Regulation of cancer cell proliferation and mitosis by NF-κB

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Abstract

The NF-κB family of transcription factors can induce or repress target gene expression by binding DNA as homo- or hetero-dimers. In some cancer cells the p52 (NF-κB2) subunit, which is derived by proteolytic processing of its precursor p100, regulates the expression of genes having a role in cell proliferation, such as Cyclin D1 and is also involved in the regulation of G2/M phase. Depletion of p100/p52 by siRNA leads to an increase of cells in G2/M phase and defects in mitosis. These defects in mitosis can be visualised as aberrant chromosome segregation, disruption of the microtubule network and poor alignment of chromosomes on the metaphasic plate. Moreover, p100/p52 depletion results in an increase in multinucleate cells, as well as aberrant centrosome structures.

To elucidate this mechanism, I have investigated p100/p52 regulation of various genes involved in the cell cycle and centrosome duplication. I discovered that p100/p52 siRNA depletion reduces the expression of a number of cell cycle regulators, including Polo-like kinase 4 (PLK4) or Spindle assembly abnormal protein 6 (SAS6), a member of the SAS proteins family. PLK4, in conjunction with the cyclin-dependent kinase CDK2 and the PLK4 effector SAS6, is a key regulator of centriolar duplication. I demonstrated by reporter-gene and chromatin-immunoprecipitation analyses that the PLK4 promoter is a direct target for multiple NF-κB subunits and its activity depends upon NF-κB expression. Moreover PLK4 and p100/p52 mRNA and protein expression are cell cycle regulated and NF-κB subunits bind the PLK4 promoter in a cell cycle dependent manner.

These results reveal PLK4 as a new NF-κB target, providing a direct link between NF-κB and centrosome duplication with implications for the role of p52 in tumorigenesis.

Abbreviations

Ank	Ankyrin
APC/C	Anaphase Promoting complex/cyclosome
ATM	Ataxia Telangiectasia Mutated
ATR	ATM- and Rad3-related
Bp	base pairs
BSA	Bovine Serum Albumin
CBP	CREB Binding protein
CDK	Cyclin Dependent Kinase
ChIP	Chromatin ImmunoPrecipitation
CK	casein kinase
CLL	chronic lymphocytic leukemia
CREB	Cyclin Adenosine Monophosphate Response Element Binding Protein
DBM	DNA Binding Mutant
DD	Death Domain
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECM	extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ER	oestrogen receptor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-Associated Cell Sorting
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GRR	Glycine Rich Region
GSK	Glutathione S Transferase
HA	Haemagluttinin
HAT	Histone Acetyltransferase
HDAC	Histone deacetylase
HU	Hydroxy-urea
IKK	IκB Kinase
IL	Interleukin
IκB	Inhibitor of NF-κB
JNK	c-Jun N-Terminal Kinase
LMP1	Latent membrane protein
LPS	Lipopolysaccharide
LT-βR	Lymphotoxin β Receptor
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
mRNA	messenger RNA
MTOC	Microtubule Organising Centre
NEMO	NF-κB modulator
NES	nuclear export signal
NF-κB	Nuclear Factor κB
NIK	NF-κB Inducing Kinase
NK	natural killer
NKAP	NF-κB activating protein
NLS	Nuclear Localisation Sequence
ORC	origin recognition complex
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCM	Pericentriolar Material

PCR	Polymerase Chain Reaction
PEST	Proline, Glutamine Acid, Serine, Threonine
PID	Processing-Inhibitory Domain
PLK	Polo-Like Kinase
Pol	Polymerase
q-PCR	quantitative PCR
RANK	Receptor activator of NF-κB
ReChIP	ChIP reverese
RHD	Rel Homology Domain
RNA	ribonucleic acid
Rpm	rotation per minute
rRNA	ribosomal RNA
RSK2	ribosomal S6 kinase 2
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
SCF	Skp1/Cul1/F-Box
SDS	Sodium Dodecylsulphate
shRNA	short hairpin RNA
siRNA	small interfering RNA
Skp2	S-phase kinase associated protein 2
ssDNA	salmon sperm DNA
SUMO	small ubiquitin-like modifier
TAD	Transactivation Domain
TAK	TGF-β activated kinase
TBS	Tris Buffered Saline
TE	Tris-EDTA-Buffer
TGF	tumor growth factor
TLR	Toll like receptor

TNF	Tumor Necrosis Factor
TNFR	TNF receptors
TRAF	TNF associated receptor
tRNA	transfert RNA
UV	Ultra Violet
VEGF	vascular endothelial growth factor
Wt	Wild Type

1 - Introduction

1.1 The transcription factor NF-κB

1.1.1 Transcription and transcription factors

Transcription is the cellular process used to convert information encoded within the DNA into RNA, which can then act as the template for protein synthesis or fulfill structural or regulatory functions. The machinery to allow transcription in eukaryotes is extremely complex and well regulated but mainly relies on three different RNA polymerase enzymes. These three enzymes share structural similarities and have some common subunits, although others are unique to the enzyme (Sekine et al, 2012). RNA polymerase I, responsible for 70% of transcription, is specific to the production of large ribosomal RNA (rRNA), whereas RNA polymerase III is in charge of ~10% of transcriptional activity and creates small RNAs such as transfer RNA (tRNA) or the 5S rRNA. The most studied of RNA polymerases is RNA polymerase II, involved in 20% of the cellular transcriptional activity and responsible for the transcription of genes translated into proteins (Sekine et al, 2012).

Usually, a group of proteins called transcription factors binds the DNA on a specific sequence within the promoter or enhancer of a target gene and thereby regulates its expression. An enhancer is a short DNA sequence recognized by transcription factors for regulation of the gene. However, in contrast to a regular promoter, it can be localized at the further distance from the gene it regulates (Arnosti & Kulkarni, 2005). Besides some general transcription factors (TFIIA, TFIIB, TFIID for example) required for accurate basal transcription, sequence specific DNA binding proteins (transcription factors) are required for regulated, gene specific transcriptional activity. Transcription factors can activate or repress transcription via the recruitment or interaction with co-regulator proteins (Sekine et al, 2012). These co-regulators do not bind directly the DNA, but facilitate the transcription factor dependent recruitment of the general transcription machinery, or possess chromatin remodeling functions, to regulate the accessibility of the gene's regulatory regions. Co-regulator proteins can act as co-activators (such as p300 and CBP

(CREB Binding Protein)) or co-repressors (such as Sin3 or TIF1). Transcription factor activity requires extensive regulation, through various mechanisms such as abundance, post-translational modifications, cellular localization, dimerization or proteolytic degradation (Sekine et al, 2012).

1.1.2 Overview of the NF-κB pathway

Nuclear factor κB (NF-κB) was discovered twenty-six years ago as a nuclear protein that binds to the immunoglobulin κ light chain enhancer in B cells (Sen & Baltimore, 1986). NF-κB corresponds to a family of transcription factors, which regulate the expression of a wide variety of genes. Perfect example on inducible transcription factor, NF-κB's main role is in the development and regulation of immune system (Hayden & Ghosh, 2012). NF-κB can also be found in other species such as mouse, dog and chicken. NF-κB has been well studied in *Drosophila melanogaster*, where three subunits have been described, Dorsal, Dif and Relish, which are involved in embryonic pattern formation, immunity, muscle development and hematopoiesis (Govind, 1999). NF-κB can also be found in organisms such as Xenopus (Kao 1991) and Zebrafish (Correa et al, 2004). Furthermore, a homologue of the NF-κB activating protein (NKAP) has been identified in *Caenorhabditis elegans* (C. elegans) although this organism seems to have lost NF-κB subunits (Chen et al, 2003). This broad range of organisms demonstrates that the NF-κB pathway is an ancient and well-conserved evolutionary pathway, which exists in many organisms.

In mammalian cells, the NF-κB family is constituted of 5 members: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (Figure 1.1), which can induce or repress the expression of target genes by binding DNA as homo- or hetero-dimers (Perkins, 2007). The avian viral oncogene v-Rel is also an NF-κB protein (Joyce et al, 2001). In unstimulated cells, NF-κB localizes in the cytoplasm in an inactive complex with proteins from the IκB (Inhibitors of the NF-κB proteins) family (see section 1.1.3). Upon exposure to various stimuli such as cytokines, growth factors or viral products such as tumor necrosis factor (TNF)-α, interleukin-1 (IL-1) or lipopolysaccharide (LPS) or viral proteins (such as Tax or LMP1), IκB is phosphorylated by the IκB kinase (IKK)

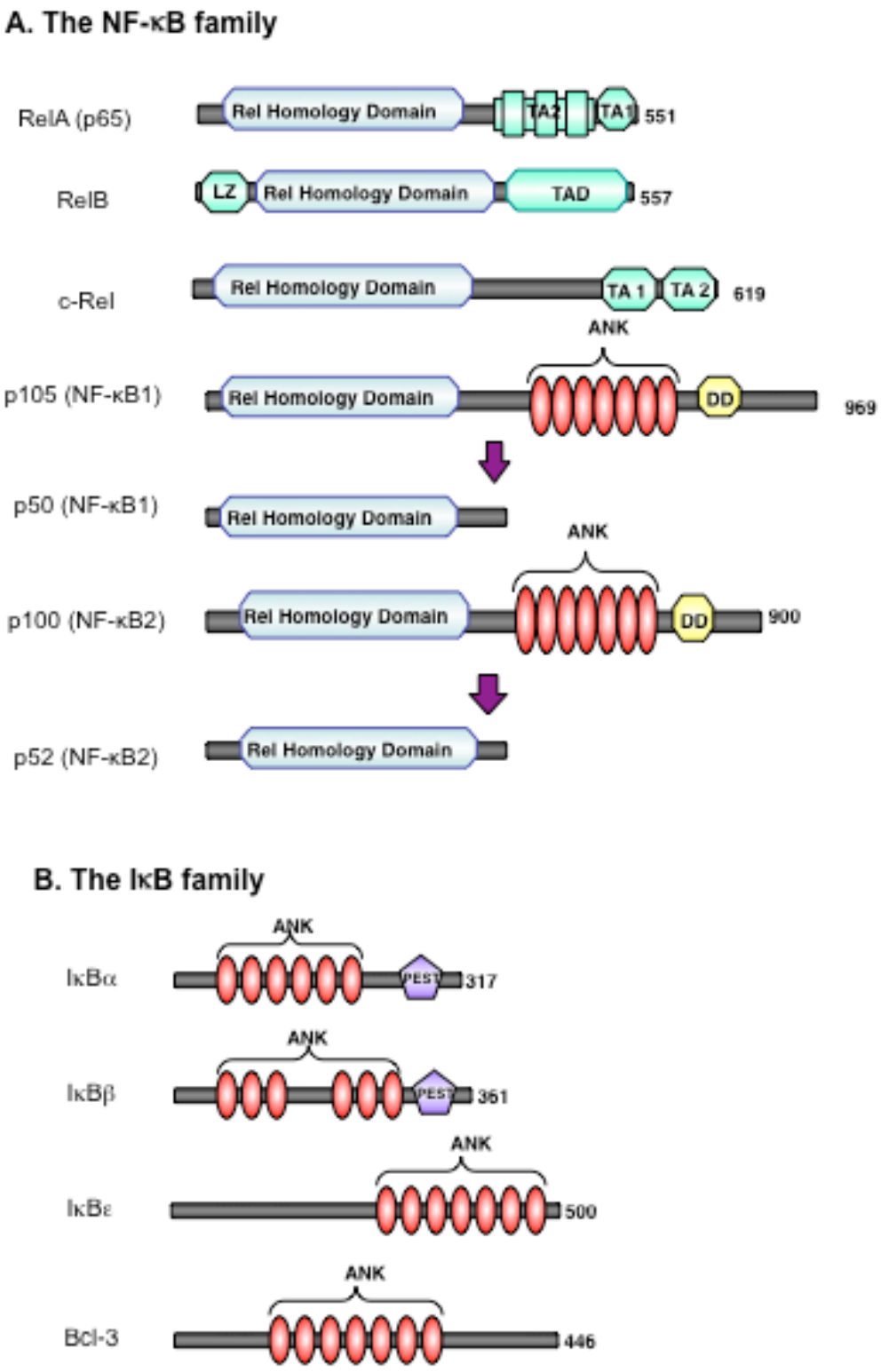


Figure 1-1.1: the members of the NF-κB and IκB families

A. All the members of the NF-κB family contain a Rel Homology Domain, which allows DNA binding and dimerization. RelA, RelB and c-Rel contain, in their C termini, a transactivation domain (TAD), which has sub-domains TA1 and TA2 for RelA and c-Rel. NF-κB1 is processed from p105 into p50 and NF-κB2 from p100 into p52, which results in two shorter proteins lacking the 7 ankyrin repeat domains and the Death Domain (DD).
B. The inhibitor of NF-κB (IκB) family is constituted of IκBα, IκBβ, IκBε and Bcl-3. They all contain ankyrin repeat domains. IκBα and IκBβ have a PEST domain (a domain rich in Proline, Glutamate, Serine, and Threonine). Adapted from (Perkins, 2007)

complex and then degraded, unmasking the nuclear localization signal (NLS) of the NF-κB dimers leading to its activation. NF-κB complexes can be induced by various stimuli such as DNA damage, cell stress, bacterial products, inflammatory cytokines, viral proteins and infection.

NF-κB subunits act as dimers and almost all combinations of dimers are possible. In the literature NF-κB usually refers to the RelA/p50 hetero-dimer, as the most commonly observed complex. RelA homo- and hetero-dimers are responsible for much of the observed NF-κB transactivation activity, due to a strong transactivation domain in RelA (Karin et al, 2002). RelB can have an activating or repressing function (Karin et al, 2002) and can be sometimes associated with p50 (Joyce et al, 2001). However, RelB mainly binds to p100 in the cytoplasm before translocating to the nucleus once p100 is processed to p52 (Karin et al, 2002). RelB/c-Rel association is rare (Joyce et al, 2001) and does not bind to the DNA, as it is the case for RelA/RelB heterodimers and RelB homo-dimers (Joyce et al, 2001; Karin et al, 2002). c-Rel/p50 hetero-dimers are less abundant and have a slower response (Karin et al, 2002). p50/p50 and p52/p52 homo-dimers, as well as p50/p52 hetero-dimers therefore do not act as transcriptional activators by themselves and need the recruitment of co-activators (Hoffmann & Baltimore, 2006). Despite the extensive research, the biological function of different dimers combinations is still unknown.

