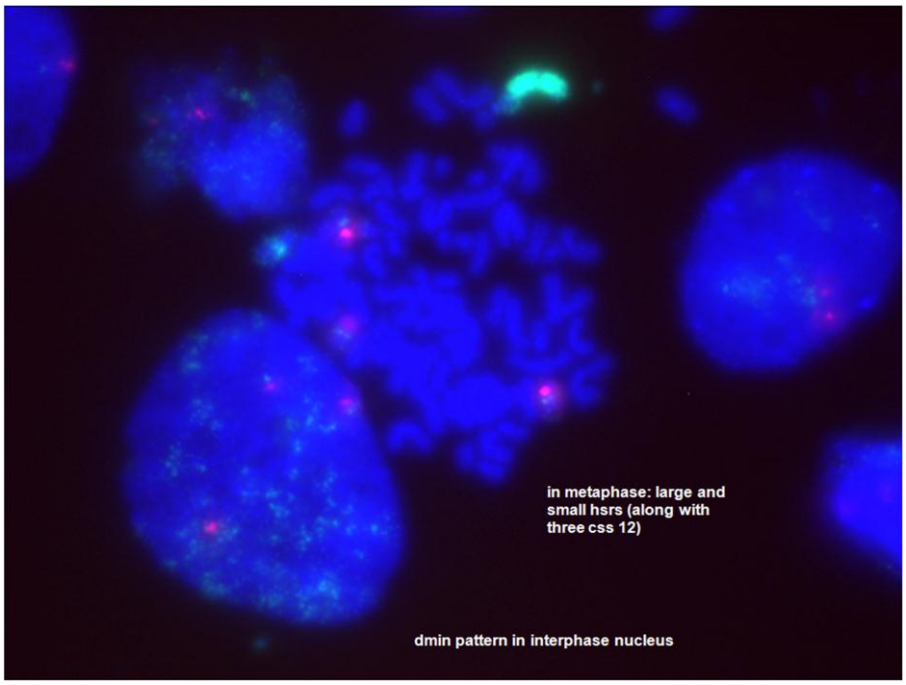


Figure 4.19. **RH18 cell line. a)** Karyogram of RH18 cell line. **b)** FISH analysis of a interphase cell, showing HSR pattern of amplification, **c)** FISH analysis of a metaphase spread showing HSR in metaphase, and DM in interphase nucleus. Images generated by Dr Nick Bown.

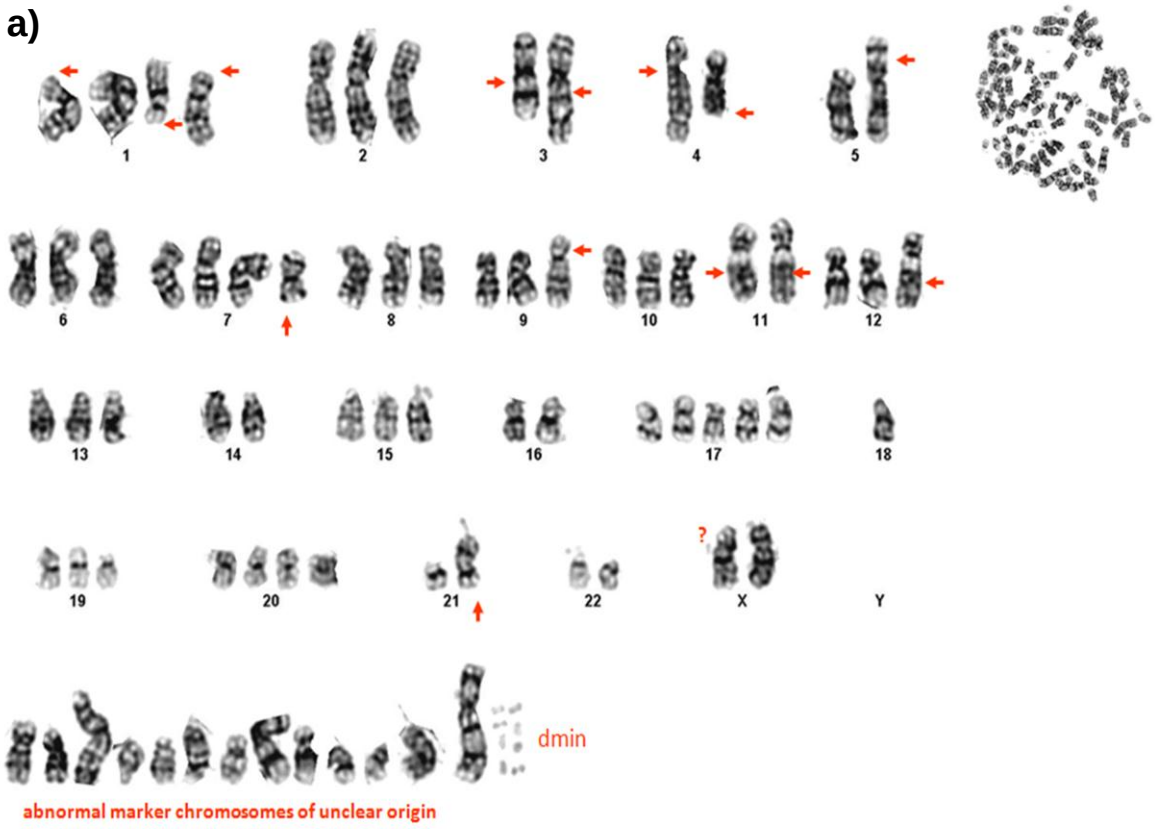
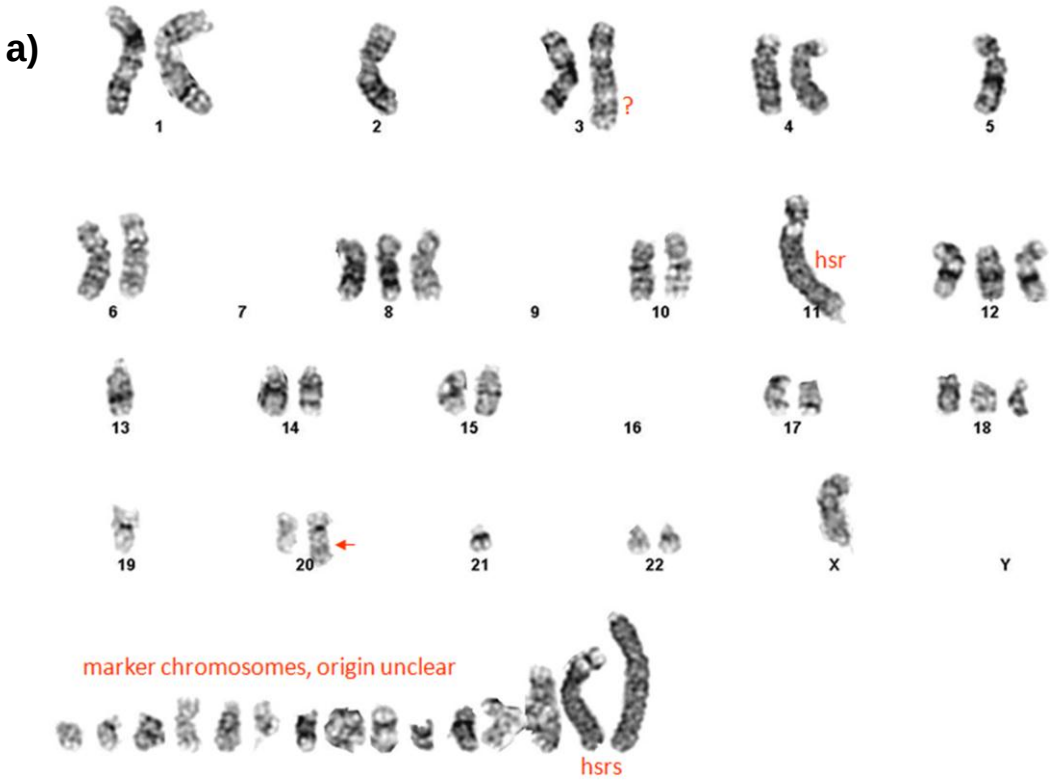


Figure 4.20. **MHM cell line. a)** Karyogram of MHM cell line. **b)** FISH analysis of a metaphase spread, showing an HSR for MDM2. Image generated by Dr Nick Bown.



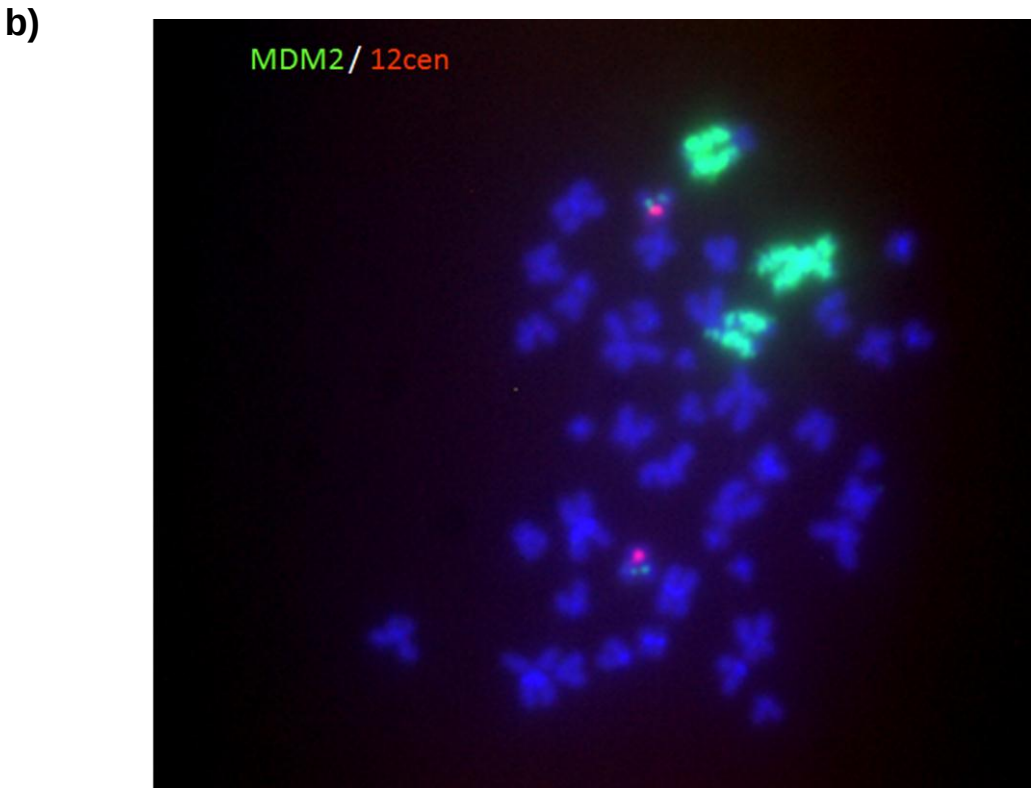


Figure 4.21. **T449 cell line. a)** Karyogram of T449 cell line **b)** FISH analysis of metaphase spread showing HSRs. Image generated by Dr Nick Bown.

a)

b)

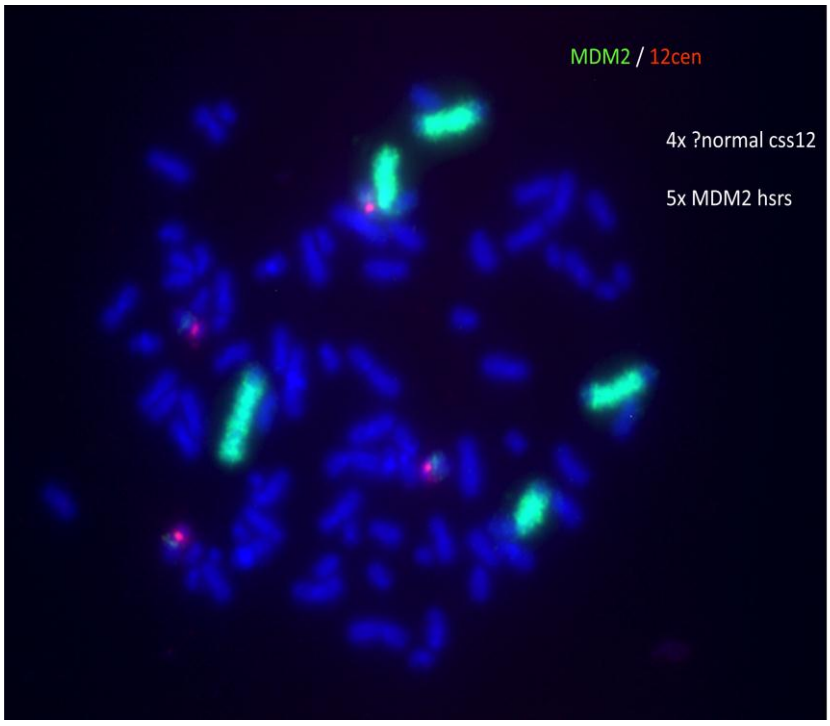


Figure 4.22. **T778 cell line. a)** Karyogram of T778 cell line **b)** FISH analysis of metaphase spread showing HSRs. Image generated by Nick Bown.

4.4.10 Comparison of GI_{50} values in *MDM2*-amplified neuroblastoma cell lines and *MDM2*-amplified sarcoma cell lines, following MDM2-p53 antagonist treatment

MDM2, MDMX, and p53 expression across the *MDM2*-amplified neuroblastoma and sarcoma cell lines are shown in Figure 4.23a. IMR32 was used as a non-*MDM2*-amplified control. GI_{50} values were determined and are shown in Table 4.4. As shown in Figure 4.23b and 4.23c, the neuroblastoma cell lines have significantly higher median GI_{50} values compared to the sarcoma cell lines ($p = 0.016$ for Nutlin-3; $p = 0.027$ for MI-63). As shown in Figure 4.23a, MDM2 and MDMX levels are similar across the *MDM2*-amplified cell lines, but the 4 *MDM2*-amplified neuroblastoma cell lines are co-amplified for *MYCN* and have high levels of MYCN protein expression.

Cell Line	GI_{50} (μM)	
	Nutlin-3	MI-63
NGP	2.53 ± 0.43	1.21 ± 0.04
LS	2.95 ± 0.12	0.98 ± 0.06
NB1691	2.80 ± 0.17	0.87 ± 0.22
TR14	2.91 ± 0.28	1.09 ± 0.25
SJSA-1	1.60 ± 0.12	0.90 ± 0.06
RH18	2.18 ± 0.35	0.75 ± 0.05
MHM	2.22 ± 0.10	0.99 ± 0.13
T449	1.37 ± 0.10	0.61 ± 0.04
T778	1.36 ± 0.05	0.55 ± 0.03

Table 4.4. **GI_{50} values in the panel of *MDM2*-amplified cell lines.**

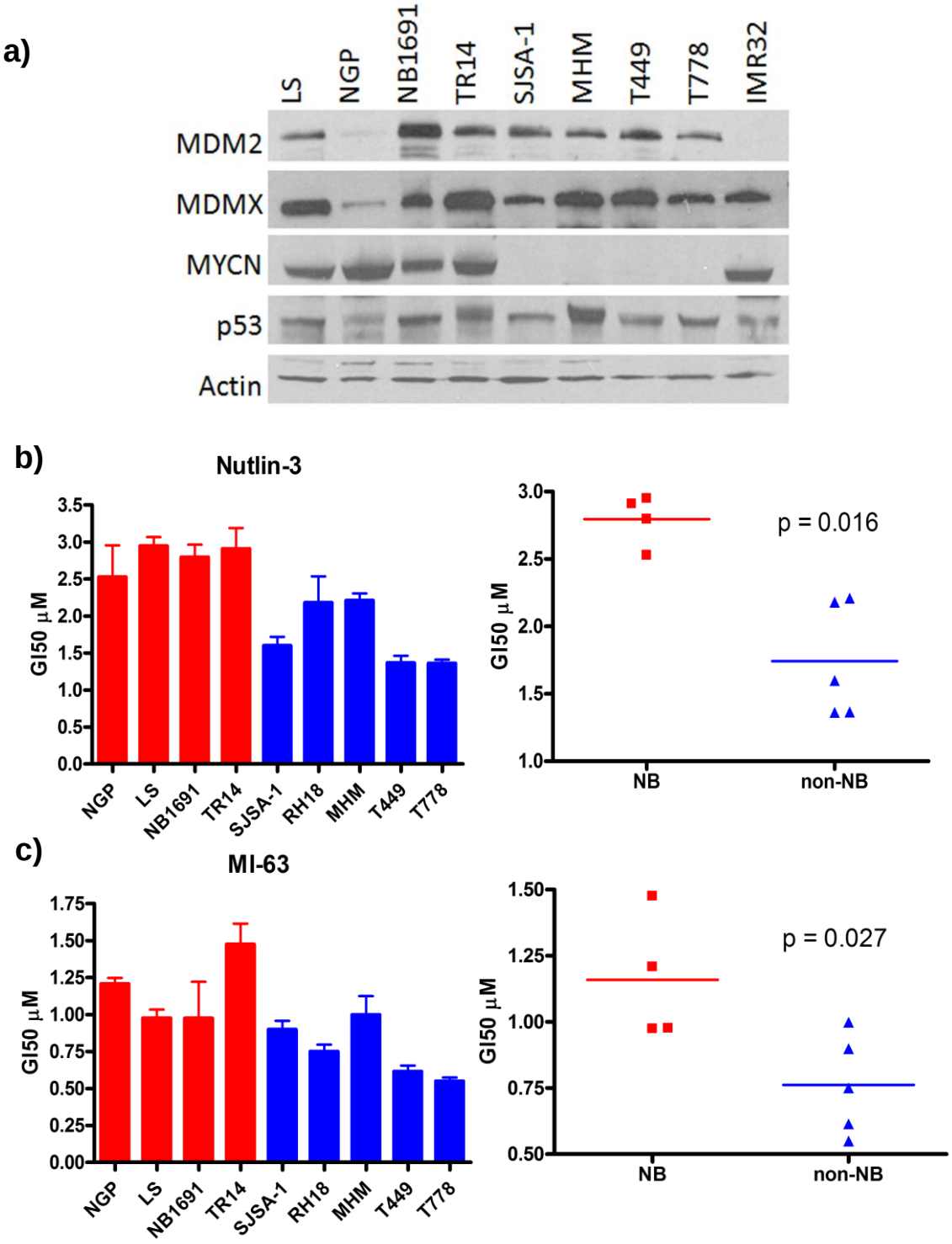


***Figure 4.23. MDM2*-amplification in neuroblastoma compared to sarcoma cell lines. a)** Western blot of *MDM2*-amplified neuroblastoma and sarcoma cell lines. Median GI_{50} values of *MDM2*-amplified neuroblastomas are higher than those for *MDM2*-amplified sarcomas following **b)** Nutlin-3 and **c)** MI-63 treatment) (Nutlin-3, p = 0.016; MI-63, p = 0.027; Mann-Whitney test).

4.5 Discussion

Despite the use of intense multimodal therapies, high risk neuroblastoma still has a 5 year survival rate of less than 40%, outlining an urgent need for new therapies. Unlike other cancer types, the majority of neuroblastomas have wildtype p53 at both diagnosis (>98%) and relapse (85%), and therefore therapies aimed at reactivating p53 might be ideal for this tumour type. Here the effect of MDM2 and MDMX on the response to MDM2-p53 antagonists in neuroblastoma cell lines was investigated. A number of studies have shown that pathways downstream of p53 are intact in neuroblastoma and that p53 can induce apoptotic responses (Hogarty, 2003; Tweddle et al., 2003; Hosoi et al., 1994; Vogan et al., 1993).

4.5.1 The effect of MDM2 on the apoptotic response to MDM2-p53 antagonists

Previous reports suggest that in various cancer types amplification or overexpression of MDM2 sensitizes cells to MDM2-p53 antagonists (Gu et al., 2008b; Tovar et al., 2006; Kojima et al., 2005), whilst other groups report no effect (Liu et al., 2009; Kojima et al., 2006; Van Maerken et al., 2006). However, these studies have only compared a small number of cell lines and have not addressed this question systematically with a significant number of cell lines in a given cancer type. In neuroblastoma, work described in Chapter 3 on a panel of 18 cell lines showed *MYCN*-amplified neuroblastoma to be more sensitive to MDM2-p53 binding antagonists than non-amplified cell lines, but that within the *MYCN*-amplified set, the *MDM2*- and *MYCN*-co-amplified cell lines had a higher average GI_{50} value for Nutlin-3 and MI-63 than the subset with *MYCN*-amplification alone. In the study presented in this chapter, MDM2 knockdown in two *MDM2*-amplified cell lines did not alter the sensitivity of these cells to Nutlin-3 or MI-63 mediated p53 activation or apoptosis. In Chapter 3, increased caspase 3/7 activity was identified in *MDM2*-amplified neuroblastoma cell lines following Nutlin-3 and MI-63 treatment compared to other neuroblastoma cell lines (Gamble et al., 2011a), and although the average caspase activity was higher in MDM2 amplified cell lines compared to *MYCN* and *MDM2* co-amplified cell lines, the difference was not significant. Since knockdown of MDM2 did not alter sensitivity to apoptosis, it may be that this is a result of the increased $p14^{ARF}$ in these cell lines (Carr et al., 2006), as in other studies within this thesis, $p14^{ARF}$ was found to be important in determining the response to MDM2-p53 antagonists (Chapter 5).

4.5.2 MDMX and the response to MDM2-p53 antagonists

As previous studies have shown that MDMX removal may be necessary to fully activate the p53 response in various cancer types, the effect of MDMX expression in neuroblastoma on the response to two MDM2-p53 antagonists, Nutlin-3 and MI-63, both of which have much greater affinity for MDM2 over MDMX, was investigated.

MDMX is a paralogue of MDM2 and is also a negative regulator of p53, but unlike MDM2 its transcription is not driven by p53 and does not appear to be so tightly regulated. In previous studies in various cancer types, MDMX overexpression decreases the sensitivity to MDM2-p53 antagonists, promoting tumour formation, and suggesting that MDMX removal may be necessary to fully activate the p53 response by MDM2-p53 antagonist treatment (Hu et al., 2006; Marine et al., 2006; Patton et al., 2006; Wade et al., 2006). Many cancer cell lines have been shown to overexpress MDMX (Ramos et al., 2001). MDMX expression has not been previously explored in neuroblastoma, and shown here is that MDMX protein expression is highly variable across a panel of 21 cell lines, regardless of *MYCN* status, but appears to be expressed at lower levels in *MDM2*-amplified cell lines. The effect of MDMX expression in neuroblastoma on the response to MDM2-p53 antagonists was explored and interestingly, and in contradiction to previous reports in other tumour types, siRNA-mediated MDMX knockdown resulted in decreased levels of apoptosis following Nutlin-3 and MI-63 treatment. Furthermore, MDMX protein expression positively correlated with increased caspase 3/7 activity in response to MDM2-p53 antagonists. However, there was no correlation between MDMX expression and GI_{50} values, despite a weak inverse correlation between GI_{50} values and caspase 3/7 activity across the cell lines. Since the SRB growth inhibition assays take into consideration both cell cycle arrest and apoptosis, this may suggest that the cell lines with high MDMX expression (which show high induction of caspase activity) did not arrest and that high MDMX expression may therefore be associated with defective cell cycle arrest.

The unexpected effect of MDMX on the response to MDM2-p53 antagonists in neuroblastoma highlights the requirement for better understanding of the functions and mechanisms of MDMX action. Interestingly, siRNA-mediated knockdown of MDMX and MDM2-p53 antagonist treatment had a much greater effect on the expression of p53 and p53 related proteins in the cell line without *MDM2*-amplification, suggesting that MDMX may play a more important role in cells that have lower levels of MDM2.

4.5.3 MDMX expression is linked with cell type

It was found that the two cell lines derived from SKNSH cells, SHEP (a substrate-adherent S-type) and SHSY5Y (a neurite-bearing N-type) had very different MDMX protein expression patterns, with high levels of MDMX in SHSY5Y cells, and barely detectable MDMX in SHEP cells. On further investigation, it was found that N-type neuroblastoma cells had a significantly higher median MDMX expression compared to S-type and N/S-type cells. Cells lines consisting of a mixture of N and S type cells had a median MDMX expression in-between the two. This may be why neuroblastoma cell lines with increased MDMX expression are more sensitive to MDM2-p53 antagonist mediated apoptosis, as N-type neuroblastomas have been found to be more sensitive to chemotherapy than S-type cells which are relatively resistant, and in response to DNA-damage, N-type cells preferably undergo apoptosis whereas S-type cells undergo senescence (Isaacs et al., 2001; Rodriguez-Lopez et al., 2001).

4.5.4 MDMX and the cell cycle response

To determine if high MDMX expression was associated with a cell cycle arrest, MDMX was knocked down and the effect on the cell cycle response investigated. Following MDMX knockdown and MDM2-p53 antagonist treatment in a cell line that normally G_1 arrested in response to MDM2-p53 antagonists (NGP), and in a cell line that did not G_1 arrest in response to MDM2-p53 antagonists (LS), no alteration on the cell cycle distribution was observed. This is consistent with cell cycle data presented in Chapter 5 (Figure 5.13), where the panel of 21 neuroblastoma cell lines were investigated for their cell cycle response to Nutlin-3. The cell lines that underwent a G_1 arrest in response to Nutlin-3 were NGP, TR14, PER-108, GIMEN, SKNRA, SHEP and NBLS. All of these cell lines had varying levels of MDMX protein expression; SKNRA had high levels, NGP and PER-108 had intermediate levels, and TR14, GIMEN, SHEP and NBLS had low levels. These results suggest no relationship between MDMX expression and the ability of a neuroblastoma cell line to G_1 arrest.

A possible explanation for the lack of relationship between GI_{50} values and MDMX protein levels, but the increased caspase activity correlating with MDMX expression, is that high MDMX expression is associated with N-type cells, and low MDMX expression with S-type cells. In response to irradiation, N-type cells have been shown to undergo apoptosis, but fail to G_1 arrest (Carr-Wilkinson et al., 2011; Mergui et al., 2008; Berthold et al., 2005). It may therefore be the cell type that dictates the apoptotic

or cell cycle response to MDM2-p53 antagonists. Cell cycle response and cell type is investigated in Chapter 5.4.

4.5.5 A possible relationship between MYCN and MDMX

It was found that MDMX expression in neuroblastoma may be indirectly related to MYCN expression, and it was previously shown that *MYCN*-amplification or expression sensitizes neuroblastoma cell lines to MDM2-p53 antagonists (Chapter 3 and (Gamble et al., 2011a)). In the Tet21N MYCN regulatable cell line, MYCN protein expression was associated with MDMX protein expression, with MYCN induction or removal corresponding with MDMX expression levels. However, the same effect was not observed at the mRNA level, suggesting an indirect mechanism of regulation. However, upon MYCN knockdown in 4 *MYCN*-amplified neuroblastoma cell lines, either no change or an increase in MDMX expression (at 72 hours) was observed and therefore any link between MYCN and MDMX may be cell line specific.

4.5.6 Degradation of MDMX upon MDM2-p53 antagonist treatment

In agreement with previous reports, a reduction in MDMX protein upon MDM2-p53 antagonist treatment was observed, which may be a result of p53-dependent upregulation of MDM2, known to ubiquitinate and degrade MDMX following DNA damage (Xia et al., 2008; Patton et al., 2006; Wade et al., 2006). This also suggests that the ubiquitin ligase activity of MDM2 and proteosomal targeting of MDM2-MDMX heterodimers is not affected by Nutlin-3 and indeed may be promoted when p53 is prevented from binding to MDM2. Additionally, MDMX is a substrate for caspase 3, and is degraded during apoptosis, and may provide an alternative explanation for MDMX degradation upon MDM2-p53 antagonist treatment (Gentiletti et al., 2002). It was also found that *MDM2*-amplified cell lines had low MDMX protein expression compared to *MYCN*-only amplified cell lines, suggesting that MDM2 may also promote MDMX degradation in the absence of DNA damage. These cell lines have increased $p14^{ARF}$ expression, and $p14^{ARF}$ has been shown to promote MDM2-mediated ubiquitination of MDMX, and may be the reason these cell lines have low MDMX expression (Pan and Chen, 2003).

4.5.7 MDMX and $p14^{ARF}$

Within the p53-MDM2/MDMX-$p14^{ARF}$ network, there are also links reported between MDMX and $p14^{ARF}$. There is evidence that $p14^{ARF}$ promotes MDMX ubiquitination by MDM2 (Pan and Chen, 2003). $p14^{ARF}$ binding of MDM2 can regulate its ability to

ubiquitinate both p53 and MDMX, as $p14^{ARF}$ stimulates MDMX ubiquitination by MDM2 through the N-terminal domain that normally inhibits MDM2 ubiquitination of p53 (Pan and Chen, 2003; Midgley et al., 2000). There are also some p53-independent functions of MDMX such as an ability to inhibit E2F1 transactivation, suggesting MDMX may repress E2F1 regulated genes such as $p14^{ARF}$ (Wunderlich et al., 2004). These further functions of $p14^{ARF}$ and MDMX highlight the complexity of this network, and the need for increased understanding.

4.5.8 *MDM2*-amplified neuroblastoma compared to *MDM2*-amplified sarcoma

MDM2 is amplified in 13% of neuroblastoma tumours, and up to 20% of sarcomas (Carr-Wilkinson et al., 2010; Momand et al., 1998; Oliner et al., 1992). Since it was found that knockdown of MDM2 results in an unaltered response to MDM2-p53 antagonists in *MDM2*-amplified neuroblastoma cell lines, and since early studies suggest that MDM2-p53 antagonists may be particularly effective in sarcomas (Vassilev, 2007; Freedman et al., 1999; Momand et al., 1998; Florenes et al., 1994), the effect of MDM2-p53 antagonist mediated growth inhibition in *MDM2*-amplified neuroblastoma cell lines compared to *MDM2*-amplified sarcomas was investigated. It was found that *MDM2*-amplified neuroblastomas had significantly higher mean GI_{50} values compared to sarcomas, consistent with *MDM2*-amplification possibly having a sensitising effect in sarcomas and not in neuroblastomas. This may be a result of *MYCN* co-amplification in the *MDM2*-amplified neuroblastoma cell lines, which is not present in the sarcoma cell lines, but this is unlikely as I previously reported that *MYCN*-amplification sensitises neuroblastomas to the effects of MDM2-p53 antagonists (Gamble et al., 2011a). This data suggests that the effect of *MDM2*-amplification on response to MDM2-p53 antagonists is cancer-type specific and would need to be assessed for individual cancer types. It would be interesting to see if *MDM2*-amplified sarcomas respond in the same way as non-MDM2-amplified sarcomas to MDM2-p53 antagonists.

4.5.9 Conclusions and Future Work

In conclusion, in this chapter it has been shown that a) MDM2 levels do not affect the p53 or apoptotic response to MDM2-p53 antagonists in neuroblastoma, b) that MDM2-p53 antagonists induce higher levels of apoptosis in neuroblastoma cell lines expressing high levels of MDMX, and that knockdown of MDMX desensitizes cells to their

apoptotic effect and c) *MDM2*-amplified neuroblastoma cell lines are less sensitive to MDM2-p53 antagonist mediated growth inhibition compared to *MDM2*-amplified sarcoma cell lines.

The status of MDMX and MDM2 in the p53-MDM2/MDMX-p14^{ARF} network may therefore be important in determining the response to MDM2-p53 antagonists, and the effects of increased MDMX expression should be investigated further in pre-clinical models.

Chapter 5. $p14^{ARF}$ expression in neuroblastoma and the response to MDM2-p53 antagonists

5.1 Introduction

This chapter focuses on the role of $p14^{ARF}$ in the p53/MDM2/MYCN network, and the effect of $p14^{ARF}$ expression on the response to MDM2-p53 antagonists. *$p14^{ARF}$* is a tumour suppressor gene located at 9p21-22 and plays a role in regulating cell proliferation, and in cancer development. $p14^{ARF}$ is an upstream regulator of p53. It directly interacts with MDM2 to suppress MDM2 ubiquitin ligase activity, and also sequesters MDM2 in the nucleolus preventing interaction of MDM2 with p53, leading to p53 stabilisation and accumulation (Gallagher et al., 2006; Zhang and Xiong, 2001; Honda and Yasuda, 1999). p53 can be activated through induction of $p14^{ARF}$ which is expressed in response to aberrant proliferative signals and activated oncogenes such as MYCC (Zindy et al., 1998), E2F (de Stanchina et al., 1998), and Ras (Palmero et al., 1998), and through its activity on p53, activates pathways involved in tumour suppression and cell cycle control. There is also evidence that p53 downregulates $p14^{ARF}$ expression, creating a negative feedback loop between p53, MDM2 and $p14^{ARF}$ (Robertson and Jones, 1998).

However, $p14^{ARF}$ has both p53-dependent and p53-independent functions and interacts with several proteins involved in proliferation, the ATM/ATR/CHK signalling pathway and transcription factors in the retinoblastoma pathway. It plays roles in inducing p53-independent G_2 arrest, to delay M-phase progression and can induce G_1 arrest in cells lacking functional p53 and Rb (Eymin et al., 2003; Yarbrough et al., 2002; Martelli et al., 2001). Weber *et al* demonstrated that in mice lacking both *$p19^{ARF}$* and *p53*, the resulting primary tumours are multiple and of a wider spectrum than mice null for either gene alone (Weber et al., 2000). They also showed that mice lacking *p53*, *MDM2* and *$p19^{ARF}$* develop tumours with a wider spectrum and a higher frequency than mice lacking just p53 and MDM2, or mice lacking just p53 alone. This indicates that $p14^{ARF}$ has important MDM2 and p53-independent functions (Weber et al., 2000). Mice lacking $p19^{ARF}$, but expressing functional $p16^{INK4a}$ are viable but develop tumours early in life (Kamijo et al., 1997).

5.1.1 $p14^{ARF}$ overexpression in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines

A study by Carr *et al.* showed that when p53 is inactivated by *MDM2*-amplification, levels of $p14^{ARF}$ mRNA and protein are higher in neuroblastoma cell lines which are co-amplified for *MYCN*, compared to non-*MDM2*-amplified cell lines (Carr et al., 2006).

Two non-neuroblastoma cell lines, with either *MDM2*-amplification (SJSA-1) or *MDM2*-gain (JAR), also expressed decreased levels of $p14^{ARF}$ mRNA and protein compared to *MDM2*-amplified neuroblastoma cells. MDM2 is reportedly a direct transcriptional target of MYCN in neuroblastoma (Slack et al., 2005a) and because *MYCN* is co-amplified in the neuroblastoma cell lines, it is possible that MDM2 and MYCN have a co-operative effect on $p14^{ARF}$.