Several mechanisms have been proposed to explain the processing of p105 into p50. The best described is the constitutive generation of p50 in un-stimulated cells resulting from co-translational proteolysis in the C-terminus of p105 by the 26S proteasome, liberating p50 (Hayden & Ghosh, 2012). Processing of p100 into p52 will be described later. The 26S proteasome is a high molecular weight complex responsible for most of protein degradation by ATP-dependent proteolysis and is constituted of a 20S catalytic core and a 19S regulatory subunit (Frankland-Searby & Bhaumik, 2012).

The NF-κB family is involved in the regulation of thousands of genes, controlling various cellular processes such as the immune and inflammatory responses, cell death or cell survival, stress responses, cell adhesion and proliferation (Perkins, 2004b). NF-κB itself does not have intrinsic enzymatic activity and thus needs to recruit co-activators such as p300 and CBP or co-repressors such as histone deacetylase (HDAC) proteins to its target genes in

order to regulate their transcriptional activity (Hayden & Ghosh, 2012). More than 200 regulators or inducers of NF-κB activity have been described. Constitutive activation or loss of expression of one or several proteins in the κB pathway can cause multiple diseases such as cancer, muscular and neurodegenerative diseases and diabetes (type I and II) (Kumar et al, 2004). So far, NF-κB has been found involved in 79 diseases, many of those resulting from inflammatory responses, such as atherosclerosis and arthritis. Viral infection is also well described to induce a NF-κB response. A growing literature associates dysfunctional NF-κB activity with cancer, as will be discussed later (see section 1.4.2) (www.nf-kb.org).

The NF-κB subunits contain a number of conserved motifs. DNA binding is mediated by a domain called the RHD (Rel Homolgy Domain), which is highly conserved between all NF-κB subunits in different species. The RHD domain is approximately 300 amino-acids and also permits the dimerization of NF-κB sub-units. It contains a nuclear localization sequence (NLS) and allows members of the IκB family to bind to the Rel proteins in order to keep them inactivated. The DNA binding activity of NF-κB dimers via the RHD domains can be controlled by modifications such as phosphorylation, oxidation and acetylation (Perkins, 2006).

The main difference between all the members of the NF-κB family is in their C terminal domains. RelA, RelB and c-Rel have transactivation domains (TAD) able to activate the transcription of NF-κB target genes (Karin et al, 2002). p50 and p52 do not contain a transactivation region and can only efficiently regulate gene expression if complexed with Rel sub-units or other proteins.

The ankyrin (Ank) repeat motif is a sequence of 33 amino acids that mediates specific protein-protein interactions, discovered for the first time in the SW16 protein of *Saccharomyces cerevisiae* (May & Ghosh, 1997). p105 and p100 contain, in their C termini extensions, 7 ankyrin repeat motifs. This domain is also contained within the IκBs and allows the binding to the NLS of NF-κB and consequently its inactivation by sequestration in the cytoplasm (Karin et al, 2002). However, in case of IκBα-RelA-p50 complexes, cytoplasmic sequestration is not absolute, with constant shuttling occuring between the

cytoplasm and nucleus. NF-κB NLS is only partially masked, resulting in the possible nuclear translocation. Once in the nucleus, the nuclear export signal (NES) of IκBα causes the complex to be rapidly exported back to the cytoplasm (Hayden & Ghosh, 2012). In p100 and p105, this ankyrin repeat domain also interacts with the RHD domain in other NF-κB proteins and acts as a IκB-like protein, masking the NLS and sequestering the NF-κB dimers in the cytoplasm (Joyce et al, 2001).

1.1.3 The IκB family

The NF-κB complexes are retained in an inactive cytoplasmic form by a family of inhibitor proteins. This family of IκB proteins includes IκBα, IκBβ, IκBγ, IκBε and Bcl-3 (Figure 1B) (Perkins, 2007). These proteins possess a series of ankyrin repeat motifs that interact with the DNA binding domain of NF-κB dimers, as described above (Joyce et al, 2001). However, the number of ankyrin repeat motifs does not seem to be involved in the mechanism of NF-κB inhibition (May & Ghosh, 1997). IκBγ is a splice variant encoding only the p105 C-terminus, is specific to lymphoid cells and is able to interact only with p105/p50 and p100/p52 homodimers, whereas IκBε is specific to RelA and c-Rel complexes (Whiteside et al, 1997). The N- and C-terminal regions of IκB do not have effects on the ability to bind NF-κB but the deletion of the C-terminal domain inhibits the dissociation of DNA-NF-κB dimers and the repression of DNA binding by NF-κB (May & Ghosh, 1997). Bcl-3, even though it structurally belongs to the IκB family, is nuclear, can bind to p50 and p52 homo-dimers and functions as a transcriptional co-activator (Joyce et al, 2001). The function of the IκB proteins can be independent from NF-κB activation. Atypical members of the IκB family, like Bc-3 or IκBζ, can bind and recruit other co-activators and then affect their transcription response (Ghosh & Hayden, 2008; Hayden & Ghosh, 2012). IκB proteins are generally expressed in every cell type. As NF-κB generates a negative feedback loop by inducing IκBα expression, in the absence of IκBα, the termination NF-κB activation after stimulation by the cytokine TNFα is delayed compared to normal cells (Klement et al, 1996).

Indeed, IκBα contains a nuclear export sequence, which by binding to NF-κB in the nucleus, will return it to the cytoplasm (Perkins, 1997).

IκBα degradation is induced via phosphorylation of the serine residues 32 and 36, which leads to lysine K48-linked ubiquitination at K20 and K21 and degradation via the proteasome. IκBβ and IκBε are activated via similar, conserved serine residues. An alternative mechanism has been described for the activation via IκBα: in response to the re-oxygenation of hypoxic cells, tyrosine Y42 phosphorylation does not induce a proteolytic degradation but leads to the dissociation of IκBα from NF-κB and thus its activation (Perkins, 1997). IκBα can also be responsible for NF-κB inactivation following induction.

Similar to IκBα, upon stimulation IκBε is phosphorylated at Ser157 and Ser161, which induces its degradation by the proteasome (Whiteside et al, 1997). IκBε is believed to be specific for RelA homo-dimers and c-Rel/RelA hetero-dimers. A combined depletion of IκBα and IκBε in mice induces a reduction in splenic natural killer (NK) cells numbers, as well as incomplete NK cell maturation. This is linked to a hyperactivation of NF-κB. However, single depletion of either of these IκB proteins does not affect NK cell production (Samson et al, 2004). This has been confirmed with IκBα and IκBε double deficiency increasing NF-κB activity and inducing neonatal death, associated with a disruption in lymphocyte production (Goudeau et al, 2003).

IκBβ, despite homologies to IκBα, possesses functional differences. IκBβ is subject to slower degradation and resynthesis (Scheibel et al, 2010) and does not affect the kinetic of the NF-κB response, in contrast to IκBα (Hoffmann et al, 2002). In contrast to IκBα, which when complexed with NF-κB dimers, shuttles between the cytoplasm and nucleus, IκBβ-NF-κB complexes stay in the cytoplasm (Scheibel et al, 2010).

Bcl-3 was discovered as a proto-oncogene present in B-cell chronic lymphocytic leukemia. Although it has the structure of an IκB protein, it is localized to the nucleus and acts as a transcriptional co-activator with p50 or p52 homo-dimers. Bcl-3, by association with the p52 protein, forms a functional transcription factor complex (Bours et al, 1993) and binds, for example, to the promoter of the cyclin D1 gene, regulating its expression. But when Bcl-3 levels decrease (e.g. with UV stimulation or activation of p53), the p52/p52 homo-

dimer associates with HDAC1, leading to the repression of cyclin D1 transcription and then a decrease in cyclin D1 expression (Rocha et al, 2003). The activation of transcription by Bcl-3 depends of its localization in the nucleus, which is regulated by ubiquitination (Hayden & Ghosh, 2012). The deubiquitinase CYLD has been shown to promote Bcl-3 deubuquitination, thereby keeping it inactive in cytoplasm. With CYLD deficiency, Bcl-3 accumulates in the nucleus with p50 or p52 dimers, activating transcription of genes such as cyclin D1 (Massoumi et al, 2006). Bcl-3 has also been shown to act as an inhibitor of NF-κB activity, by stabilizing inactive p50 dimers in a DNA bound state on a target gene promoter, blocking the binding of more transcriptionally active dimers (Hayden & Ghosh, 2012). This system of regulation is seen, for example, with the LPS-tolerance. Indeed, Bcl-3 is a negative regulator of Toll-like receptor (TLR) signaling. After LPS stimulation, Bcl-3 associates with p50 complexes negatively regulating target gene transcription, by blocking p50 ubiquitination. In Bcl-3 deficient mice, the absence of Bcl-3-p50 complexes induces hypersensitivity to the TLR response following LPS stimulation. This makes Bcl-3 regulation the basis of LPS-tolerance (Carmody et al, 2007).

IκBζ (also called MAIL or INAP) has a sequence similar to Bcl-3 (Kitamura et al, 2000). Associated with p50 complexes, it activates transcription of target genes such as IL-6 (Hayden & Ghosh, 2012). After stimulation by some stimuli (MyD88- or TRAFF6-dependent signaling pathways), its expression can be induced by NF-κB (Eto et al, 2003). IκBζ does not have a TAD and can act as a regulator for RelA/p50 dimers upon interaction with the p50 subunit (Motoyama et al, 2005).

Another IκB protein, called IκBNS, is less studied. It was first characterized as a promoter of apoptosis in T cells under negative selection but can also be found in various cell lines (Fiorini et al, 2002). IκBNS overexpression inhibits NF-kB in the same manner as Bcl-3. IκBNS expression is induced by IL10 and it binds mainly to p50 and stabilizes p50 homo-dimers at the κB sites (Hayden & Ghosh, 2012).

p105 and p100, in addition to their role as precursors to p50 and p52, have also been described acting as IκB-like proteins. Their interaction with NF-κB dimers requires either direct binding to the RHD of NF-κB proteins or

interaction between the p105 and p100 Ankyrin repeats and preformed dimers (Savinova et al, 2009). Processing of p105 and p100, by losing their IκB-like C terminus, releases specific NF-κB complexes (Xiao et al, 2001). RelB associates mainly with p100 and p52 and binding to p100 stabilizes the complex (Fusco et al, 2008; Solan et al, 2002). Activation and processing of p100 facilitates the exchange on κB sites from the RelA heterodimer to a RelB/p52 dimer during dendritic cell maturation. As different NF-κB dimers have different transcriptional activity, this induces a different response at later time point (Saccani et al, 2003). p100 associates with RelA/p50 dimers in its IκB-like C terminus and regulates their activation in response to non-canonical stimuli such as LTβR signaling (Basak et al, 2007). p100 also retains NF-κB complexes in the cytoplasm, keeping them inactive and influences the pool of NF-κB subunits available. Indeed, an increase in p100/p52 levels will enhance formation of RelB/p52 complexes, whereas an increase in p52/RelA or p52/p50 will induce these “canonical” NF-κB dimers activation (Hayden & Ghosh, 2012).

p105’s IκB-like function was identified as early as 1992 (Rice et al, 1992). p105, after binding to NF-κB dimers, is phosphorylated and constitutively processed to p50 via the ubiquitin-dependent proteasome pathway (Moorthy et al, 2006). p105, to be recognized by the proteasome and proteolytically processed, needs a Glycine Rich Region (GRR) that acts as a stop signal, inducing p50 generation instead of a full degradation of p105 (Hayden & Ghosh, 2012; Xiao et al, 2001). p105 requires a quick processing tp produce active p50 dimers. However, in case of slower processing, the p105 C terminus binds to stabilize NF-κB dimers and maintains them inactive in the cytoplasm, acting as IκB-like protein (Shih et al, 2009).

1.1.4 The IKK family

As discussed above, to release the NF-κB dimers, IκB proteins are phosphorylated by the IκB kinase complex (IKK), which results in their ubiquitination and degradation by the proteasome. The IKK complex constitutes of two catalytic subunits (IKKα and IKKβ) and a regulatory protein NEMO (NF-κB essential modulator, also called IKKγ). The IKKα and IKKβ proteins have distinct functions: IKKβ is involved in NF-κB activation through the canonical

pathway, whereas IKKα is required for the activation of the non-canonical pathway (see below) (Perkins, 2007). After stimulation, IKKα is phosphorylated at serines 176 and 180 by NF-κB inducing Kinase (NIK) (Karin et al, 2002), inducing the processing of p100 to p52 (Senftleben et al, 2001). IKKβ is activated by phosphorylation on serines 177 and 181 by kinases such as the TGF-β activated-kinase1 (TAK1) resulting from TAK1 recruitment following NEMO-ubiquitination (Perkins, 2007).

Recently, new functions for IKKs, independent from NF-κB and IκB, have been discovered, suggesting that IKK proteins could have a more widespread role. Activation of IKKβ promotes survival and proliferation, inducing anti-apoptotic (Tang et al, 2001) and proliferative pathways (Widera et al, 2006) in response to TNF-α. For example, IKKβ can also inhibit apoptosis and promote cell growth by phosphorylation and inhibition of the tumour suppressor FOXO3 (Hu & Hung, 2005; Tezil et al, 2012). This promotes tumorigenesis and enhances chemoresistance (Hu & Hung, 2005), by inducing FOXO3 sequestration in the cytoplasm after cisplatin treatment in breast cancer cells (Tezil et al, 2012). IKKβ can also activate the MAP kinase (MAPK) pathway, promoting the cell proliferation. This is achieved by inducing the proteolytic degradation of p105 (Perkins, 2007), a subset of which is associated with the Tpl2 kinase, which upon p105 degradation is released, leading to MAP kinase activation (Perkins, 2007).

IKKα also has a variety of other substrates. IKKα is notably able to regulate cyclin D1 expression, resulting in effects on cell proliferation, independently of NF-κB. After UVB stimulation, IKKα activates the p38 and ERK MAPK pathways, which lead to repression of cyclin D1 transcription by ERK1/2 in addition to p38-dependent proteolysis of cyclin D1 (Song et al, 2010). By contrast, IKKα is also able to induce its expression, so promoting cell proliferation. Indeed, IKKα associates with oestrogen receptor α (ERα) and is then able to activate transcription of oestrogen-responsive genes such as cyclin D1 or c-myc in breast cancer cells (Park et al, 2005). IKKα can also regulate chromatin structure through association with the co-activator complex or by functioning as a histone H3 kinase (Perkins, 2007).

1.1.5 Canonical pathway of NF-κB activation

Although various pathways have been shown to activate NF-κB, the two most common and best described are: the canonical (classical) and the non-canonical (alternative) pathway. The first to be described here is the canonical pathway and results in the degradation of IκBα and release of RelA/p50 or c-Rel/p50 heterodimers, which translocate to the nucleus, where they regulate the transcription of target genes (Perkins, 2007) (Figure 1.2). Upon stimulation by pro-inflammatory cytokines the IKK complex is activated and phosphorylates IκBα on the serine 32 and 36 residues. This leads to IκBα polyubiquitination on Lysine 21 and 22 residues by the SCF (Skp1/Cul1/F-box) ubiquitin ligase family, resulting in its degradation by the proteasome (Karin et al, 2002), (Perkins & Gilmore, 2006b). The canonical pathway allows a fast and transient response to various stimuli, independent of protein synthesis (Sun, 2011).

NF-κB classical activation is mainly dependent on IKKβ (Senftleben et al, 2001). By contrast, IKKα contributes to NF-κB activation but does not seem indispensable for the resistance to apoptosis (Baldwin, 2012). To terminate canonical pathway activation, NF-κB induces a negative feedback loop involving the transcription of target genes such as IκBα and A20. Resynthetized IκBα binds NF-κB dimers and promotes their translocation to the cytoplasm as an inactive dimer (Perkins & Gilmore, 2006b). In parallel, induction of A20 deubiquitinase expression results in inhibition of IKK activity (Krikos et al, 1992). A20 interacts TRAF6 and RIP1, inactivating IKK complexes and thus stopping IκBα degradation, leading to NF-κB inactivation (Shembade & Harhaj, 2012).

Although the mechanism for NF-κB translocation to the nucleus is not yet fully described, it has been suggested to result from the binding with karyopherins, proteins responsible for transport between the nucleus and cytoplasm (Rothwarf & Karin, 1999). In 2005, Fagerlund et al demonstrated that RelA/p50 heterodimers and p50/p50 homodimers could be imported to the nucleus by the importin α3 and α4 proteins. Importin α3 and α4 are members of the importin α family, constituted of 6 members: α1 (hSRP1α), α3 (Qip1), α4 (hSRP1γ), α5 (NPI1), α6 and α7 (Fagerlund et al, 2005). Importin α proteins contain armadillo repeat motifs that recognize NLS sequence of target proteins, and form a heterodimer with the importin β protein that will enable nuclear

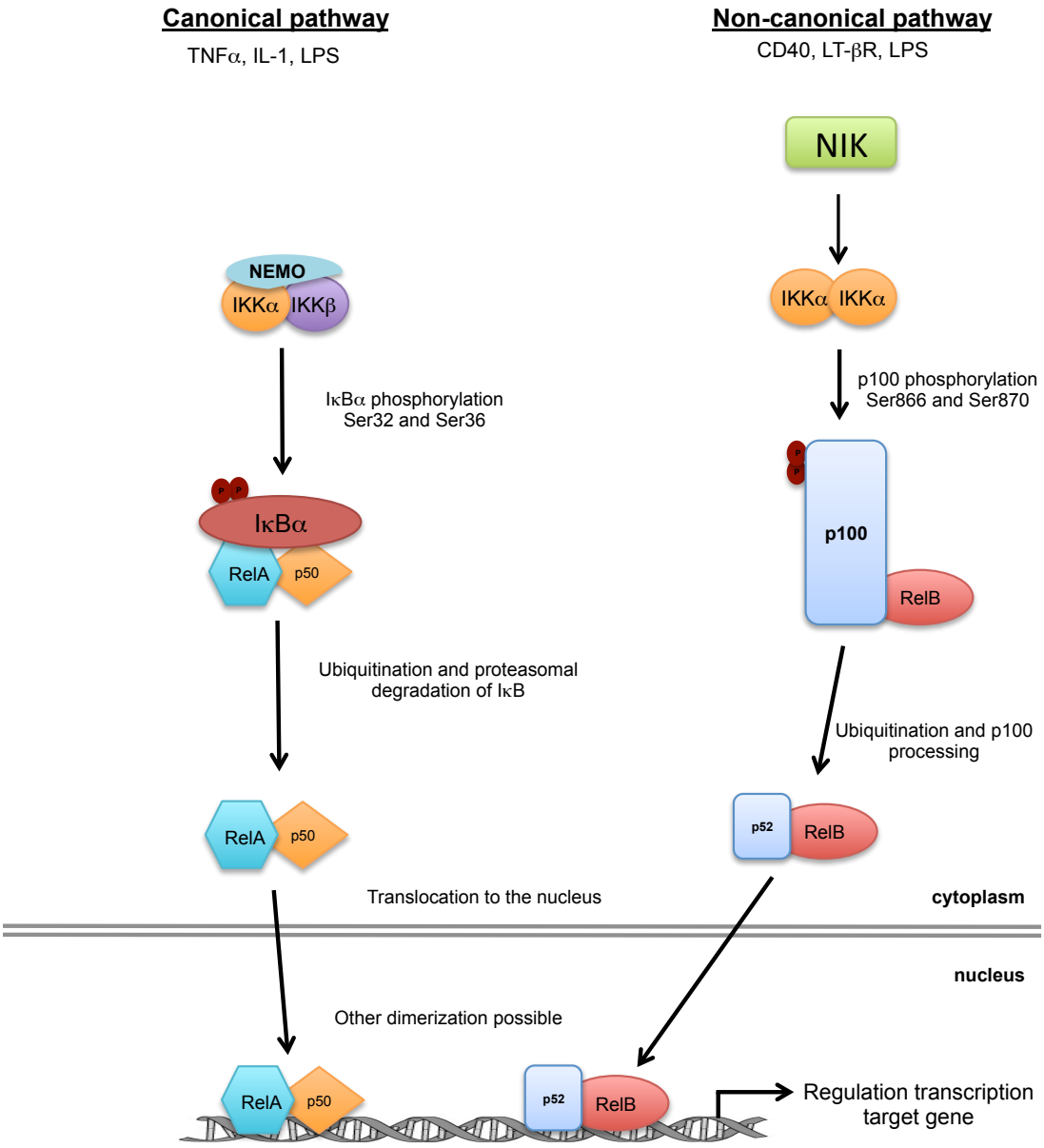


Figure 1-2: Canonical and non-canonical pathways for NF-κB activation

Schematic overview of the canonical and non-canonical pathways leading to NF-κB activation. The canonical pathway (left side) leads to the activation of RelA-p50 heterodimers. After stimuli such as TNFα or IL-1, the IKK complex containing NEMO, IKKα and IKKβ phosphorylates IκBα on Ser32 and Ser36, inducing ubiquitination and proteasomal degradation of IκBα, thus releasing RelA-p50 dimers, which then translocate to the nucleus to regulate the target gene transcription. The non-canonical pathway (right side) is responsible for p100 processing and p52 activation. Following stimuli by CD40 or LPS, NIK phosphorylates a IKKα complex. These kinase then phosphorylate p100 on Ser866 ad Ser870, leading to its ubiquitination and p100 processing into p52. p52, forming a dimer with RelB, translocates to the nucleus and then can regulate the transcription of target gene. Other combinations of NF-κB dimerization than RelA-p50 or RelB-p52 are also possible.

translocation of the target proteins. Importin α3 directly binds to the RelA and p50 NLS region and facilitates nuclear translocation only if NF-κB complexes are free from IκBα (Fagerlund et al, 2005).

Aberrant activation of the canonical NF-κB pathway is critical for lymphocyte proliferation and survival (Jost & Ruland, 2007) and mediates tumorigenesis following inflammation in animal models (Pacifico & Leonardi, 2006; Pikarsky et al, 2004).

1.1.6 Activation of the non-canonical NF-κB pathway

The second NF-κB activation pathway, called the non-canonical or alternative pathway, results in the processing of p100 to p52 and release of p52/RelB hetero-dimers (Figure 1.2). Discovered about 10 years ago, it has been the subject of extensive research, as it is significantly different from the classical pathway in the composition of IKK complexes and the signaling mechanisms involved (Perkins & Gilmore, 2006b; Sun, 2011). As it is also the case for the classical pathway, the non-canonical NF-κB activation pathway can be aberrantly activated in many tumours. NF-κB subunits can be mutated or aberrantly active in tumours, and NF-κB2, is particularly associated with a number of lymphomas, notably through chromosomal translocation that generates a truncated constitutively active form of the protein (Karin et al, 2002).

Stimuli known to induce the non-canonical pathway include CD40 ligand (Coope et al, 2002), Lymphotoxin β Receptor (LT-βR) (Dejardin et al, 2002), LPS (Mordmuller et al, 2003), viral proteins including Latent membrane protein (LMP1) from the Epstein Barr Virus (Atkinson et al, 2003) and the transcription factor Stat3 (Nadiminty et al, 2006). Although most of these stimuli are specific for the non-canonical pathway, some are also able to activate NF-κB via the classical pathway. After stimulation by these inducers, IKKα complexes are activated by phosphorylation on Ser177 and Ser188 via NIK (Karin et al, 2002; Senftleben et al, 2001). The exact composition of IKKα complexes is still unknown but previous studies demonstrated no requirement for IKKβ or NEMO (Perkins, 2006). Activated IKKα complexes are involved in p100 processing, mechanism described later (see section 1.1.7.).

Among the stimuli for the non-canonical pathway (Table 1.3), a common characteristic is the role of TNFR superfamily members. TNFR superfamily members, also called Death Receptors, are cytokine receptors binding signals. It cooperates with adaptator protein such as TRAF or TRADD and can induce various responses depending on the stimuli (survival, cell proliferation, apoptosis, etc.) (Sun, 2011).

- The CD40 ligand is a TNF family member expressed on various cell types such as B cell, dendritic cells or endothelial cells. It is important for the promotion of cell survival and homotypic B cells aggregation, which is dependent upon stimulation of p100 processing (Coope et al, 2002; Sun, 2011).

- The LTβ receptor is a TNFR superfamily member expressed in myeloid and epithelial cells and is specific for the lymphotoxin and LIGHT ligands (discussed later, see section 1.1.12). Its activation leads to the induction of a number of cytokines and secondary lymphoid organogenesis of the spleen, lymph nodes and Peyers patches (Dejardin et al, 2002).

- The RANK receptor, with its ligand RANKL (Receptor activator of NF-κB Ligand) activates p100 processing to regulate osteoclast differentiation (Novack et al, 2003), suggesting a role for the non-canonical NF-κB pathway in bone formation. This has been confirmed with the demonstration of IKKα involvement in skeletal, tooth and skin development using knockout mice (Hu et al, 2001; Ohazama et al, 2004; Takeda et al, 1999). Interestingly, the same phenotype was observed in *nfkb1/nfkb2* double knockout mice (Gerondakis et al, 2006).

The regulation of the non-canonical pathway is dependent on the regulation of NIK levels. Indeed, NIK is naturally low (and maintained as such by TRAF3) in most cells. De novo protein synthesis is then required to produce and accumulate NIK protein (Sun, 2011). In contrast to the classical pathway, whose main feature is the rapidity and temporary nature of its response, activation of the non-canonical pathway is slow and persistent and can be described as context-specific, in response to TNFR stimuli such as CD40 (Coope et al, 2002), LTβR (Dejardin et al, 2002) or RANK (Novack et al, 2003).

Name	Receptor/Mediator	Cell type	References
BAFF	BAFF Receptor	Splenic B cells	(Coope et al, 2002)
BLyS	BAFF R3 (BR3)	Diffuse, large B cell lymphoma	(Pham et al, 2011)
CD30L	CD30	Lymphoma cell lines	(Nishikori et al, 2005)
CD40L	CD40 / TRAF	Murine splenic cells	(Coope et al, 2002)
CD70	CD27	Peripheral Blood Mononuclear cells	(Ramakrishnan et al, 2004)
Human β-defensin-3	TLR 1/2	Human monocytes	(Funderburg et al, 2011)
IFN	TRAF2	Human fibrosarcoma cells	(Yang et al, 2005)
LPS	TLR4	Human dendritic cells B cells	(Saccani et al, 2003); (Mordmuller et al, 2003)
LTβ	LTβ-receptor TRAF3 LIGHT	MEF; Hela Colorectal adenocarcinoma cells Endothelial cells	Dejardin 2002; (Mordmuller et al, 2003); (Bista et al, 2010)
Notch	Notch receptor	Lymphoma cells	(Schwarzer et al, 2012)
RANKL	RANK	Osteoclast precursors	(Novack et al, 2003)
Stat3	N/A	Breast cancer cell lines	(Nadiminty et al, 2006)
TWEAK	TRAF2/TRAF5	MEF	(Saitoh et al, 2003)
TNF-α	TNFR1 / RIP1 TNFR2	Human dendritic cells MEF T cells	(Saccani et al, 2003); (Kim et al, 2011) (Rauert et al, 2010)
Viral products			
LMP1	TRAF2/3	B cells, epithelial cells MEF	(Atkinson et al, 2003); (Song et al, 2010)
Tax	N/A	T cells	(Xiao et al, 2001)
vFLIP K13	N/A	PEL cell lines	(Matta & Chaudhary, 2004)

Table 1-3: Stimuli inducing p100 processing to p52

List of stimuli known to activate the NF-κB non-canonical pathway and induce p100 processing to p52

1.1.7 Processing of p100 in p52

The NF-κB2 protein is produced as the precursor p100 but needs to be processed to a shorter version, p52, to be activated and trans-located to the nucleus. This p100 processing, in addition to producing p52, removes the IκB-like activity of p100, leading to the nuclear translocation of RelB/p52 complexes (Sun, 2011). By contrast to p105, whose degradation to produce p50 happens at a high rate constitutively, p100 processing is relatively slow and mainly inducible. To process p100 to p52, stable dimers have to be formed. If p100 is not able to dimerize, it is completely degraded, with a significant reduction in p52 formation (Betts & Nabel, 1996). The activation of IKKα by various stimuli leads to the phosphorylation of p100 on serines 866 and 870, resulting in the creation of a binding site for β-TrCP, a component of the SCF E3 ubiquitin ligase. Mutations on one or both of the Serine 866 and 870 residues completely inhibit p100 (Sun, 2011). This site is homologous to the ones found in IκBα and p105 (Perkins, 2006). Binding with β-TrCP induces p100 K48 linked ubiquitination, on Lysine 856 at the N-terminal region of the β-TrCP binding site (Amir et al, 2004). This Lysine 856 residue is analogous to the ubiquitination site of IκBα (K22), and mutation of K856 decreases p100 ubiquitination and processing (Sun, 2011).