In this chapter the mechanism of increased $p14^{ARF}$ expression in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines is investigated using knockdown studies, and $p14^{ARF}$ protein expression in an increased number of *MDM2*-amplified non-neuroblastoma cell lines is examined.

5.1.2 $p14^{ARF}$ inactivation in neuroblastoma

Neuroblastoma cell lines, particularly those established after treatment, have been found to have alternate mechanisms of p53 functional inactivation such as *MDM2*-amplification and $p14^{ARF}$ inactivation (reviewed by (Tweddle et al., 2003)). Mice null for $p19^{ARF}$, the mouse homologue of $p14^{ARF}$, are highly tumour prone, developing a wide spectrum of tumours, suggesting an important role in carcinogenesis for $p14^{ARF}$ (Kamijo et al., 1997). $p14^{ARF}$ methylation or deletion occurs frequently in neuroblastoma, and inactivation of $p14^{ARF}$ can increase levels of MDM2, which results in decreased p53 activation.

DNA promoter methylation is particularly common in tumour suppressor and DNA repair genes (Das and Singal, 2004). In a study by Carr-Wilkinson *et al.*, $p14^{ARF}$ was inactivated in 29% of neuroblastoma tumours (Carr-Wilkinson et al., 2010). This was associated with stage 4 disease, and 9 of 12 patients died of disease. In the same study, methylation was detected in 7% (3/41) of tumours at both diagnosis and relapse, compared to another study where $p14^{ARF}$ methylation was detected in 14% (6/44) of diagnostic tumours (Gonzalez-Gomez et al., 2003). Homozygous deletion of $p14^{ARF}$ was reported at a frequency of 22% in neuroblastoma tumour samples in the study by Carr-Wilkinson *et al*, which is higher than previously reported, with 9% (4/46) of cell lines $p14^{ARF}$ deleted in a study by Thompson *et al*, and none in a study of tumour samples by Omura-Minamisawa *et al* (Omura-Minamisawa et al., 2001; Thompson et al., 2001). Two other studies looked for homozygous deletion of $p16^{INK4a}$ in neuroblastoma, which is usually associated with $p14^{ARF}$ co-deletion. In the first study, 1 of 19 neuroblastoma cell lines were homozygously deleted for $p16^{INK4a}$ (Bassi et al.,

2004), and in the other study no cases were found in patient samples (Diccianni et al., 1996).

5.1.3 $p14^{ARF}$ inactivation in cell lines used in this study

Four cell lines inactivated for $p14^{ARF}$ were used in this study. Two are methylated for $p14^{ARF}$; GIMEN and PER-108 (Carr et al., 2006), and two are homozygously deleted for $p14^{ARF}$; SHEP and LAN6 (Carr et al., 2006; Thompson et al., 2001). GIMEN and PER-108 are derived from relapsed tumours and are methylated at the $p14^{ARF}$ gene promoter, independently of $p16^{INK4a}$ (Carr et al., 2006). Homozygous deletion of exon 1β was detected in LAN6 and SHEP cells, and in both these cell lines $p16^{INK4a}$ is also deleted. Interestingly, in SHEP cells, 1 copy of the 190kb region was deleted in only 66% of cells, but since there was no genomic DNA PCR product a smaller 9p deletion affecting the $p14^{ARF}$ locus may be present in the remainder (Carr et al., 2006).

5.1.4 $p14^{ARF}$ and chemoresistance

Previous studies using mouse models found that *CDKN2A* mutations resulted in chemoresistance to cyclophosphamide (Schmitt et al., 1999). Homozygously deleted LAN6 cells were found to be resistant to melphalan, carboplatin and etoposide (Keshelava et al., 2001), and SHEP cells were resistant to irradiation and adenoviral therapy compared to the related SHSY5Y and SKNSH cell lines (Van Maerken et al., 2009b; Makin et al., 2001). Loss of $p19^{ARF}$, the mouse homologue of $p14^{ARF}$, limited sensitivity to imatinib, a tyrosine kinase inhibitor (Ohgaki et al., 1993). Interestingly, restoration of $p14^{ARF}$ expression in methylated GIMEN cells did not alter the sensitivity of this cell line to cisplatin (Carr et al., 2006).

5.1.5 $p14^{ARF}$ and response to Nutlin-3 in neuroblastoma

One study has previously investigated the effect of $p14^{ARF}$ on the response to Nutlin-3 in neuroblastoma. Van Maerken *et al.* found that despite having wildtype p53, LAN6 and SHEP cells were relatively resistant to nutlin-3 and in fact IC_{50} values were comparable to those cells with mutant p53 (Van Maerken et al., 2011). Despite being closely related to SKNSH and SHSY5Y cells, SHEP cells were especially resistant to apoptosis from Nutlin-3, but did undergo a G_1 arrest suggesting intact checkpoint control mechanisms. The homozygous deletion of the *CDKN2A* gene in SHEP cells is not present in SKNSH and SHSY5Y cells (Van Maerken et al., 2011) and is proposed as the mechanism by which SHEP cells gain resistance to Nutlin-3. However, induced overexpression of the *CDKN2A* gene in SHEP cells did not restore sensitivity to Nutlin-

3 (Van Maerken et al., 2011). Knockdown of the *CDKN2A* gene in IMR32 and NGP cells resulted in a moderate reduction in sensitivity to Nutlin-3, and *CDKN2A* overexpression in modified tetracycline regulatable IMR32 cells resulted in a reduction in cell viability and increased apoptosis (Van Maerken et al., 2011). This effect was a result of $p14^{ARF}$ deletion, and not $p16^{INK4a}$ deletion. The status of $p14^{ARF}$ may therefore be particularly important and clinically relevant in neuroblastoma.

5.2 Hypotheses and Aims

Hypotheses:

- Increased $p14^{ARF}$ expression in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines is a co-operative effect of MYCN and MDM2.
- $p14^{ARF}$ knockdown or impairment through methylation or homozygous deletion results in decreased apoptosis following MDM2-p53 antagonist treatment.

Aims:

1. To investigate the mechanism of increased $p14^{ARF}$ expression in *MYCN*- and *MDM2*-co-amplified neuroblastoma cell lines using siRNA to knockdown MYCN and/or MDM2.
2. To determine mRNA and protein levels of $p14^{ARF}$ in *MDM2*-amplified neuroblastomas and *MDM2*-amplified non-neuroblastoma cell lines.
3. To examine levels of apoptosis following MDM2-p53 antagonist treatment in $p14^{ARF}$ impaired neuroblastoma cell lines, and in neuroblastoma cell lines expressing high levels of $p14^{ARF}$.
4. To determine the cell cycle response to MDM2-p53 antagonists in a panel of neuroblastoma cell lines, and to investigate the extent of G_1 arrest in response to Nutlin-3.
5. To investigate the effect of $p14^{ARF}$ knockdown on the p53 response and apoptosis in the presence and absence of MDM2-p53 antagonist treatment.

5.3 Specific Materials and Methods

5.3.1 Investigating increased levels of $p14^{ARF}$ in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines

MDM2 and/or MYCN was knocked down using siRNA in *MYCN* and *MDM2* co-amplified NGP, LS, NB1691 and TR14 cells as described in Chapter 2.3. The effect on $p14^{ARF}$, p53 and $p21^{WAF1}$ was determined using Western blotting.

5.3.1.1 MDM2 knockdown

MDM2 knockdown in NGP and LS cells was previously optimised and is described in Chapter 4.3.1. Concentrations of 40nM were used, and optimal knockdown was at 24 hours. This concentration was tested in TR14 cells, and high levels of knockdown were achieved. MDM2 knockdown required further optimisation in NB1691 cells, and as shown in Figure 5.1, optimal knockdown was again at a 40nM concentration but at 48 hours, where 100% knockdown was achieved when quantified using densitometry and normalised to actin (as described in Chapter 2.4.10).

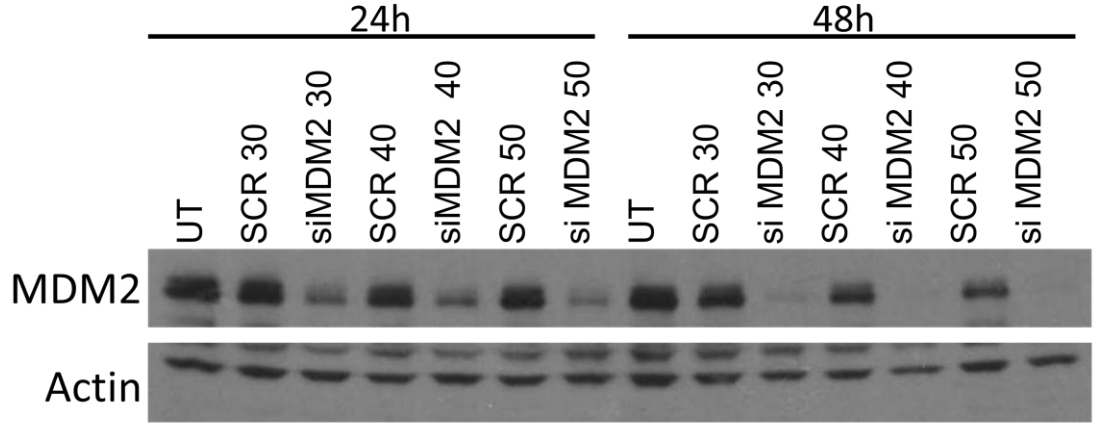


Figure 5.1. **Optimisation of MDM2 knockdown in NB1691 cells at 24 and 48 hours.**

5.3.1.2 MYCN knockdown

MYCN knockdown was previously optimised in NGP, TR14 and LS cells as described in Chapter 3.3.2. As shown in Figure 5.2, an optimal MYCN knockdown of 83% was achieved in NB1691 cells at 30nM siRNA treatment for 48 hours.

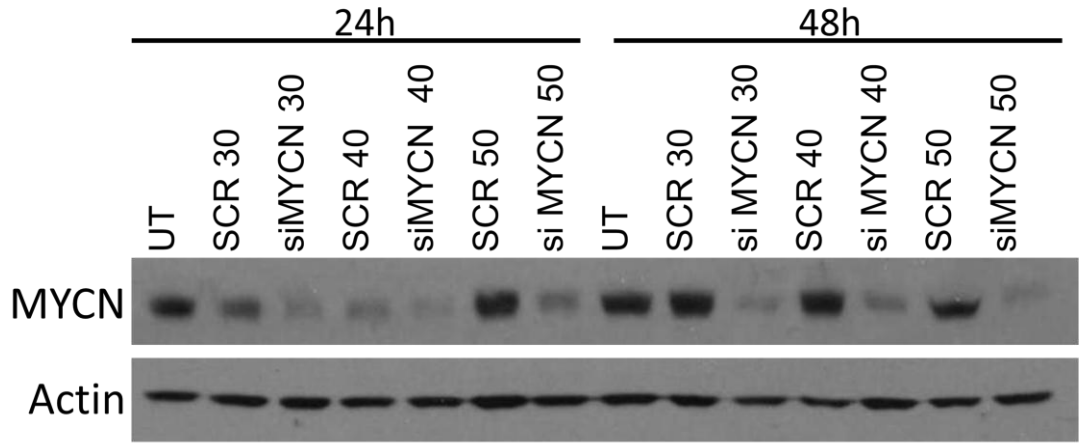


Figure 5.2. **MYCN knockdown in NB1691 cells at 24 and 48 hours.**

5.3.1.3 Double knockdown of MYCN and MDM2

Double knockdown of MYCN and MDM2 was optimised as shown in Figure 5.3 for the 4 cell lines. Concentrations shown are final concentrations from combining equal concentrations of MYCN and MDM2 siRNA. Optimal knockdown was as follows; NGP 80nM at 48 hours (66% MDM2 knockdown, 94% MYCN knockdown), LS 40nM at 48 hours (84% MDM2 knockdown, 81% MYCN knockdown), NB1691 40nM at 48 hours (79% MDM2 knockdown, 100% MYCN knockdown) and TR14 40nM at 48 hours (97% MDM2 knockdown, 92% MYCN knockdown).

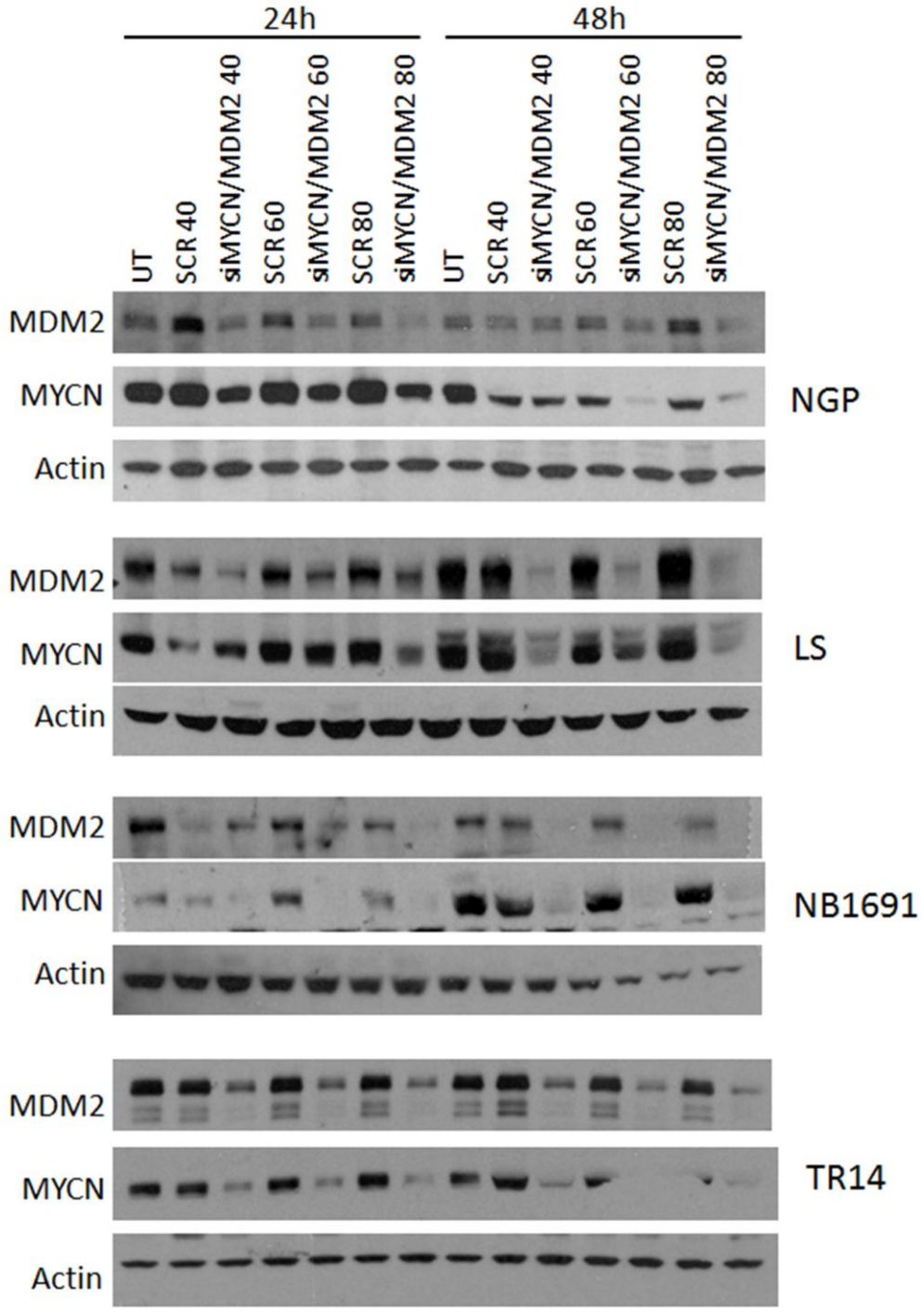


Figure 5.3. **Double knockdown of MYCN and MDM2 in 4 *MYCN* and *MDM2* co-amplified cell lines.**

5.3.2 $p14^{ARF}$ knockdown

$p14^{ARF}$ knockdown was performed using the method and siRNA sequence described in Chapter 2.3. $p14^{ARF}$ was knocked down in LS, NB1691 and NGP cells. These cells express high levels of $p14^{ARF}$, so $p14^{ARF}$ protein is detectable by Western blot. As shown in Figure 5.4, 3 concentrations of siRNA were tested at 24 and 48 hours. Optimal knockdown was achieved in NGP cells at 40nM for 48 hours (70%), in LS cells at 40nM for 24 hours (95%) and in NB1691 cells at 40nM for 24 hours (96%).

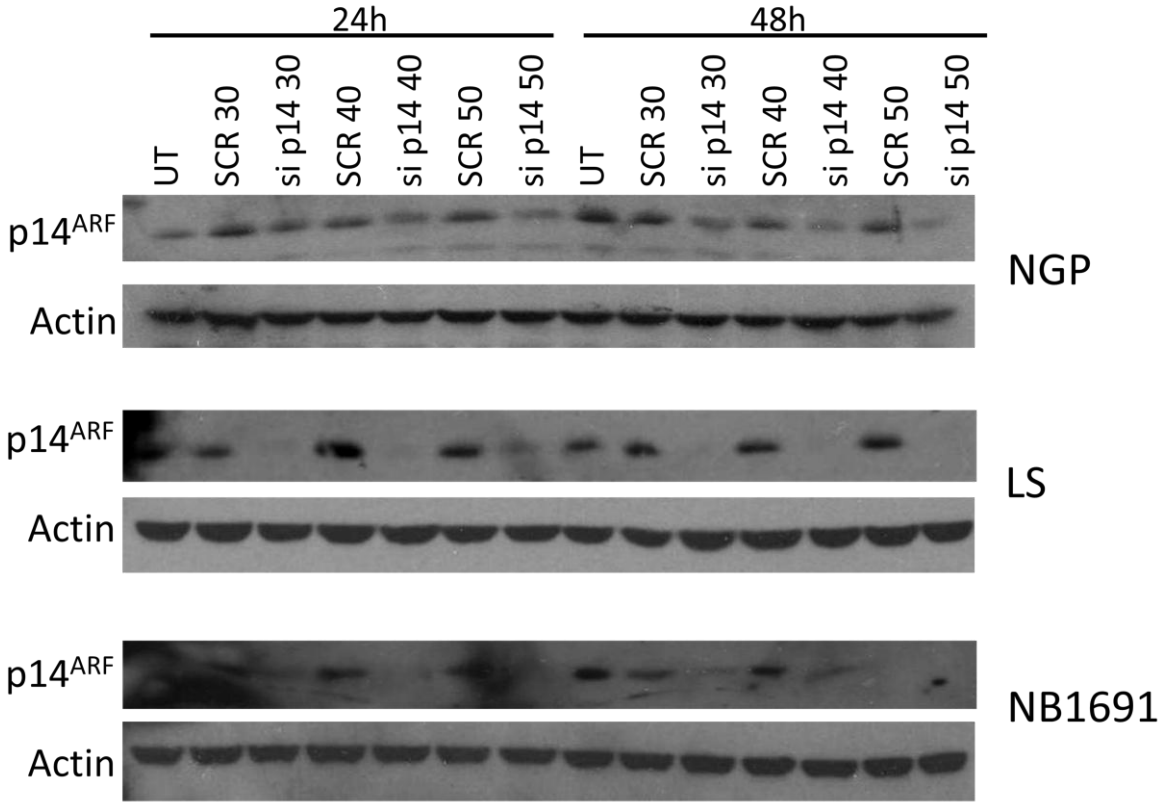


Figure 5.4. **Optimisation of $p14^{ARF}$ knockdown in NGP, LS and NB1691 cells.**

5.3.3 Immunofluorescence

5.3.3.1 Preparation of cells

Cells were seeded at a density of $4x10^5$ cells/well in 6-well plates containing coverslips for 48 hours. Media was then aspirated off and cells washed with cold PBS. Cells were fixed in 4% paraformaldehyde (pH 7.4) for 10 minutes at room temperature, which was then replaced with ice cold methanol to permeabilise cells for a further 15 minutes. Methanol was removed and cells washed for 3 x 5 minutes in PBS.

5.3.3.2 Antibody Detection

Coverslips were gently removed from the 6-well plate and placed in a petri dish, with the cell layer facing up. Damp paper towel was placed around the edges of the petri dish to prevent evaporation. To block non-specific antibody binding, 200µl of 3% BSA (dissolved in PBS) was added to the coverslip, and incubated for 30 minutes at room temperature. The primary $p14^{ARF}$ antibody used was p14 ARF (ARF 4C6/4): sc-53392 (Santra Cruz). HeLa cells were used as a positive control, as the use of this antibody in immunofluorescence has been previously published in these cells. The blocking solution was replaced with a 1:200 dilution of either the primary antibody in 3% BSA and 1% Triton-X, or just 3% BSA and 1% Triton-X as the no primary control, and incubated overnight at 4°C. Cells were then washed in PBS for 3x 10 minutes, and secondary antibody, Goat-anti mouse (Dako), added at a concentration of 1:200 for 1 hour at room temperature. Cells were again washed in PBS for 3x 10 minutes, mounted with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Labs), and left to dry.

5.3.3.3 Fluorescent microscopy

Images were captured using a Nikon Eclipse E600 microscope, with NIS-elements F 3.0 (Nikon) software.

5.3.4 Flow cytometry

The panel of 21 neuroblastoma cell lines were treated with 10µM Nutlin-3 or DMSO, and samples harvested at 24, 48, 72 and 96 hours. FACs was performed and analysed as previously described (Chapter 2.5). The sub G_1 peak was determined at each time point, and cell cycle analysis carried out at 24 hours. The degree of G_1 arrest was determined using the ratio of cell numbers in G_1 and S phases of the cell cycle, and compared to DMSO control. An increase in the G_1:S ratio indicates a G_1 arrest.

5.4 Results

5.4.1 Investigation of the increased $p14^{ARF}$ levels in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines

Carr *et al.* previously found that both $p14^{ARF}$ mRNA and protein levels are increased in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines compared to *MYCN*-only amplified neuroblastoma cell lines, non-amplified neuroblastoma cell lines, and also non-neuroblastoma *MDM2*-amplified cell lines (Carr et al., 2006). Only two *MDM2*-amplified non-neuroblastoma cell lines were tested in this previous study; SJSA-1 and JAR. Here siRNA was used to knockdown MDM2 and/or MYCN in the 4 *MYCN* and *MDM2* co-amplified, high $p14^{ARF}$ expressing neuroblastoma cell lines.

5.4.1.1 The effect of MDM2 knockdown on $p14^{ARF}$ and activation of p53

First of all, to assess the effect that *MDM2*-amplification has on $p14^{ARF}$ protein levels, MDM2 was knocked down using siRNA in the 4 cell lines, and samples collected 24 and 48 hours later. As shown in Figure 5.5, high levels of MDM2 knockdown were achieved in the 4 *MYCN* and *MDM2* co-amplified cell lines NGP, TR14, NB1691 and LS. The effect on $p14^{ARF}$ protein expression was highly variable, ranging from 0-48% reduction following 24 hours knockdown across the cell lines, and 20-42% reduction following 48 hours knockdown. This indicates that whilst knockdown of MDM2 is having some effect on $p14^{ARF}$, other factors may be involved.

As expected and consistent with what we have already reported, knockdown of MDM2 results in increased p53 and $p21^{WAF1}$ protein levels at both 24 and 48 hours (Figure 5.5). MDM2 is a negative regulator of p53, and therefore p53 is released from this negative control, allowing levels to increase (due to decreased degradation) and for activity to increase (due to decreased MDM2-mediated repression of transcription). This is reflected in the increased induction of the p53 transcriptional target, $p21^{WAF1}$ observed following MDM2 knockdown.

5.4.1.2 The effect of MYCN knockdown on $p14^{ARF}$ and activation of p53

MYCN was knocked down to determine if there was an effect on $p14^{ARF}$ and if so, whether or not it was greater than that after MDM2 knockdown. As shown in Figure 5.6, 24 hours following MYCN knockdown, a 0-42% reduction in $p14^{ARF}$ was seen across the cell lines, but at 48 hours a greater reduction between 52% and 77% was observed across the cell lines. This indicates that MYCN is contributing to the

overexpression of $p14^{ARF}$ in these cells, and to a greater extent than MDM2, despite *MYCN*-amplification alone having no effect on $p14^{ARF}$ levels.

Interestingly, MYCN knockdown had little effect on p53, MDM2 and $p21^{WAF1}$ protein levels (shown in Figure 3.11), despite both p53 and MDM2 being reported as direct transcriptional targets of MYCN (Chen et al., 2010b; Slack et al., 2005a). p53 levels did decrease in TR14 cells, but not in the other 3 cell lines and the effect on MDM2 and $p21^{WAF1}$ was variable.

5.4.1.3 The effect of double knockdown of MYCN and MDM2 on $p14^{ARF}$ and activation of p53

Since both MDM2 and MYCN knockdown resulted in decreased $p14^{ARF}$ levels, double knockdown should result in further reduction if amplification of both these proteins is contributing to the increased $p14^{ARF}$ levels in the *MYCN* and *MDM2* co-amplified cell lines. As shown in Figure 5.7, after 48 hours knockdown of both MYCN and MDM2, the decrease in $p14^{ARF}$ levels ranged from 65-92% across the cell lines, with the greater decreases in $p14^{ARF}$ corresponding with the highest levels of knockdown. This data suggests that *MDM2* and *MYCN* amplification have a co-operative effect on the expression of $p14^{ARF}$. Since MYCN knockdown had a more pronounced effect on $p14^{ARF}$, it is interesting that $p14^{ARF}$ is not increased upon *MYCN*-amplification alone.

Following double knockdown, there is no effect on p53 except in TR14 cells where an increase is seen. However, $p21^{WAF1}$ expression increases in all 4 cell lines suggesting that p53 is activated (Figure 5.7). This may suggest that MDM2 has a more dominating effect on p53 activation than MYCN, as is expected since MDM2 is the major negative regulator of p53. However, following DNA damage, MYCC is directly recruited to the $p21^{WAF1}$ promoter where it blocks $p21^{WAF1}$ induction by p53 and promotes apoptosis (Seoane et al., 2002). In addition, ectopic expression of MYCC has been shown to repress the $p21^{WAF1}$ promoter (Gartel et al., 2001). Evidence suggests that MYCN functions in a similar way. Bell *et al* found that following irradiation, $p21^{WAF1}$ was expressed at higher levels in non-*MYCN*-amplified neuroblastoma cell lines compared to *MYCN*-amplified cell lines, and the non-amplified cell lines G_1 arrested whereas the *MYCN*-amplified cell lines did not (Bell et al., 2006). Therefore in this study, knockdown of MYCN may result in increased expression of $p21^{WAF1}$. However, this was not observed after MYCN knockdown alone.

5.4.1.4 The effect of MDMX knockdown on p14^{ARF} and p53 activation

Within the p53-MDM2/MDMX-p14^{ARF} network, there are reports of links between MDMX and p14^{ARF}. There is evidence that p14^{ARF} promotes MDMX ubiquitination through MDM2 (Pan and Chen, 2003). p14^{ARF} binding of MDM2 can regulate its ability to ubiquitinate both p53 and MDMX as p14^{ARF} stimulates MDMX ubiquitination by MDM2 through the N-terminal domain that normally inhibits MDM2 ubiquitination of p53 (Pan and Chen, 2003; Midgley et al., 2000). There are also some p53-independent functions of MDMX such as an ability to inhibit E2F1 transactivation, suggesting MDMX may repress E2F1 regulated genes such as p14^{ARF} (Wunderlich et al., 2004). As shown in Figure 5.8, knockdown of MDMX in the 4 *MYCN* and *MDM2* co-amplified cell lines did not have an effect on p14^{ARF} protein levels. However, only a 24 hour time point was examined, so there may have been an effect at later time points, although following MDM2 and MYCN knockdown, an effect on p14^{ARF} was seen within 24 hours. MDMX is unlikely to contribute to the overexpression of p14^{ARF} in these cell lines, and in addition it has previously been shown that these cells lines have low MDMX expression (Chapter 4).

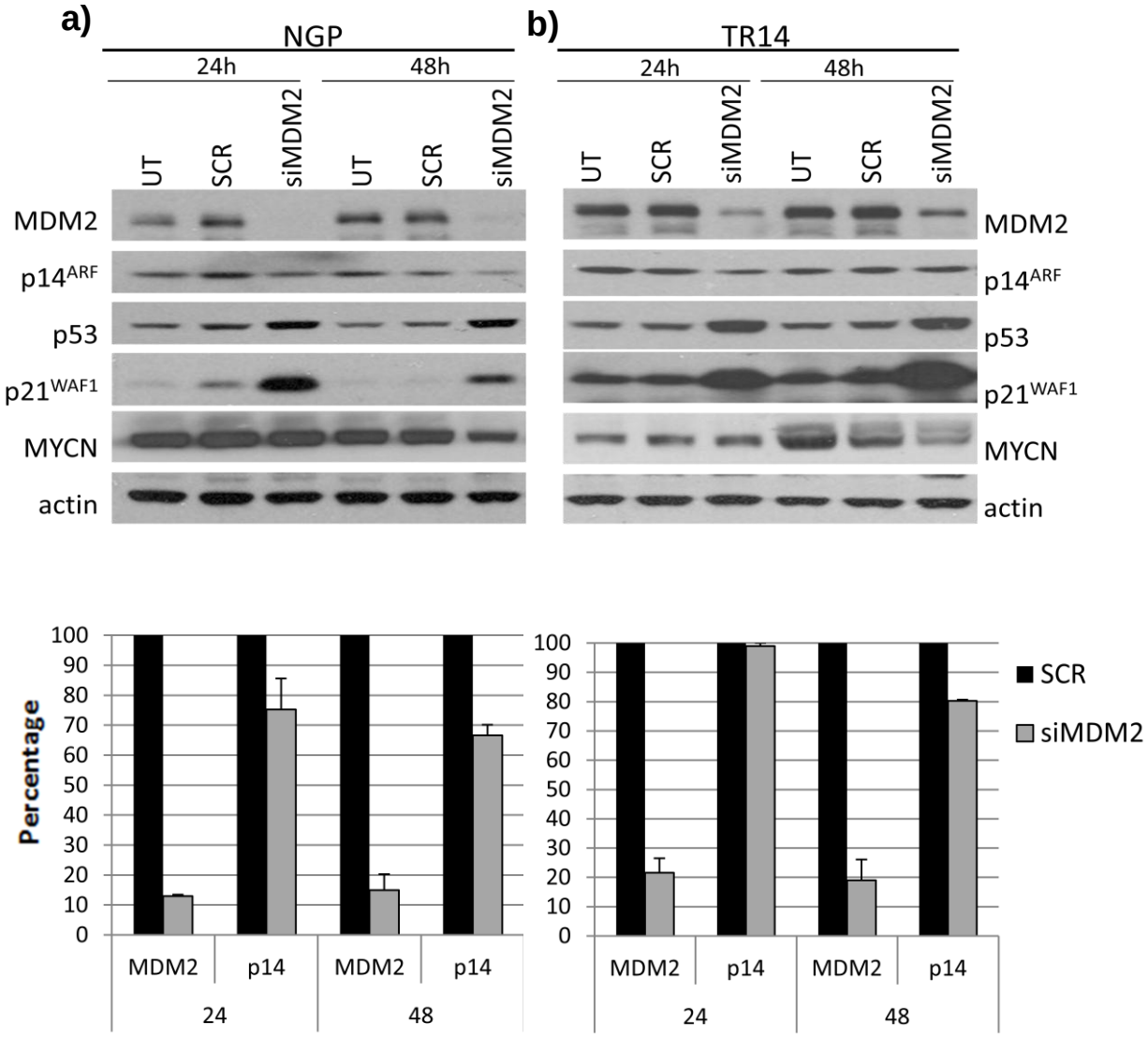
The effect on p53 was minimal; however there was induction of p21^{WAF1} indicating p53 activation. Previously pronounced effects on p53 and p21^{WAF1} have been observed following MDMX knockdown (Figure 4.11a).

5.4.2 p14^{ARF} levels in *MDM2*-amplified cell lines

Carr *et al* also looked at p14^{ARF} mRNA and protein expression in *MDM2*-amplified non-neuroblastoma cell lines and found that increased p14^{ARF} mRNA and protein expression did not occur in these cell lines (Carr et al., 2006). We have since increased the number of *MDM2*-amplified non-neuroblastoma cell lines, all of which are sarcomas. As shown in Figure 5.9, both *MDM2*-amplified neuroblastomas and sarcomas have high and comparable levels of p14^{ARF} expression compared with non-*MDM2*-amplified cell lines. This suggests that *MDM2*-amplification in the absence and presence of *MYCN*-amplification results in increased p14^{ARF} expression, whereas *MYCN*-amplification alone does not. Alternatively, it may be that p14^{ARF} expression is cancer type specific and it would be interesting to see what p14^{ARF} expression levels are in non-*MDM2*-amplified sarcomas.