NF-κB Inducing Kinase (NIK) has been shown to induce p100 processing to p52 via stimulation of site-specific phosphorylation and then ubiquitination. NIK is naturally mutated in alymphoplasia and *nik* -/- or *nfkb2* -/- mice present the same immune deficiencies (Xiao et al, 2001). NIK was first described as a MAP kinase kinase kinase (MAP3K), which interacts with the TNF receptor associated factor 2 (TRAF2). NIK phosphorylates IKKα in its activation loop, activating the phosphorylation and processing of p100 (Sun, 2011; Xiao et al, 2001). The post-ubiquitination events leading to p100 cleavage to p52 and not full degradation are yet to be fully understood, although some elements are described. p100 ubiquitination triggered by NIK induces its recruitment to the proteasome via the interaction of the p100 Death Domain (DD) with S9, a non ATPase subunit located in the lid of the 19S proteasome (Fong et al, 2002b; Sun, 2011). Even though the final mechanism for p100 processing remains unclear, Fong et al demonstrated in 2002 that the DD is essential for NIK-induced p100 processing, making a further link between the processing and S9

binding (Fong et al, 2002b). Disruption of the Ankyrin repeat domain or the DD induces constitutive processing of p100 (Liao & Sun, 2003). It is still unknown how the DD and Ankyrin repeats inhibit p100 processing but one hypothesis is that through the Ankyrin repeats interacting with the RHD, p100 adopts a conformation that blocks processing by masking p100 NLS (Sun, 2011).

Heusch demonstrated that inhibition of the proteasome by agents such as MG115 blocks p52 generation (Heusch et al, 1999). Recent results show that p100 sumoylation is involved in ubiquitination and processing (Vatsyayan et al, 2008). SUMO (for Small Ubiquitin-like Modifier) modifications are reversible and SUMO proteins can be removed by SUMO-specific proteases. In some human cell lines and mouse fibroblasts, a small quantity of p100 is naturally found conjugated to SUMO1 and a mutation in the sumoylation site decreases inducible processing. Moreover, basal sumoylation has been found to be necessary for p100 phosphorylaton but the detailed mechanism of how SUMO proteins regulate p100 processing still has to be fully understood (Vatsyayan et al, 2008).

An additional domain, the Glycine Rich Repeat Region (GRR), from amino acids 346 to 377, acts as a “stop-transfer signal” and is also necessary for the cleavage, which happens after the amino acid 405. This GRR, also found in p105, has been shown to be essential for p105 processing in p50 (Lin & Ghosh, 1996). The poor efficiency of p100 processing compared to p105 can also be explained by the peptide sequence around the cleavage site, situated downstream GRR and responsible for p52 production, and rather than the GRR itself (Heusch et al, 1999).

As mentioned above, constitutive p100 processing is extremely low. p100 contains a domain, called the Processing-Inhibitory Domain (PID), situated downstream the Ankyrin repeat region, in the same region than DD (amino acids 777 to 859), which negatively regulates p100 processing to p52 (Xiao et al, 2001).

1.1.8 Other NF-κB activation pathways

In addition to the canonical and non-canonical NF-κB pathways, some atypical activation mechanisms have been described, although their efficiency

is slower and weaker than the classical pathways (Rothwarf & Karin, 1999). These atypical pathways, in response to stimuli such as genotoxic stress, UV or hypoxia can be both IKK dependent or independent and usually activate the "classical" NF-κB complex of RelA/p50 hetero-dimers (Perkins & Gilmore, 2006b).

After genotoxic stress resulting from treatment with drugs such as etoposide or exposure to ionizing radiation, NEMO protein alone translocates to the nucleus where it is sumoylated. There, the sumo modification is replaced by mono-ubiquitination at the same site in an ATM (ataxia telangiectasia mutated)-dependent manner before relocation back to the cytoplasm, where together, NEMO and ATM activate IKK complexes. However, the cell type distribution of this activation pathway remains unclear (Perkins, 2007).

Different mechanisms have been described for the atypical activation of NF-κB in an IKK-independent manner, After stimulation by ultra-violet (UV) light or expression of the Her2/Neu oncogene, the C terminus of IκBα can become phosphorylated by the casein kinase II (CK2) leading to its degradation (Perkins & Gilmore, 2006b), activating NF-κB dimers. After UV stimulation, IκBα phosphorylation by CK2 is dependent on the p38-MAP kinase pathway. Inhibition of the p38-MAP pathway increases UV-induced cell death, demonstrating its importance during the UV-stress response (Kato et al, 2003). In 2010, Tsuchiya et al showed that after UV irradiation, IκBα degradation happens after phosphorylation in its C terminus PEST domain by CK2, through interaction with p38. IKKβ is required for UV-induced IκBα proteolysis and NF-κB activation but not its kinase function. In this particular case, IKKβ acts as an adaptor protein for IκBα degradation, as it can associate either with β-TrCP to promote N terminal ubiquitination of IκBα or with the kinases CK2 and p38 to promote its degradation through C terminal phosphorylation (Tsuchiya et al, 2010).

Amplification or overexpression of the Her2/Neu oncogene is altered in 30% of breast cancers (Pianetti et al, 2001) and results in PI3K-Akt activation of CK2, which phosphorylates IκBα, inducing its degradation by calpain (Romieu-Mourez et al, 2002). This IKK-independent NF-κB activation can be blocked by

the receptor or tumor suppressor PTEN in breast cancer cells (Pianetti et al, 2001).

IκBα can also be phosphorylated on Tyr42 by the c-Src kinase after hypoxia/reoxygenation and hydrogen peroxide stimulation, inducing its degradation (Perkins & Gilmore, 2006b; Rothwarf & Karin, 1999). Surprisingly, in bone marrow cells, NF-κB activation after TNF-α stimulation comes from this Tyr42 phosphorylation and atypical pathway, and not from the canonical pathway (Abu-Amer et al, 1998).

Finally, late time point activation of NF-κB can occur in response to drugs such as doxorubicin in an IKK-independent manner but the exact mechanism still has to be elucidated (Perkins & Gilmore, 2006b; Tergaonkar et al, 2003).

1.1.9 DNA binding

To regulate their target genes, NF-κB subunits bind to a specific DNA sequence of 9-11 base pairs called a κB site: GGG RNN YYC C, where R is a purine (adenine or guanine), N can be any base pair and Y represents a pyrimidine (cytosine or thymidine) (Natoli et al, 2005). Although this sequence is considered as the classical κB site, it is highly degenerate, which considerably increases the number of κB sites possible in the genome. For example, c-Rel homo-dimers show a high affinity for the sequence AGA AAT TCC found in the CD28 response element of IL2 enhancer. RelB/p52 hetero-dimers bind specifically on the κB site RGG AGA YTT R on genes encoding chemokines such as CXCL13 or CCL19 (Natoli et al, 2005). RelA/p52 hetero-dimers show specificity for sequences such as GG RNN YYC C (Perkins, 1997). The p50 subunit has a high affinity for the κB sites GGG ACT TTC C (for the Ig κ chain enhancer and HIV-1 LTR) and GGG ATT CCC C in the MHC class I H2-K enhancer, whereas p52 only recognizes the latter and RelA homo-dimers have a very low affinity for both. By contrast, RelA binds preferentially to sequences ending with TTT CC, such as the palindrome GGA AA TTC C (Perkins, 1997). Alteration of the κB consensus binding site by mutation causes changes in the structure and affinity of the site therefore attracting different NF-κB dimers (Natoli et al, 2005). This specificity of binding has the potential to allow genes with variant κB sites to be regulated by different NF-κB complexes, although the

binding of a specific NF-κB complex is not encoded in the κB site itself (Hoffmann et al, 2003).

Natoli reported an estimation of 1.4 x 10^4 potential κB sites in the human genome but admitted that this was probably inaccurate and the final number should be closer to 1.5 x 10^5, although not all would be localized in promoters (Natoli et al, 2005). This large variability of potential κB sites, which can differentially respond to different stimuli, has yet to be fully understood and explained. However, work in *Drosophila melanogaster* has suggested one possibility (Minakhina & Steward, 2006). In this species, sites with weak affinity were found to require a high quantity of Dorsal, whereas κB sites with a high affinity only required a low quantity of protein available. Since, different quantities of NF-kB subunits are found in the various organs of Drosophila, therefore a difference in affinity will induce a different response (Minakhina & Steward, 2006).

1.1.10 Post transcriptional modifications of NF-κB

Multiple post-transcriptional modifications that regulate NF-κB activity have been described. For example phosphorylation of RelA on S276, which can be carried out by PKA, MSI or MSII, is required for the interaction with the p300/CBP complex and the activation of transcription (Hayden & Ghosh, 2012). IKKα and IKKβ act as kinases for RelA and phosphorylate it on S536 (Sakurai et al, 1999). Interestingly, RelA S536 phosphorylation, occurring mainly in the cytoplasm (Moreno et al, 2010), allows transcription of specific target genes, such as IL-8, in cells stimulated by PMA and ionomycin (Buss et al, 2004; Sasaki et al, 2005). S536 phosphorylation is independent of IκBα (Sasaki et al, 2005) but can be dependent on IKKβ in human multiple myeloma cells upon HDAC inhibitor stimulation (Dai et al, 2011). This promotes translocation to the nucleus and enhances RelA acetylation (Dai et al, 2011). RelA S536 can be used as a prognostic marker for colorectal cancer (Lewander et al, 2012) and is linked to epileptic vulnerability in rat (Ryu et al, 2011). Phosphorylation of T505 by the checkpoint kinase Chk1 inhibits RelA transactivation activity, which leads to the repression of the anti-apoptotic gene Bcl-xL by inducing association with HDAC1 (Rocha et al, 2005b). This T505 phosphorylation is maximal during the S phase of the cell cycle, as Chk1 is activated by the DNA replication

checkpoint (Barre & Perkins, 2007). Interestingly, RelA S536 modification can be linked to the cell cycle. In unstimulated U20S cells, this phosphorylation occurs specifically during the G2 phase of the cell cycle (S536 phosphorylation is low during G1 phase), whereas IKKβ is also responsible for RelA Ser468 phosphorylation in G1 phase. This change of phosphorylation sites during the cell cycle is thought to be involved in the switch from RelA dependent activation to repression of target gene expression (Barre & Perkins, 2007).

RelA can also be subject to many other modifications, such as acetylation on lysine residues such as K310 by the p300 and CBP co-activators. However, this only occurs if S276 is phosphorylated, making the S276 residue mandatory for HAT recruitment (Hayden & Ghosh, 2012; Zhong et al, 2002).

Only a few phosphorylation sites (and no other modifications) have so far been found in the RelB subunit. Phosphorylation on T84 and S552 initiates RelB degradation by the proteasome (Marienfeld et al, 2001). RelB S368 phosphorylation, not required for nuclear translocation, is involved in RelB/p100 dimerization (Maier et al, 2003). Cells expressing a non-phospho mutant of S368 RelB displayed a shorter p100 half-life compared to wild-type RelB, suggesting than RelB is involved in regulating p100 processing (Maier et al, 2003). Phospho-proteomic studies identified a few more phosphorylation sites on the Ser37 and Ser73 residus (Dephoure et al, 2008; Olsen et al, 2010). Some lysine residus (K295, 297, 327 and 330) have been involved in the regulation of RelB activity and stability (Leidner et al, 2008).

The post-transcriptional modifications described to date for p100/p52 have been shown to mainly regulate p100 processing to p52 and can be found described in section 1.1.7. It has also been shown that Ser866 phosphorylation, required for p100 processing, is inhibited in S phase and is dependent on IKKα for G1 and G2 phase basal phosphorylation (Barre & Perkins, 2007). It can also be induced by DNA damaging agents such as cisplatin or UV in U2OS cells (Barre et al, 2010c). In addition, some phospho-proteomic studies found additional p100/p52 phosphorylation sites during mitosis (figure 1.4), on serine residues 22, 23, 222, 226, 429, 759, 762, 811, 816 (Dephoure et al, 2008; Olsen et al, 2010).

Modification sites on p100/p52:

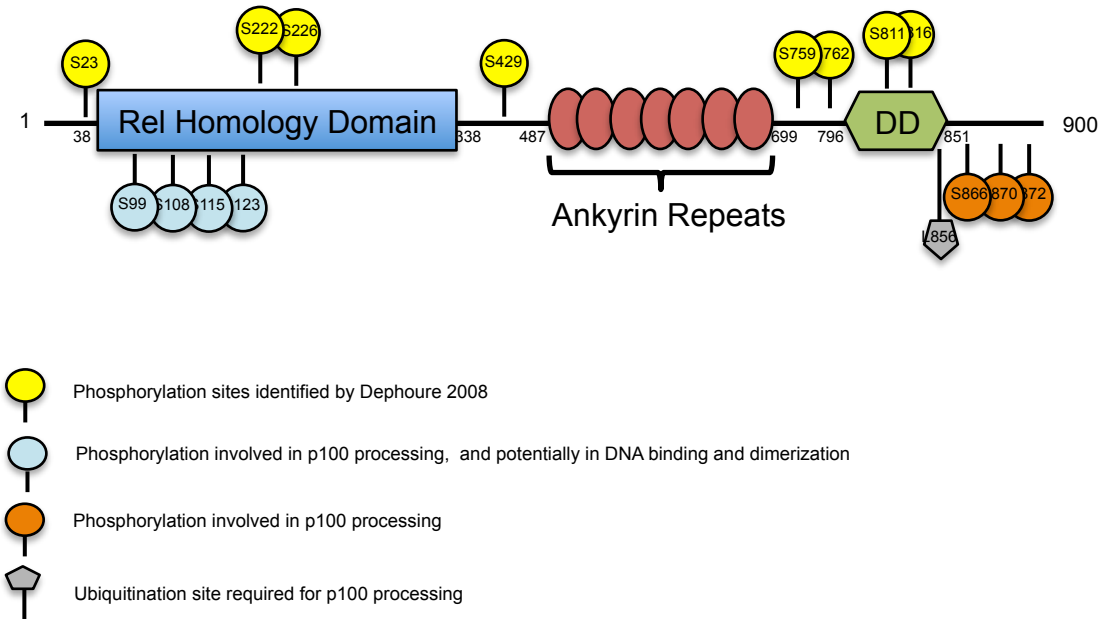


Figure 1-4: Modification sites in p100/p52

Schematic representation of the modifications sites identified in p100/p52. The phosphorylation sites that are targets of IKKα, required for p100 processing are represented in orange. In grey is the ubiquitination site in the β-TrCP binding site that is required for processing. The mitotic phosphorylation sites identified by (Dephoure et al, 2008) are represented in yellow. Adapted from (Perkins, 2006).

Although the full functionality of these phosphorylation sites is not yet studied, mostly due to the recentness of the reports, some elements are known. For example Olsen et al suggested possible kinases for some phosphorylation sites: Ser23 is part of a motif for the kinase CK2, while Ser816 might be target for the kinases CK1 or GSK3β (Olsen et al, 2010). Interestingly, our laboratory also published in 2010 that GSK3β is responsible for Ser222 phosphorylation, regulating the p52 dependent activation or repression of the Skp2 promoter and p52 dimerization (also discussed later) (Barre & Perkins, 2010). This Ser222 phosphorylation by GSK3β would require primary phosphorylation at Ser226, but the kinase involved is still unknown (Barre et al, 2010c) Some further analysis will be necessary to fully understand the influence of p52 phosphorylation sites and how post-transcriptional modifications affect the NF-κB response (Barre & Perkins, 2010b).

Modifications of the c-Rel and p105/p50 subunits have been reported (Perkins, 2007) but they will not be discussed here, as they have not been linked to the cell cycle, the major topic of this thesis.

1.1.11 NF-κB target genes

In the past 25 years of research on NF-κB, hundreds of target genes have been identified and described. These can be categorized as 14 types of target genes: chemokines/cytokines and modulators, immunoreceptors, proteins involved in antigen presentation, cell adhesion molecules, acute phase proteins, stress response genes, cell surface receptors, regulators of apoptosis, growth factors/ligands and modulators, early gene responses, transcription factors and their regulators, viruses, enzymes and others (www.nfkb.org). However, in 2002, Karin suggested that most of these could be grouped and proposed the existence of only four types of target genes for NF-κB (Karin et al, 2002). The first group concerns genes involved in cell proliferation, such as Skp2 (Schneider et al, 2006) or cyclin D1 (Schumm et al, 2006). The second includes target genes involved in the regulation of apoptosis such as Bcl-2 (Catz & Johnson, 2001) or Bcl-xL (Chen et al, 1999). The third group contains genes with an immunoregulatory function and that are involved in the inflammatory response, such as TNF-α (Shakhov et al, 1990) or IL-6

(Libermann & Baltimore, 1990). The final group of NF-κB target genes is those actually involved in the positive, such as RelB (Bren et al, 2001) or negative regulation of NF-κB activity, which includes IκBα (Sun et al, 1993), *nfkb1* and *nfkb2* (Lombardi et al, 1995; Ten et al, 1992) and A20 (Shembade & Harhaj, 2012).

Although these hundreds of NF-κB target genes, only some are known to be p52 specific target genes. p52 can regulate the expression of genes having a role in cell proliferation, such as Cyclin D1 (Barre & Perkins, 2007; Schumm et al, 2006). NF-κB and specifically p52 protein/protein interactions have been described in abundance in the literature but will not be discussed here.

1.1.12 Opposing roles for NF-κB subunits

As more studies about NF-κB are published, it is becoming clearer that NF-κB dimers can act as activators or repressors of the same target genes, depending on the conditions (Campbell & Perkins, 2004b).

The first reports of NF-κB dependent repression of transcription concerned RelA regulation of apoptosis. Following activation via the canonical pathway, in response to stimuli such as TNF-α, LPS or ionizing radiation, RelA/p50 complexes associate with co-activator complexes containing p300/CBP, which leads to the induction of anti-apoptotic genes and promotes cell survival (Sheikh & Huang, 2003). However, it has been shown that following stimulation by UV-C, drugs such as daunorubicin, doxorubicin, cisplatin or activation by the tumour suppressor ARF, RelA complexes associate with the co-repressor HDAC1 and induce the repression of anti-apoptotic genes, promoting cell death (Campbell et al, 2004; Rocha et al, 2003).

Previous works in our laboratory proposed a switch in function from proto-oncogene to tumour suppressor-like activity for the p52 subunit, depending on its phosphorylation at Ser222 after DNA damage (Barre & Perkins, 2010). It is however important to note that p52 has not yet been identified as a tumor suppressor but its behavior can be consistent with a tumor-suppressor-like function. After stimulation by cisplatin in U2OS cells, Akt is active, which inhibits GSK3β activity and thus allows the formation of unphosphorylated Ser222 p52/Bcl-3 complexes. These complexes, with the

help of p53, increase Skp2 (S-phase kinase associated protein 2) expression, repressing $p27^{kip1}$ and ultimately suppressing autophagy (Barre & Perkins, 2010). By contrast, in UV-C stimulated cells, the inactivation of Akt allows GSK3β to phosphorylate p52 at Ser222, disrupting p52/Bcl-3 complexes and enabling the formation of p52/c-Rel complexes, which repress Skp2 expression in association with HDAC1, increasing cell death via autophagy (Barre & Perkins, 2010). Barre et al showed in 2010 that this p52 duality does not only apply to autophagy but can also be found in the regulation of genes involved in the cell cycle and cell death (Barre et al, 2010c). This was illustrated with the cyclin D1 promoter, where p52 can change from an activator to a repressor, depending on the induction of p53 (Rocha et al, 2003b; Schumm et al, 2006).

Similar results demonstrating the duality of NF-κB function have been reported by other laboratories. For example, another study recently illustrated the dual role of the RelB subunit. This showed that the non-canonical pathway could be activated by the binding to the LTβ receptor of LIGHT, a member of the TNF super-family. This led to the activation of RelB/p52 dimers and the regulation of various genes, including the chemokine pro-inflammatory CXCL12. LIGHT ultimately leads to the activation of CXCL12. However, TNF-α upregulates p100 and RelB expression through the canonical pathway. This activation of RelB by TNF reduces the basal expression of CXCL12, inhibiting the upregulation of CXCL12 by LIGHT via RelB (Madge & May, 2011).

In 2006, Strozyk et al demonstrated that NF-κB's anti-apoptotic or pro-apoptotic role relies on the type of DNA damage. When epithelial carcinoma cells were irradiated with UV-B, NF-κB was activated by IL-1, thus inducing an increase in TNF-α expression followed by an increase of apoptosis (Strozyk et al, 2006). By contrast, in the case of DNA strand breaks, no IL-1-dependent NF-κB activation occurred, and the usual anti-apoptotic role for NF-κB ensued (Strozyk et al, 2006). This difference in NF-κB activation was confirmed in another study. Here, DNA damage inducing agents such as aphidicolin or hydroxyl-urea induced ATM activation, which induced pro-apoptotic NF-κB (Habraken 2006). By contrast, replication stress had also been shown to activate the ATR (ATM- and Rad3- related) kinase, which was shown to compete with ATM and associate with but not activate NEMO, thus delaying NF-κB activation (Wu & Miyamoto, 2008b). However, in this study replication

stress was still found to lead to a pro-apoptotic role for NF-κB, notably through the repression of the anti-apoptotic gene Bcl-xL, whereas double strand breaks induced the anti-apoptotic role of NF-κB. The differences in the NF-κB response to DNA damage reported in different studies likely result from changes in cell context. For example, the growth conditions used and the activity of parallel signaling pathways such as p53. A definitive explanation for what regulates the outcome of NF-κB activation after DNA damage has still to be provided.

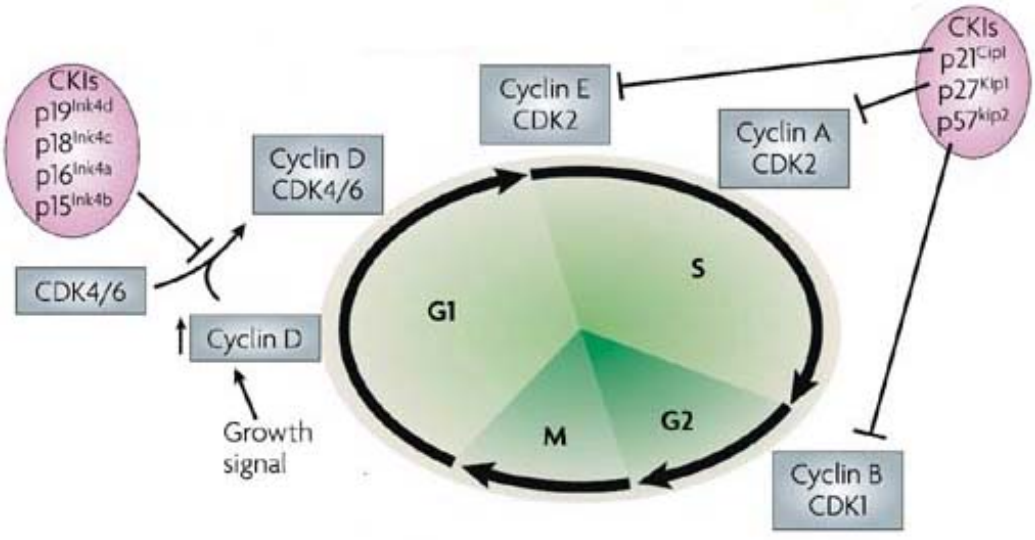
All of these articles and other demonstrate that, despite the large amount of research into the role NF-κB role in cells after stimulation, more is necessary, since it is becoming clearer that NF-κB effects can vary upon the cellular context and nature of the stimulation.

1.2 The cell cycle

One of the main features of tumour cells is their ability to proliferate rapidly and in an uncontrolled way. To understand how this can happen, it is necessary to understand first how cells normally replicate their genetic material and divide into viable daughter cells. After mitogenic stimuli, cells leave their quiescent stage (G0) and enter into the cell cycle. In mammalian cells, the cell cycle is separated four phases: a gap phase G1, follows by the DNA synthesis phase (S phase). A second gap phase (G2 phase) precedes the mitosis (M phase). Once in the cell cycle, the cell progresses through G1 phase to S phase, when DNA replication occurs. Then, the cell enters into G2 phase, which allows a checkpoint to ensure DNA is correctly replicated, before the entry into M phase (Figure 1.5.A).

During M phase, mitosis occurs, which ends with the separation of the two daughter cells through a mechanism called cytokinesis. Mitosis can be separated into five distinct phases, based on cellular morphology. In prophase, cells organize their cytoskeleton in order to have a microtubule network called the mitotic spindle (Figure 1.5.B). At the same time, the DNA duplicated during S phase begins to condense into chromatids. Nuclear envelope break down signals the end of this phase.

A.



B.

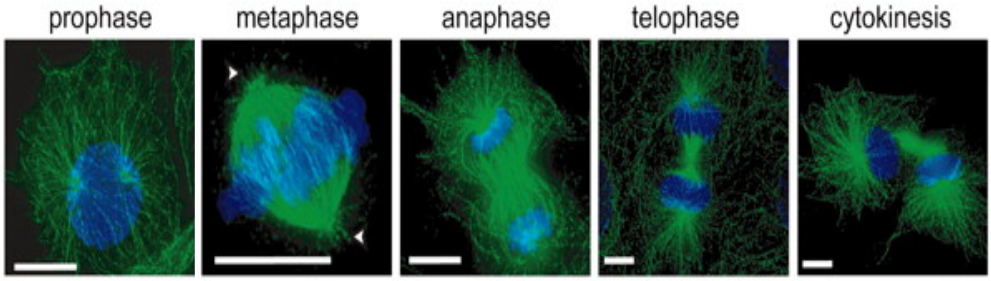


Figure 1-5: Cell cycle and mitosis

A. Schematic overview of the cell cycle and the Cyclin-CDK complexes involved in its regulation. Adaptation from (Herrup & Yang, 2007)

B. Hela cells were immunostained for α-tubulin (green) to detect the mitotic spindle and DAPI (blue) to detect DNA. The images shown are a representation of the five phases during mitosis. Images taken from (Bakal et al, 2005)

The next step is prometaphase, a sub-step defined by kinetochore attachment to the spindle. This is followed by proper metaphase, during which the chromosomes align in a “metaphase-plate” (Bharadwaj & Yu, 2004). Anaphase is the step where the two chromatids separate and moves to the opposite pole. The chromatids decondense during the next step, telophase, while the nuclear envelope re-forms and the mitotic spindle disassembles. At this stage, a ring of actin-myosin begins to form. The last step of mitosis, which some researchers consider to be still within telophase, is cytokinesis, with the separation of the two daughter cells (Jackson et al, 2007).

1.2.1 The regulation of the cell cycle

A family of protein kinases, the cyclin-dependent-kinases (CDKs) drive progression through the cell cycle. CDK activity is modulated by mitogens and growth factors or by signalling pathways, which includes, in some contexts, NF-κB (Joyce et al, 2001). The CDKs become active after forming complexes with regulator proteins, called the cyclins (Figure 1.5.A) (Herrup & Yang, 2007).

The cyclin D family contains cyclin D1, cyclin D2 and cyclin D3. Cyclin D1 is widely expressed in mammalian cells and its expression can vary during the cell cycle. This is controlled by the rate of gene transcription and is induced following exposure to mitogenic stimuli, through an extensive range of transcription factors including NF-κB. The other cyclins, D2 and D3, are more tissue-specific and are required in late G1 or S phase respectively (Joyce et al, 2001). After detection of proliferative signals, Cyclin D associates with CDK4 or CDK6 and allows progression through G1 phase. Here, the Cyclin D1-CDK4 complex phosphorylates the retinoblastoma protein Rb. This relieves repression of the E2F transcription factor which is required for synthesis of cyclin E and entry into S phase. The accumulation of CDK2-Cyclin E allows the G1/S transition (Malumbres & Barbacid, 2001) and the accumulation of cyclin A regulates S phase. Cyclin A and cyclin E are both under the control of the E2F transcription factor. Cyclin A continues to increase in S phase and in the beginning of G2 phase, followed by an increase in the cyclin B1 level, which is involved in the start of M phase (Joyce et al, 2001).

In order to make sure that the cell cycle produces two viable daughter cells identical to the mother cell, checkpoints are required during the cell cycle to ensure mistakes are corrected prior to cell division. Two families of inhibitor proteins regulate the activities of the CDKs: the CDK Interacting Protein / CDK Inhibitory Proteins (CIP/KIP) and the Inhibitors of CDK4 (INK4) family. Members of the INK4 family (p16INK4a, p15INK4b, p18INK4c and p19 INK4d) specifically inhibit CDK4 and CDK6 (Denicourt & Dowdy, 2004) (Figure 1.5.A). The CIP/KIP family is composed of p21Cip1, p27Kip1, and p57Kip2 and can inhibit a large spectrum of cyclin-CDK complexes (Denicourt & Dowdy, 2004). Among this family, p21 is one of the most studied, since it is a target gene of the p53 tumour suppressor, and one of the main effectors of cell cycle arrest following DNA damage (Weiss, 2003).