5.4.3 $p14^{ARF}$ localisation in neuroblastoma cell lines

Immunofluorescence was used to investigate $p14^{ARF}$ localisation in the $p14^{ARF}$ overexpressing cell lines, NGP and LS. HeLa cells were used as a positive control. As shown in Figure 5.10, $p14^{ARF}$ is localised in the nucleolus in NGP, LS and HeLa cells. Interestingly, whereas $p14^{ARF}$ was detected in every HeLa cell, the majority of NGP and LS cells did not have levels of $p14^{ARF}$that were detectable.


a)
NGP
24h
48h
UT
SCR
siMDM2
UT
SCR
siMDM2
MDM2
p14ARF
p53
p21WAF1
MYCN
actin
b)
TR14
24h
48h
UT
SCR
siMDM2
UT
SCR
siMDM2
MDM2
p14ARF
p53
p21WAF1
MYCN
actin
Percentage
100
90
80
70
60
50
40
30
20
10
0
MDM2
p14
MDM2
p14
24
48
SCR
siMDM2

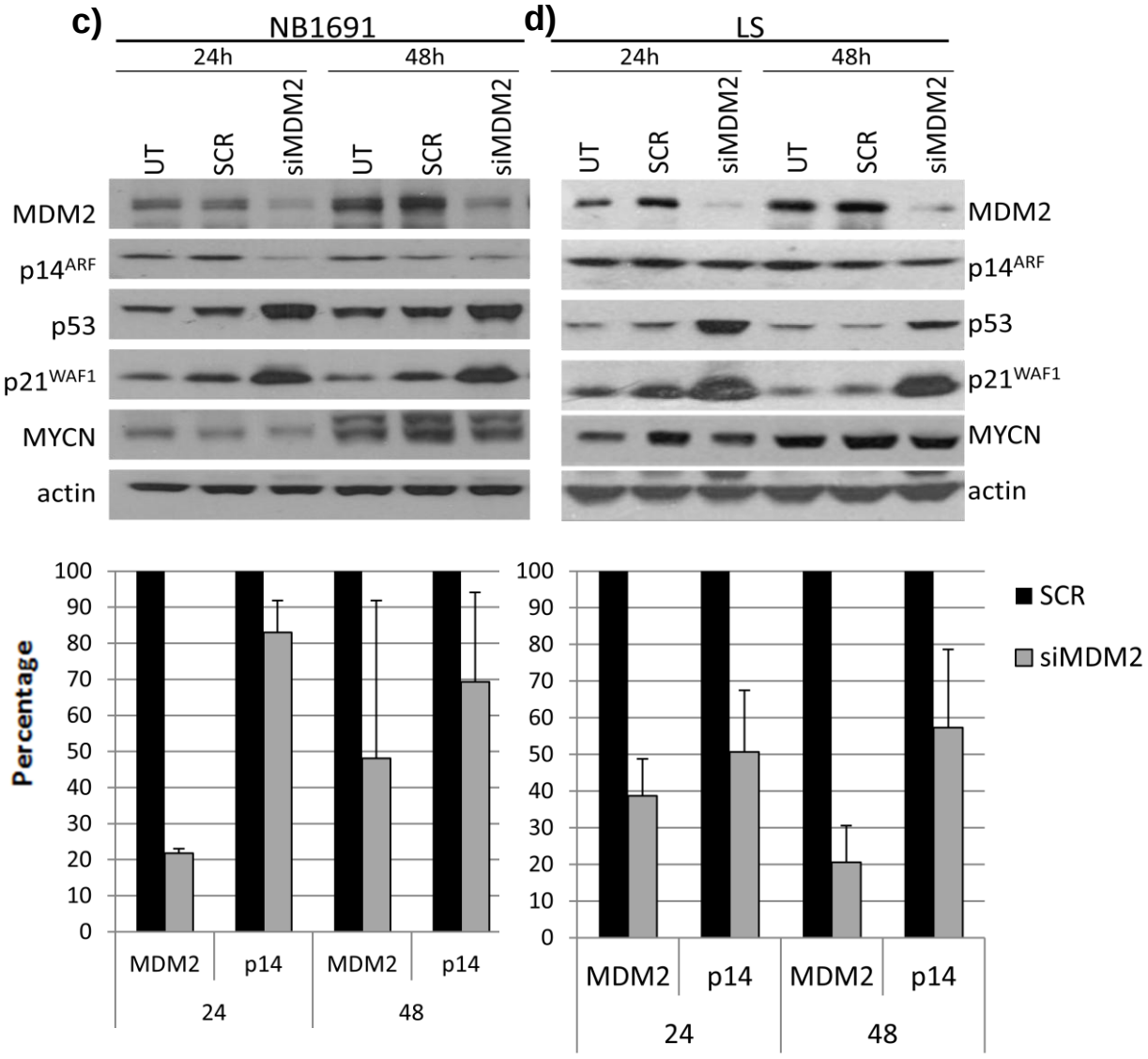


Figure 5.5. **MDM2 knockdown in 4 *MYCN* and *MDM2* co-amplified neuroblastoma cell lines and the effect on $p14^{ARF}$, p53 and $p21^{WAF1}$. a)** NGP, **b)** TR14, **c)** NB1691, **d)** LS. Actin was used as a loading control. Densitometry was used to generate bar charts from n=3 blots.

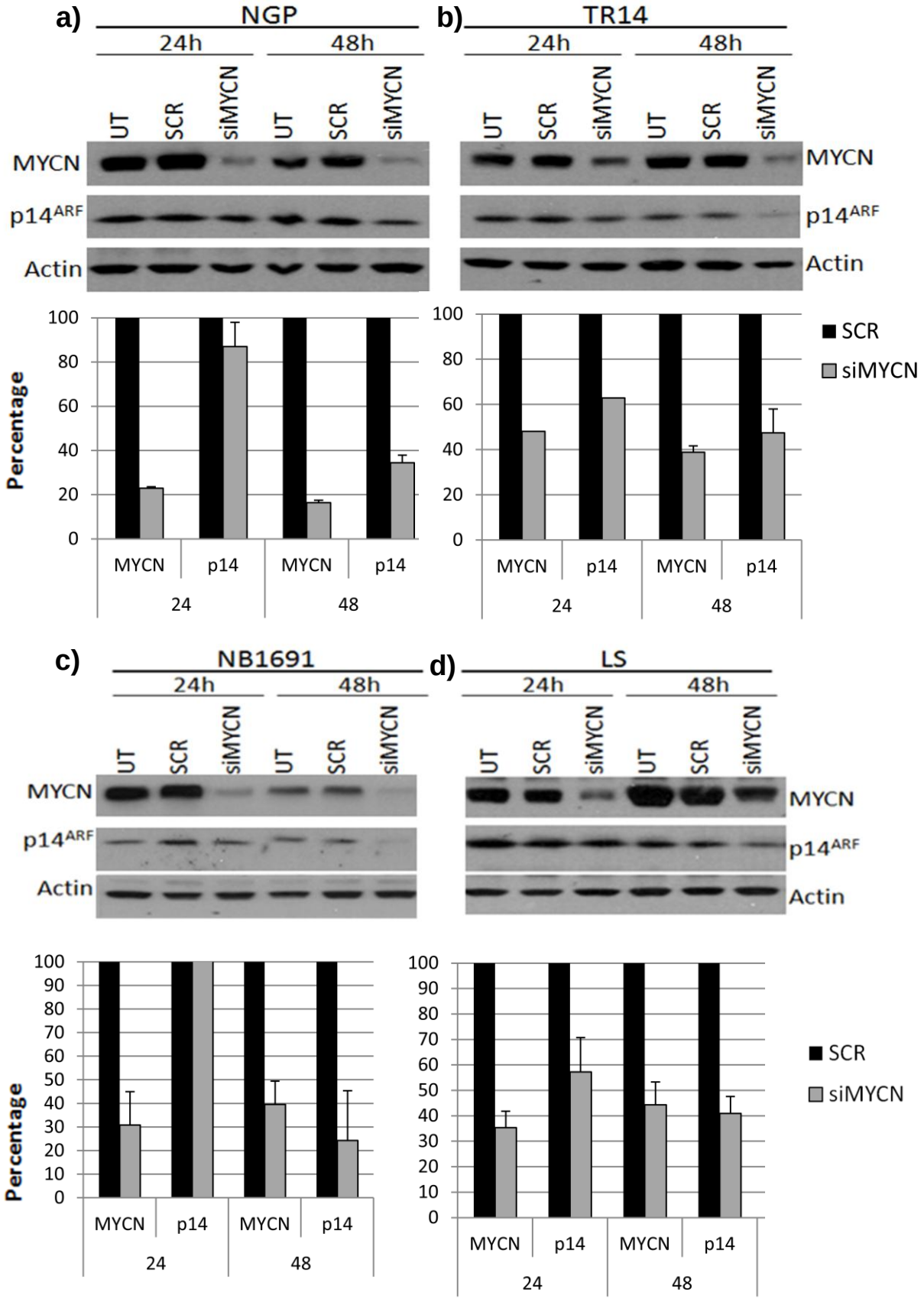


Figure 5.6. **MYCN knockdown in 4 *MYCN* and *MDM2* co-amplified neuroblastoma cell lines and the effect on p14^{ARF}. a)** NGP, **b)** TR14, **c)** NB1691, **d)** LS. Actin was used as a loading control. Densitometry was used to generate bar charts from n=3 blots.

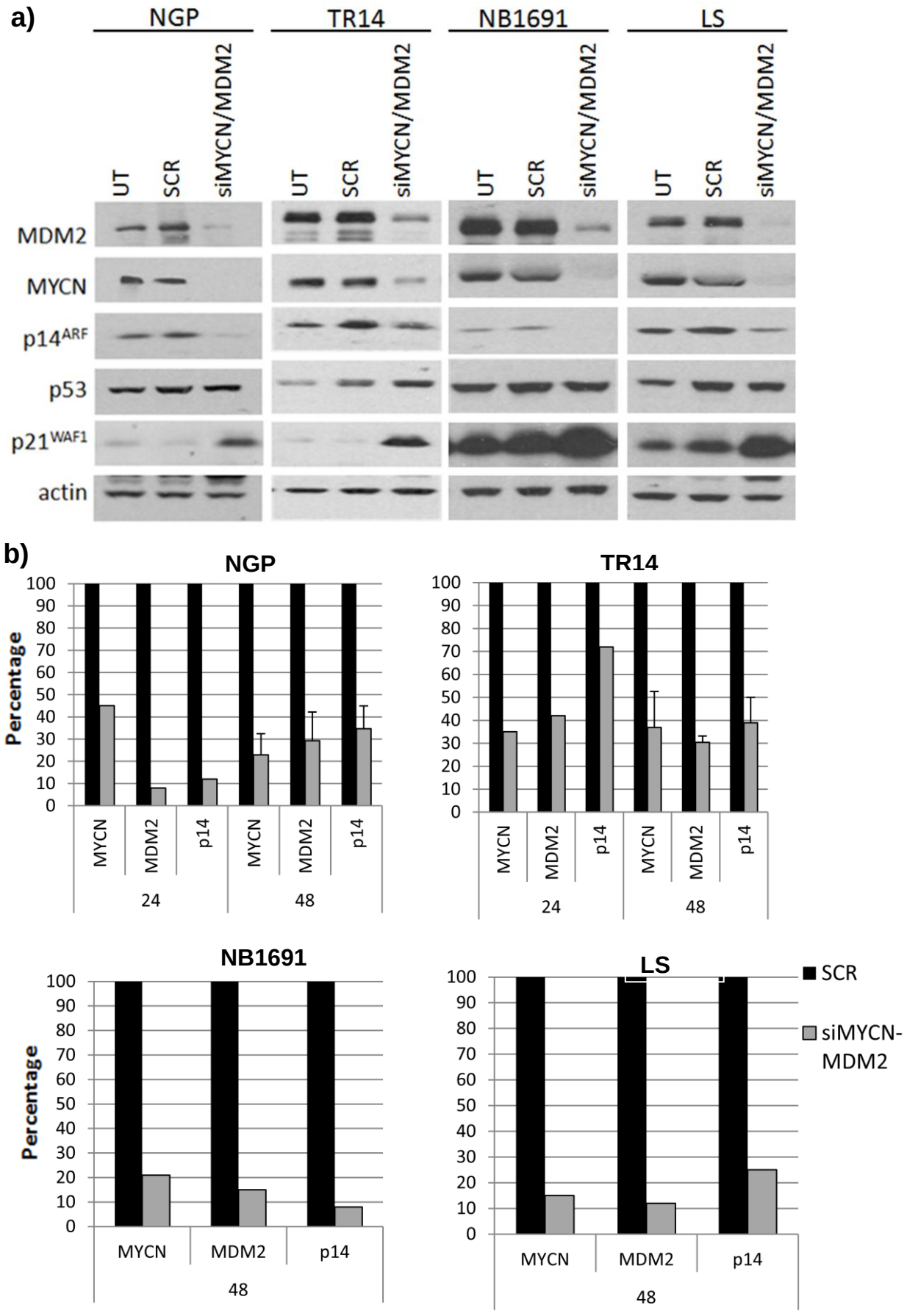

a)
NGP
TR14
NB1691
LS
UT
SCR
siMYCN/MDM2
MDM2
MYCN
p14ARF
p53
p21WAF1
actin
b)
NGP
TR14
NB1691
LS
Percentage
MYCN
MDM2
p14
24
48
SCR
siMYCN-MDM2

Figure 5.7. **Double knockdown of MYCN and MDM2 in 4 *MYCN* and *MDM2* co-amplified neuroblastoma cell lines, NGP, TR14, NB1691 and LS, and the effect on $p14^{ARF}$, p53 and $p21^{WAF1}$. a)** Western blots showing MYCN and MDM2 knockdown at 48 hours and the effect on p53 and $p21^{WAF1}$. Actin was used as a loading control. **b)** Graphs generated from densitometry of the Western blots. For NGP and TR14, n=3 at 48 hours, and n=1 at 24 hours. For NB1691 and LS, n=1 at 48 hours only.

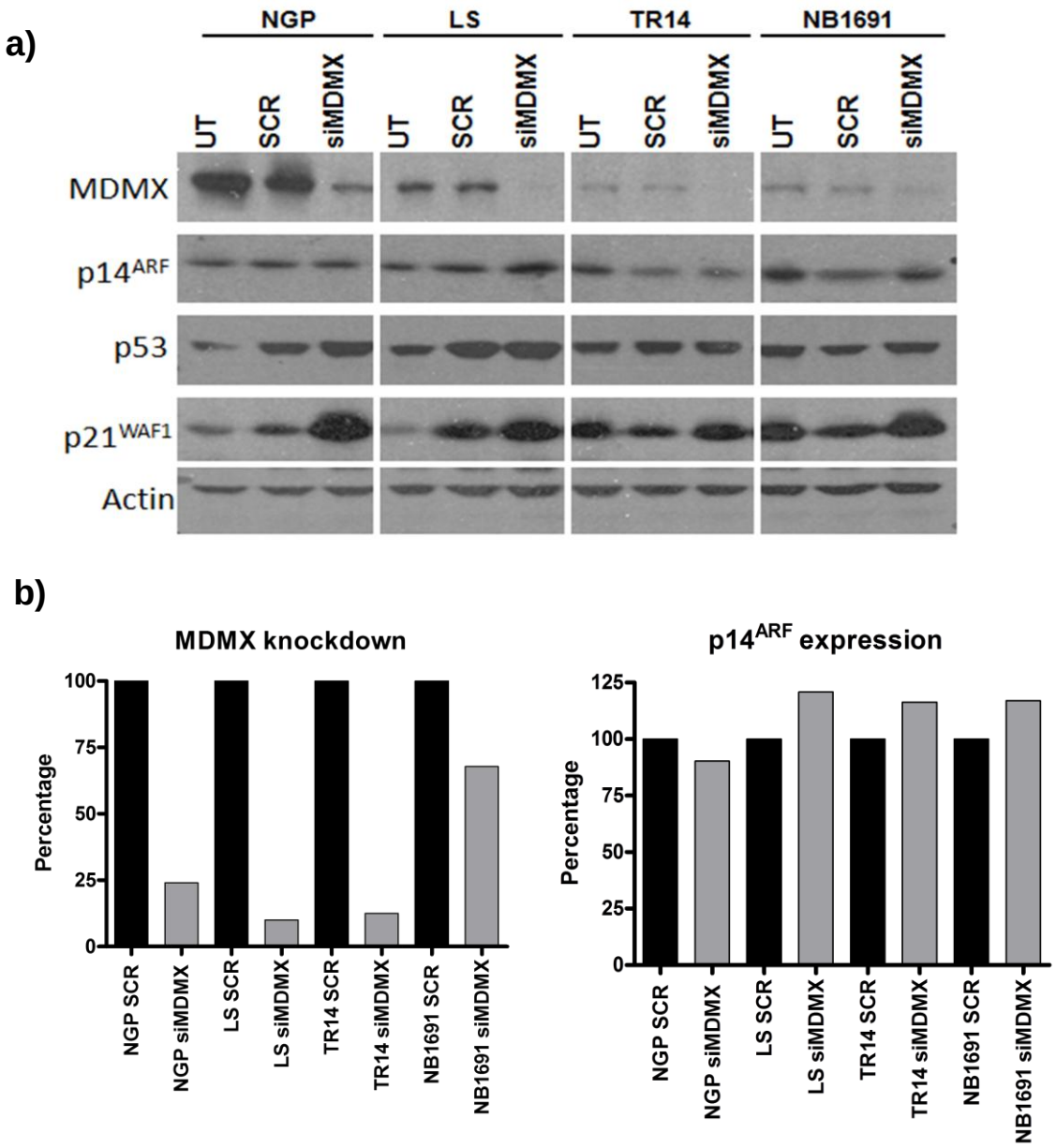


Figure 5.8. **Knockdown of MDMX in 4 *MYCN* and *MDM2* co-amplified neuroblastoma cell lines, NGP, TR14, NB1691 and LS, and the effect on $p14^{ARF}$, p53 and $p21^{WAF1}$ at 24 hours. a)** Western blots showing levels of MDMX knockdown and the effect on $p14^{ARF}$, p53 and $p21^{WAF1}$. Actin was used as a loading control. **b)** Graphs generated from densitometry of the blots (n=1).

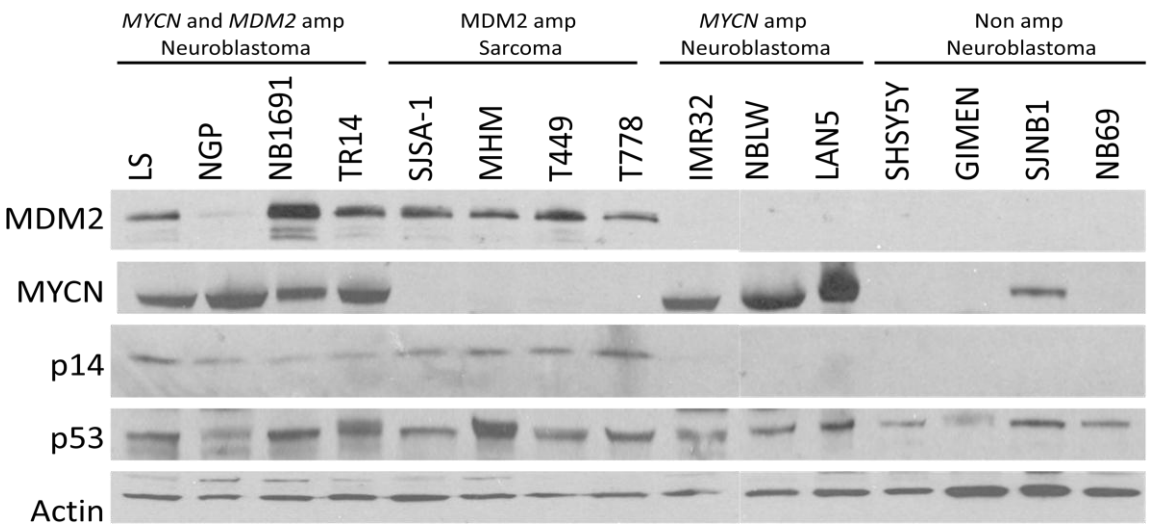


Figure 5.9. **$p14^{ARF}$ expression levels in *MDM2*-amplified neuroblastoma and sarcoma cell lines, and non-*MDM2*-amplified neuroblastoma cell lines.**

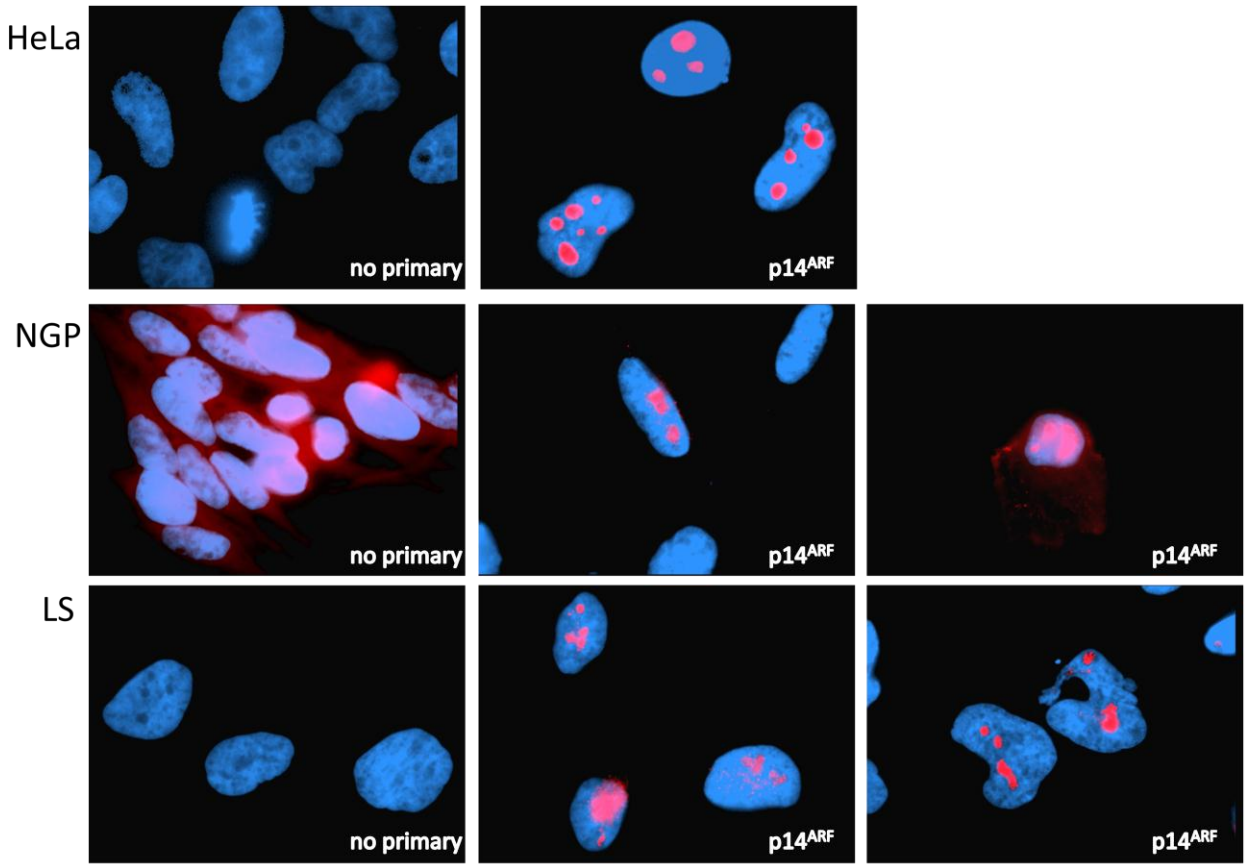


Figure 5.10. **$p14^{ARF}$ localisation in *MDM2*-amplified neuroblastoma cell lines NGP and LS. High $p14^{ARF}$ expressing HeLa cells were used as a positive control.**

5.4.4 Cell death and apoptosis in a panel of 21 neuroblastoma cell lines following Nutlin-3 treatment

A panel of 21 neuroblastoma cell lines were treated with 10μM Nutlin-3 or DMSO and samples harvested for FACs analysis at 24, 48, 72 and 96 hours. The panel of cell lines included 4 $p14^{ARF}$ impaired cell lines: *MYCN*-amplified PER-108 (methylated), and non-*MYCN*-amplified SHEP (homozygous deletion), LAN6 (homozygous deletion) and GIMEN (methylated). Cell cycle profiles for all the cell lines at 24, 48, 72 and 96 hours treatment are shown in Figure 5.11. The sub G_1 DNA fraction was determined for all samples, and the percentage increase from baseline sub G_1 levels (from DMSO control) determined after Nutlin-3 treatment (Figure 5.12). At the 24 hour time point, all 4 $p14^{ARF}$ impaired cell lines had very little sub G_1 DNA following Nutlin-3 treatment. At 48 hours the *MYCN*-amplified PER-108 cell line had high levels of sub G_1, whereas the non-*MYCN* amplified cells did not, and similar results were seen at 72 and 96 hours. SHEP cells were especially resistant to Nutlin-3 and had as little sub G_1 fraction as p53 mutant cells. p53 mutant cell lines were resistant to Nutlin-3 treatment, and the cell cycle distribution had not changed by 96 hours showing no evidence of a cell cycle arrest. Interestingly, the wildtype p53 SKNRA cell line was also very resistant to MDM2-p53 antagonist mediated cell death and the mechanism for this is unknown.

Previously, the caspase 3/7 activities in this panel had been determined at 2.5μM MI-63 and 5μM Nutlin-3 (Figure 3.22), and there was a significant reduction in caspase 3/7 activity in $p14^{ARF}$ impaired cell lines. Despite seeing no difference in the sub G_1 population between $p14^{ARF}$ overexpressing *MDM2*-amplified cell lines and the other cell lines (Figure 5.12), there was increased caspase 3/7 activity in these cell lines (Figure 3.22). Again the SKNRA cell line was resistant to MDM2-p53 antagonist mediated caspase activation.

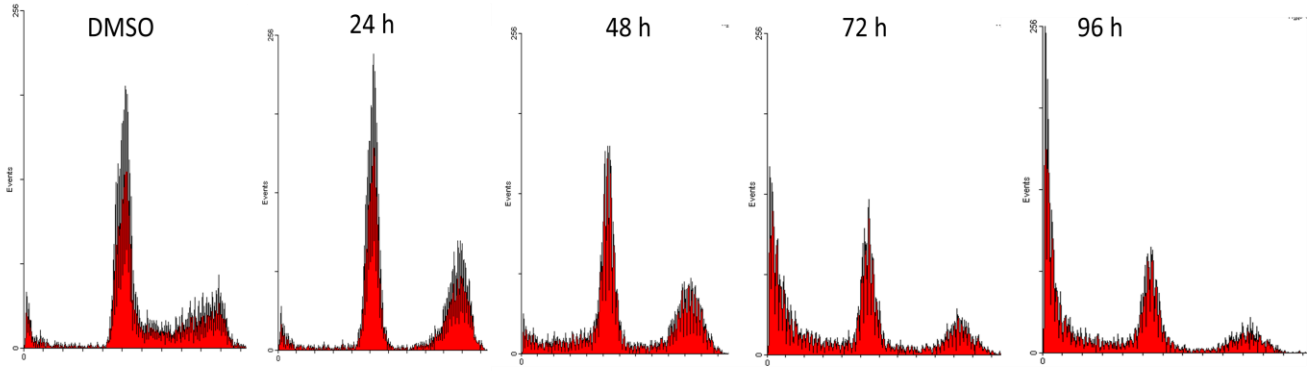
These data suggest that $p14^{ARF}$ impaired cell lines undergo very little apoptosis following Nutlin-3 and MI-63 treatment, particularly those without *MYCN*-amplification.

5.4.5 Cell cycle analysis in a panel of 21 neuroblastoma cell lines following Nutlin-3 treatment

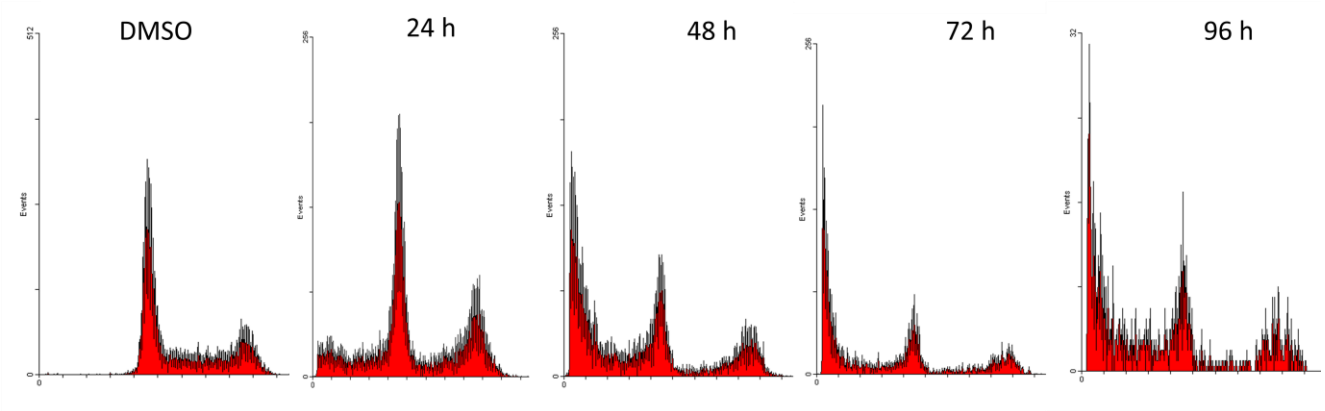
$p14^{ARF}$ impaired cell lines were analysed for their cell cycle response to MDM2-p53 antagonists compared to cell lines with intact $p14^{ARF}$. Following 10μM Nutlin-3 treatment, 3 of the 4 $p14^{ARF}$ impaired cell lines (SHEP, PER-108 and GIMEN)

underwent a G_1 arrest (Figure 5.13) indicating a strong G_1/S checkpoint response, with the LAN6 cell line neither undergoing a G_1 arrest nor apoptosis (at 24 hours). Unlike p53 mutant cell lines, LAN6 did undergo increasing levels of apoptosis at later time points. GIMEN also showed an increase in the G_2 peak (Figure 5.11). $p14^{ARF}$ impairment might therefore protect cells against Nutlin-3 mediated apoptosis as the cells are more likely to G_1 arrest. The p53 wildtype SKNRA cell line is also particularly resistant to MDM2-p53 antagonist mediated apoptosis and this cell line shows a strong G_1 arrest following Nutlin-3 treatment.

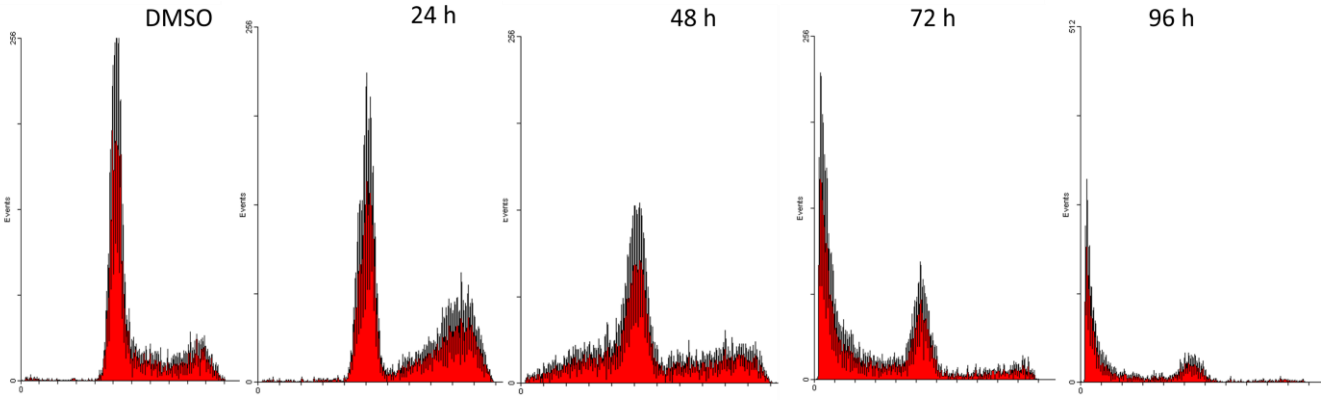
NGP



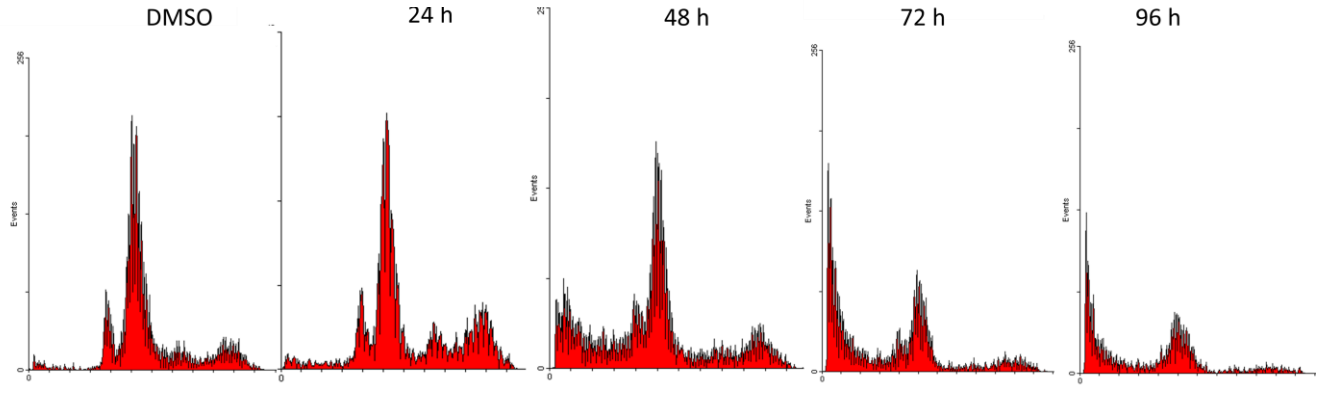
LS



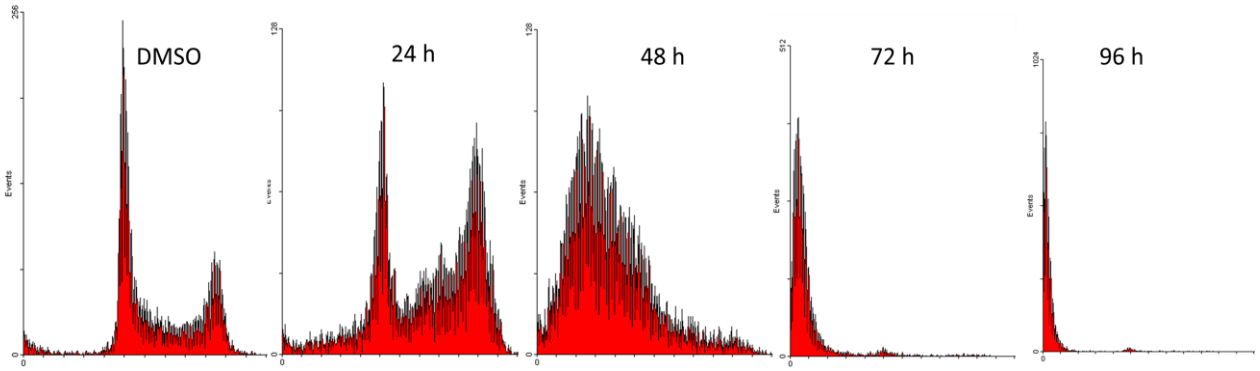
NB1691



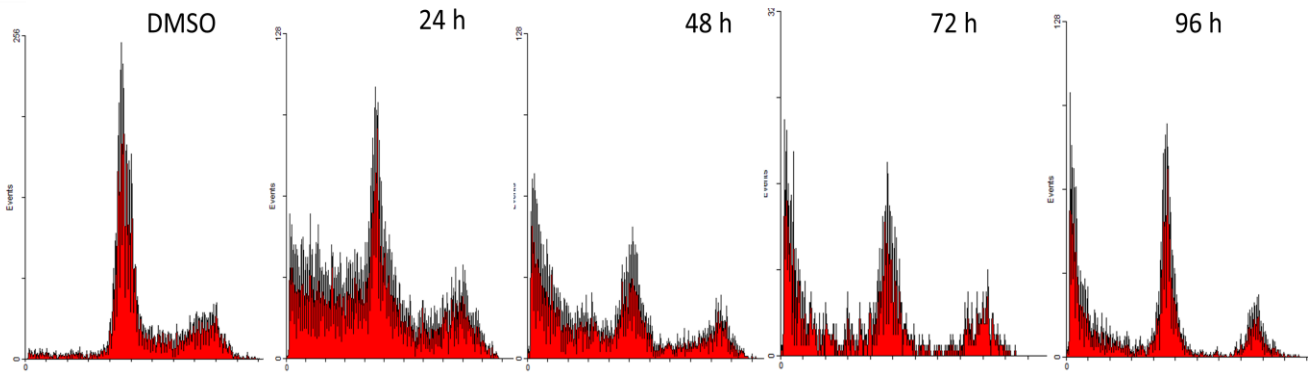
TR14



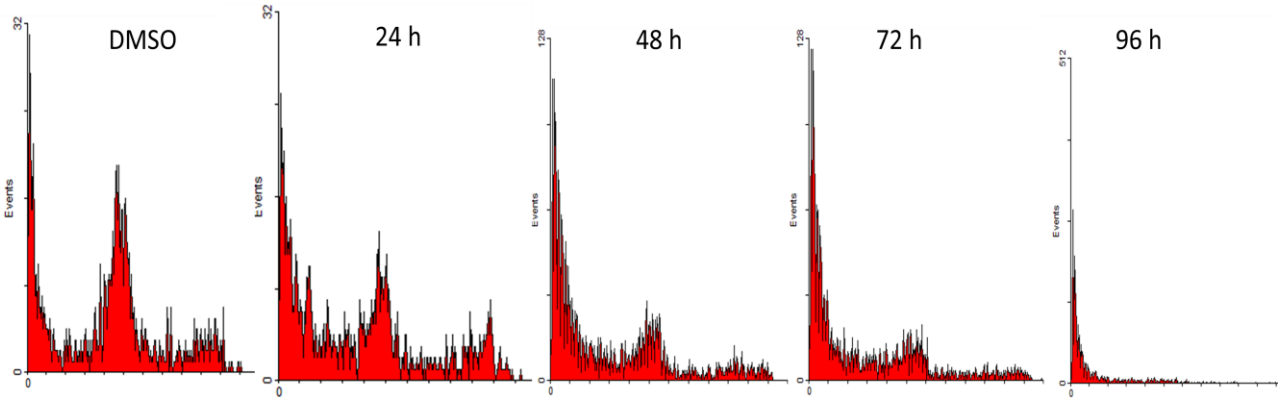
IMR32



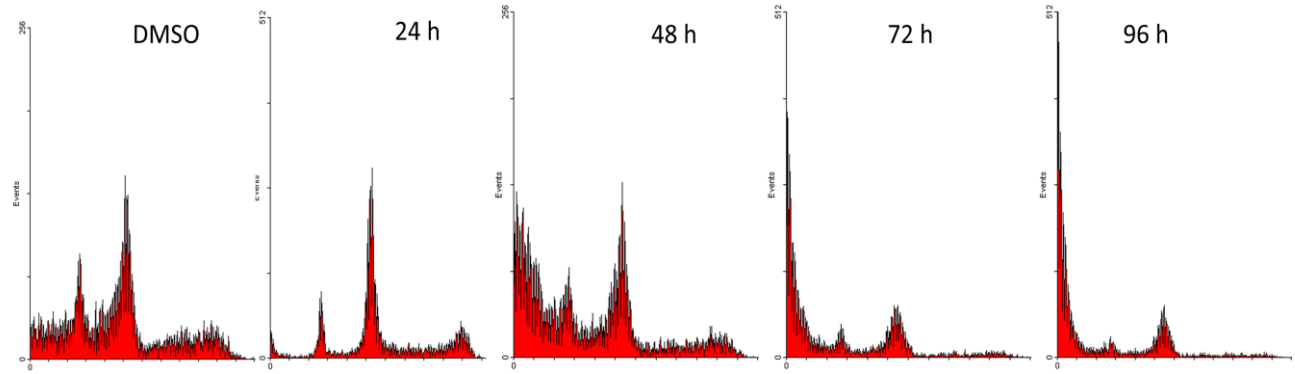
NBLW



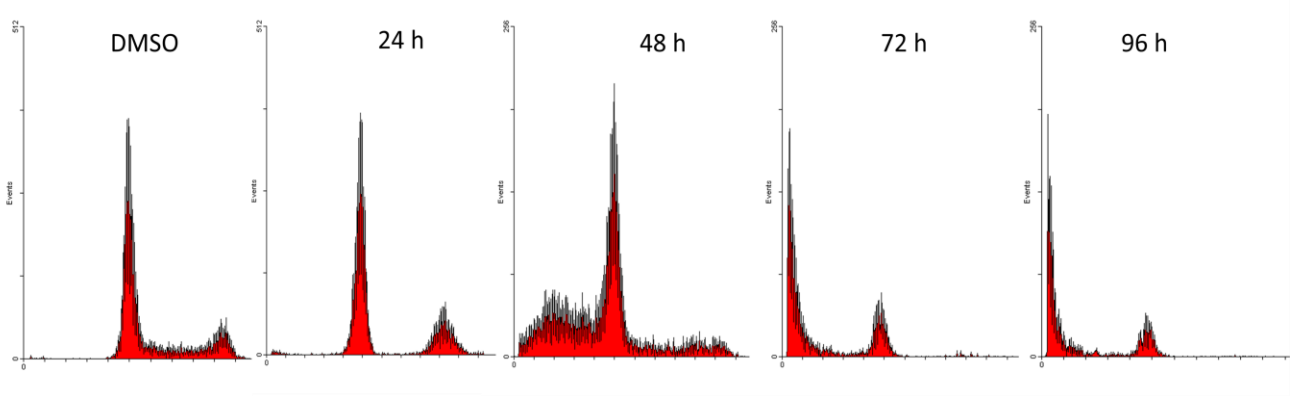
SMSKCNR



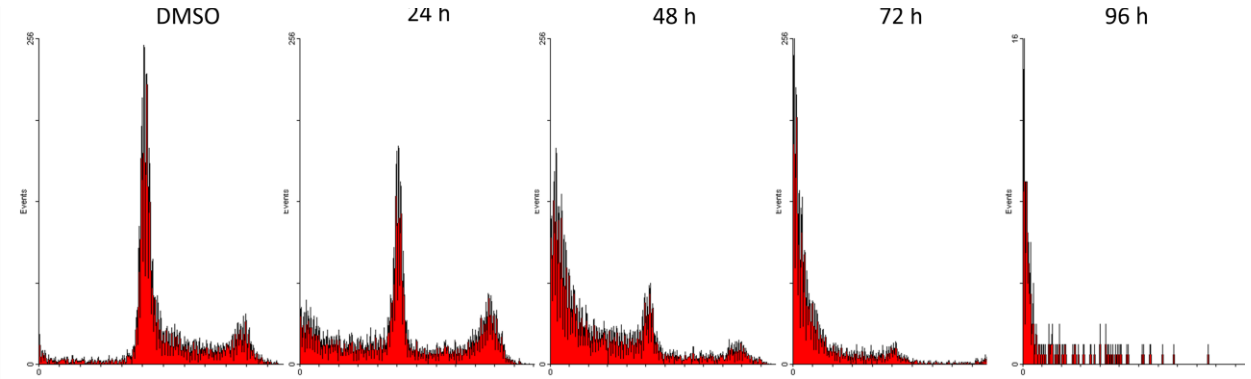
LAN5



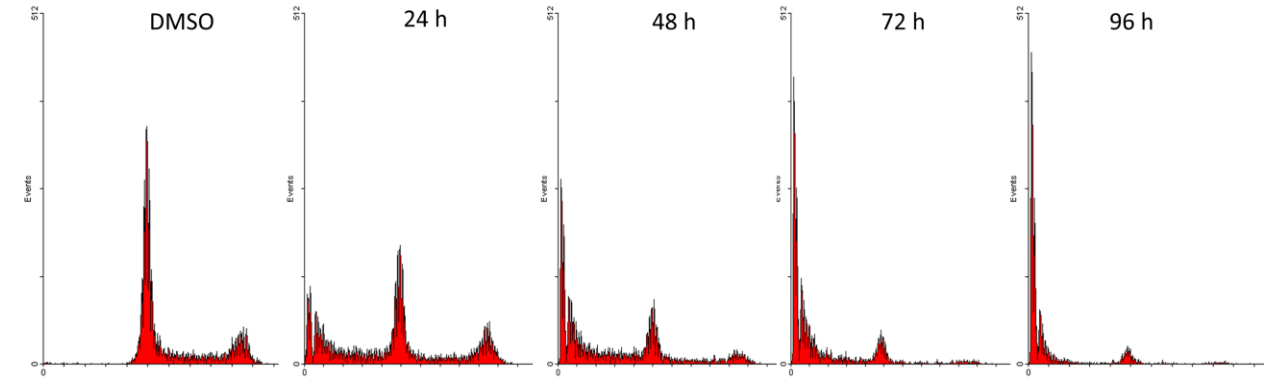
PER-108 - p14^{ARF} methylated



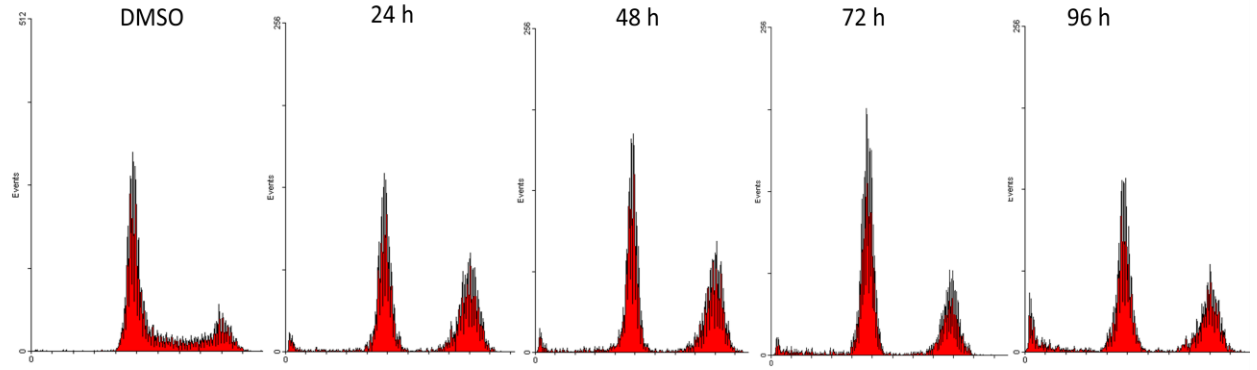
CHLA136



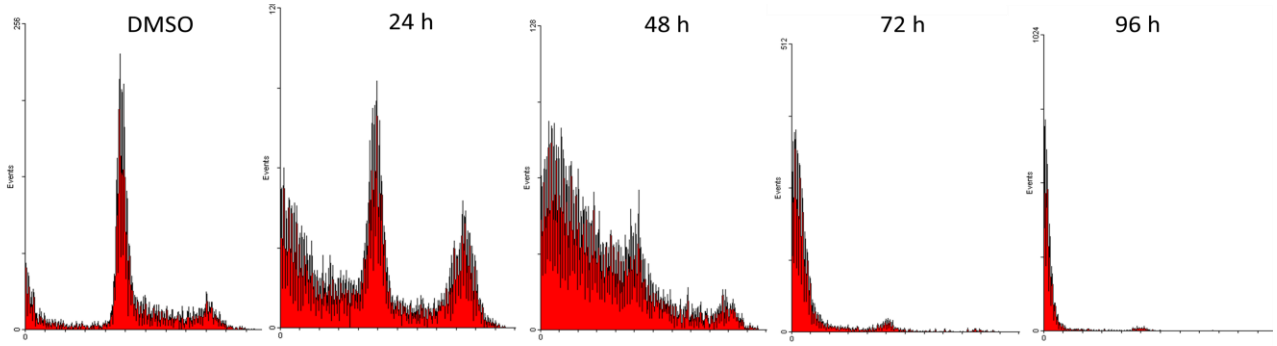
SHSY5Y



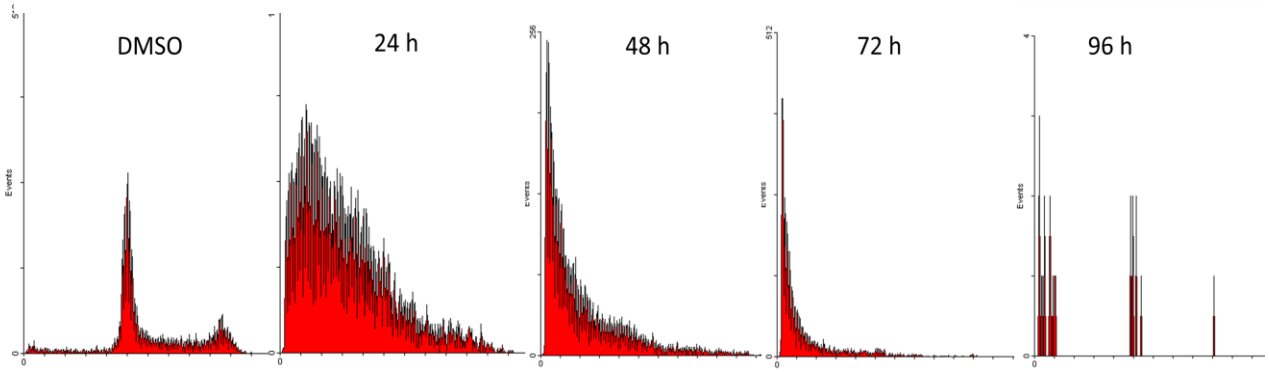
GIMEN - p14^{ARF} methylated



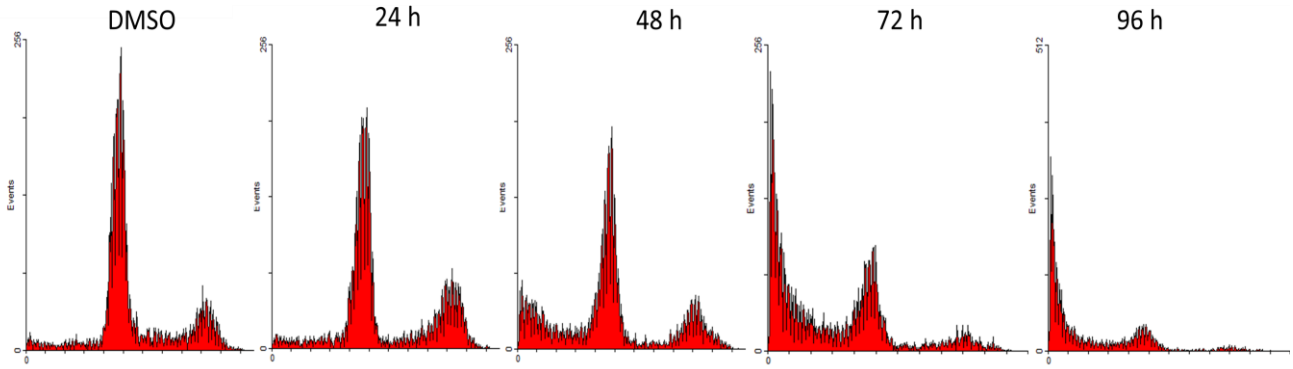
SJNB1



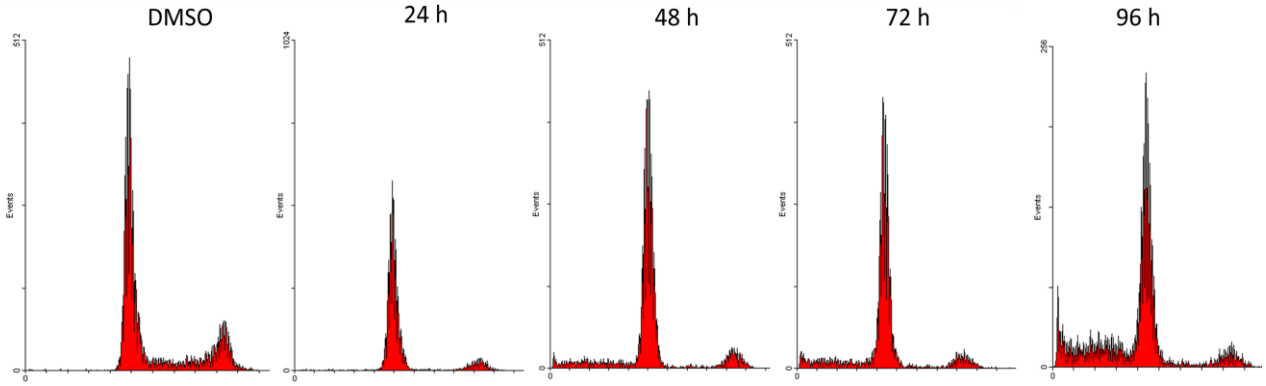
NB69



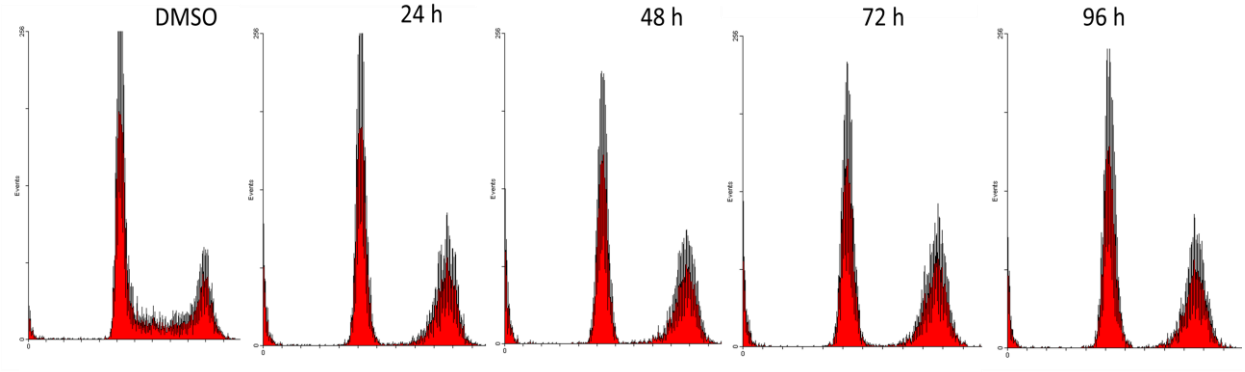
PER-108 – homozygous deletion of p14^{ARF}



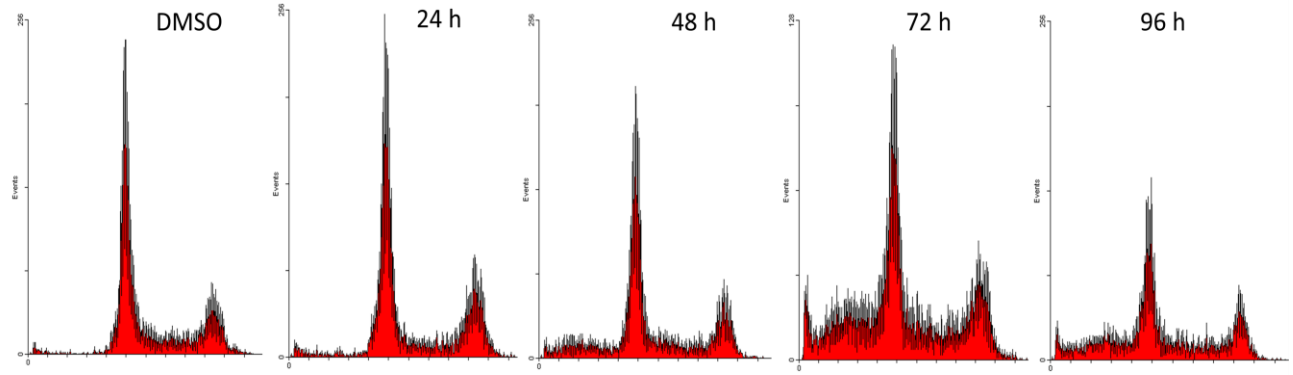
SKNRA



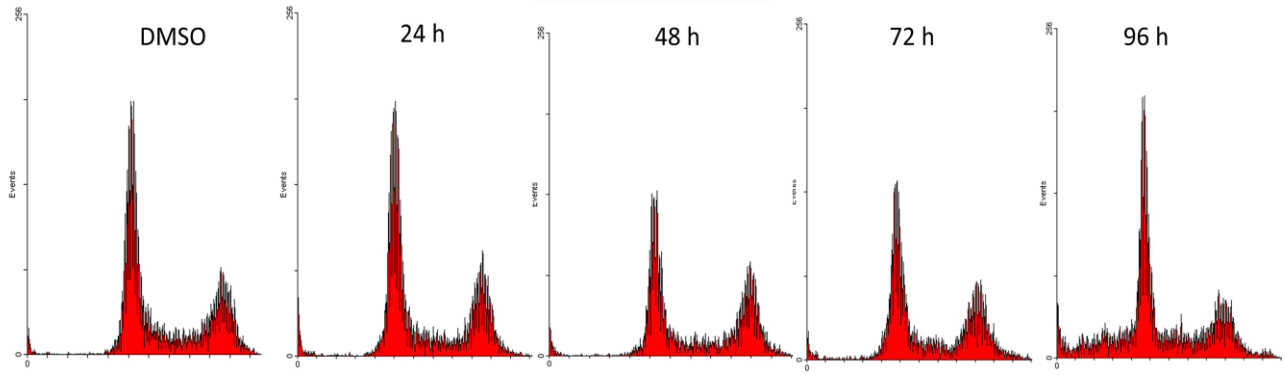
SHEP – homozygous deletion of p14^{ARF}



IGNR91



SKNBe2C



SKNAS

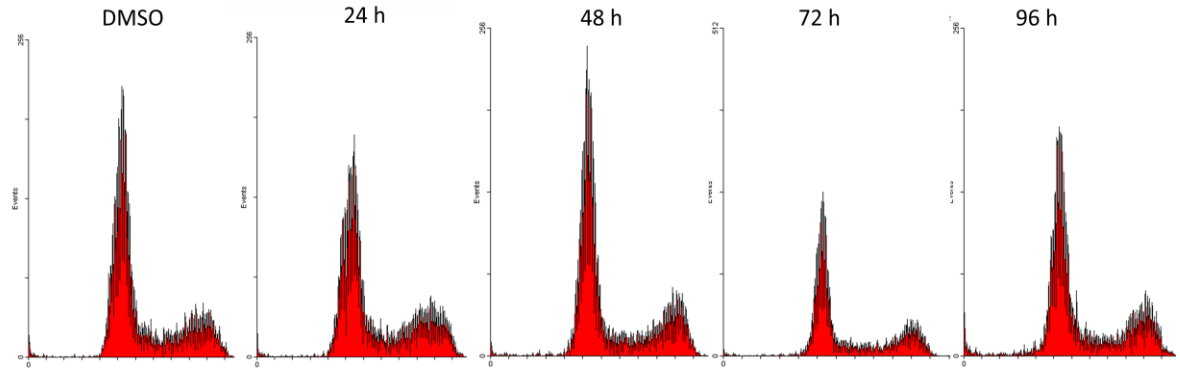


Figure 5.11. **Representative cell cycle profiles following 10μM Nutlin-3 treatment in a panel of neuroblastoma cell lines at 24, 48, 72 and 96 hours (n=3).** DMSO controls were used to place markers for the sub G_1 fraction at each time point.

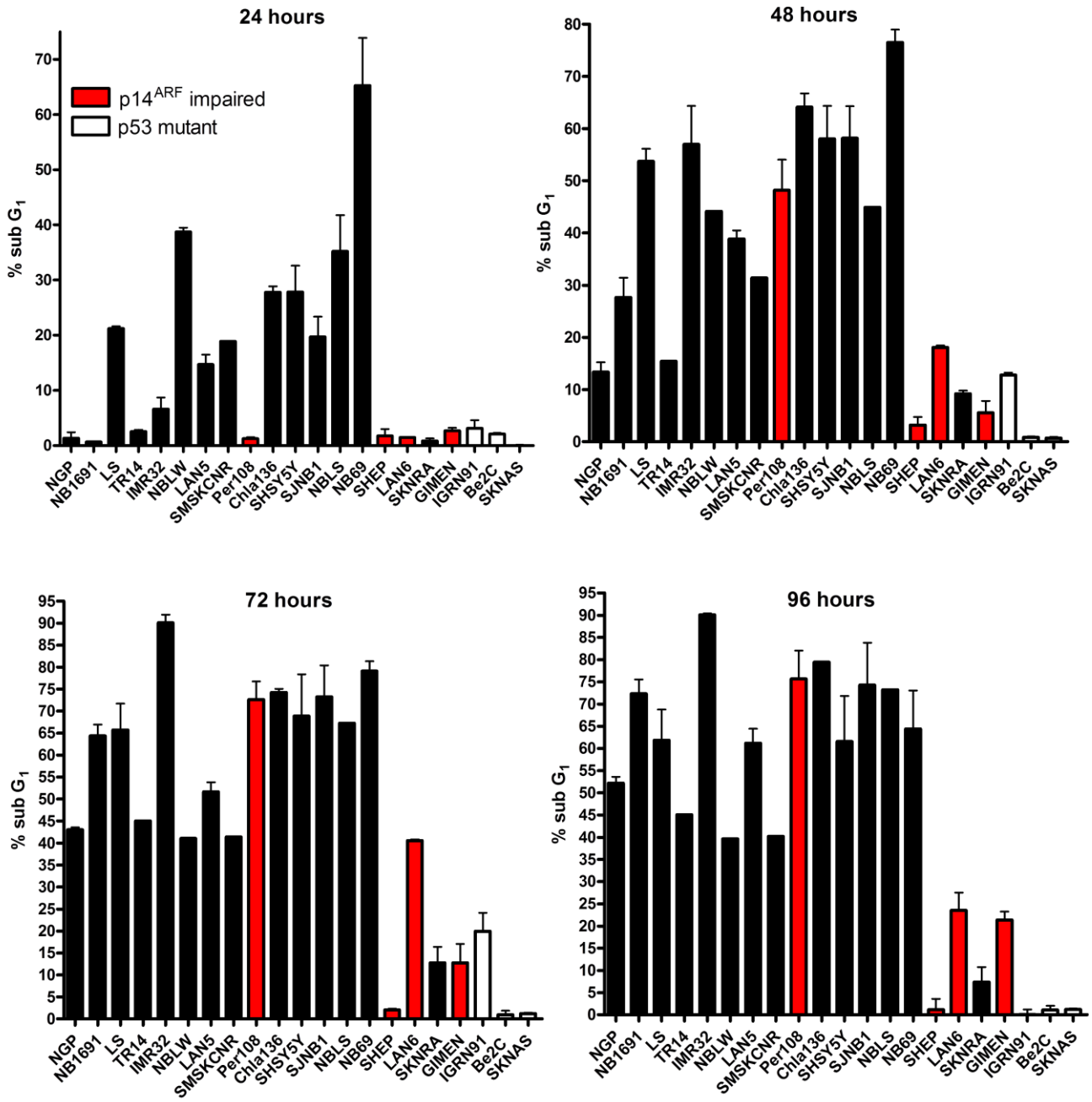


Figure 5.12. **The sub G_1 DNA fraction at 24, 48, 72 and 96 hours in a panel of 21 neuroblastoma cell lines.** p14^{ARF} impaired cell lines are highlighted in red, and p53 mutant cell lines are white.

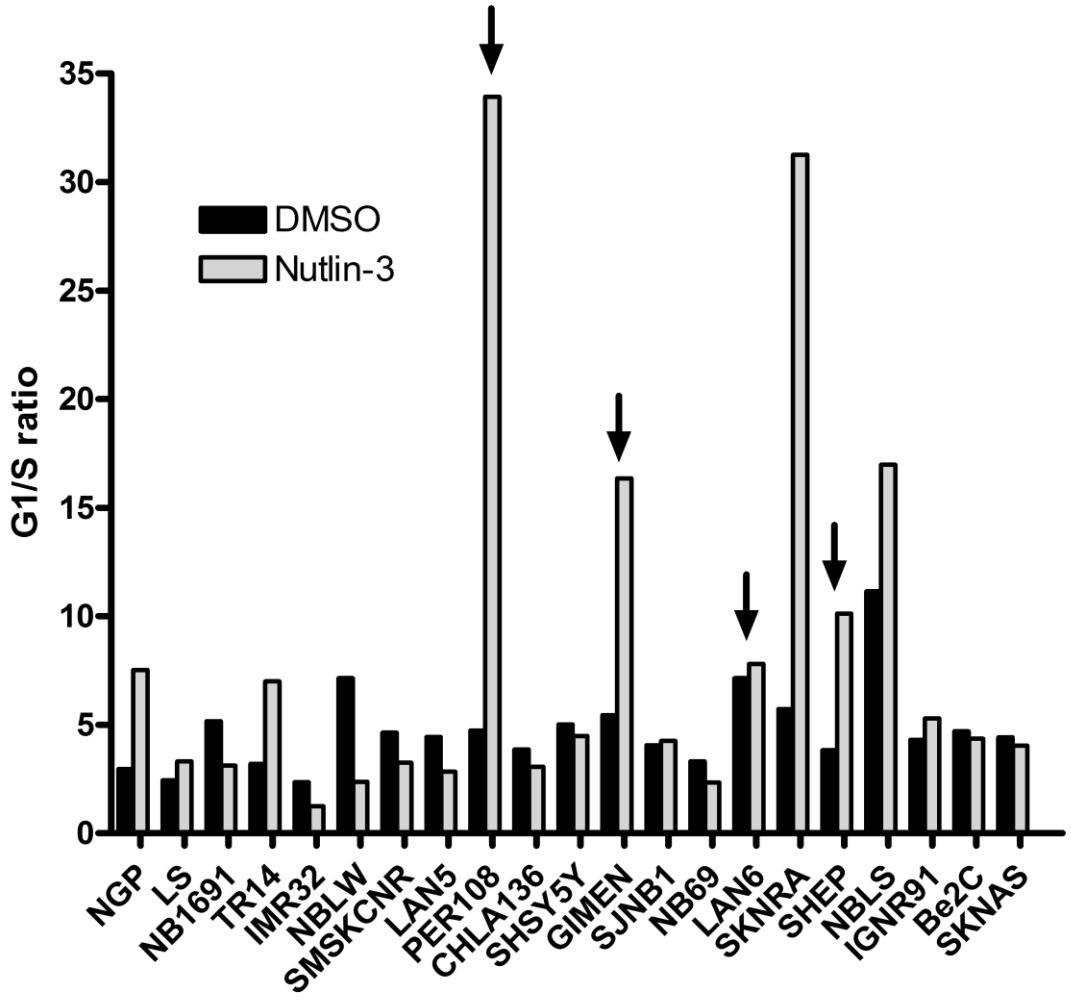


Figure 5.13. **G_1/S ratios following 10µM Nutlin-3 treatment for 24 hours in a panel of 21 neuroblastoma cell lines.** $p14^{ARF}$ impaired cell lines are indicated by arrows.

5.4.6 $p14^{ARF}$ knockdown decreases caspase activity but does not affect p53 levels

Since $p14^{ARF}$ impaired cell lines have low levels of apoptosis following MDM2-p53 antagonist treatment, it can be predicted that $p14^{ARF}$ knockdown would result in reduced levels of apoptosis also. $p14^{ARF}$ was knocked down in NGP, NB1691 and LS cells, all of which have detectable $p14^{ARF}$ by Western blot. As shown in Figure 5.14a, following $p14^{ARF}$ knockdown little or no effect was observed on p53 or MDM2 protein expression. However, as shown in Figure 14b, caspase 3/7 activity was decreased following $p14^{ARF}$ knockdown at both 24 and 48 hours. This is expected as $p14^{ARF}$ negatively regulates MDM2. There is no increase in MDM2, but there is evidence that $p14^{ARF}$ regulates MDM2 by removing it from its site of action in the nucleoplasm, into the nucleolus. Upon $p14^{ARF}$ knockdown, MDM2 may relocate to the nucleolus where it inhibits p53, reducing levels of apoptosis.

5.4.7 $p14^{ARF}$ knockdown followed by p53-MDM2 antagonist treatment results in reduced apoptosis

Knockdown of $p14^{ARF}$ followed by MDM2-p53 antagonist treatment resulted in reduced levels of cleaved caspase 3 and cleaved PARP compared to SCR control (Figure 5.15; lanes 7-10 compared to 2-5). Interestingly, no change in p53 induction and induction of p53 target genes, including the pro-apoptotic marker PUMA, was observed following $p14^{ARF}$ knockdown, suggesting that p53-independent functions of $p14^{ARF}$ may be involved. In addition, Nutlin-3 and MI-63 appeared to reduce $p14^{ARF}$ expression in NGP cells, but increased expression in LS cells. In Chapter 3 it was found that MDM2-p53 antagonists reduced MYCN expression, and MDMX expression in NGP cells compared to other cell lines.

Following treatment with Nutlin-3 or MI-63, a decrease in caspase 3/7 activity was generally observed following $p14^{ARF}$ knockdown compared to SCR control, although this was not statistically significant in all cases (Figure 5.16). Surprisingly, in LS and NB1691 cells, the decrease in caspase activity was not as great following MDM2-p53 antagonist treatment as $p14^{ARF}$ knockdown alone, whereas in NGP cells the decrease was greater than $p14^{ARF}$ knockdown alone. This suggests that addition of Nutlin-3 or MI-63 is rescuing cells from $p14^{ARF}$ knockdown mediated-apoptosis in some cell lines, but enhancing the effect in others.