After cellular stress or DNA damage, the cell cycle can also be arrested by other types of proteins, specific to the type of damage. For example, the ATM kinase is activated following double-strand DNA breaks whereas the related ATR kinase is more specific to the arrest of DNA replication forks (Shiloh, 2001). Following DNA damage, ATM/Chk2 and ATR/Chk1 kinase pathways are activated and can lead to cell cycle arrest, either at the G1/S transition or G2/M checkpoint. This regulation occurs in a manner dependent or independent of the tumor suppressor p53. Indeed, DNA damage response leads to activation of p53, which induces the expression of CDK inhibitor $p21^{waf1}$, resulting in arrest in G1 or G2 phases (Poehlmann & Roessner, 2010). However Chk1 and Chk2 kinases are also able to directly phosphorylate Cdc25a and Cdc25c, leading to an accumulation of inactive CDK2/cyclin E and CDK1/cyclin B complexes, arresting the cell cycle in G1 and G2 phases respectively (Niida & Nakanishi, 2006; Poehlmann & Roessner, 2010).

The DNA damage response can induce cell cycle arrest through the MAPK (mitogen activated protein kinases) pathways. ERK induces arrest in G1 phase through a direct, p53-independent, activation of $p21^{waf1}$. It also inactivates Cdc25c, blocking entry in mitosis (Poehlmann & Roessner, 2010). JNK signaling effects on the G1/S transition, following DNA damage, involves p53 phosphorylation and $p21^{waf1}$ activation, whereas JNK-mediated G2/M arrest requires either CDC25c activity regulation, or $p21^{waf1}$ activation (Poehlmann & Roessner, 2010). Finally, the p38 MAPK arrests cells in G2 phase through p53 phosphorylation, whereas multiple mechanisms are involved in its G1/S arrest,

including p53 and $p21^{waf1}$ activation, or even a direct phosphorylation of cyclin D1, regulating its degradation (Poehlmann & Roessner, 2010).

1.2.2 NF-κB and the regulation of the cell cycle

Several studies show that NF-κB can have an effect on cellular growth by inducing cell cycle arrest (Joyce et al, 2001). Overexpression of RelA or c-Rel arrests cells at the G1/S transition in pro-B cells and HeLa cells, respectively (Bash et al, 1997; Sheehy & Schlissel, 1999), although these effects can be due to p53 activation following NF-κB over-expression. RelA and p50 are able to induce cell cycle arrest in epithelial cells, notably through the induction of p21 (Seitz et al, 2000) whereas p21 is induced in lymphoma cells by RelB over-expression and in HeLa cells by RelB/p50 heterodimers (Bren et al, 2001).

However, NF-κB can also stimulate cell proliferation. The cyclin D1 gene is one of the best-known NF-κB target genes involved in cell cycle regulation during G1 phase (Barre & Perkins, 2007). This gene contains three κB sites in its promoter, where all the NF-κB sub-units can bind to up- or down-regulate cyclin D1 expression, depending on p53 activation (Guttridge et al, 1999), (Rocha et al, 2003). Cyclin D2 and D3 also have κB response elements in their promoters (Bharti et al, 2004). Interestingly, cyclin A also appears to be a potential RelA target gene (Joyce et al, 1999). Cyclin B1 also possesses a κB site in its promoter and its expression has been shown to correlate with NF-κB expression, even if the exact mechanism or binding by NF-κB is not yet demonstrated (Cude et al, 2007).

NF-κB has also been shown to indirectly regulate cell proliferation. For example, c-Myc promotes proliferation and can be regulated by the RelB/p52 heterodimer (Demicco et al, 2005). Similarly, IKKα, which is responsible for the activation of the NF-κB non-canonical pathway, promotes the transcription of the *skp2* gene through RelB/p52 dimers. Skp2 promotes the degradation of the CDK inhibitor p27, allowing cell cycle progression (Schneider et al, 2006). IKKα is also involved in the regulation of cyclin D1 independently of NF-κB, through being recruited to its promoter by oestrogen receptor α (ERα) in breast cancer cells, which leads to increase cyclin D1 expression (Park et al, 2005).

An active role for NF-κB in mitosis has not yet been identified, although some studies suggest that NF-κB can regulate mitosis either, directly or indirectly. For example NF-κB is activated after stimulation by several drugs acting on the microtubule cytoskeleton, such as taxol (stabilization of the microtubules) or nocodazole (depolymerization) (Das & White, 1997; Mistry et al, 2004; Rosette & Karin, 1995). These drugs act on NF-κB through the activation of IKKα and IKKβ (Mistry et al, 2004). However the exact mechanism involved is still unclear. Furthermore, a few articles link IKK proteins to mitotic progression. In 2006, Prajapati et al demonstrated that IKKα, but not IKKβ, siRNA treatment induces an increase in cell number in G2/M and this effect involves cyclin B1 and PLK1 regulation. They also demonstrated that IKKα associates and phosphorylates AuroraA on T288, indicating a possible mechanism for regulation of G2/M progression regulation (Prajapati et al, 2006). However, in 2007, Irelan et al found that IKKβ depletion deregulates AuroraA stability and leads to the formation of multipolar spindle and defective chromosome segregation (Irelan et al, 2007). This has been confirmed using an IKK inhibitor, resulting in blockage of AuroraA and CDK1 activity and induction of cyclin B1 degradation, as well as defective chromosome segregation (Blazkova et al, 2007). Together, these studies have defined a role for NF-κB in G1/S but more literature is emerging also linking NF-κB to mitosis, although definite mechanisms have not yet been explained.

1.3 The centrosome

1.3.1 Centrosome duplication cycle

Centrosomes are small organelles, near the centre of the cell in the periphery of the nucleus (Doxsey, 2001). Centrosomes are composed of two centrioles and pericentriolar material (PCM), which permits the nucleation of microtubules (Figure 1.6.A and 1.6.B) (Doxsey, 2001). During most of the cell cycle, centrioles are perpendicular to each other but are not completely similar: although they both are composed of nine triplets of microtubules, at the end of the mother centriole, appendages can be observed that are not present in the daughter centriole (Nigg, 2002).

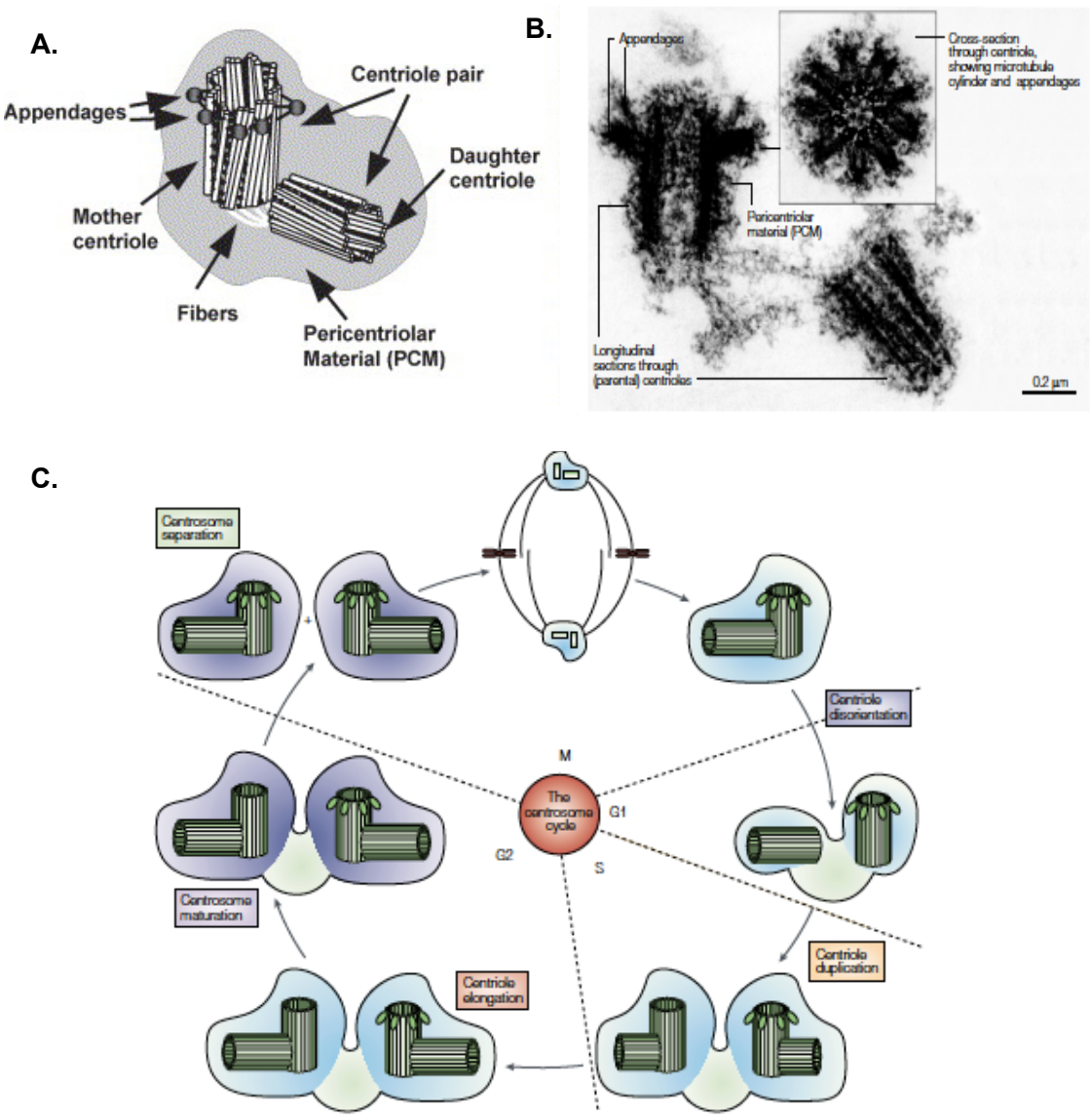


Figure 1-6: Centrosome structure and centrosome cycle

A.The centrosome. The centrosome is constituted of two centrioles perpendicular to each other and some pericentriolar material (PCM). Each centriole is composed of nine triplets of microtubules. At the end of one centriole, the mother centriole, can be observed appendages allowing anchoring of the microtubules and this is not present in the daughter centriole. Schematic representation taken from (Fukasawa, 2005).

B. **Electron microscopy picture of a centrosome**. Taken from (Nigg, 2002).

C. The centrosome duplication cycle. The centrosome duplication cycle can be separated in various phases linked to the cell cycle. During the G1 phase, the two centrioles are separated, before being duplicated in S phase. In G2 phase the newly formed centrioles elongate and mature to form two functional centrosomes, before the centrosomes separate in mitosis. Taken from (Nigg, 2002).

These appendages allow anchoring of the microtubules. During the cell cycle, the centrioles duplicate and appendages appear on the daughter centriole in G2 phase (Figure 1.6.C) (Nigg, 2002). During mitosis, centrosomes are involved in the formation of the mitotic spindle, while during interphase, they form a structure called the Microtubule Organising Centre (MTOC) (Cuschieri et al, 2007). The MTOC is involved in vesicle transport and the formation of cell shape, as well as cellular polarity. Several proteins are involved in the following processes: anchoring (ninein, centriolin, dynactin), nucleation (γ-tubulin, pericentrin, polo-kinases, aurora-kinases) and release (katanin) (Badano et al, 2005).

Regulatory mechanisms are required to make sure that centrosomes duplicate only once per cell cycle. Maintaining the right number of centrosomes is necessary for genomic stability, although the formation of the mitotic spindle can occur without centrosomes under some circumstances (Doxsey, 2001). Among these mechanisms, various proteins involved in centriole duplication have been described (Table 1.7). These include γ-tubulin whose depletion blocks centriole duplication or induces problems in centriolar structure (Raynaud-Messina et al) and the Ser/Thr kinases CDK2 and PLK4, which also affect centrosome duplication (Bettencourt-Dias & Glover, 2007).

Mechanisms are required to make sure that centrosomes duplicate only once per cell cycle. Indeed, deregulation of the centrosome duplication cycle leads to multipolar spindle and increases chromosome abnormalities and multinucleate cells. One of these mechanisms involves the enzyme separase being required during anaphase to separate the sister chromatids. This link between centrosomal duplication and anaphase is important, as deregulation could lead to excess number of centrosomes and dysfunctional spindles (Tsou & Stearns, 2006).

A ring of γ-tubulin forms the junction between the PCM and the microtubules. The microtubules are mainly composed of α- and β- tubulin proteins organised in a polarised tube with the negative extremity bound to the PCM and the positive one to the kinetochores. This tube is very dynamic and it alternates between polymerization and depolymerization, which permits microtubule movements in the cell (O'Connell & Khodjakov, 2007).

Protein	Function	Assays and phenotypes	Reference
PLK4	centriole duplication	RNAi, mutations => no duplication Overexpression => amplification	(Habedanck et al, 2005)
SAS6	Centrosome duplication	RNAi => no duplication Overexpression => amplification	(Leidel et al, 2005), (Strnad et al, 2007)
CDK2	cell cycle regulation	Dominant negative mutant, inhibition => no duplication Hyperactivation => amplification	(Meraldi et al, 1999), (Duensing et al, 2006) (Adon et al, 2010)
Centrin	Centriole duplication	RNAi => no duplication	(Salisbury et al, 2002)
CP110	CDK substrate	RNAi => no duplication	(Chen et al, 2002)
Nucleophosmin	Substrate for CDK2/cyclin E	RNAi => amplification	(Lim et al, 2002)
γ-tubulin	Nucleation and orientation of microtubules	RNAi => no duplication	(Dammermann et al, 2004)
CEP135	Organization of microtubules	Inhibition, RNAi => disorganization of microtubules Overexpression => accumulation of particules	(Ohta et al, 2002)
p53	tumor suppressor	Mutations => Amplification	(Shinmura et al, 2007)

Table 1-7: Proteins involved in centrosome duplication in Homo Sapiens

Non-exhaustive list summarizing the main proteins for which a change in expression affects centrosome duplication in human cells. Adapted from (Bettencourt-Dias & Glover, 2007)

The contact with the kinetochore halts the microtubule dynamics and leads to the capture of a chromosome, as well as the beginning of the growth of the mitotic spindle (O'Connell & Khodjakov, 2007).

Mitosis is subject to a high level of regulation in order to permit its correct resolution. Indeed, aneuploidy and chromosome rearrangements are common in most human cancers (Nigg, 2002) and, usually, a deficiency in cell cycle checkpoints leads to faulty chromosome segregation, followed by the development of genomic rearrangements (Gisselsson, 2005). It is well known that several mitotic kinases are involved in tumorigenesis, due to their central role in the regulation of the cell cycle checkpoints. For example, the mitotic kinase Aurora-A, which in normal cells is required for centrosome maturation, mitotic spindle organization, G2/M transition and cytokinesis, is over-expressed in many primary tumours and is involved in cellular transformation (Nigg, 2002). Furthermore, mutations have been detected in genes involved in the spindle checkpoint, in a number of cancers (Bharadwaj & Yu, 2004).