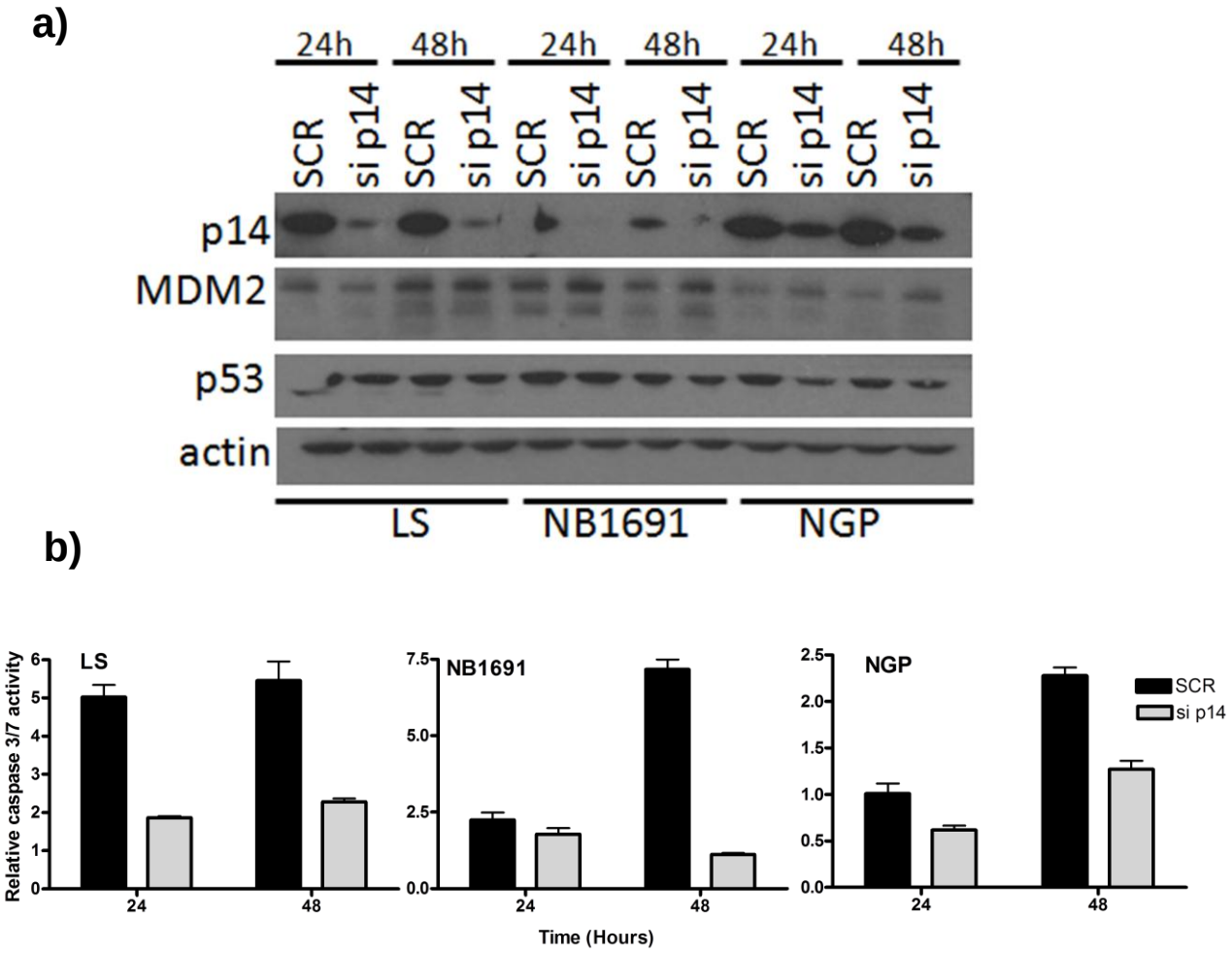


Figure 5.14. **Knockdown of p14^{ARF} in LS, NB1691 and NGP cells at 24 and 48 hours. a)** Western blot showing p14^{ARF} knockdown, and the effect on MDM2 and p53. Actin was used as a loading control. **b)** The basal level of Caspase 3/7 activity was reduced by p14^{ARF} knockdown.

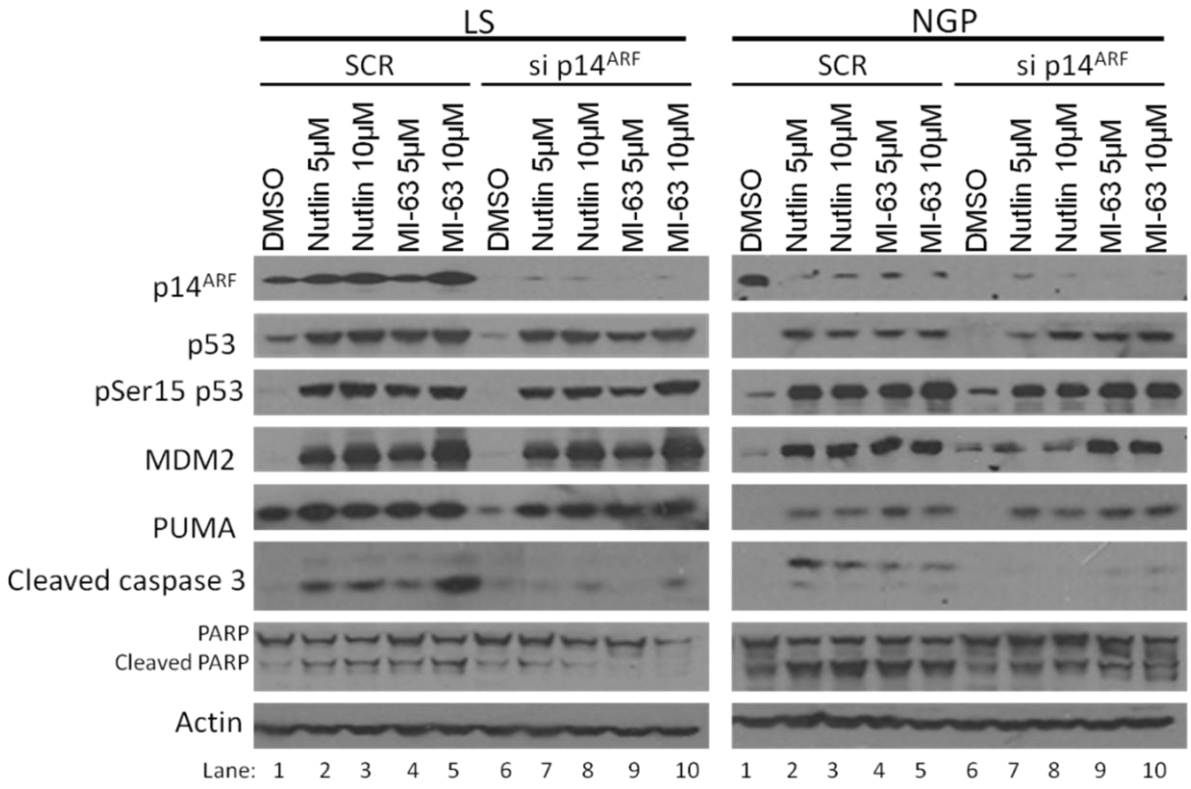


Figure 5.15. **p14^{ARF} knockdown followed by MDM2-p53 antagonist treatment (5μM and 10μM) in NGP and LS cells, compared to SCR control.** The effects on p53 and p53 responsive genes, and apoptotic markers were detected. Actin was used as a loading control.

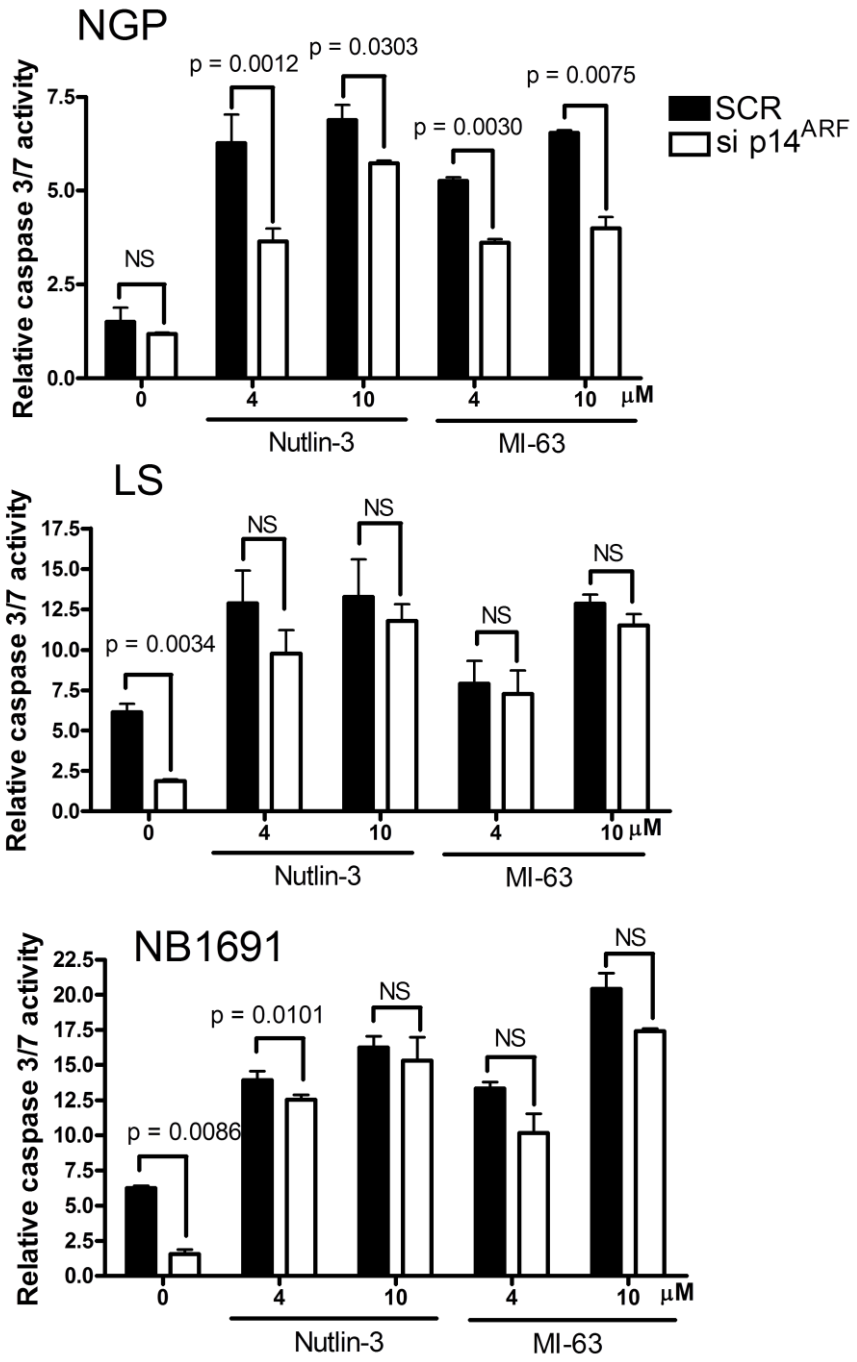


Figure 5.16. **Caspase 3/7 activity in NGP, LS and NB1691 cells, following p14^{ARF} knockdown and DMSO, Nutlin-3 or MI-63 treatment, compared to SCR control.** P values were generated using 2-tailed paired t-tests and are displayed where significant. NS – not significant.

5.5 Discussion

p14^{ARF} inactivation frequently occurs in neuroblastoma and was found to be impaired through methylation or homozygous deletion in several studies (Carr-Wilkinson et al., 2010; Caren et al., 2008; Gonzalez-Gomez et al., 2003; Thompson et al., 2001). Previous data suggests that p14^{ARF} may be an important determinant of response to MDM2-p53 antagonists and hence a potentially useful biomarker to use in combination with p53 status for identifying patients that are likely to respond to these agents.

5.5.1 MYCN and MDM2 both contribute to the p14^{ARF} overexpression seen in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines

p14^{ARF} has previously been found to be overexpressed in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines (Figure 5.17). Here the effect of siRNA-mediated knockdown of MDM2 or MYCN was investigated, or simultaneous knockdown of both MYCN and MDM2, on p14^{ARF} protein expression. Both MDM2 and MYCN knockdown lead to a decrease in p14^{ARF} expression, with a greater decrease effect seen following MYCN knockdown. Double knockdown resulted in a further reduction in p14^{ARF} protein levels of up to 92%. This suggests that co-amplification of *MYCN* and *MDM2* has a co-operate effect on p14^{ARF} protein levels. In support of this data, there is evidence that both MDM2 and MYCN independently affect p14^{ARF}.

First of all, *MDM2*-amplified non-neuroblastoma cell lines that are not amplified for *MYCN* also have levels of p14^{ARF} protein expression comparable to that of *MDM2*-amplified neuroblastoma (Figure 5.9), despite Carr *et al* reporting no difference when just two cell lines were compared (Figure 5.17) (Carr et al., 2006). p14^{ARF} forms part of the p53/MDM2/p14^{ARF} negative feedback loop, and is ubiquitously expressed and has increased levels in p53 null cells, suggesting that p53, when activated, downregulates and limits p14^{ARF} expression (Sharpless, 2005). In support of this Carr *et al* found that p53 mutant Be2C had increased p14^{ARF} compared to the paired p53 wildtype Be1n at both the mRNA and protein level (Carr et al., 2006). This situation is reflected in *MDM2*-amplified cell lines where p53 function is attenuated and is not downregulating p14^{ARF} expression. However in the p53 mutant cell lines, the effect on p14^{ARF} is not as great as *MDM2*-amplification.

Secondly, MYCC has been shown to activate the p14^{ARF} tumour suppressor, resulting in p53 activation and apoptosis through Bcl-X_L and Bcl-2 dependent and independent pathways (Hosoi et al., 1994). There are many similarities between MYCN and MYCC

and although it has not been investigated, it is possible that MYCN works in a similar way and may explain why *MYCN*-amplification results in $p14^{ARF}$ induction. However, in contrast to this, *MYCC* and *MYCN* amplification is usually associated with TWIST1 overexpression, which has been shown to impair $p14^{ARF}$ activity (Valsesia-Wittmann et al., 2004; Maestro et al., 1999). Since *MYCN*-amplified cell lines do not have $p14^{ARF}$ levels comparable to *MYCN* and *MDM2* co-amplified cell lines, and it is possible that MDM2 may interfere with this pathway in *MYCN*-amplified neuroblastoma resulting in increased $p14^{ARF}$ expression. Although *MYCN*-amplified neuroblastoma cell lines do not overexpress $p14^{ARF}$ compared to *MYCN*- and *MDM2*-co-amplified cell lines, the original figure from Carr *et al* showing mRNA levels in the panel of neuroblastoma cell lines ((Carr et al., 2006), Figure 3a, and here reproduced as Figure 5.17) suggests that $p14^{ARF}$ mRNA levels are increased in *MYCN*-amplified compared to non-amplified neuroblastoma cell lines, but this difference is not as obvious as the difference between *MYCN*- and *MDM2*-co-amplified compared to the other cell lines. There is evidence that $p14^{ARF}$ interacts with MYCN *in vivo* inhibiting MYCN-mediated transcriptional activation (Amente et al., 2007), and this is also true of MYCC (Amente et al., 2006). If MYCN does activate $p14^{ARF}$ this suggests a negative feedback loop between these two proteins. In addition, MDM2 is a direct transcriptional target of MYCN, further linking MYCN with the p53/MDM2/$p14^{ARF}$ network.

Taken together this data suggests that *MYCN* and *MDM2* co-amplification must have a co-operative effect on $p14^{ARF}$, and that in this circumstance MYCN is a more dominant driver of $p14^{ARF}$ expression than MDM2, but only in the presence of *MDM2*-amplification.

5.5.1.1 Limitations of using siRNA to investigate increased levels of $p14^{ARF}$ expression

When interpreting changes in the expression of a particular protein following knockdown of different genes in a number of cell lines, the level of knockdown should be taken into consideration. The transfection efficiency of different cell lines varies, and levels of knockdown achieved with different siRNAs vary. Ideally, more than 1 siRNA should be used. In this study, we sought to determine the effect on $p14^{ARF}$ of MYCN, MDM2 and MYCN/MDM2 knockdown in 4 cell lines. High levels of knockdown were achieved in each cell line with each siRNA, allowing easy comparison of $p14^{ARF}$ levels in this case. Following MDM2 knockdown, the greatest effect on $p14^{ARF}$ is seen in NGP and LS cells which also have the highest levels of MDM2 knockdown.

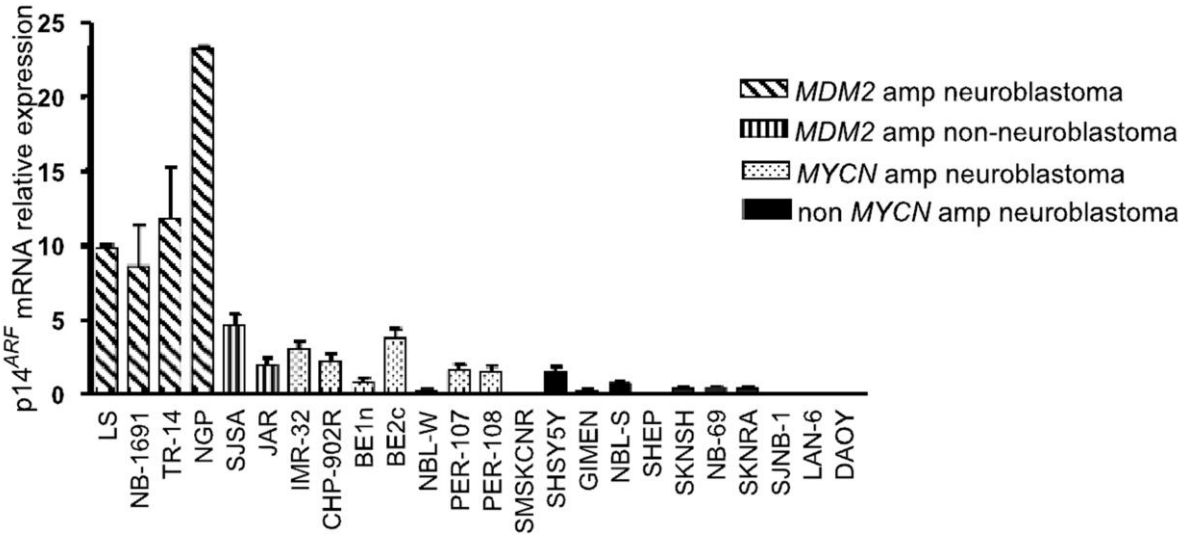


Figure 5.17. **$p14^{ARF}$ mRNA levels across a panel of neuroblastoma and non neuroblastoma cell lines, taken from (Carr et al., 2006).**

5.5.2 $p14^{ARF}$ is located in the nucleolus but not expressed at detectable levels in every cell in *MYCN* and *MDM2* co-amplified cell lines

Immunofluorescence was used to determine localisation of $p14^{ARF}$ in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines. $p14^{ARF}$ was found to be localised in the nucleolus. It would be interesting to see if MDM2 co-localised in the nucleolus with $p14^{ARF}$, as relocalisation of MDM2 from the nucleoplasm to the nucleolus is the chief mechanism by which $p14^{ARF}$ inhibits MDM2, in addition to binding and preventing interaction with p53 (Wang et al., 2001; Xirodimas et al., 2001). It is likely that some MDM2 will be co-localised with $p14^{ARF}$ and inactive in the nucleolus, but that MDM2 levels are in such excess that the effect of increased $p14^{ARF}$ on reactivating p53 is negligible.

Interestingly, it was found that in NGP and LS cells, not all the cells had detectable levels of $p14^{ARF}$ in the nucleolus, and $p14^{ARF}$ was only detectable in about 10% of NGP and LS cells. This was unexpected, as $p14^{ARF}$ was present in every HeLa cell which was used as a positive control. Previously, FISH in LS cells (Figure 4.18) showed *MDM2*-amplification in every cell, and if $p14^{ARF}$ is overexpressed partly as a result of *MDM2*-amplification increased $p14^{ARF}$ would be expected in every cell. It is also a possibility that the antibody is not binding efficiently to $p14^{ARF}$ in the nucleolus. The cells had been fixed and permeabilised, but not denatured and if $p14^{ARF}$ was bound to

another protein, such as MDM2 this may interfere with antibody binding. This could be investigated further in Be2C cells which have higher levels of $p14^{ARF}$ but are not amplified for *MDM2*.

5.5.3 $p14^{ARF}$ impaired cell lines are resistant to apoptosis but undergo a G_1 arrest following Nutlin-3 treatment

$p19^{ARF}$ null mice are highly tumour prone (Kamijo et al., 1999). Mice hemizygous for $p19^{ARF}$ were generated by crossbreeding ARF null mice with Eμ-myc transgenic mice and intercrossing the offspring (Eischen et al., 1999). These mice display progressive disease and 80% of tumours lose their wildtype $p19^{ARF}$ allele. This protein must therefore be important in tumour prevention. Previously it has been shown that *MDM2*-amplified, $p14^{ARF}$ overexpressing cells have increased caspase 3/7 activity compared to other neuroblastoma cell lines. Here it was shown that *$p14^{ARF}$* impaired cell lines are resistant to MDM2-p53 antagonist mediated apoptosis. The four *$p14^{ARF}$* impaired cell lines; SHEP (homozygous deletion), LAN-6 (homozygous deletion), GIMEN (methylated) and PER-108 (methylated), had significantly decreased levels of caspase 3/7 activity compared to $p14^{ARF}$ wildtype cell lines, and also had a decreased sub G_1 fraction after 24 hours of Nutlin-3 treatment. This resistance was lost in the PER-108 cell line at 48 hours but was maintained in the other *$p14^{ARF}$* impaired cell lines through to 96 hours treatment. PER-108 is the only $p14^{ARF}$ impaired cell line that is amplified for *MYCN*. However, *MYCN*-amplification is probably not responsible for the sensitivity to Nutlin-3 following 48 hours treatment. Carr *et al.* have previously reported that methylation in GIMEN cells was associated with transcriptional silencing of *$p14^{ARF}$* and p53 function is compromised (Carr et al., 2006). This was not the case with the PER-108 cells, where $p14^{ARF}$ mRNA expression was comparable to that of its partner cell line PER-107, which has functional $p14^{ARF}$ (Figure 5.17). The PER-108 cells may therefore have some heterogeneity, with some cells containing methylated $p14^{ARF}$ and others expressing functional $p14^{ARF}$. Alternatively, it may be that the degree of methylation affects gene expression (Carr et al., 2006).

Despite being resistant to apoptosis, 3 of the 4 $p14^{ARF}$ impaired cell lines underwent a pronounced G_1 arrest. LAN6 cells did not arrest. Due to the low levels of apoptosis observed in these cells following MDM2-p53 antagonist treatment, this G_1 arrest may be protecting cells against apoptosis, and this should be investigated further to determine if the cell cycle arrest can be prevented. Preventing the cell cycle arrest may

potentiate the apoptotic effects of Nutlin-3, as treated cells would continue through the cell cycle and be unable to escape the pro-apoptotic effects of the MDM2-p53 antagonists. Cells may be G_1 arresting instead of undergoing apoptosis, as occurs after DNA damage in some types of p53 wildtype cancer cells.

The mTOR pathway plays a role in determining the outcome of p53-induced cell cycle arrest, and there is evidence that cell cycle arrest is reversible as long as mTOR is also inhibited (Korotchkina et al., 2010). mTOR status in these $p14^{ARF}$ impaired cell lines would therefore be interesting to determine.

5.5.3.1 Limitations of flow cytometry to measure sub G_1 DNA and cell cycle

The sub G_1 peak is a measure of fragmented DNA that separates into apoptotic nuclei, and has DNA content of less than a normal 2n cell, where n is the haploid complement. It is not an ideal method of investigating levels of apoptosis. First of all it takes into account all types of cell death, not just apoptosis and should therefore be backed up by other methods such as caspase activity. Secondly, it measures fragmented DNA within a cell, but cells undergoing apoptosis eventually break up, and if the DNA is not contained within a single cell the different fragments are measured individually. The sub G_1 peak is therefore not necessarily a measure of the number of dead or dying cells, but a measure of the amount of fragmented DNA within the cell suspension. In this study, basal levels of sub G_1 DNA were also taken into account when making comparisons between cell lines, as levels vary across cell lines and notably *MYCN*-amplified cell lines had higher levels of basal sub G_1.

When comparing flow cytometry results across a panel of cell lines, results should be interpreted with caution as some cell lines respond to Nutlin-3 more rapidly than others. No given time-point would give maximum levels of sub G_1 for all the cell lines, so a number of time-points have been studied. Similarly, some cell lines do not arrest at 24 hours treatment but do at 48 hours, and yet some cell lines are still cycling at 96 hours. However, this is also a measure of how sensitive the cells are to the compounds and for this reason, a time-point of 24 hours was used for cell cycle analysis.

5.5.4 Cell Type and $p14^{ARF}$ status and the cell cycle response

There does not appear to be a relationship between cell type and $p14^{ARF}$ status; PER-108 and LAN6 $p14^{ARF}$ impaired cell lines are N-type cells, SHEP is S-type, and GIMEN is predominantly S-type. There also appears to be no relationship between cell cycle response and cell type. The following cell lines underwent a G_1 arrest in response

to Nutlin-3; NGP, TR14, PER108, GIMEN, SKNRA, SHEP and NBLS. In response to MDM2-p53 antagonists, N-type cells did have increased apoptosis, as previously reported for irradiation treatment (Carr-Wilkinson et al., 2011; Mergui et al., 2008; Bell et al., 2006; Tweddle et al., 2001b; Isaacs et al., 1998). However, in response to irradiation, N-type cells fail to undergo a G_1 arrest. NGP, TR14 and PER-108 cells are all N-type and therefore this is not true for Nutlin-3. However, NGP and TR14 cells are *MDM2*-amplified, and PER-108 cells are $p14^{ARF}$ impaired both of which result in overactive MDM2. This increased MDM2 in these 3 cell lines may alter the cell type specific response, since none of the other N-type cells underwent a G_1 arrest. When compared with a previous study with some of these cell lines in response to irradiation (Bell et al., 2006), 5/6 of the cell lines that arrested in response to Nutlin-3 also arrested in response to irradiation, but many of the cell lines that did not arrest in response to Nutlin, did arrest in response to irradiation. This suggests that activated p53 from irradiation and MDM2-p53 antagonists do not always initiate the same response, and this is likely to be cell type specific.

5.5.5 The SKNRA cell line is resistant to apoptosis, but undergoes a G_1 arrest

Interestingly the SKNRA cell line is especially resistant to MDM2-p53 antagonists, with low levels of induced apoptosis, comparable to p53 mutant cell lines and some of the $p14^{ARF}$ impaired cell lines. However, whereas p53 mutant cell lines did not G_1 arrest, following 24 hours of Nutlin-3 treatment SKNRA cells underwent a pronounced G_1 arrest similar to $p14^{ARF}$ impaired cell lines, suggesting $p14^{ARF}$ may not be intact. However, this cell line has been previously investigated for $p14^{ARF}$ impairment through methylation or deletion and also for p53 mutation, all of which were wildtype (Carr et al., 2006). The cell line has not been investigated for *CDKN2A* mutation. *CDKN2A* mutations have not been previously reported in neuroblastoma, but do occur in other cancer types such as melanomas (Hayward, 2003), and $p14^{ARF}$ mutations in mice induced chemoresistance in lymphomas (Schmitt et al., 1999). Alternately, this cell line may be defective in the apoptotic pathway downstream of p53, but it should first be determined if p53 is activated. It is also worth noting that this cell line is S-type, and S-type cells are shown to be more chemoresistant than N-type cells (Carr-Wilkinson et al., 2011; Mergui et al., 2008). However this has not been investigated in relation to MDM2-p53 antagonist treatment, and in this study it does appear that N-type cells are

more sensitive to MDM2-p53 antagonist mediated apoptosis, but that cell type does not influence the ability of the cell to G_1 arrest.

5.5.6 $p14^{ARF}$ knockdown and the effect of MDM2-p53 antagonists

Van Maerken *et al.* previously found that $p14^{ARF}$ knockdown results in decreased cell viability and apoptosis, and overexpression increased cell survival and apoptosis following MDM2-p53 antagonist treatment (Van Maerken et al., 2011). Surprisingly, in the present study knockdown of $p14^{ARF}$ alone did not affect p53 levels, but did result in decreased caspase 3/7 activation. Since $p14^{ARF}$ negatively regulates MDM2, an increase in MDM2 activity is expected upon $p14^{ARF}$ knockdown which in turn will result in inhibition of p53 and decreased apoptosis.

In the current study, $p14^{ARF}$ knockdown followed by treatment with MDM2-p53 antagonists again did not alter the p53 response with p53, phosphorylated p53, MDM2 and PUMA expression levels comparable to those seen with the SCR control. A decrease in the apoptotic markers caspase 3 and PARP cleavage was observed by Western blot, but no change in the apoptotic marker and p53 transcriptional target PUMA.

Despite seeing a significant decrease in caspase activity upon $p14^{ARF}$ knockdown alone, upon addition of MDM2-p53 antagonists there were variable effects on caspase 3/7 activity. In LS and NB1691 cells, MDM2-p53 antagonist treatment in cells knocked down for $p14^{ARF}$ had reduced caspase 3/7 activity compared to cells with $p14^{ARF}$ knockdown alone. However the opposite effect was seen in NGP cells. This suggests that knockdown of $p14^{ARF}$ is protecting from Nutlin-3/MI-63 mediated apoptosis in LS and NB1691 cells, but is co-operating with Nutlin-3/MI-63 in NGP cells to induce greater levels of apoptosis, indicating that the mechanisms by which $p14^{ARF}$ work by are complicated and variable. More cell lines need to be tested, including non-*MDM2*-amplified cells, as this would eliminate any effect of *MDM2*-amplification. The three cell lines tested in this study were *MYCN* and *MDM2* co-amplified and were chosen because $p14^{ARF}$ is overexpressed and protein expression is detectable by Western blot. Van Maerken *et al* have previously shown that non-*MDM2*-amplified IMR32 cells had decreased cell viability following $p14^{ARF}$ knockdown, and increased caspase activity following stable $p14^{ARF}$ overexpression (Van Maerken et al., 2011).

Since p53 levels or activity did not change, shown by no change in the p53-transcriptional target MDM2, following $p14^{ARF}$ knockdown, p53-independent functions

of $p14^{ARF}$ may be responsible for the decrease in caspase 3/7 activity, especially since PUMA (a p53 transcriptional target) levels did not change, but levels of caspase and PARP cleavage did following MDM2-p53 antagonist treatment. $p14^{ARF}$ interacts with proteins involved in proliferation, the ATM/ATR/CHK signalling pathway and transcription factors in the retinoblastoma pathway, through which it may additionally affect cell cycle regulation and apoptosis if altered. $p19^{ARF}$ has also been shown to act independently of p53 in response to oncogenic signals to suppress cell proliferation. Reintroduction of $p19^{ARF}$ into p53 and ARF null mice resulted in a G_1 cell cycle arrest (Weber et al., 2000).

5.5.7 Conclusions and future directions

Here it has been shown that a) $p14^{ARF}$ overexpression in *MYCN* and *MDM2* co-amplified cell lines is a result of co-amplification, and that each protein plays a role, b) that $p14^{ARF}$ impaired cell lines undergo G_1 arrest and show low levels of apoptosis following MDM2-p53 antagonist treatment, c) that despite $p14^{ARF}$ knockdown resulting in decreased caspase 3/7 activity, the effect of subsequent MDM2-p53 antagonist treatment is ambiguous, and does not appear to alter p53 or p53 target gene protein expression. Van Maerken *et al* found that knockdown of $p14^{ARF}$ resulted in a moderate reduction in the sensitivity of IMR32 and NGP cells to Nutlin-3, shown in cell viability assays, and apoptosis in NGP cells (Van Maerken et al., 2011).

Despite $p14^{ARF}$ knockdown having inconclusive results on apoptosis, there is a large amount of evidence to support the importance of $p14^{ARF}$ in cancer prevention, and functional $p14^{ARF}$ is important for the response to anticancer agents, such as MDM2-p53 antagonists. In neuroblastoma van Maerken *et al.* provided evidence of a co-stimulatory effect of $p14^{ARF}$ expression and Nutlin-3 response (Van Maerken et al., 2011). This data together with our data from studying the $p14^{ARF}$ impaired cell lines suggests that $p14^{ARF}$ inactivation may limit the response to MDM2-p53 antagonists in neuroblastoma, and that further investigations are required, including *in vivo* studies.

Since there is no effect on p53 and p53 targets, p53-independent induction of apoptosis may occur. It has been shown that $p14^{ARF}$ impaired cell lines undergo G_1 arrest following Nutlin-3 treatment, so one would expect to see a G_1 arrest following $p14^{ARF}$ knockdown and MDM2-p53 antagonist treatment, and this should be confirmed using cell cycle studies. In addition, experiments should be carried out to see if the cell cycle arrest observed in the $p14^{ARF}$ impaired cell lines is reversible. Long-term survival could

be determined using clonogenic survival assays, and arrested cells could be isolated and cultured to see if they divide. The mTOR status may also be important in determining whether the G_1 arrest in these cells is reversible, and should be determined for these cell lines. The mutational status of $p14^{ARF}$ should be investigated in the panel of neuroblastoma cell lines, particularly SKNRA to eliminate this as a mechanism of resistance to MDM2-p53 antagonists.

Chapter 6. Identification of p53 mutations in 2 *MYCN*-amplified neuroblastoma cell lines

6.1 Introduction

Two neuroblastoma cell lines, BCH-N-AD and BCH-N-NS were obtained from Dr Carmel McConville, University of Birmingham, to add to the panel of *MYCN*-amplified neuroblastoma cell lines already used in this study. However, these cell lines did not respond to MDM2-p53 antagonists, arousing suspicions that they may be mutant for p53. In this chapter, the response of the 2 cells lines to Nutlin-3 and MI-63 was investigated and the sequencing of their p53 gene is described.

6.1.1 p53 mutations in cancer

The *p53* gene is located on the short arm of chromosome 17p13.1 and contains 11 exons, spanning 20kb (Mercer et al., 2007). p53 plays a critical role in maintaining genomic stability and cancer prevention by preventing cells from accumulating mutations. Mutations can provide cells with growth advantages, leading to abnormal proliferation (Agarwal et al., 1998; Lane, 1992). p53 reacts to cellular stresses such as DNA damage and induces a number of anti-cancer responses including cell cycle arrest and apoptosis. Therefore inactivation of p53 results in the rapid accumulation of mutations as cells cannot respond to stress. As a result, cells gain selective advantages such as a high tolerance to growth arrest and apoptosis (Petitjean et al., 2007; Levine, 1997). The function of p53 and its mechanisms of action are described in more detail in Chapter 1.

p53 is mutated in over 50% of human cancers types, and is therefore the most commonly mutated gene in cancer (Levine, 1997). In the remaining p53 wildtype cancers, it is thought that abnormalities in p53 regulatory proteins occur (Brown et al., 2009). The importance of p53 is shown in p53-deficient mice, which have a significantly higher rate of tumour formation than wildtype mice. Donehower *et al* reported that 74% of mice with homozygous mutation of the *p53* gene developed tumours within 6 months, whereas only 2/96 heterozygous mice and no p53 wildtype mice developed tumours within 9 months (Donehower et al., 1992).

p53 is a 393 amino acid protein, and the 3 major domains are the acidic NH_2-terminal transactivation domain containing a nuclear export signal (amino acids 1-44), a central sequence-specific DNA binding domain (DBD) (amino acids 102-292) and the COOH-terminal oligomerisation domain (amino acids 325-359) (reviewed by (Joerger and Fersht, 2007)). It also contains 3 nuclear localisation signals (amino acids 305-322, 369-375, and 379-384) and a proline rich domain containing 5 PXXP motifs which allow for rapid protein protein interaction (Joerger and Fersht, 2007). About 85% of

tumour associated p53 mutations occur within the central DBD (exons 5-8) and often result in an inability of p53 to recognise sequence-specific response elements within target promoters, inhibiting the ability of p53 to activate target genes (Joerger and Fersht, 2007; Ryan and Vousden, 2002; Hollstein et al., 1999; Hollstein et al., 1996). The mutations within the DBD are often contact or structural mutations, which often lead to destabilisation of p53 and/or a disruption of the proper conformation of the DBD, resulting in loss of sequence specific transactivation. The sequence-specific DNA-binding ability of p53 is also tightly linked to its pro-apoptotic activity (El-Deiry, 2003; Pietenpol et al., 1994), so mutations within this domain may result in a failure to induce apoptosis. Tumours with missense p53 mutations frequently have characteristically higher levels of basal p53 in cells, with a much longer half-life than wildtype p53. This is because some mutant forms of p53 accumulate as a result of an inability to transactivate MDM2, or due to conformational change so cannot bind MDM2, and as a result are not degraded (Crawford et al., 1984).

Cells with wildtype p53 function are usually chemoresponsive, but the inactivation of p53 is thought to contribute to chemoresistance (reviewed by (Tweddle et al., 2003)). Many cytotoxic agents act via p53-dependent pathways, and when p53 mutates, patients become resistant to therapy. p53 mutations confer resistance of tumour cells to anticancer drugs by inhibiting p53-dependent pro-apoptotic pathways (Vogelstein et al., 2000; Velculescu and El-Deiry, 1996; Vogelstein and Kinzler, 1992).

6.1.2 p53 mutations and neuroblastoma

p53 mutations are rare in neuroblastoma and only occur in about 15% of cases of refractory or relapsed disease and less than 2% of *de novo* tumours (Tweddle et al., 2003; Hosoi et al., 1994). A study by Carr *et al.* revealed that 53% (9/17) of neuroblastoma cell lines established from relapse tumour samples had inactive p53 whereas no pre-treatment cell lines did (Carr et al., 2006). However, surprisingly only 1 of these cell lines studied was mutant for p53. Another study found that p53 mutations occurred in neuroblastoma cell lines at a higher frequency of 26% (9/34) (Van Maerken et al., 2011). In the panel of cell lines used in this thesis, 14% (3/21) were mutant for p53 (IGNR91, SKNBe2C, SKNAS). Tweddle *et al* demonstrated that the neuroblastoma cell line, SKNBE(1n), had functional and wild-type p53 at diagnosis but the corresponding cell line, SKNBE(2c), taken from the same patient at relapse 5 months later after treatment with cytotoxic therapy, had inactive mutant p53 (Tweddle et al., 2001a). However, some primary tumours and most relapsed tumours have

developed alternative mechanisms of inactivating p53 in neuroblastoma, contributing to chemoresistance (Carr-Wilkinson et al., 2010; Carr et al., 2006; Tweddle et al., 2003).

The p53/MDM2/p14^{ARF} network plays a major role in tumourigenesis, as it is important in genetic stability and oncogene activation. This pathway is defective in many neuroblastoma tumours, and often changes status in relapsed disease after cells have undergone cytotoxic therapies which frequently results in p53 inactivation (Carr-Wilkinson et al., 2010; Carr et al., 2006; Tweddle et al., 2003). These include p14^{ARF} homozygous deletion or methylation, and *MDM2*-amplification which in cell lines occurs with *MYCN*-amplification (Carr et al., 2006). In the cell lines used in this study, three have mutant p53, two have *p14^{ARF}* methylation, two have *p14^{ARF}* deletion and four have *MDM2*-amplification. In primary neuroblastoma, one study found variable p14^{ARF} expression, infrequent MDM2 overexpression and mutation of p53 in 3/40 cases (Omura-Minamisawa et al., 2001). In a study by Carr-Wilkinson *et al* p53 mutation was found in 6 of 41 cases, and just one was at diagnosis, *MDM2* was amplified in 3 of 23 cases both at diagnosis and relapse, and p14^{ARF} was inactivated in 12 of 41 cases, 9 at diagnosis and relapse, and 3 at relapse only (Carr-Wilkinson et al., 2010). Unlike cell lines, there are reports of *MDM2* or *12q* amplification in the absence of *MYCN*-amplification (Carr-Wilkinson et al., 2010; Su et al., 2004).

As the inability of cancer cells to die or enter cell cycle arrest reduces the effectiveness of radiation and chemotoxic drugs, methods of restoring the p53 response are required in order to improve treatment response rates. MDM2-p53 antagonists have been shown to activate p53 and induce apoptosis in neuroblastoma and other cancer types, providing wild-type p53 is present.

Patients with high risk neuroblastoma relapse in >50% of cases, and relapsed disease, being very difficult to treat, carries a high mortality rate. Treatment of these patients often involves using chemotherapeutic agents such as topotecan and irinotecan, and topoisomerase inhibitors in combination with cyclophosphamide to improve efficacy (Maris et al., 2007).

Current therapy regimes are insufficient and patients with aggressive disease still have poor survival rates and suffer from dose-related toxicity (Laverdiere et al., 2005). Treatment for this disease is a challenge and new non-genotoxic approaches are needed (Friedman and Castleberry, 2007). There is a requirement for tumour specific therapies

for patients with high risk disease. A promising molecular target is inhibition of MDM2-p53 binding and is currently an intense area of research.

6.1.3 p53 mutations and response to MDM2-p53 antagonists

MDM2-p53 antagonists inhibit the MDM2-p53 interaction by mimicking p53, restoring the p53 pathway both *in vitro* and *in vivo* (Vassilev, 2004). Nutlin-3 has previously been shown to be highly effective at reactivating p53 in p53 wildtype neuroblastoma cell lines (Van Maerken et al., 2006). Many studies have shown that Nutlin-3 is not effective in cell lines or patient samples with mutant p53. This has been shown in rhabdomyosarcomas (Miyachi et al., 2009), in patient CLL samples (Kojima et al., 2006) and in AML samples (Kojima et al., 2005) and in neuroblastoma cell lines (Van Maerken et al., 2006). Interestingly, in neuroblastoma, whilst Nutlin-3 had no effect on p53 mutant cell lines, both Nutlin-3a and the inactive enantiomer 3b strongly increased the efficacy of vincristine in p53-mutated P-glycoprotein (P-gp) overexpressing cell lines. This was as a result of Nutlin interfering with P-gp, and MRP-1, drug efflux, preventing the P-gp/MRP-1 chemotherapy drugs from being removed from the cell (Michaelis et al., 2009).

6.2 Hypotheses and Aims

Hypothesis

- The *MYCN*-amplified neuroblastoma cell lines BCH-N-AD and BCH-N-NS are p53 mutant

Aims

1. To determine the growth inhibitory and apoptotic response of BCH-N-AD and BCH-N-NS cells lines to the MDM2-p53 antagonists Nutlin-3 and MI-63.
2. To sequence exons 4-9 of the p53 gene in BCH-N-AD and BCH-N-NS cell lines for p53 mutations.

6.3 Specific Materials and Methods

6.3.1 Analysis of Nutlin-3 and MI-63 treatment in BCH-N-AD and BCH-N-NS cells

Caspase activity was measured as previously described (Chapter 2.7) and GI_{50} values calculated as described in Chapter 2.6. For Western analysis to look at activation of p53 and p53 target genes, BCH-N-NS, BCH-N-AD and p53 wildtype NGP cells were treated with 5 or 10µM Nutlin-3 for 4 hours.

6.3.2 DNA extraction for sequencing

$5x10^6$ BCH-N-AD, BCH-N-NS and LAN5 cells were spun down for 5 minutes at 1200rpm. DNeasy Blood and Tissue kit (Qiagen; cat no. 69504) was used to extract DNA which was eluted in 200µl of AE Buffer.

6.3.3 PCR

The mastermixes for exons 4, 5, 6, 7, and 8/9 were prepared as shown in Table 6.1. Primer sequences are shown in Table 6.2 and were stored at a stock concentration of 10µM. 23µl of mastermix was added to 4 wells in a 96-well plate for each exon, and 2µl of DNA (BCH-N-AD, BCH-N-NS, LAN5 (wildtype p53 positive control)) or nuclease-free water (negative control). PCR conditions are displayed in Table 6.3.

Reagent	Volume per reaction (µl)	MasterMix volume (µl)
PCR Gold Buffer	2.5	12.5
MgCl2	2.5	12.5
dNTP's	2.5	12.5
Primer SN	1	5
Primer ASN	1	5
dH2O	13.25	66.25
Amplitaq Gold	0.25	1.25

Table 6.1. **Reaction mix for PCR.** Mastermix was made up for each exon (4 reactions).

Exon	Sense/Antisense	Sequence
4	Sense	5'-GTTCTGGTAAGGACAAGGGT-3'
	Antisense	5'-ATACGGCCAGGCATTGAAGT-3'
5	Sense	5'-ATCTGTTCACTTGTGCCCTG-3'
	Antisense	5'-CAACCAGCCCTGTCGTCTCTC-3'
6	Sense	5'-GCCTCTGATTCCTCACTGAT-3'
	Antisense	5'-GGAGGGCCACTGACAACCA-3'
7	Sense	5'-AAGGCGCACTGGCCTCATCTT-3'
	Antisense	5'-CAGGGGTCAGCGGCAAGCAGA-3'
8/9	Sense	5'-TTTAAATGGGACAGGTAGGAC-3'

	Antisense	5’-GCCCCAATTGCAGGTAAAACAG-3’

Table 6.2. **Sense and antisense primers for p53 exons 4-9.**

	Hotstart	14 cycles			26 cycles			End
Temperature (° C)	94	94	62*	72	94	55	72	72
Time (minutes)	10	20 sec	1	1	20 sec	1	1	5

Table 6.3. **PCR cycling conditions.** * decreases by 0.5°C each cycle

6.3.4 Agarose gel

2% Agarose gel was made up by adding 100ml of 0.5x TBE (see Appendix 1) and 2g of agarose (Genetic Analysis grade, Fisher Scientific), melted in a microwave oven until clear. 10µl of GelRed™ Nucleic Acid Gel Stain (Biotium) was added, mixed and poured into the gel apparatus and allowed to set. The gel was placed in the gel tank, filled with 0.5x TBE and 5µl of PCR product was mixed with 2µl of loading buffer (see Appendix 1) and samples loaded onto the gel. Gel electrophoresis of the samples was carried out for 20-25 minutes at 130V.

6.3.5 Purification of PCR product

The band of the correct size on the gel was excised and the PureLink™ PCR Purification kit (Invitrogen; cat no. K3100-02) was used to purify the PCR product, followed by elution of the PCR product in 30µl of nuclease-free water. For PCR products that displayed more than one band on the agarose gel, 20µl of PCR product was loaded and gel extraction was carried out to purify the appropriate band using the QIAquick® Gel Extraction kit (Qiagen; cat no. 28706).

6.3.6 Sequencing

Samples were sent away for sequencing to DBS genomics (Durham University, School of Biological and Biomedical Sciences, Science Site, South Road, Durham, DH1 3LE) along with 3µM concentration of primers (2.88µl of primer, 6.12µl water). Sequencing was performed using an Applied Biosystems 3730 Genetic Analyzer.

6.3.7 Sequence Analysis

DNASTAR software (DNASTAR, Inc.) was used for sequence analysis. Within DNASTAR, SeqMan was used to generate chromatograms, and MegAllign for comparison to standard sequences.

6.4 Results

6.4.1 Karyotype and origin of BCH-N-AD, and BCH-N-NS cells

BCH-N-AD and BCH-N-NS cells were originally obtained from Dr Carmel McConville, School of Cancer Sciences, University of Birmingham, to add to the existing panel of *MYCN*-amplified cell lines used in this study. The karyotype, short-tandem-repeats (STR) fingerprint and origin of these cells are displayed in Table 6.4. The STR fingerprint is a method of DNA profiling, and uses highly polymorphic regions that have short repeated sequences of DNA. The STR loci are targeted with sequence-specific primers and amplified using PCR. The resulting DNA fragments are separated using electrophoresis. As shown in the karyotypes in Table 6.4, both cell lines have monosomy of chromosome 17, as do most of the other neuroblastoma cell lines with mutant p53. BCH-N-AD also has a translocation involving chromosomes 3 and 17, and BCH-N-NS has translocations involving chromosome 17 and chromosome 1, and a translocation of chromosome 17 material to derivative chromosome 16.

Cell line ID	Karyotype	STR fingerprint	Origin
BCH-N-AD	78-86XXY,-Y,+1,add(1)(p13)x2,der(1)dic(1)9q?44)del(1)(p?22),der(3)t(3;17)(p21;q21)x2,der(4)t(4;17)(p14;q21)x2,-6,-10,-11,-12,-17,-17,-18,del(19)(p13)x2, idic(22)(q13)x2,50-100dmin[cp5]	D13S317 – 10,11 D16S539 – 11,12 D5S818 – 9,12	Bone marrow metastasis (relapse) from 4y old male; Post chemotherapy. *MYCN* amplified
BCH-N-NS	43~45,XX,+1,del(1)(p36),der(1)t(1;17)(p36;q?1),der(1)(7qter->q1::?17q25->q?1::1p36->qter),idic(3)(p1),add(3)(q2),ins(7;?)(q11;?),-10,der(15;17)(q10;q10),der(16)t(16;17)(q24;q?1),dic(16;19)(p13;q1),-17,-17,+1~2mar,8~120dmin[cp7) /89~100,idem x2, inc[cp2]	D13S317 – 10,13 D16S539 - 12, D5S818 – 10,11	Bone marrow metastasis from 2y old male at diagnosis (pre-chemo); *MYCN* amplified

Table 6.4. **Karyotype and origin of BCH-N-AD and BCH-N-NS *MYCN*-amplified neuroblastoma cell lines.**

6.4.2 BCH-N-AD and BCH-N-NS have caspase 3/7 activity and growth inhibition values following MDM2-p53 antagonist treatment comparable to those of p53 mutant cell lines

As shown in Figure 6.1, BCH-N-AD and BCH-N-NS have low caspase 3/7 activities of 0.8759 ± 0.06391 and 1.123 ± 0.2239 respectively. These values are comparable to

those of other p53 mutant neuroblastoma cell lines IGNR91, SKNAS and Be2C. Similarly, the levels of growth inhibition following 20μM Nutlin-3 (BCH-N-AD, 90.67% ± 3.46%; BCH-N-NS, 89.21% ± 1.46%) and 10μM MI-63 (BCH-N-AD, 81.36% ± 16.36%; BCH-N-NS, 77.79% ± 4.22%) treatment were also comparable to those of other p53 mutant cell lines (Figure 6.2), with considerably less growth inhibition at these concentrations than seen for p53 wildtype cell lines. These data indicate that BCH-N-AD and BCH-N-NS have a defective growth inhibitory and apoptotic response to Nutlin-3, strongly suggestive of a mutation within the p53 gene.

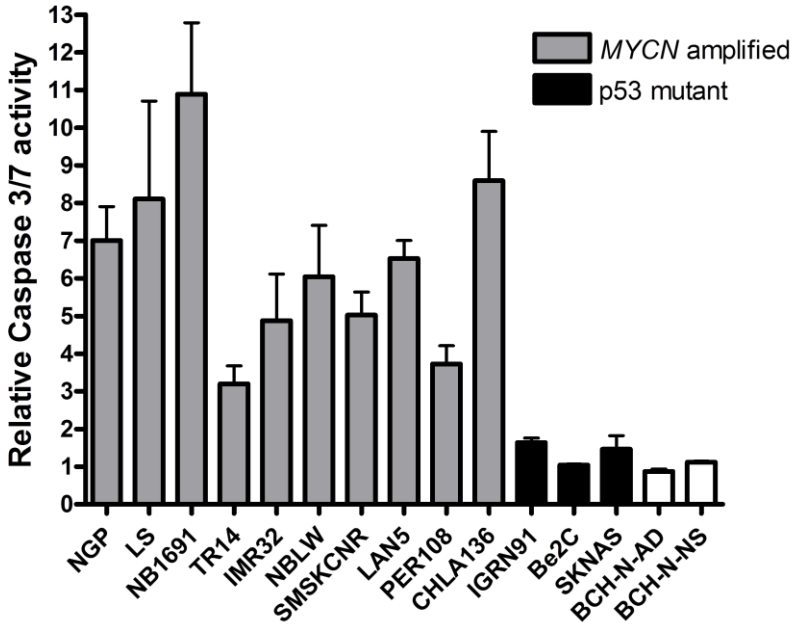


Figure 6.1. **Caspase 3/7 activity following 5μM Nutlin-3 treatment in BCH-N-AD and BCH-N-NS *MYCN*-amplified cell lines, compared to other *MYCN*-amplified cell lines and p53 mutant cell lines (5μM Nutlin-3, treated for 24 hours).**

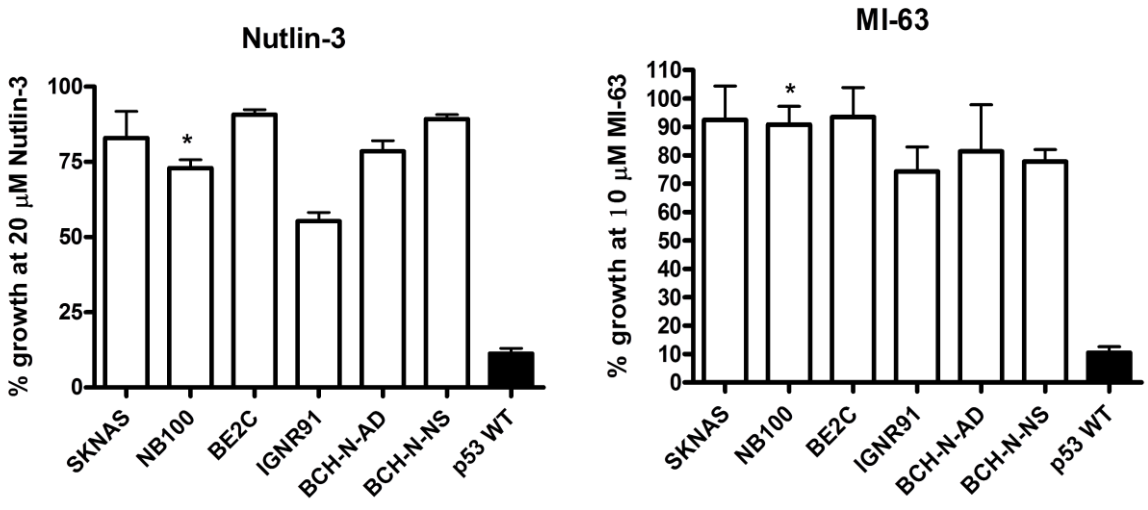


Figure 6.2. **Growth inhibition in BCH-N-AD and BCH-N-NS cells compared to other p53 mutant neuroblastoma cell lines (*PNET cell line), and the mean value of all the p53 wildtype cell lines from Figure 3.19 and 3.20 at 20μM Nutlin-3 and 10μM MI-63.**

6.4.3 BCH-N-NS and BCH-N-AD are resistant to Nutlin-3 mediated p53 activation

A characteristic of p53 mutant cell lines following MDM2-p53 antagonist treatment is a lack of induction of p53 target genes. BCH-N-AD, BCH-N-NS, and NGP cells (as a control for wildtype p53) were treated with 0, 5 or 10μM Nutlin-3 for 4 hours, and levels of p53 and induction of the well-characterised p53 transcriptional targets $p21^{WAF1}$ and MDM2 detected by Western blot. As shown in Figure 6.3, NGP cells have increased p53, and increased levels of p53 target genes, $p21^{WAF1}$ and MDM2. Despite seeing a small degree of p53 induction in BCH-N-AD and BCH-N-NS cells, there was no induction of p53 transcriptional targets, suggesting that these cell lines do not have functional p53. These cell lines also have increased basal levels of p53 compared to NGP cells, another characteristic of p53 mutant cells. Tumours with missense mutant p53 that results in full length protein cannot usually bind to DNA and upregulate MDM2, therefore p53 is not ubiquitinated or degraded and accumulates. p53 may also undergo a conformational change that prevents binding to MDM2.

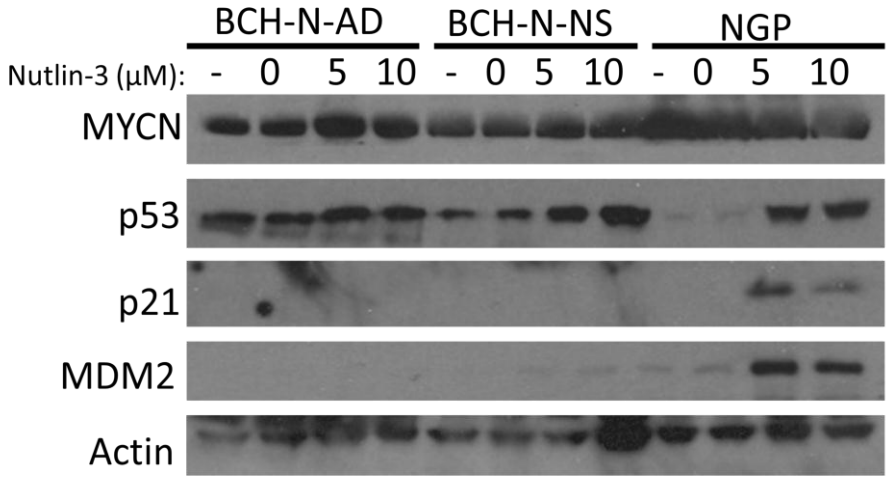


Figure 6.3. **BCH-N-AD and BCH-N-AD were treated with 0, 5 and 10μM of Nutlin-3 for 4 hours and induction of p53 and p53 target genes ($p21^{WAF1}$ and MDM2) detected by western blot.** NGP cells were used as a positive control for wildtype p53. Actin was used as a loading control. (-, UT; 0, DMSO control.)

6.4.4 BCH-N-NS is mutated at exon 7, codon 241.

PCR was carried out with BCH-N-NS DNA with exon 7 primers, and the products analysed on a 2% agarose gel. As shown in Figure 6.4, multiple bands/products were detected. These were gel extracted and sent for sequencing The chromatograms shown in Figure 6.5 show that compared to the wildtype sequence, BCH-N-NS cells have a single base change at codon 241, resulting in a change in amino acid from a serine (TCC) to a bulky phenylalanine (TTC). This was confirmed in both sense and antisense sequences

6.4.5 BCH-N-AD is mutated at exon 8, codon 277.

PCR was carried out with BCH-N-AD DNA with exon 8/9 primers, and the products analysed on a 2% agarose gel, as shown in Figure 6.4. A single clear band was detected so the PCR product was purified and sent for sequencing. The chromatograms shown in Figure 6.6 show that compared to the wildtype sequence, BCH-N-AD cells have a single base change at codon 277, resulting in an amino acid change from a cysteine (TGT) to a phenylalanine (TTT). This is confirmed in both sense and antisense sequences.

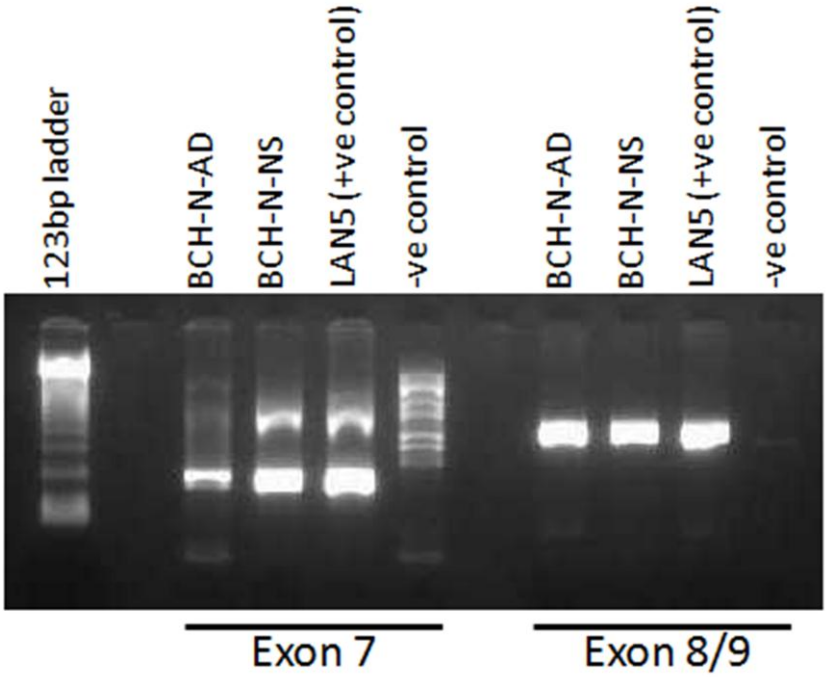


Figure 6.4. **PCR products for Exon 7 and Exon 8/9 in BCH-N-AD, BCH-N-NS, LAN5 (positive control) and negative control (DNase-free water).** Exon 7 was gel extracted as there are multiple bands, exon8/9 DNA was purified before sending off for sequencing

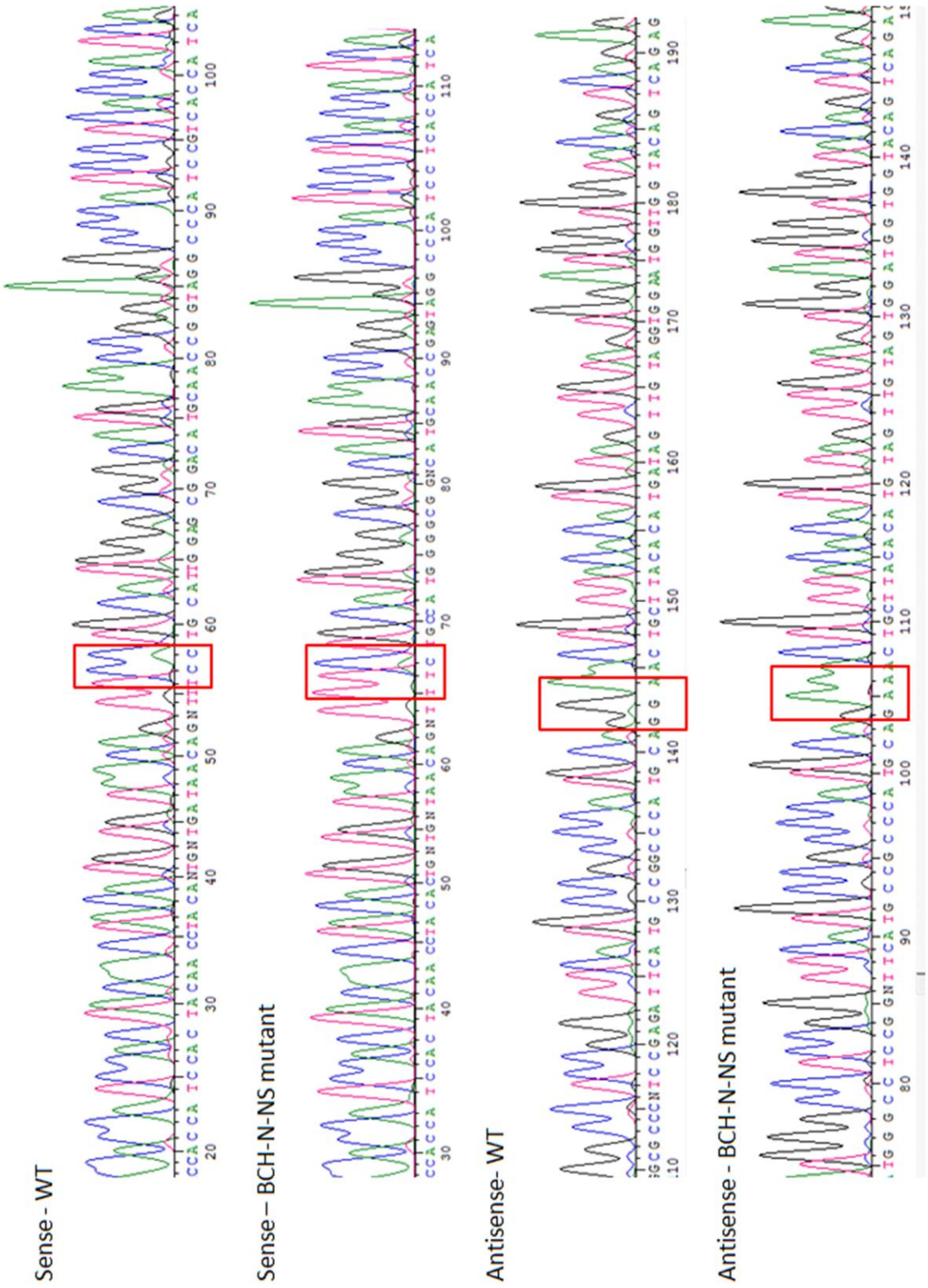


***Figure 6.5*. BCH-N-NS has a p53 mutation in exon 7, at codon 241. The TCC (serine) → TTC (phenylalanine) change is shown next to wildtype sequences in both sense and antisense directions.**

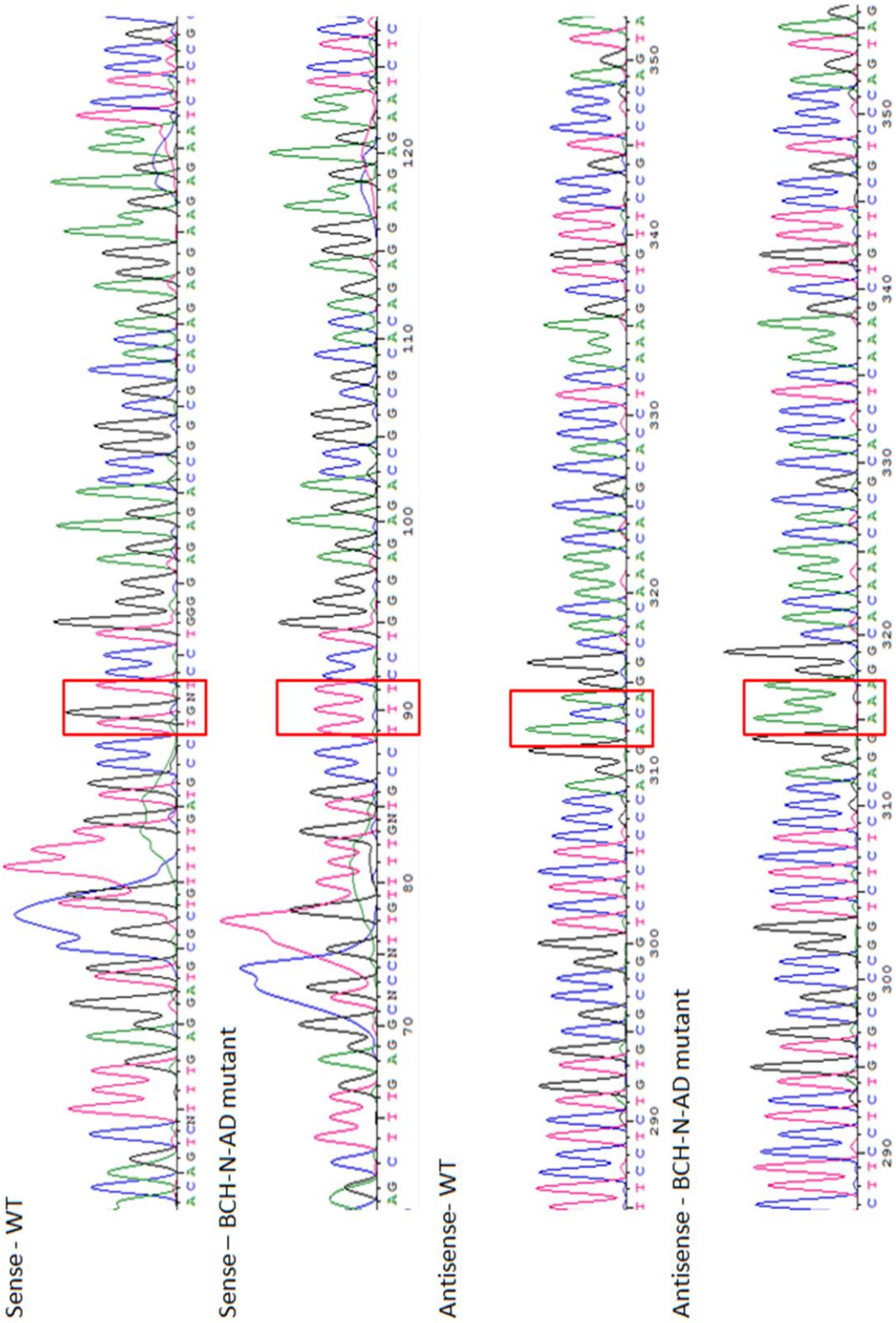


Figure 6.6. **BCH-N-AD p53 mutation in exon 8, at codon 277. The TGT (cysteine) → TTT (phenylalanine) change is shown next to wildtype sequences in both sense and antisense directions.**

6.5 Discussion

p53 mutations in neuroblastomas are relatively rare, occurring at a frequency of 14% in neuroblastoma cell lines used in this study so far, and at a frequency of 15% (5/41 following chemotherapy, 1//41 at diagnosis) in a previous study of paired tumour samples from patients at diagnosis and relapse (Carr-Wilkinson et al., 2010).