1.3.2 The spindle checkpoint

As described previously, mitosis can be separated into five phases: prophase, metaphase, anaphase, telophase and cytokinesis (Figure 1.5.B). At the end of metaphase, the cells have to pass a checkpoint before anaphase can begin, to be sure that the chromosome segregation only occurs when the two daughter cells can receive the same partition of genetic material (Bharadwaj & Yu, 2004). This mechanism is called the spindle checkpoint, which detects a lack of attachment or tension at kinetochores and if activated prevents chromosome segregation (Bharadwaj & Yu, 2004). The spindle checkpoint is well characterized and requires the proteins Mad1, Mad2, BubR1 (also named Mad3 in yeast), Bub1, Bub3 and Mps1 (Figure 1.8) (Bharadwaj & Yu, 2004). If a lack of attachment or tension is detected, protein complexes containing Bub1, BubR1 and Mad2 bind to kinetochores and induce a stop in anaphase. These complexes bind and inhibit Cdc20, which is essential for the activation of the anaphase promoting complex/cyclosome (APC/C).

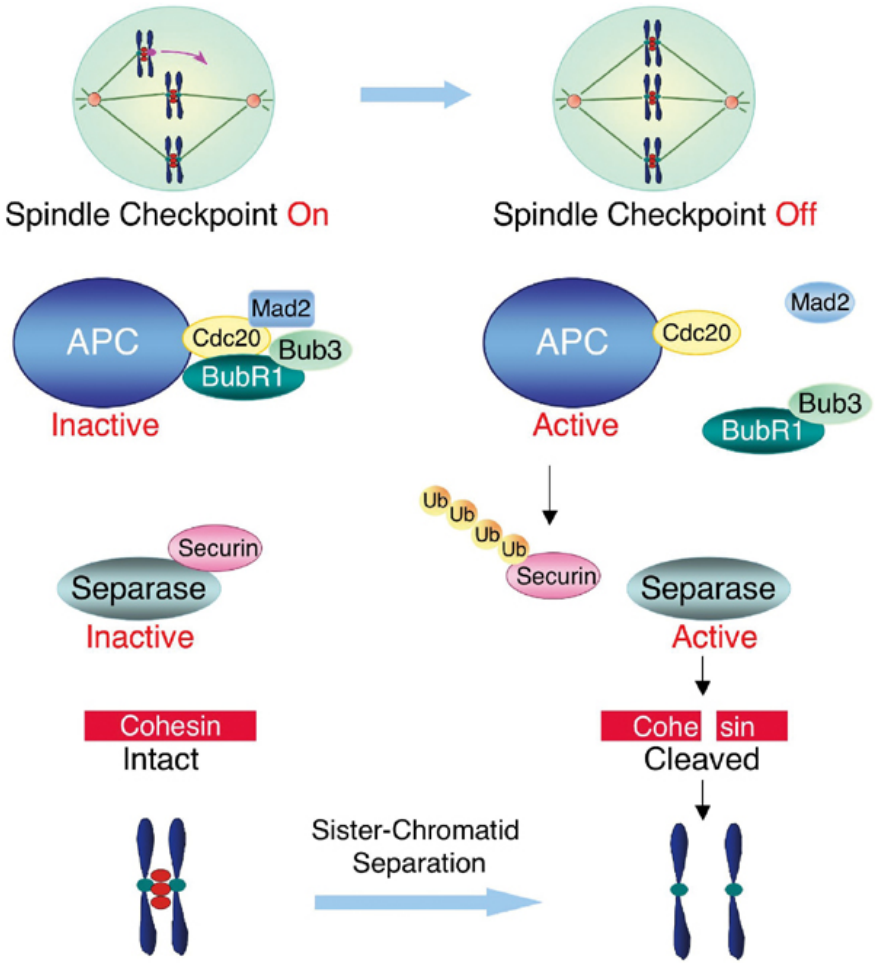


Figure 1-8: The spindle checkpoint

During the metaphase-anaphase transition, APC/C is activated by release from a complex with BubR1, Bub3 and Mad2, leading to Securin ubiquitination. The degradation of securin activates separase, which cleaves cohesin keeping the sister chromatids together, thus allowing their separation. If the sister chromatids are not properly attached to the mitotic spindle, APC/C is kept inactive, and securin is stabilized to preserve chromatid cohesion and delay mitotic progression into anaphase.

Taken from (Bharadwaj & Yu, 2004).

APC/C is a multi-subunit E3 ubiquitin ligase that allows the proteosomal degradation of a large number of cell cycle regulators through their ubiquitination. This ubiquitination permits the activation of the enzyme separase, which induces the destruction of the link between sister chromatids. APC/C can also trigger the degradation of cyclins, promoting the exit from mitosis (Malmanche et al, 2006). Once the spindle checkpoint is passed, each set of sister chromatids migrate to the opposite spindle pole (anaphase). The ring's assembly in telophase is mediated by the serine/threonine kinase ROCK, an effector protein of the GTPase RhoA (Glotzer, 2005). In animal cells, around 20 conserved proteins, found in the contractile ring, regulate cytokinesis. These proteins participate in the RhoA pathway and are responsible for expansion of the membrane and breakage of the cytoplasmic bridge (Glotzer, 2005). A failure in cytokinesis can lead to multinucleate cells, with an abnormal number of centrosomes.

1.3.3 The PLK family

Named after the polo gene of Drosophila melanogaster, the members of the polo-like kinase (PLK) family have been identified in many organisms, from yeast to mammals (Nigg, 1998).

The PLK family is constituted of five serine - threonine kinases in mammalian cells, all sharing a high sequence homology in their N-terminal kinase catalytic domain and usually containing two polo-box domains (only one in PLK4's case) (Figure 1.9) (de Carcer et al, 2011). PLK1, the most studied member of the family, has been found in multiple organisms, from yeast (called Cdc5) to Drosophila (called Polo) and is an essential gene as null mutants in yeast and Drosophila are lethal (de Carcer et al, 2011). PLK1 and PLK4 are mostly expressed in highly proliferative tissues, whereas PLK2, PLK3 and PLK5 can also be found in non-proliferative cells such as the nervous system (for PLK2 and PLK5) or respiratory organs such as lungs, trachea or bronchus (for PLK3) (de Carcer et al, 2011). Preliminary results show that PLK1 can act as an oncogene in human hepatocellular carcinoma, while PLK2, PLK3 and PLK4 (also known as SAK) are more likely to act as tumour suppressors (Pellegrino et al, 2010).

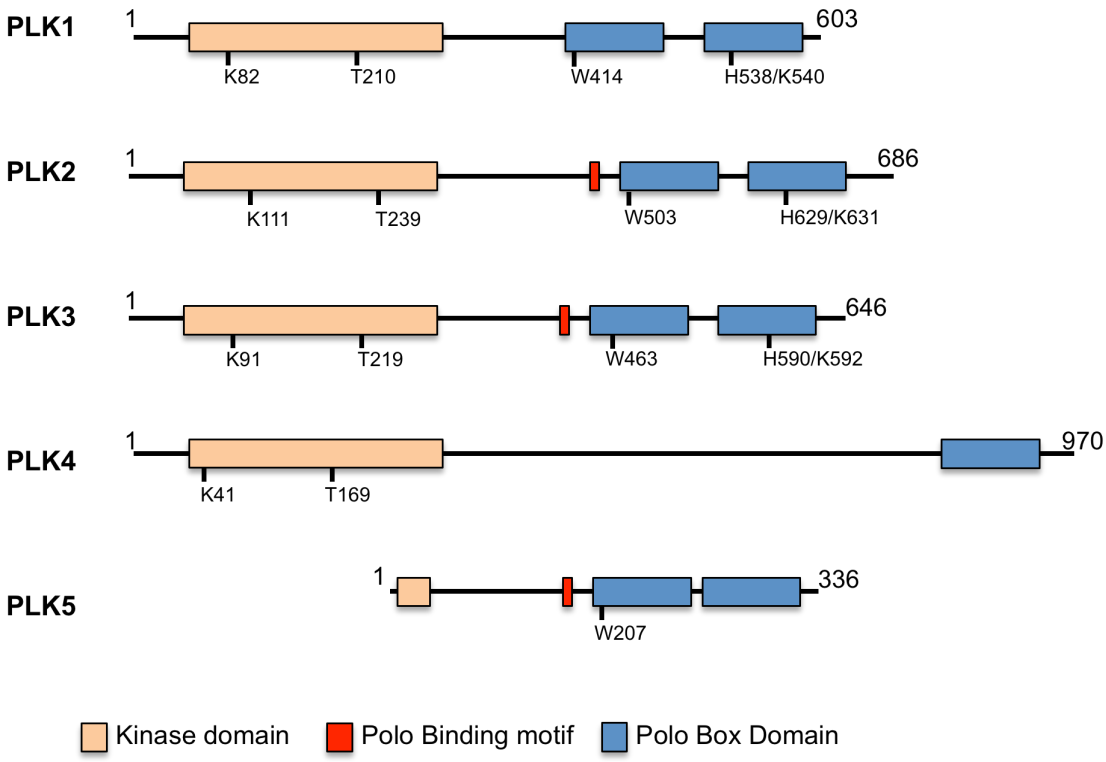


Figure 1-9: The PLK family member

Schematic representation of the five PLK family members. Kinase domains are shown in yellow, polo box domains in blue and the Polo Binding motif in red. Key residues such as the Lysine acceptor and T-loop Threonine in the kinase domain are represented, as well as the key residues for substrate recognition within the Polo Box domain. Based on (de Carcer et al, 2011)

The PLK proteins are involved in centrosome maturation and bipolar spindle formation, the activation and inactivation of CDK1/cyclin B1 complexes, co-ordinating the spindle assemble and promoting the exit from mitosis (Figure 1.10) (Nigg, 1998). PLK proteins are essential regulators of the APC/C complex and PLK absence blocks the destruction of mitotic cyclins (Kotani et al, 1998). PLK mRNA and protein levels vary during the cell cycle, mainly in abundance, localization and activity: PLK1 levels increase to reach a maximum in activity and phosphorylation during M phase (Winkles & Alberts, 2005). PLKs possess multiple phosphorylation sites whose functions are not yet fully understood (Nigg, 1998). PLK1 substrates are found during most of the cell cycle (Luo & Liu, 2012). Indeed Hbo1, a histone transferase, (Wu & Liu, 2008) and Orc2, a component of the ORC complex facilitating DNA replication, (Song et al, 2011) are both involved in S phase progression and are published PLK1 substrates (Luo & Liu, 2012). G2/M PLK1 target proteins are either involved in centrosome duplication, for the centrioles elongation, maturation and separation, or the regulation of the cell cycle (Luo & Liu, 2012). Two of these substrates are essential G2/M regulators whose phosphorylation by PLK1 promotes their nuclear translocation during prophase: Cdc25 (Toyoshima-Morimoto et al, 2002) and cyclin B1 (Toyoshima-Morimoto et al, 2001). Specific mitotic PLK1 substrates involve proteins required for the recruitment to the kinetochores (Luo & Liu, 2012), such as INCENP (Goto et al, 2006) and BubR1 (Elowe et al, 2007).

PLK2 is localized to the centrosome and reaches a maximum in expression and activity during the S phase of the cell cycle. By contrast PLK3 mRNA peaks in G1 phase, however its protein expression remains stable during the whole cell cycle, suggesting an alternative regulation not yet elucidated (de Carcer et al, 2011). PLK3 allows cyclin E accumulation and Cdc25a activation, leading to DNA replication. PLK3 has been shown to be activated after genotoxic stress, in a p53-dependent manner (de Carcer et al, 2011).

PLK5 is the most recent PLK protein described. In humans a stop codon mutation changes the reading frame, removing the kinase domain, whereas the mouse PLK5 gene encodes the full length protein.

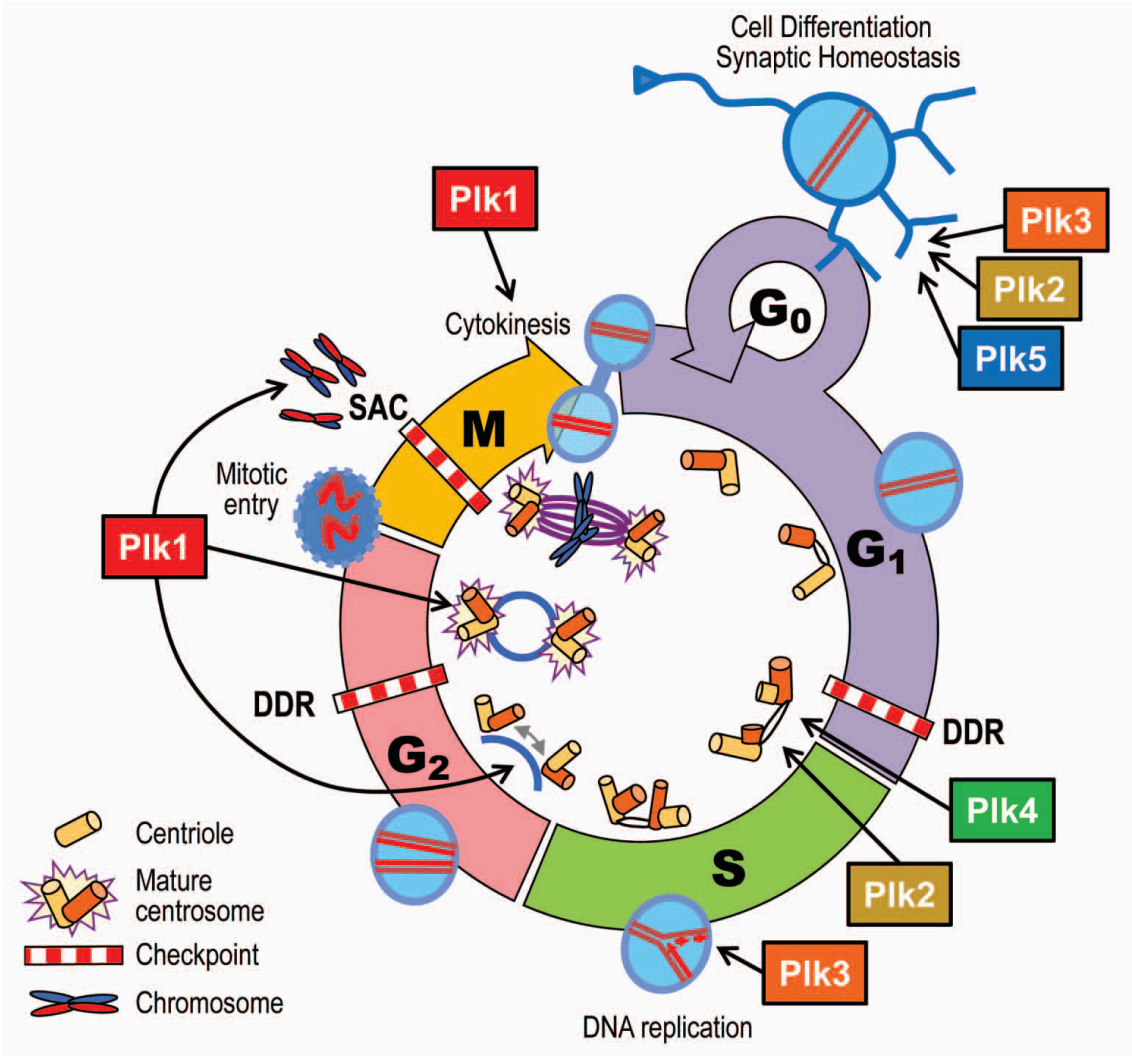


Figure 1-10: Functions of the PLK proteins during the cell cycle

Schematic overview of the PLK proteins and their moment of action during the cell cycle. PLK1 is involved in mitotic entry, centrosome separation and maturation, spindle formation, chromosome segregation and cytokinesis. PLK2 and PLK4 regulate centriole duplication, whereas PLK3 is involved in DNA replication. PLK2, PLK3 and PLK5 also have non-proliferative roles, such as neuron differentiation and synaptic homeostasis. DDR = DNA damage response; SAC = spindle assembly checkpoint.