6.5.1 Identification of p53 mutations in BCH-N-AD and BCH-N-NS

The wildtype p53 gene contains 11 exons that encode 393 amino acids. p53 mutant cell lines used in this study are SKNBe2c, IGNR91 and SKNAS. In SKNBe2c cells, there is a missense mutation in p53 codon 135 (shown in Figure 6.7), converting cysteine to phenylalanine. In IGNR91 cells, a duplication of exons 7-8-9 adds 107 amino acids to a total of 500, and in SKNAS cells, a mutation due to alternative splicing downstream of exon 9 leads to a protein of 341 amino acids (Goldschneider et al., 2006) (Figure 6.7). In the newly identified p53 mutant BCH-N-AD and BCH-N-NS cell lines, homozygous point missense mutations were found in the DNA binding domain which according to the p53 mutation database (http://www-p53.iarc.fr/p53main.html) lead to a non—functional protein. The p53 mutation in the BCH-N-AD cell line occurred within the DNA binding domain in exon 8 at codon 277 (shown in Figure 6.7), resulting in a TGT → TTT change. This resulted in an amino acid substitution from cysteine to phenylalanine (Figure 6.8). Codon 277 is an hotspot for p53 mutations, with 112 mutations recorded in the p53 mutation database, 5.1% of which are in tumours derived from nerve tumour sites (http://www-p53.iarc.fr/p53main.html), and has been previously reported in neuroblastoma (Manhani et al., 1997), where it was also accompanied by *MYCN*-amplification. The p53 mutation in the BCH-N-NS cell line occurred within the DNA binding domain in exon 7 at codon 241 (shown in Figure 6.7), resulting in a TCC → TTC change. This resulted in an amino acid substitution from a serine to a phenylalanine. Within the p53 mutation database, there are 246 mutations reported at this hotspot, but none of which are of neuronal origin and there are no previous reports of this mutation in neuroblastoma.

In BCH-N-AD cells, the resulting amino acid change was from a cysteine to a phenylalanine (Figure 6.8), and in BCH-N-NS cells a serine was changed again to a phenylalanine. Cysteine residues are small and slightly polar, and are usually critical to protein structure and function due to their ability to form disulphide bonds which

usually function to stabilise protein structure (Betts and Russell, 2003). Serine is small and slightly polar, and contains a reactive hydroxyl group that can form hydrogen bonds. Phenylalanine is a large bulky hydrophobic amino acid. It is relatively unreactive and rarely directly involved in protein function. It is therefore likely that this mutation results in p53 protein destabilisation and conformational change in these cell lines as a result of addition of a bulky amino acid, and loss of critical bonds that hold the protein together (Betts and Russell, 2003).

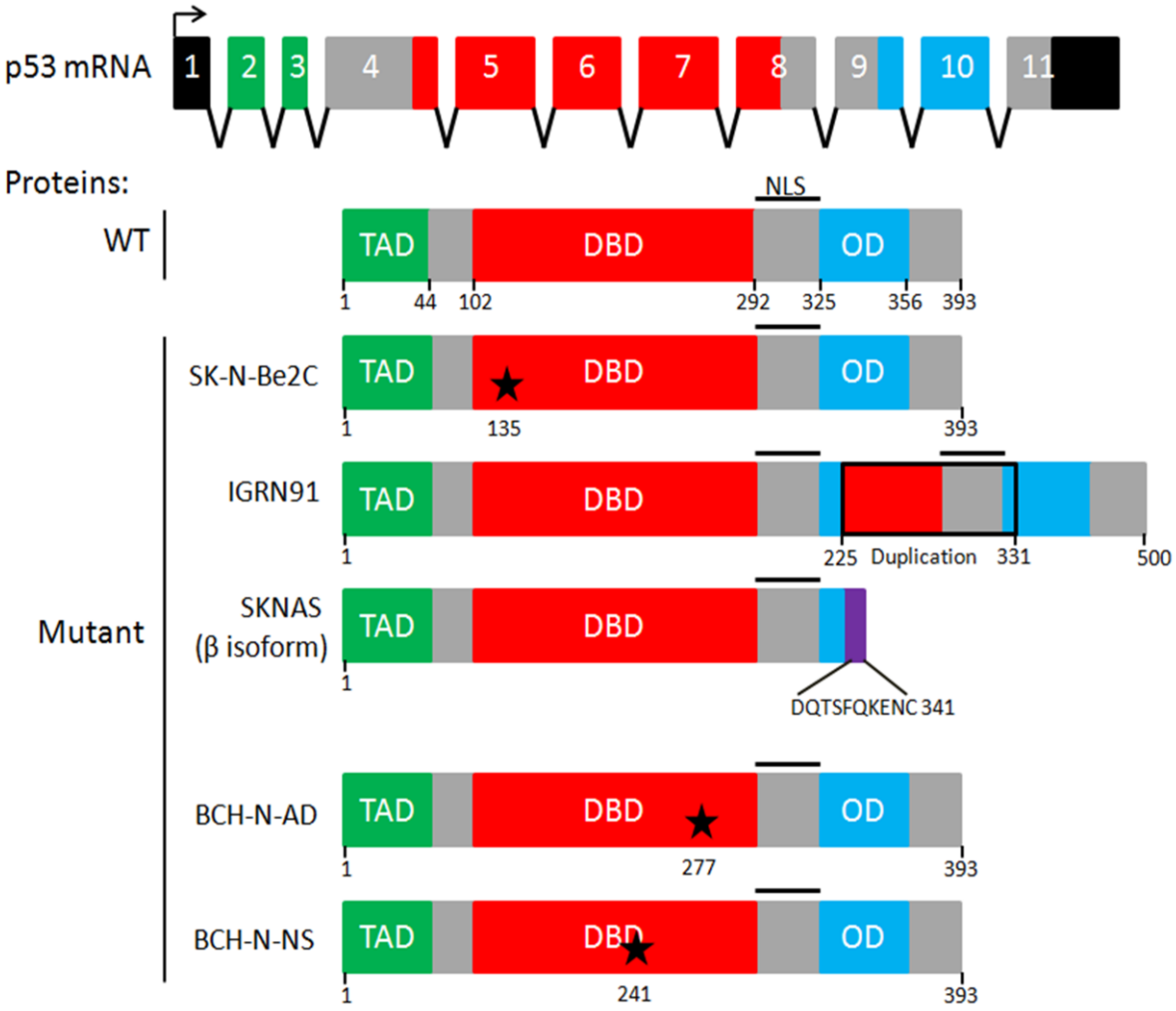


Figure 6.7. **Structure of p53 mRNA, and p53 proteins in p53 mutant neuroblastoma cell lines, including BCH-N-AD and BCH-N-NS.** p53 mRNA is made up of 11 exons and the three functional domains within the 393 amino acid protein are the transactivation domain (TAD), the DNA-binding domain (DBD) and the oligomerisation domain (OgD). The p53 mutations in SKNBe2C, IGNR91 and SKNAS cell lines were previously reported. The p53 mutations in BCH-N-AD and BCH-N-NS were identified in this study. Figure adapted from (Goldschneider et al., 2006).

6.5.2 The response of BCH-N-AD and BCH-N-NS cell lines to MDM2-p53 antagonists

p53 mutant cell lines are resistant to the effects of MDM2-p53 antagonists (Van Maerken et al., 2011; Vassilev, 2004). The loss of function associated with p53 mutation was indicated by high level nuclear accumulation of p53 protein in untreated cells and the failure of MDM2-p53 antagonists to induce a p53 response, apoptosis or growth inhibition.

In comparison to NGP cells, both BCH-N-AD and BCH-N-NS have high basal levels of p53 due to a much longer half-life, as the p53 protein does not induce MDM2 transcription and is not degraded (Crawford et al., 1984). Upon MDM2-53 antagonist treatment, compared to NGP cells, neither BCH-N-AD not BCH-N-NS induce any p53 target genes (Figure 6.3). Caspase 3/7 activity is not induced and levels are comparable to the other p53 mutant cell lines used in this study. Furthermore, growth inhibition assays at high concentrations of Nutlin-3 or MI-63 failed to achieve 50% growth inhibition, showing resistance comparable with other p53 mutant cell lines.

6.5.3 BCH-N-NS is a diagnostic neuroblastoma cell line, and p53 mutations are rare

BCH-N-NS cells were established from a patient at diagnosis. p53 mutations in neuroblastoma cell lines are very rare from diagnostic neuroblastoma and all the other p53 mutant cell lines used within this study (SKNBe2C, SKNAS, IGNR91 and BCH-N-AD) were established at relapse. Unfortunately, at present it is not known if the original tumour sample had a p53 mutation, as cell culture can select for p53 mutations. Follow-up studies are planned to sequence the corresponding primary tumour directly from a diagnostic sample.

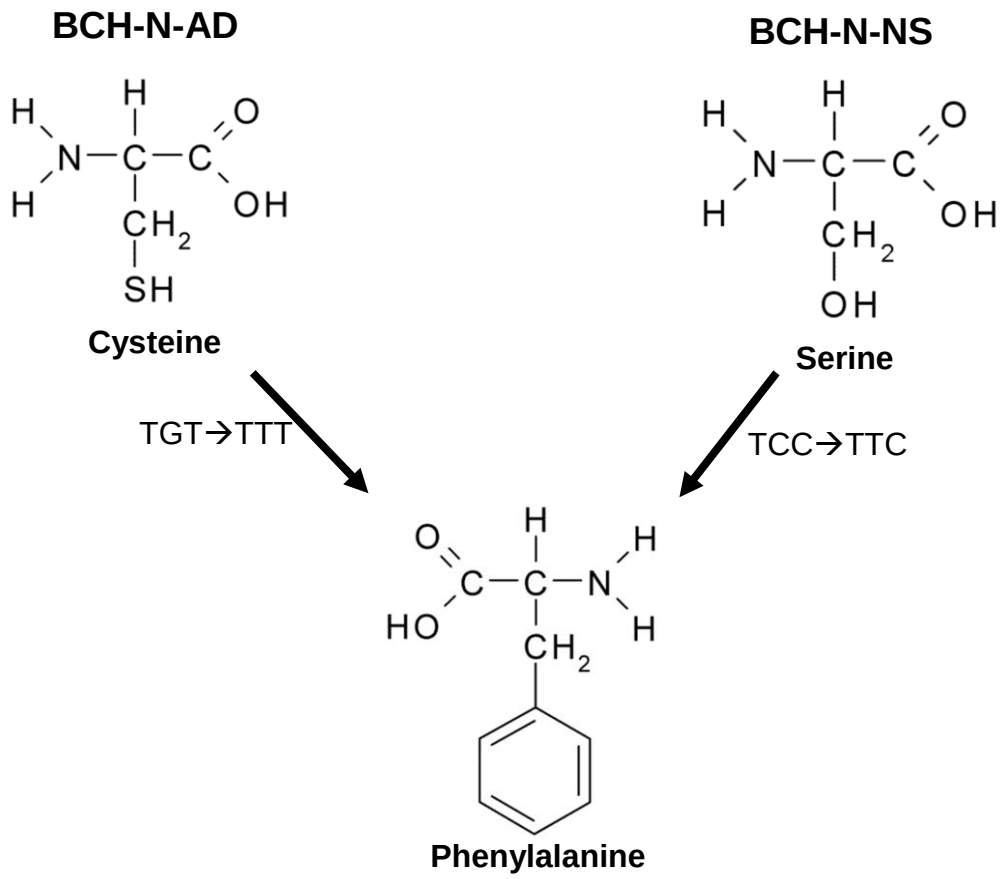


Figure 6.8. **The amino acid changes for BCH-N-AD and BCH-N-NS.**

6.5.4 Treatment of p53 mutant cancers

6.5.4.1 Restoration of p53 function in p53 mutant cancers

Tumours with p53 mutations are usually resistant to conventional therapies, particularly those involving DNA damaging agents. MDM2-p53 antagonists also rely on wildtype p53 and p53 mutated tumours would therefore be unresponsive. In p53 mutated cell lines, restoration of p53 function is being investigated. The p53 structure includes one zinc ion as an important cofactor that stabilises the second and third loops of the DBD, and is required for wildtype p53 function (Joerger and Fersht, 2008; Hainaut and Milner, 1993). p53 mutant proteins are prone to the loss of the Zn^{2+} atom that is bound to the wildtype core, and this results in the unfolding of p53 and loss of DNA binding (Butler and Loh, 2003). Mutations that change the conformation of p53 can also result in loss of this zinc. A study by Puca *et al.* showed that addition of zinc (100μM $ZnCl_2$) modified the equilibrium between p53 mutant and wildtype conformations in some cancer cell lines, positively reactivating some of the most frequently p53 mutated

residues (Puca et al., 2011). This lead to reactivation of target genes in response to DNA damage and also inhibition of some pro-oncogenic functions such as interaction with p73 and inhibition of MDR1, resulting in drug sensitivity and inhibition of tumour growth.

More recently, another strategy for p53 reactivation has been investigated, which involves suppressing glucosylceramide synthase (GCS), an enzyme involved in glycosylating ceramine, decreasing its pro-apoptotic activity in cancer cells (Liu et al., 2011). GCS silencing sensitised mutant p53 ovarian cancer cells (that were resistant to DNA-damage induced apoptosis) to doxorubicin, increasing the p53 response and apoptosis. Therefore restoration of active ceramide co cells can resuscitate wildtype p53 function in p53 mutant cells.

As of yet, nothing very convincing as a therapeutic has come out of investigations into restoring p53 activity in p53 mutant cells.

6.5.4.2 p5- independent therapies

p53 mutated tumours require p53-independent therapies, but most chemotherapeutic drugs function by inducing p53, as the majority of p53 mutated cancers response poorly to chemotherapy. Paclitaxel, or taxol, is a mitotic inhibitor that is used in cancer chemotherapy and has p53-independent functions. Paclitaxel stabilises microtubules and interferes with normal breakdown of microtubules during cell division. Paclitaxel treated cells have defects in mitotic spindle assembly, chromosome segregation and cell division.

Interestingly, a number of studies have reported anti-cancer effects of Nutlin-3 in p53 mutant cell lines. Nutlin-3 treatment potentiated the ability of doxorubicin to block cell proliferation and induce apoptosis in a p53 mutant cell line, as a result of TAp73 and E2F1 release from MDM2 (Peirce and Findley, 2009b). In line with this, another study found that Nutlin increased cytotoxicity of carboplatin and doxorubicin in a series of p53 mutant cell lines in an E2F dependent manner (Ambrosini et al., 2007). In addition both Nutlin-3a and the inactive enantiomer 3b strongly increased the efficacy of vincristine in p53-mutated P-gp or MRP-1 overexpressing cell lines. This was as a result of Nutlin interfering with P-gp and MRP-1 drug efflux, preventing the P-gp/MRP-1 from removing the chemotherapy drugs from the cell (Michaelis et al., 2009). In another study, nutllin in combination with doxorubicin or cisplatin in sarcomas reduced the IC50 of Nutlin in p53 mutant cell lines (Ohnstad et al., 2011).

Chapter 7. General Discussion

7 MYCN and the p53-MDM2/MDMX-p14^{ARF} network in neuroblastoma and response to MDM2-p53 antagonists

The studies presented in this thesis have focused on the use of MDM2-p53 antagonists for the treatment of neuroblastoma, and the effects of amplification, impairment or manipulation of MYCN and the components of the p53-MDM2/MDMX-p14^{ARF} network. A panel of 21 cell lines with varying status of this network and MYCN was investigated for their growth inhibitory and apoptotic response to two structurally different MDM2-p53 antagonists Nutlin-3 and MI-63. In addition, siRNA-mediated knockdown and a MYCN-regulatable cell line was used to assess effects on p53 activation, cell cycle and apoptosis. *MYCN*-amplification or induced expression sensitised neuroblastoma cell lines to MDM2-p53 antagonist-mediated apoptosis and growth inhibition (Chapter 3 and (Gamble et al., 2011a)). Manipulation of MDM2 did not affect the apoptotic response to Nutlin-3 or MI-63 whereas MDMX expression correlated with caspase activity and was associated with cell type (Chapter 4 and (Gamble et al., 2011b), submitted). p14^{ARF} knockdown resulted in a modest decrease in apoptosis following MDM2-p53 antagonist treatment, whilst p14^{ARF} impaired cell lines tended to G_1 arrest following Nutlin-3 treatment, and were resistant to apoptosis (Chapter 5 and (Gamble et al., 2011b), submitted). Consistent with previous studies and as expected if the action of MDM2-p53 antagonists is specific, p53 mutant cell lines were not responsive to these compounds.

7.1 Nutlin-3 induces apoptosis in neuroblastoma

A number of studies found that Nutlin-3 induces apoptosis in leukaemia cells, but only reversible cell cycle arrest in a wide array of solid tumours in both pre-clinical and clinical studies (Demidenko et al., 2010; Saha et al., 2010; Huang et al., 2009; Vassilev, 2007; Tovar et al., 2006). In this study and others, the majority of neuroblastoma cells underwent apoptosis when treated with Nutlin-3, with some cell lines being more sensitive than others. Previous studies have shown that in comparison to other cancers including CLL, multiple myeloma, lung cancer and osteosarcoma, neuroblastomas have much more rapid and robust levels of p53 induction and rates of apoptosis after 24-48 hour treatment, and apoptosis was induced in all cell lines tested (Barbieri et al., 2006; Cao et al., 2006; Kojima et al., 2006; Stuhmer et al., 2005; Vassilev et al., 2004).

p21^{WAF1} has been shown to affect induction of apoptosis (Abbas and Dutta, 2009), and may have a role in inhibiting apoptosis (Janicke et al., 2007; Gartel and Tyner, 2002).

HCT116 cells undergo cell cycle arrest following DNA damage, but when $p21^{WAF1}$ is removed, undergo apoptosis (Chan et al., 2000; Bunz et al., 1999). In other cancer types, upregulation of $p21^{WAF1}$ was associated with altered expression of pro-apoptotic genes, and may explain why these tumours undergo a reversible cell cycle arrest in response to Nutlin, instead of apoptosis. A recent study however, found that the increased $p21^{WAF1}$ levels did not protect solid cancers against Nutlin induced apoptosis (Xia et al., 2011) and in this study, $p21^{WAF1}$ was induced upon Nutlin-3 and MI-63 in a number of cell lines, all of which underwent apoptosis. Although not specifically tested in this study, data suggests that increased levels of $p21^{WAF1}$ induction do not inhibit induction of apoptosis in neuroblastoma following MDM2-p53 antagonist treatment, and is not necessarily indicative of cell cycle arrest. Previous studies have investigated the role of $p21^{WAF1}$ following DNA damage in neuroblastoma. Despite evidence of $p21^{WAF1}$ induction, Tweddle *et al* found that following irradiation induced DNA damage, there was a lack of G_1 arrest in the *MYCN*-amplified cell lines compared to non-amplified cell lines, suggesting that MYCN amplification is associated with a defective G_1 checkpoint (Bell et al., 2006; Tweddle et al., 2003). However, the *MYCN*-amplified cells were shown to undergo increased levels of apoptosis compared to non-amplified cells (Bell et al., 2006). Another study found that despite $p21^{WAF1}$ induction in a *MYCN*-amplified cell line, cells continued cycling (McKenzie et al., 2003), and whilst CDK2 function was not attenuated, there was no formation of the $p21^{WAF1}$-CDK2 complex required to induce a G_1 arrest. This complex also did not form in 2 non-*MYCN*-amplified cell lines, SJNB1 and NBLS. In the present study, just 6 of the 21 cell lines G_1 arrested in response to Nutlin-3, 3 of which were *MYCN*-amplified, and 3 of which were S-type non-MYCN-amplified cells (shown to arrest preferentially over apoptosis in response to irradiation).

7.2 Targeting MYCN in neuroblastoma

MYCN-amplification is a major negative prognostic marker occurring in 23-35% of neuroblastomas (Maris et al., 2007; Maris et al., 2000). MYCN plays roles in proliferation, differentiation and apoptosis. Expression is essential during normal neural crest development but is downregulated as the tissues terminally differentiate (Thomas et al., 2004). Ectopic MYCN expression increases DNA synthesis and drives cell cycle progression, resulting in increased cell proliferation (Lutz et al., 1996). In a transgenic mouse model, MYCN expression alone is necessary and sufficient for transformation (Weiss et al., 1997). Induced MYCN expression in non-*MYCN*-

amplified cell lines inhibited cell growth and induced the pro-apoptotic functions of MYCN and differentiation within neurones (Kang et al., 2006; Fulda et al., 2000; Nesbit et al., 1999). A number of studies have shown that *MYCN*-amplification potently sensitises cells to TNF-related apoptosis-inducing ligand (TRAIL), chemotherapy and irradiation induced apoptosis (Petroni et al., 2011; Bell et al., 2006; Cui et al., 2005; Fulda and Debatin, 2004; Fulda et al., 2000; Cole and McMahon, 1999; Lutz et al., 1998). It appears that induction of MYCN increases the sensitivity to apoptotic stimuli, but also increases G_1-S progression. To provide a selective advantage for the tumour, defects in apoptotic pathways are proposed as a mechanism by which *MYCN*-amplified neuroblastoma cells circumvent MYCN-induced apoptosis (Hogarty, 2003).

Both p53 and MDM2 have been identified as transcriptional targets of MYCN (Chen et al., 2010b; Slack et al., 2005a) , both of which were also identified in a ChIP-chip array study (Westermann et al., 2008). However, whereas both p53 and MDM2 are expressed at higher levels in neuroblastomas that express high MYCN (He et al., 2011; Chen et al., 2010b), induced expression of MYCN resulted in increased p53 expression but not MDM2 (He et al., 2011). In addition Chen *et al.* found that the increased MDM2 detected in the presence of MYCN decreased upon p53 knockdown suggesting that increased expression in these cell lines may be as a result of MYCN-driven p53 expression and not MYCN-driven MDM2 expression (Chen et al., 2010b). However, in the same study that MDM2 was confirmed as a transcriptional target of MYCN, decreased MYCN expression was associated with decreased MDM2 expression, stabilisation of p53 and apoptosis (Slack et al., 2005a). No studies have looked for an association between MYCN and MDM2 expression in neuroblastoma patient samples (partly because reliable antibodies to MDM2 are not available for IHC on paraffin sections), whilst *MYCN*-amplification is associated with enhanced p53 expression (Chen et al., 2010b).

MDM2 is considered to be important for MYCN-driven tumourigenesis, to overcome the tumour suppressive functions of p53, and MDM2 deficiency has been shown to suppress MYCN-driven neuroblastoma *in vivo* (Chen et al., 2009). However, in a study by Carr-Wilkinson *et al*, *MDM2*-amplification was not associated with either stage or overall survival (Carr-Wilkinson et al., 2010). However, inactivation of the negative regulator of MDM2, $p14^{ARF}$ was associated with stage, but not survival. MDM2 has been recently identified as a translational activator of MYCN, as cytoplasmic MDM2 binds AU-rich elements of the MYCN 3'UTR (Gu et al., 2011) indicating yet another

negative feedback loop within the p53 network (He et al., 2011) as shown in Figure 7.1. This may be a mechanism by which MDM2 promotes MYCN-driven tumourigenesis, but may also support a tumour suppressive role for MDM2 as a result of MYCN-induced p53. This study suggests that MDM2 and MYCN are mutually regulated, and reciprocally regulate p53 in neuroblastoma (He et al., 2011).

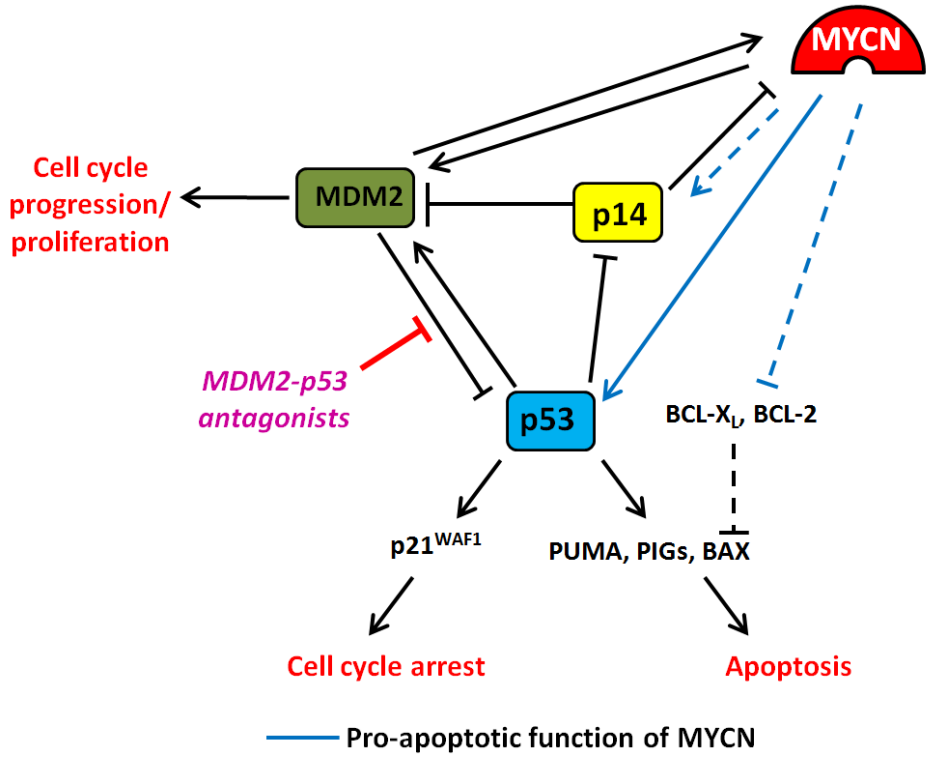


Figure 7.1. **Targeting the MYCN/p53/MDM2/p14^{ARF} network with MDM2-p53 antagonist to enhance the pro-apoptotic function of MYCN through MDM2 inhibition and subsequent p53 activation. Dashed lines indicate where the function has only been investigated for MYCC.**

7.2.1 Targeting the pro-apoptotic functions of MYCN

In this study the use of MDM2-p53 antagonists in activating the p53-dependent pro-apoptotic function of MYCN was investigated, indicated by blue arrows in Figure 7.1. In a panel of *MYCN*-amplified and non-*MYCN*-amplified neuroblastoma cell lines, and in MYCN-inducible Tet21N cells, *MYCN*-amplification or induced expression sensitised to MDM2-p53 antagonist-mediated apoptosis and growth inhibition. In agreement, siRNA-mediated knockdown of MYCN resulted in resistance to MDM2-p53 antagonist-mediated p53 activation and apoptosis. These findings are in line with a study by Barbieri *et al* who found a trend towards an increased IC_{50} for Nutlin-3 in MYCN(+) compared to MYCN(-) Tet21Ns (Barbieri et al., 2006). Similar to the results presented in this study, van Maerken *et al*, found that both *MYCN*-amplified and non-

MYCN-amplified cell lines were responsive to Nutlin-3 treatment (Van Maerken et al., 2006). However, they did not find a significant association between *MYCN*-amplification and sensitivity to Nutlin-3, reporting no significant difference in cell viability and caspase activity in a panel of 7 p53 wildtype cell lines (3 of which were *MYCN*-amplified) (Van Maerken et al., 2006). This group however did not use any isogenic systems to investigate the effect of MYCN.

7.2.2 Destabilising MYCN

Using transgenic mouse models, it has been found that MYC-induced tumours remain dependent on MYC after they are established, and therefore strategies that interfere with MYC function are another therapeutic approach for the treatment of *MYCN*-amplified neuroblastomas. Tumours that depend on MYCN might also depend on upstream regulatory functions or downstream target genes that are less essential for the growth of MYCN-independent tumours. A number of kinases have been identified as potential targets that result in the destabilisation of MYCN and are shown in Figure 7.2. Potential targets include Aurora A kinase, phosphatidylinositol 3-kinase (PI3-kinase) and mTOR (Figure 7.2).

7.2.2.1 Aurora A kinase (AurKA) inhibitors

Aurora kinases are cell cycle regulated, and are important for transition through mitosis (Marumoto et al., 2005; Keen and Taylor, 2004). AurKA binds to and stabilises MYCN independently of its kinase activity via inhibition of the E3 ubiquitin ligase, FBXW7, which is responsible for degrading MYCN in neuronal progenitor cells. This interferes with cell-cycle exit in developing peripheral neurones (Otto et al., 2009; Sjostrom et al., 2005; Yada et al., 2004). AurKA is overexpressed in human neuroblastoma cell lines and is associated with poor clinical outcome and decreased progression free survival in patients. 194 genes that are expressed in a manner dependent on *MYCN*-amplification in neuroblastoma, or are direct target genes of MYC were analysed in a shRNA screen to identify synthetic lethal interactions (Otto et al., 2009). AurKA was identified as a gene required for the growth of *MYCN*-amplified neuroblastoma cell lines. Knockdown of AurKA inhibited cell proliferation and enhanced chemosensitivity in neuroblastoma cell lines that are dependent on high MYCN protein levels, but had no effect in those that are not (Otto et al., 2009). Small molecules have been developed that inhibit AurKA. In preclinical models, the AurKA inhibitor MLN8237 showed efficacy against neuroblastoma (Maris et al., 2010). Recently, a highly selective and bioavailable AurKA and B inhibitor, CCT137690 has been tested in neuroblastoma and inhibited

growth of *MYCN*-amplified neuroblastoma cell lines, dramatically reducing tumour mass in MYCN-driven transgenic tumours (Faisal et al., 2011). Aurora kinase inhibitors may therefore significantly enhance the treatment of MYCN-dependent neuroblastomas.

7.2.2.2 Targeting the PI3K pathway

Most primary neuroblastomas display activation of critical mediators involved in the PI3K/AKT/mTOR signalling pathway and this is associated with resistance to apoptosis (Boller et al., 2008; Johnsen et al., 2008; Opel et al., 2007). PI3Ks function as signal transducers downstream of cell-surface receptors. Both PI3K and mTOR are kinases involved in regulating cell growth, proliferation and translation, and play a role in phosphorylating AKT as shown in Figure 7.2, subsequently activating both mTOR and GSKβ. MYCN is stabilised through PI3K signalling, and inhibition of this pathway represents a potential strategy to promote degradation of MYCN protein, resulting in loss of both MYCN-dependent proliferation and apoptosis. In neuroblastoma, studies have shown that inhibition of key molecules of this pathway have profound effects on the survival of neuroblastoma cells both *in vitro* and *in vivo* (Bender et al., 2011; Li et al., 2010; Johnsen et al., 2008; Opel et al., 2007; Chesler et al., 2006).

The PI3K/mTOR inhibitor PI-103, and the PDK1 inhibitor OSU03012, impair neuroblastoma growth *in vitro* and downregulate cyclin D1 and MYCN protein levels (Segerstrom et al., 2011). These compounds significantly inhibited the growth of *MYCN*-amplified neuroblastomas in nude-mice and were more effective in *MYCN*-amplified cells. Similar effects were found with the pan PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin (Johnsen et al., 2008; Chesler et al., 2006). In addition, inhibitors of PI3K/Akt and mTOR synergised with chemotherapeutic drugs (Doxorubicin, Etoposide, Topotecan, Cisplatin, Vincristine and Taxol) in neuroblastoma (Bender et al., 2011; Johnsen et al., 2008).

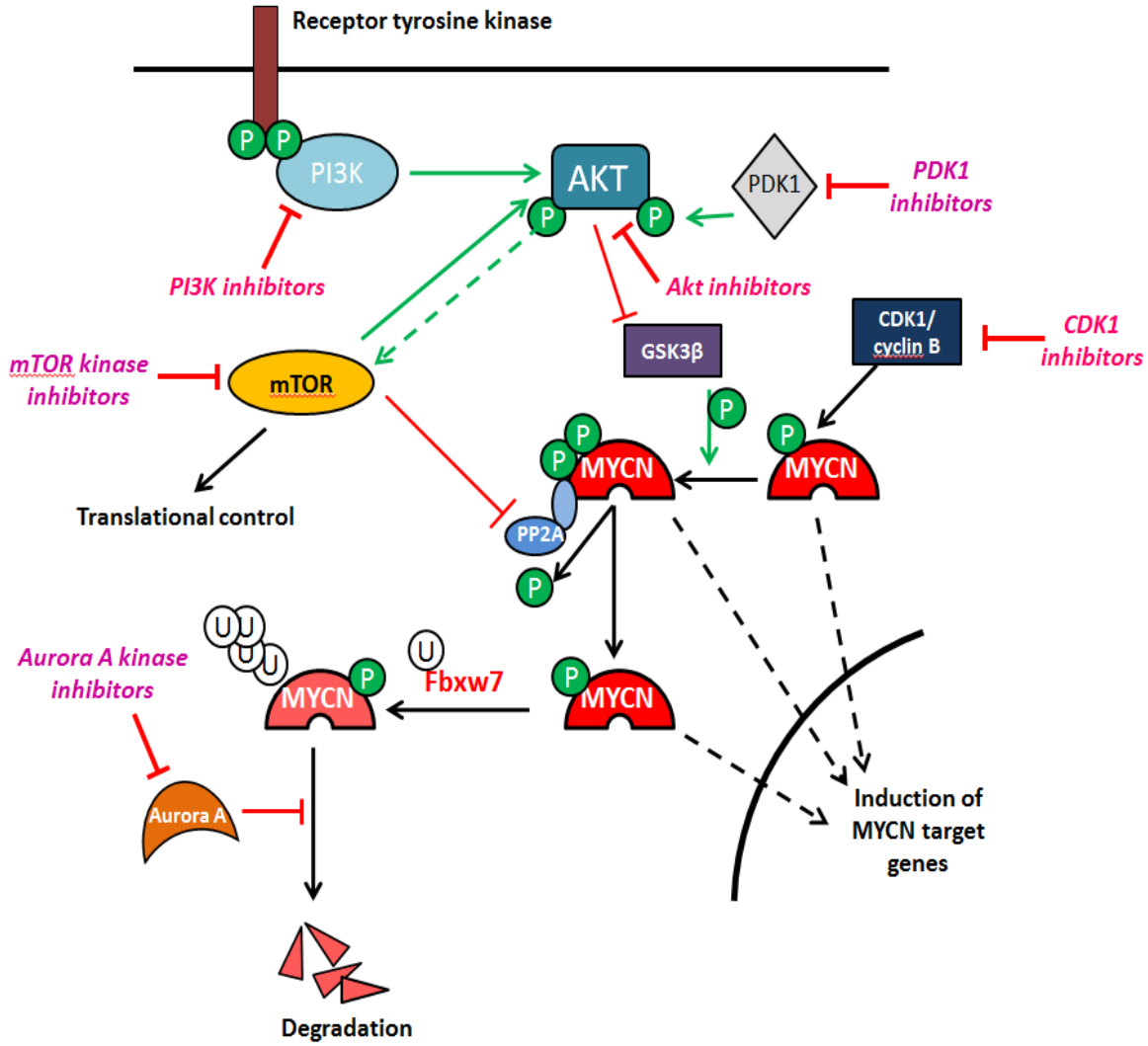


Figure 7.2. **The PI3K/AKT/mTOR pathway, and Aurora kinase A and the interactions with MYCN. Inhibitors that are currently being investigated for the treatment of cancer are displayed (Gustafson and Weiss, 2010).**

7.2.3 Advantages and disadvantages of these targets

Both MDM2 inhibitors and inhibitors of the PI3K/AKT/mTOR network are being investigated in a number of cancer types, including neuroblastoma and both are potential promising new therapeutic approaches for the treatment of *MYCN*-amplified disease. In addition, since these compounds are showing activity in preclinical early phase studies in various cancer types, their development is appealing to pharmaceutical companies.

PI3K plays a role in a wide range of normal biological processes and organ systems, and is responsible for inhibiting a number of other kinases. This raises concerns about the potential toxicities associated with inhibiting this pathway and until recently presented a major hurdle (Workman et al., 2006). Several subunits of PI3K and AKT

exist making drug design difficult. Conventional PI3K inhibitors such as wortmannin or LY294002 do not discriminate between different PI3K isoforms and are toxic to normal tissues. The toxicity of PI3K inhibitors can be overcome by the use of isoform-specific PI3K inhibitors, which were well tolerated in mice for the treatment of systemic lupus erythematosus (Marone et al., 2008). Most toxicities of mTOR inhibitors are mild to moderate in severity but can usually be managed clinically by dose modification and supportive measures (Sankhala et al., 2009). A number of recent studies have reported no toxicity with new specific compounds, including the PI-103 PI3K/mTOR inhibitor (Fan et al., 2006), and combined PI3K/mTOR inhibitors (Mazzoletti et al., 2011). Another option is to target PDK1, as with OSU03012, for which only 1 isoform exits which is capable of activating all AKT isoforms (Garcia-Echeverria and Sellers, 2008).

Nutlin-3 and other MDM2-p53 antagonists have shown no toxicity to normal cells despite inducing p53 (Shangary et al., 2008; Vassilev et al., 2004). The MDM2-p53 antagonist MI-147 resulted in no weight loss or toxicity to normal tissues in a mouse model (Yu et al., 2009), and MI-219 treatment induced p53 but no apoptosis in normal cells, and was selectively toxic to tumour tissues (Shangary et al., 2008). This compound also completely inhibited tumour growth when used in combination with irinotecan.

The disadvantage of MDM2-p53 antagonists is that they require wildtype p53 for their mechanism of action, and this study and many previous studies have found them to be inactive in mutant cells. However, targeting the p53 pathway is particularly attractive in neuroblastoma as it is usually wildtype, even at relapse, and since MDM2 is proposed as a mechanism by which *MYCN*-amplified tumours induce tumourigenesis, inhibition of MDM2 may tip the balance from MYCN-mediated cell proliferation in favour of MYCN-induced cell death. However, a recent study found that SJSA-1 osteosarcoma cells that were exposed to repeated Nutlin treatment acquired somatic mutations within p53, suggesting that Nutlin selects for p53-mutations (Aziz et al., 2011), and this has also been found in in-house studies both in the SJSA-1 cell lines and in the neuroblastoma cell lines NGP. This suggests a requirement for p53-independent therapies, or therapies that result in complete eradication of the tumour. The use of MDM2-p53 antagonists in combination with low doses of chemotherapeutic agents looks promising.

7.3 Nutlin-3 in combination with chemotherapeutic agents

Previous studies have shown that MDM2-p53 antagonists synergise with chemotherapeutic drugs in various cancer types including neuroblastoma, and therefore MDM2-p53 antagonists may be useful as an adjuvant to chemotherapy.

In neuroblastoma cell lines, response to genotoxic drugs has been reported to be significantly enhanced upon disruption of the MDM2-p53 interaction (Barbieri et al., 2006). Nutlin-3 combined with stress-inducing cisplatin significantly reduced cell growth compared to cisplatin alone, with an 8-10 fold change in $IC_{50,}$ and apoptosis occurred in all cell lines tested. Petroni *et al* found that Nutlin-3 sensitised *MYCN*-amplified neuroblastoma cell lines to the effect of the DNA damaging agent bleomycin (Petroni et al., 2011). Doxorubicin enhanced the sensitivity to Nutlin-3 induced apoptosis, further decreasing cell viability, and was associated with increased MYCN expression indicating that MYCN-driven apoptosis is important in neuroblastoma (Peirce and Findley, 2009a).

Modern chemotherapy for the treatment of high risk *MYCN*-amplified neuroblastoma subjects patients to a high genotoxic burden that often presents long-term complications and late side effects (Laverdiere et al., 2009). Combining established therapy with MDM2-p53 inhibitors would allow much lower doses of genotoxic drugs to be used since the genotoxic damage itself would not be potentiated.

7.4 Do Nutlin-3 and MI-63 cause a p53-mediated cell cycle arrest and induce DNA damage?

It has been previously reported that in a panel of neuroblastoma cell lines, most of which were used in this study, *MYCN*-amplification was significantly associated with a failure to G_1 arrest following irradiation-induced DNA damage (Bell et al., 2006). In the present study, the changes in cell cycle distribution following Nutlin-3 and MI-63 treatment was cell line dependent; 3 of 10 *MYCN*-amplified cell lines G_1 arrested in response to MDM2-p53 antagonists, compared to 4 of 8 non-*MYCN* amplified cell lines. The cell cycle response of several *MYCN*-amplified cell lines were investigated in more depth, and it was found that NGP cells and Tet21N (MYCN+ and MYCN-) cells G_1 arrested at very low concentrations of MDM2-p53 antagonists, TR14 partially arrested, and LAN5 and IMR32 cells remained in the cell cycle with concentrations of up to 10μM Nutlin-3 or MI-63. In addition, siRNA-mediated knockdown of MYCN did not alter the cell cycle response of any of these cell lines to MDM2-p53 antagonists. In cell

lines that did arrest (NGP and Tet21N), the proportion of cells in G_2 increased at increasing concentrations of compound, particularly with the more potent MI-63. The G_1 checkpoint is p53-dependent and a G_1 arrest is more commonly associated with p53 activation. Activation of G_2, however, can be p53-dependent or p53-independent and G_2 arrest can be activated in response to DNA damage (Xiao et al., 2003).

p53 is activated following DNA damage through phosphorylation, preventing binding to MDM2 (Shieh et al., 1997). There are conflicting reports about whether MDM2-p53 antagonists induce phosphorylation of p53, and whether Nutlin-3 induces DNA damage. In this study, phosphorylation of p53 at serine 15 was observed, and a difference in p53 phosphorylation depending on *MYCN* status. Another group report serine 15 phosphorylation of p53, but at very low levels compared to doxorubicin, supporting a non-genotoxic mode of p53 activation by Nutlin-3 (Drakos et al., 2007). Two studies have focused on the DNA damage response following Nutlin-3 treatment (Valentine et al., 2011; Verma et al., 2010). p53 was phosphorylated at key DNA-damage specific residues (serine 15, 20 and 37) following Nutlin-3 treatment and Nutlin-3 also induced CHK2 and ATM, required for DNA damage dependent phosphorylation and activation of p53. The DNA damage response was however, not significantly high compared to the DNA damage response initiated by other agents. The use of MDM2-p53 antagonists is therefore attractive from a therapeutic point of view, as they do not cause DNA damage or toxicity in normal tissues (Shangary et al., 2008; Vassilev et al., 2004).

The failure of *MYCN*-amplified cells to G_1 arrest in response to irradiation therapy, but not following MDM2-p53 antagonist treatment, may be because unlike irradiation, MDM2-p53 antagonists do not induce DNA damage, activating p53 in a non-genotoxic manner.

7.5 Explaining the variations in sensitivity to MDM2-p53 antagonists in neuroblastoma cell lines

In the panel of 18 p53 wildtype neuroblastoma cell lines investigated for their response to MDM2-p53 antagonist initially in Chapter 3, and again in Chapters 4 and 5, the sensitivity to Nutlin-3 and MI-63 was highly variable.

As shown in Chapter 3 (Figures 3.19-3.23), whilst *MYCN*-amplified cell lines were overall more sensitive to MDM2-p53 antagonist mediated growth inhibition and apoptosis, there were large variations in growth inhibitory and apoptotic responses,

particularly in non-*MYCN* amplified cell lines. The SKNRA cell line was particularly resistant to both growth inhibition and apoptosis following MDM2-p53 antagonist treatment. This may be a result of *CDKN2A* mutation, caspase activity impairment or cell type as discussed in Chapter 5.4, or it could be that this cell line has particularly high levels of ΔNp73. ΔNp73 expression levels have not been investigated in this study, but the impact of overexpression is discussed in Section 7.7. It is possible that ΔNp73 expression influences the response of other cell lines within this panel also. Other particularly resistant cell lines were those impaired for $p14^{ARF}$ (discussed in Section 7.6.4) . The PER108 cell line, which is also $p14^{ARF}$ impaired may only have partial methylation of $p14^{ARF}$ (Carr et al., 2006) and could explain why it is more sensitive than the other $p14^{ARF}$ impaired cell lines to growth inhibition and apoptosis, but is also one of the least sensitive *MYCN*-amplified cell lines to MDM2-p53 antagonist mediated apoptosis.

MDMX levels were also shown to impact on response to MDM2-p53 antagonists, and within this panel of cell lines, MDMX expression levels correlated with caspase 3/7 activity following Nutlin-3 and MI-63 treatment (discussed in Section 7.6.1). In addition, cell type has an influence on the response to MDM2-p53 antagonists, with N-type cells displaying increased sensitivity, and S-type cells being more resistant to apoptosis (discussed in Section 7.6.2).

7.6 The p53-MDM2/MDMX-$p14^{ARF}$ network and response to MDM2-p53 antagonists

During the process of neuroblastoma development and progression, *MYCN*-amplification is often associated with defects within the p53/MDM2/$p14^{ARF}$ network (shown in Figure 7.1) (Carr-Wilkinson et al., 2010; Carr et al., 2006). Functional change in this network is proposed as a mechanism by which *MYCN*-amplified neuroblastomas evade MYCN-driven p53-dependent apoptosis. MDMX expression levels and influence on treatment response have not been previously investigated in neuroblastoma.

7.6.1 MDMX and response to MDM2-p53 antagonists

As described in Chapter 1.7, MDMX is a negative regulator of p53. MDMX directly binds and inhibits p53 transcriptional function, and also forms a heterodimer with MDM2, stabilising MDM2 and promoting degradation of p53. In addition, MDMX is

reportedly involved in the cytoplasmic tethering and subsequent inactivation of p53 in some cancer types (Ohtsubo et al., 2009).

MDMX is overexpressed in up to 30% of cancers and in many cancer cell lines (Toledo and Wahl, 2007; Ramos et al., 2001). Since MDMX expression levels have not been investigated in neuroblastoma, MDMX protein expression was determined in a panel of cell lines. In contrast to MDM2, MDMX expression was highly variable and did not correlate with MYCN, p53 or $p14^{ARF}$ status, and was expressed at low levels in *MDM2*-amplified cell lines, consistent with a previous report suggesting that MDM2 and MDMX overexpression are mutually exclusive (Danovi et al., 2004), despite MDMX being required for the stabilisation of MDM2. Interestingly, MYCN(+) Tet21N cells had increased MDMX protein levels compared to MYCN(-) cells, but not RNA levels, suggesting an indirect post-transcriptional method of regulation. However, in addition to a lack of correlation between MYCN and MDMX in a panel of cell lines, siRNA-mediated knockdown of MYCN did not support this theory and therefore any link between MYCN and MDMX remains unclear.

Nutlin-3 is ineffective at disrupting the MDMX-p53 interaction, despite the close sequence and structural similarities with MDM2 (Hu et al., 2006; Wade et al., 2006). As a negative regulator of p53, MDMX may continue to suppress p53 activity following MDM2-p53 antagonist treatment. Previous studies have found that the cellular activity of MDM2-p53 inhibitors is decreased by MDMX, and Nutlin-3 does not induce apoptosis in cancer cells that express high levels of MDMX (Hu et al., 2006; Marine et al., 2006; Patton et al., 2006; Wade et al., 2006). MDMX knockdown sensitised cells to Nutlin-3 induced apoptosis (Hu et al., 2006; Wade et al., 2006), and the resistance to Nutlin-3 was completely lost upon deletion of the C-terminal RING finger of MDMX where it binds MDM2 probably as a result of subsequent MDM2-mediated degradation (Patton et al., 2006). Recently, an Hsp90 inhibitor that promotes MDMX degradation was found to dramatically enhance the apoptotic effects of Nutlin-3 both *in vitro* and in xenograft models (Vaseva et al., 2011), and a small molecule inhibitor of MDMX expression, XI-006, increased p53 activity and induced pro-apoptotic effects in an additive manner (Wang et al., 2011). However, XI-006 was also active in p53 mutant cell lines suggesting the action is not specific. In addition, another small molecule MDMX inhibitor (SJ-172550) which binds reversibly to MDMX, killed retinoblastoma cells overexpressing MDMX (Reed et al., 2010), and small peptides that disrupt the MDMX- and MDM2-p53 interaction induced both p53 and apoptosis (Hu et al., 2007a).

In the present study, knockdown of MDMX alone induced high levels of p53 and p53-responsive genes, and induced caspase 3/7 activity. Together with the literature, this data suggests that removal of MDMX may be necessary for the efficient activation of the p53 response following MDM2-p53 antagonist treatment in neuroblastoma. However, in contrast to reports elsewhere with other cell types, MDMX knockdown in neuroblastoma cell lines in this present study resulted in decreased sensitivity to Nutlin-3 and MI-63 mediated apoptosis, and MDMX protein expression positively correlated with treatment induced caspase 3/7 activity. The cell line that expressed the highest levels of MDMX, LAN5, had reduced levels of p53 and p53 responsive genes following MDMX knockdown and MDM2-p53 antagonist treatment, whereas the other two *MDM2*-amplified cell lines did not. However, apoptosis was induced at similar levels in all three cell lines. The *MDM2*-amplified cell lines may be less dependent on MDMX for the activation of p53, and the effect seen on apoptosis may be independent of, or downstream of p53. These results suggest that MDMX removal impairs the apoptotic response to MDM2-p53 antagonists in neuroblastoma, regardless of MDMX expression levels, and that tumour cells with high levels of MDMX are more sensitive to MDM2-p53 antagonist mediated apoptosis.

MDMX was degraded at the protein level upon MDM2-p53 antagonist treatment, particularly with MI-63, in all 3 cell lines tested. In the study by Hu *et al*, no MDMX degradation occurred in the U2OS cells in which the effect of MDMX knockdown and combined Nutlin-3 treatment was tested, and similarly in the MCF-7 cells used in the Wade *et al.* study (Hu et al., 2006; Wade et al., 2006). Xia *et al* tested a random panel of solid cancer cell lines for the effect of Nutlin on MDMX expression and found that in the majority of cases MDMX was degraded, but in the few cell lines it was not, then it was a major suppressor of the apoptotic response (Xia et al., 2008). This may explain the differences in response upon MDM2-p53 antagonist treatment and MDMX knockdown in this study compared to the previous two studies. In neuroblastoma, when MDM2 is inhibited, the degradation of MDMX may be MDM2-dependent as MDM2 levels increase and MDM2 promotes ubiquitination of MDMX.

This data suggests that MDMX is not a major inhibitor of the p53 response, particularly apoptosis, in neuroblastoma, and that MDMX expression co-operates with MDM2-p53 antagonists to induce apoptosis. The link between MDMX and p53, and whether the primary role of MDMX is directly inhibiting p53 transactivation function, or stabilising MDM2, is not entirely clear.

7.6.2 Cell type and response to MDM2-p53 antagonists

An alternative explanation for the MDMX associated sensitisation to apoptosis in neuroblastoma upon MDM2-p53 antagonist treatment is the strong association between MDMX expression and cell type. Neuroblastomas comprise 3 cell types; N, S and I as described in Chapter 1.4. N-type cells are more tumourigenic and are associated with increased apoptosis in response to DNA damage compared to S-type cells, which generally have increased $p21^{WAF1}$ and are more likely to G_1 arrest (Carr-Wilkinson et al., 2011; Mergui et al., 2008; Isaacs et al., 1998). Similarly, found in this study, in response to MDM2-p53 antagonists N-type cells were significantly associated with increased apoptosis compared to S-type cells. However, despite seeing a link between S-type cells and cell cycle arrest following irradiation, there was no link following MDM2-p53 antagonist treatment, with 4 of each N and S cell type cell lines showing G_1 arrest (Figure 5.13).

However, unlike irradiation, there was no link with S-type and cell cycle arrest following MDM2-p53 antagonist treatment, with 4 of each N and S cell type cell lines showing G_1 arrest (Figure 5.13). It is likely that neuroblastoma cell type (and therefore differentiation status) influences MDMX expression levels and that N-type cells have increased MDMX expression. Because of the contrast to previous reports, it may be that the response to MDM2-p53 antagonists is dominated by cell type rather than MDMX expression and that other proteins co-expressed in N-type cells are responsible for the sensitisation observed, especially since MDMX knockdown alone activates p53. In addition, N-type *MYCN*-amplified neuroblastoma cells had increased sensitivity to the AurkA inhibitor MLN8054 (Shang et al., 2009). The studies in this thesis suggest that cell type may be important in determining the response to MDM2-p53 antagonists but the link between cell type, MDMX expression and response to MDM2-p53 antagonist remains to be established.

7.6.3 MDM2 and MDM2-p53 antagonists

MDM2 is amplified in up to 13% of cases of neuroblastoma in one study, and usually occurs with *MYCN*-co-amplification (Carr-Wilkinson et al., 2010). High levels of MDM2 expression can occur even in neuroblastomas without *MDM2*-amplification, and in some cases is associated with a single nucleotide polymorphism in the MDM2 gene promoter (Cattelani et al., 2008). Previous reports have shown that in non-neuroblastoma cell types, the effect of MDM2 on the response to MDM2-p53

antagonists is variable, and may be cancer or cell type specific (Liu et al., 2009; Gu et al., 2008b; Kojima et al., 2006; Tovar et al., 2006; Van Maerken et al., 2006; Kojima et al., 2005). In this study, and in contrast to the effects seen upon MDMX knockdown, MDM2 knockdown in *MDM2*-amplified cell lines did not influence the apoptotic response to MDM2-p53 antagonists, and the effect on cell cycle remains to be investigated. On the other hand, *MDM2*-amplified cell lines had increased caspase 3/7 activity in response to MDM2-p53 antagonists. Since MDM2 knockdown had little effect, this may be as a result of $p14^{ARF}$ overexpression in these cell lines.

The relationship between MDM2 expression and cell type was not investigated in this study. The 4 MDM2-amplified cell lines were N-type, but in a previous study in non-MDM2-amplified cell lines, high MDM2 expression was associated with S-type cells (Carr-Wilkinson et al., 2011).

Interestingly, Nutlin-3 treatment has been reported to potentiate the ability of doxorubicin to block cell proliferation and induce apoptosis in a p53 mutant cell line, as a result of TAp73 and E2F1 release from MDM2, suggesting that the p53-independent effects of MDM2-p53 antagonists might be useful in p53 mutant cells for potentiating other agents (Peirce and Findley, 2009b).

7.6.4 $p14^{ARF}$ and MDM2-p53 antagonists

$p14^{ARF}$ is a key sensor of hyperproliferative signals generated by activated oncogenes, and in turn activates both p53-dependent and p53-independent pathways (Sherr, 2006). Within the p53 network, $p14^{ARF}$ is a negative regulator of MDM2, is induced by p53 and inhibits the function of MYCN as shown in Figure 7.1. *$p19^{ARF}$* null mice are highly susceptible to tumour development (Kamijo et al., 1997), and previous studies have shown that *CDKN2A* mutations induce chemoresistance by disabling p53 (Schmitt et al., 1999). *CDKN2A* mutations may affect both $p14^{ARF}$ and $p16^{INK4a}$ as they are encoded by the same gene (as described in Chapter 1.13). They are very common in melanoma (Kefford et al., 1999), but have not been reported in neuroblastoma. However, $p14^{ARF}$ is frequently inactivated through homozygous deletion or methylation in neuroblastoma (Caren et al., 2008; Thompson et al., 2001; Takita et al., 1997). Methylation of $p14^{ARF}$ occurred in 7-14% of neuroblastomas, and homozygous deletion (affecting both $p14^{ARF}$ and $p16^{INK4a}$) in up to 22% of cases (Carr-Wilkinson et al., 2010; Bassi et al., 2004; Omura-Minamisawa et al., 2001; Thompson et al., 2001; Diccianni et al., 1996). Despite MDM2 expression having been proposed as a mechanism by which *MYCN*-

amplified neuroblastomas evade p53-dependent apoptosis, $p19^{ARF}$ was lost in MYCN transgenic tumours with MDM2 haploinsufficiency (Chen et al., 2009). This suggests that either loss of $p14^{ARF}$, or increased expression of MDM2 may be required to disable the pro-apoptotic functions of MYCN.

The data presented in this thesis is in agreement with previous reports by Van Maerken *et al* (Van Maerken et al., 2011; Van Maerken et al., 2006). Cell lines that were $p14^{ARF}$ impaired (SHEP and LAN6) were resistant to MDM2-p53 antagonist mediated apoptosis. Interestingly, in this study, 3 of 4 of these cell lines were not amplified for *MYCN*, suggesting that the mechanism by which $p14^{ARF}$ inactivation suppresses the p53 response is not MYCN driven. Also, 3/4 of these cell lines (one of which was *MYCN*-amplified) underwent a pronounced G_1 arrest following Nutlin-3 treatment, suggesting that $p14^{ARF}$ impairment may protect against apoptosis. It remains to be determined whether this arrest is reversible or not. In this present study, $p21^{WAF1}$ levels upon $p14^{ARF}$ knockdown were not determined, and neither was the cell cycle response. Because of the low levels of $p14^{ARF}$ expression in neuroblastoma cells, and because of the lack of specific $p14^{ARF}$ antibodies for Western blotting, in the present study, $p14^{ARF}$ was only knocked down in 3 *MDM2*-amplified cell lines which overexpress $p14^{ARF}$. Whilst $p14^{ARF}$ knockdown alone resulted in decreased caspase activity, p53 and MDM2 levels were not affected. Upon MDM2-p53 antagonist treatment, a decrease in the apoptotic markers cleaved caspase 3 and PARP cleavage was observed, suggesting that removal of $p14^{ARF}$ impairs the apoptotic response, but again no change in p53 and p53 responsive genes including PUMA, was observed. This suggests that the apoptotic response may be p53-independent and may be due to $p14^{ARF}$ knockdown having little effect on MDM2 due to *MDM2*-amplification in these cell lines. Interestingly caspase activity following $p14^{ARF}$ knockdown and MDM2-p53 antagonist treatment did decrease compared to MDM2-p53 antagonist treatment alone, but not significantly, and therefore the effect of $p14^{ARF}$ on the response to MDM2-p53 antagonists requires further investigation. Van Maerken *et al* used both non-*MDM2* amplified IMR32 cells, and *MDM2*-amplified NGP cells for siRNA-mediated knockdown of the *CDKN2A* gene (both $p14^{ARF}$ and $p16^{INK4a}$) (Van Maerken et al., 2011). Cell viability following Nutlin-3 treatment in both cell lines increased in response to the knockdown, suggesting that the *MDM2*-amplification in NGP cell lines is not altering the apoptotic response compared to non-*MDM2* amplified IMR32 cells. However, the effect on p53 expression was not reported. CDKN2A overexpression in IMR32 derived IMR5/75

(IMR-32 cells with inducible *CDKN2A* expression) cells resulted in a decreased IC_{50} for Nutlin, and increased caspase activity. In agreement, the present study found increased caspase activity in response to MDM2-p53 antagonists in the $p14^{ARF}$ overexpressing *MDM2*-amplified cell lines. However, previous studies have found that $p14^{ARF}$ overexpression in SHEP cells did not restore sensitivity to MDM2-p53 antagonists showing cell type may also be important (Van Maerken et al., 2011), and that restoration of $p14^{ARF}$ function in GIMEN cells did not restore sensitivity to IR (Carr et al., 2006).

Taken together, this data indicate that $p14^{ARF}$ has a stimulatory effect on the Nutlin-3 response, and that $p14^{ARF}$ may signal for an apoptotic response independently of p53, for example, by increasing protein synthesis or regulating pathways that cross talk with p53 signalling (Miao et al., 2010; Rocha et al., 2005).

7.7 p73 and the response to MDM2-p53 antagonists in neuroblastoma

Some of the results observed in this study were either unexpected or difficult to explain. For example, the NGP cell line behaved differently following MYCN knockdown and MDM2-p53 antagonist treatment compared to 4 other cell lines tested, and MDMX knockdown sensitised neuroblastoma cell lines to the effects of MDM2-p53 antagonists whereas in other cancer types MDMX knockdown has been shown to desensitise to Nutlin-3. In addition, there were high degrees of variation in the 18 p53 wildtype cell lines investigated for their apoptotic and growth inhibitory response to MDM2-p53 antagonists. A possible explanation for these results may be aberrant expression of the p53 family member, p73 which was not investigated in the current study.

Both p73 and p63 are similar to p53 in amino acid sequence and function, with the transactivating isoforms (TAp63/p73) able to induce cell cycle arrest and apoptosis. However, the truncated isoforms (ΔNp63/p73) are oncogenic, promoting cell proliferation. Whilst p63 has been implicated in some cancer types, levels are very low in the central nervous system (Jacobs et al., 2005), and there are no studies reporting aberrant expression of p63, or a role for p63 in the development of neuroblastoma. p73 expression however, has been shown to be essential for neurogenesis. In primary neuroblastomas, ΔNp73 is overexpressed in up to 30% of tumours, and correlates with a poor prognosis independently of all other neuroblastoma risk factors (Casciano et al., 2002). In addition, TAp73 but not ΔNp73 is bound by MDM2, blocking activity and relocalising TAp73 to the cytoplasm (Wang et al., 2001), and may impact particularly

on the response of *MDM2*-amplified neuroblastomas to MDM2-p53 antagonists. MDMX also binds p73 and inhibits p73 transactivation, but whether this is true for both p73 isoforms is unknown (Ongkeko et al., 1999). ΔNp73 also inhibits the function of p53 and TAp73, suggesting that overexpression of ΔNp73 may decrease the activity of p53 and TAp73 released as a result of MDM2-p53 antagonist treatment.

Nutlin-3 disrupts the MDM2-p73 interaction in addition to the MDM2-p53 interaction (Lau et al., 2008). Nutlin-3 and several chemotherapeutic agents have also been shown to induce TAp73 in a p53 wildtype and p53-null cell lines in an E2F-dependent manner, which undergo apoptosis dependent on TAp73 (Peirce et al., 2009). However, increased expression of ΔNp73 may downregulate TAp73, particularly since ΔNp73 is also a transcriptional target of both p53 and TAp73. These data suggest that ΔNp73 expression levels may be important in determining the response to MDM2-p53 antagonists Nutlin-3 and MI-63, and may be responsible for the differences observed in the panel of neuroblastoma cell lines. In addition, MYCN has been shown to decrease total p73 levels, and this may play a role in sensitising *MYCN*-amplified cell lines to apoptosis as a result of decreased ΔNp73.

The expression levels of ΔNp73 should be investigated in the panel of neuroblastoma cell lines used in this study, to determine if there is any link between ΔNp73 expression and response to MDM2-p53 antagonists. In addition, 1p36 status should be determined in the panel of cell lines as 1p36 LOH is a frequent event in neuroblastoma, and loss of p73 may explain why some cell lines are more sensitive than others, as a result of reduced ΔNp73. ΔNp73 inhibitors in combination with MDM2-p53 antagonists could be potential therapeutic targets for the treatment of neuroblastoma.

7.8 Concluding remarks and future work

The work in this thesis set out to investigate the effects of varying MYCN, p53, MDM2, MDMX and $p14^{ARF}$ expression on the response to the MDM2-p53 antagonists Nutlin-3 and MI-63 in neuroblastoma. The major findings are:

- MYCN sensitises neuroblastoma cell lines to MDM2-p53 antagonist mediated apoptosis.
- MDMX expression is associated with cell type, with N-type cells having high MDMX expression and S-type low. N-type and increased MDMX expression was associated with increased sensitivity to MDM2-p53 antagonist mediated apoptosis.
- $p14^{ARF}$ expression has a sensitising effect of neuroblastoma cell lines to MDM2-p53 antagonists, and impairment results in a G_1 arrest following Nutlin-3 treatment which may be a protective effect responsible for the low levels of apoptosis induced in these cell lines.

Several lines of evidence suggest that MYCN and the status of the p53-MDM2/MDMX-$p14^{ARF}$ pathway influence the response to MDM2-p53 antagonists, and if the results were to be confirmed in *in vivo* models, they may be useful as biomarkers for predicting response to MDM2 inhibitor treatment for neuroblastoma tumours. This could be tested in due course directly on patient samples if MDM2-p53 antagonists go into clinical trials on neuroblastoma patients, as seems likely.

All of the data presented in this thesis comes from *in vitro* studies using cell lines. *In vivo* models are required to confirm the findings in a preclinical setting. This could involve the establishment of *MYCN*-amplified and non-amplified xenografts in immunodeficient nude mice to determine tumour volume index, tumour weight and tumour growth following MDM2-53 antagonist treatment after varying components of the p53-MDM2/MDMX-$p14^{ARF}$ network. MI-63 could not be used as it has poor oral bioavailability, but a number of related compounds such as MI-219 could be tested in addition to a Nutlin-3 related compound RG7112 which is currently in clinical trials for patients with solid tumours (http://clinicaltrials.gov/show/nct00559533). Xenograft models using $p14^{ARF}$ impaired cell lines would be useful in determining the long term *in vivo* effects of $p14^{ARF}$ impairment and the G_1 arrest observed following Nutlin-3 treatment. In addition, the MYCN transgenic mouse model could be used to assess the effect of MYCN on the sensitisation to Nutlin. MYCN transgenic mice express varying

levels of MYCN, and MYCN overexpressing mice can be interbred to produce mice with increased dosage of the MYCN transgene. Since Nutlin-3 may select for p53 mutants, the long-term effects of MDM2-p53 antagonist treatment needs to be assessed, and the possibility of combining MDM2-p53 antagonists with low doses of cytotoxic agents.

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Appendix I – Buffers and Instrument Settings

Cell Culture buffers

Carnoy's fixative

1 part glacial acetic acid (BDH)
3 parts methanol (Fisher Scientific, HPLC grade)

Western Blotting buffers

Laemmli (lysis)Buffer

0.0625M Tris-HCl pH 6.8
2% SDS (Sigma)
10% Glycerol (Sigma)

Laemmli Loading Buffer

0.0625M Tris-HCl pH 6.8
2% SDS (Sigma)
10% Glycerol (Sigma)
5% β-mercaptoethanol (Sigma)
0.0025% Bromophenolblue (Biorad)

Electrophoresis Buffer

16.15g Tris
72.05g Glycine
5g SDS
Made up to 5 litres with ddH_2O

Transfer Buffer

3.03g Tris
14.14g Glycine
200ml Methanol
Make up to 1 litre with ddH_2O

10x TBS Tween

180g NaCl
120g Tris
2 litres ddH_2O
pH 7.5 with HCl
10ml Tween 20 (Sigma)

Pierce assay plate set up:

	BSA Standards			Unknowns							
water	0.2mg/ml	0.6mg/ml	1.0mg/ml	1	3	5	7	9	11	13	15
water	0.2mg/ml	0.6mg/ml	1.0mg/ml	1	3	5	7	9	11	13	15
water	0.2mg/ml	0.6mg/ml	1.0mg/ml	1	3	5	7	9	11	13	15
water	0.2mg/ml	0.6mg/ml	1.0mg/ml	1	3	5	7	9	11	13	15
water	0.4mg/ml	0.8mg/ml	1.2mg/ml	2	4	6	8	10	12	14	16
water	0.4mg/ml	0.8mg/ml	1.2mg/ml	2	4	6	8	10	12	14	16
water	0.4mg/ml	0.8mg/ml	1.2mg/ml	2	4	6	8	10	12	14	16
water	0.4mg/ml	0.8mg/ml	1.2mg/ml	2	4	6	8	10	12	14	16

FACs instrument settings (for LAN5 cells):

Parameter	Detector	Voltage	Amp gain	Mode
P1	FSC	E-1	4.26	Linear
P2	SSC	350	1	Linear
P3	FL1	150	1	Linear
P4	FL2	408	1	Linear
P5	FL3	150	1	Linear
P6	FL2-A		1	Linear
P7	FL2-W		2.15	Linear

Agarose gel electrophoresis

Loading buffer

800µl glycerol
0.2mg (0.05%) bromophenol blue
40µl 0.5M EDTA
Made up to 2ml with dH_2O

5x TBE buffer

455mM Tris
445mM Boric acid
10mM EDTA
Made up to 5L in distilled water

Appendix II – publications

1) **Gamble LD**, Kees UR, Tweddle DA, and Lunec J. (2011) MYCN sensitises neuroblastoma to the MDM2-p53 antagonists Nutlin-3 and MI-63. *Oncogene.*

2) Chen L, Iraci N, Gherardi S, **Gamble LD**, Wood KM, Perini M, Lunec J, Tweddle DA. (2010) p53 is a direct transcriptional target of MYCN in neuroblastoma. *Cancer Research* 70(4): 1377-88

3) Carr-Wilkinson J, Griffiths R, Elston R, **Gamble LD**, Goranov B, Redfern CPF, Lunec J, Tweddle DA. (2011) Outcome of the p53-mediated DNA damage response in neuroblastoma is determined by morphological subtype and MYCN expression. *Cell cycle* 10(21): 3778-87

4) **Gamble LD**, Tweddle DA, and Lunec J. The status of the p53-MDM2/MDMX-$p14^{ARF}$ network in neuroblastoma and response to MDM2-p53 antagonists. (Submitted September 2011).