Taken from (de Carcer et al, 2011)

Both human and mouse express a functional PLK5 protein, however the human gene seems to initiate translation after the codon-stop, expressing a shorter kinase domain (de Carcer et al, 2011) Overexpression of PLK5 arrests cells in G0/G1 and increases p21 expression, independently of any kinase activity (de Carcer et al, 2011b).

PLK4 is an atypical member of the polo-like kinase family. Instead of the second normal polo-box domain found in other PLK proteins, PLK4 contains a larger crypto polo-box, with a weaker homology to other polo-boxes (figure 1.9) (Sillibourne & Bornens, 2010b). Unlike other PLKs, this crypto polo-box domain is unable to dimerize, as is seen for the other PLKs, suggesting that PLK4 is not regulated in the same manner (Sillibourne & Bornens, 2010b). Maximally expressed in actively dividing tissues, PLK4 is a key regulator of centriole duplication and will be described later in more details (see Chapter 4).

1.3.4 NF-κB and regulation of centrosome duplication

So far, no published reports link NF-κB to PLK4 or PLK2 and only one study found a relationship between NF-κB and PLK3. In 2005, Li and coworkers found that the RelA subunit directly binds PLK3 promoter and activates its transcription. They previously stated that RelA induces a pro-apoptotic response via the p53-pathway in response to doxycycline-induced superoxide (Fujioka et al, 2004). In 2005, they followed by showing that in HCT116 cells, after doxyxline treatment, NF-κB is activated and induces PLK3 expression. PLK3 then associates with and phosphorylates p53, leading to p53-dependent apoptosis (Li et al, 2005b). They also demonstrated that PLK3 depletion by siRNA in HCT116 cells suppresses doxycycline-induced apoptosis; whereas the opposite effect is obtained after PLK3 overexpression (Li et al, 2005b). Although this PLK3-mediated induction of apoptosis can be either p53 dependent or independent, RelA induction of PLK3 is required in both cases (Li et al, 2005b). Some studies do link PLK1 with NF-κB but once again only from a RelA perspective, in case of cell detachment from the extra-cellular matrix (ECM) in oesophageal squamous cell carcinoma (ESCC) cells, leading to anoikis. ESCC cells detachment triggers NF-κB-dependent PLK1 induction, which through the stabilization of β-catenin, promotes anoikis resistance (Lin et

al, 2011). Interestingly, two studies by Higashimoto in 2008 and Zhang in 2010 illustrated a potential activation feedback loop, as they found that PLK1, by formation of a complex with NEMO and the phosphorylation of IKKβ, could decrease IKK activation by TNFα. This reduced IκBα phosphorylation and degradation, which then reduced NF-κB activation (Higashimoto et al, 2008; Zhang et al, 2010).

Interestingly, Bcl-3, the IκB protein that can function as a co-activator for p52 homodimers, was found to regulate cell proliferation, induce centrosome amplification and aneuploidy in Hela cells (Zamora et al, 2010). Inducible by DNA damage, Bcl-3 inhibits p53 activation (Kashatus et al, 2006). Although Zamora and coworkers did not link their study with p53 status, they demonstrated that induction of centrosome amplification; reduced cell proliferation and aneuploidy are caused by Bcl-3 depletion. These results are associated with a DNA damage response and a G2/M checkpoint delay (Zamora et al, 2010).

NF-κB, and in particular the RelA and p50 sub-units, can also regulate some genes involved in mitotic progression. Wu and co-workers suggested that NF-κB can control the expression of BRCA2, a tumour suppressor whose mutation leads to ovarian and breast cancers, by regulating its promoter (Wu et al, 2000). ERK5 has been shown to induce IκB phosphorylation and degradation via ribosomal S6 kinase 2 (RSK2) and thus promotes NF-κB activation. This ERK5-NF-κB pathway delays mitotic entry and de-regulates cyclin B1, cyclin B2 and PLK1 expression (Cude et al, 2007). Mutations in the NF-κB site in the PRC1 promoter modify its basal activity (Li et al, 2004). Since PRC1 is a regulator of cytokinesis, this provides an additional route through which NF-κB might regulate mitosis. RelA also interacts with the centrosomal P4.1-associated protein (CPAP) (Koyanagi et al, 2005), which is a component of the centrosomal complex. This interaction was identified after a yeast two-hybrid screen and then confirmed in MCF—7 breast cancer cells (Koyanagi et al, 2005).

1.4 Cancer

1.4.1 The Hallmarks of cancer

Cancer is a multi-step disease characterized by various genetic alterations, which promote the transformation of normal cells into malignant cells. More than a hundred cancers have been described, and a lot of sub-types can be found associated with specific organs (Hanahan & Weinberg, 2000) but the common characteristic is that genetic modifications induce a loss of function in tumor suppressor genes and/or a gain of function of tumor promoter genes, also called oncogenes. Hanahan and Weinberg suggested in 2000 that all cancer cells have six characteristics compared to normal cells, which are briefly described below (Figure 1.11).

- Self-sufficiency in growth signals. Some tumours, due to the activation of oncogenes such as Ras, are able to produce their own growth signals.

- Insensitivity to anti-proliferative signals: in order to pass through processes such as cellular quiescence or differentiation, cancer cells are able to modify the response to anti-proliferative signals and in this case, this results in uncontrolled growth.

- Evasion of apoptosis: this ability can sometimes be linked to uncontrolled proliferation. In normal cells, excessive proliferation results in programmed cell death. But in cancer cells, resistance to apoptosis can be acquired, through a variety of processes, such as mutation of the tumor repressor p53.

- Limitless replicative potential: normally, after a certain number of doublings, cells undergo senescence. To overcome this, tumour cells acquire limitless replicative potential. This can frequently occur by up-regulation of telomerase resulting in maintenance of telomere length and cell immortalisation.

- Angiogenesis: angiogenesis is the process necessary to grow new blood vessels. This is regulated by a balance between angiogenesis inducers (like vascular endothelial growth factor VEGF) and inhibitors (thrombospondin-1, for example). Tumor cells have the ability to shift this balance in order to increase angiogenesis.

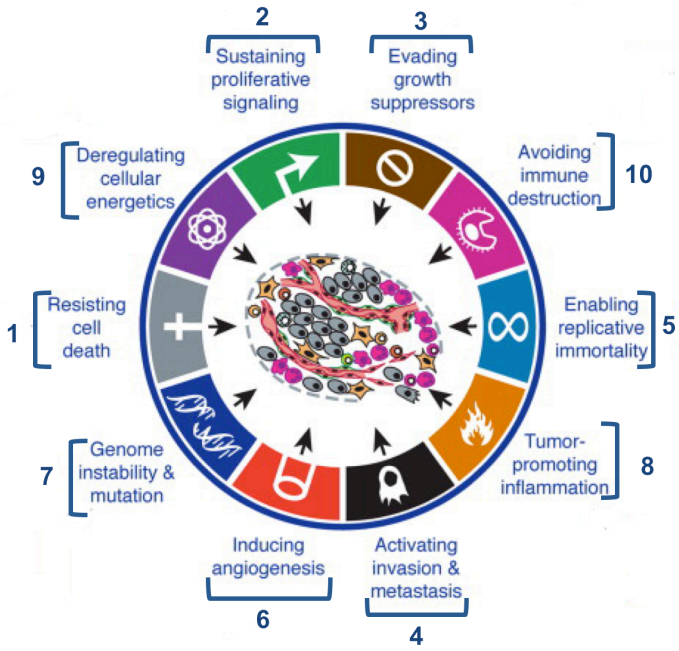


Figure 1-11: The Hallmarks of Cancer

The six first hallmarks of cancer as described by Hanahan in 2001 were constituted of: 1-evading apoptosis; 2-self sufficiency to growth signals; 3-insensitivity to anti-growth signals; 4- tissue invasion and metastasis; 5- limitless replicative potential; 6-sustained angiogenesis. To these 6 characteristics, four other hallmarks were described later: 7-Genome instability and mutations; 8- Tumour promoting inflammation; 9-Reprogramming energy metabolism; 10- Evade Immune destruction

Adapted from (Hanahan & Weinberg, 2011).

- Tissue invasion and metastasis: the ability of tumour cells to invade adjacent tissues and settle in another place, forming metastases, is a major issue in oncology and is responsible for 90% of human cancer deaths. This process frequently involves cell adhesion molecules, whose expression can become altered in malignant cancer cells.

A decade later, due to the advances in cancer research, Hanahan and Weinberg updated their first review by the addition of four more characteristics, two of which are well established (bringing the list to eight hallmarks of cancer) and two of emerging importance (Figure 1.11) (Hanahan & Weinberg, 2011). These were:

- Mutations and genome instability (established hallmark): genomic instability increases the probability that occurring mutations will enhance tumor formation and promotion (for example the mutation of the BRCA1 gene in breast cancer). Telomeric DNA loss also plays a role in the karyotype instability, by promoting the amplification or deletion of chromosomic DNA sections, which leads to a possible unlimited replication.

- Tumor promoting inflammation (established hallmark): the tumor-associated inflammatory response allows the progression and enhancement of tumor growth, by providing various essential molecules to the micro-environment, such as growth factors and cytokines

- Reprograming of energy metabolism (emerging hallmark): even with oxygen, cancer cells are able to change their glucose metabolism and then their energy production. This will support increased cell proliferation.

- Evasion of the immune destruction (emerging hallmark): cells and tissues are permanently monitored by the immune system, allowing the recognition and destruction of a majority of cancer cells. This means that the remaining tumors have found a way to bypass this surveillance, but the full mechanism involved is still to be fully understood

In addition to all these characteristics, the micro-environment has been shown to have a great influence on tumour growth. Here, different non-tumour

cell types can synthetize and provide various molecules such as growth factors to the tumour (Hanahan & Weinberg, 2011).

1.4.2 NF-κB in cancer

Usually, in normal conditions, NF-κB activation is a response mechanism to infection and inflammation. However, as described earlier in this chapter and to a larger extent in the literature, NF-κB, in association with other transcription factors such as AP-1, complex composed of pronto-oncogenes such as c-Fos and c-Jun (Kawai & Akira, 2006), can act as a tumour promoter in cancer cells. In this context, aberrantly activated NF-κB can be linked to all the "hallmarks of cancer" described by Hanahan (Hanahan & Weinberg, 2011), promoting tumor survival (Perkins, 2012). However, the effect of individual NF-κB subunits is still to be fully understood. Constant NF-κB activation in tumor cells usually derives from permanent activation of the IKK complex or a deficiency in a negative feedback loop (Perkins, 2012).

The first demonstrated link between NF-κB and cancer was when v-rel, a c-Rel homologue found in the avian reticuloendothelial virus, was shown to induce B-cells tumours in chicken (Gilmore, 1999), although this study was performed before the link between Rel and NF-κB was known. The c-Rel subunit seems to be the most oncogenic sub-unit, as c-Rel overexpression but not any of the other NF-kB subunits promotes tumour formation in chickens (Karin et al, 2002). The c-Rel gene is found amplified in approximately 23% of lymphomas. Almost half of B-cell non-Hodgkins' lymphoma have amplified c-Rel, which is usually associated with poor prognosis (Rayet & Gelinas, 1999). c-Rel mutations are found quite frequently. For example, the S525P change in B cell lymphoma, found in 2.4% of samples tested, affects an IKK phosphorylation site and increases c-Rel's in vitro transforming activity (Perkins, 2012).

Although not much mutated in cancer, NF-κB are frequently found aberrantly active. The RelA and p50 subunits are found constitutionally activated in 37% and 28% respectively of prostate cancers, whereas it is the case in almost 19% of prostate cancers for the p52 subunit, despite the fact that they could not be associated with any particular pathologic characteristics (Seo et al, 2009). Among other cancer types (Prasad et al, 2010), NF-κB was found

aberrantly activated in breast cancer (Sovak et al, 1997), and in leukemia and lymphoma (Karin et al, 2002).

Only a few mutations have been detected in the other NF-κB sub-units in cancer. One point mutation in RelA was described in a case of multiple myeloma, which changed the glutamate 495 residue to aspartate, which was reported to reduce RelA DNA-binding and transactivation activity, although the mechanism behind this is unclear (Trecca et al, 1997). Four other point mutations in RelA (T55S, E498K and Q132 in lung cancer, E127 in ovarian cancer) have been detected without any effect described yet (Perkins, 2012). Any effect of point mutations in RelB, which are mostly silent and found in ovarian cancer, are still not known (Perkins, 2012).

NFkB2 was identified as a gene found translocated in B-cell lymphoma, lyt-10-Cα (Neri et al, 1991). This involves a chromosomal translocation of the *NFkB2* locus (10q24), associated with B and T cell lymphoma, chronic lymphocytic leukaemia (CLL) and multiple myeloma (Karin et al, 2002). *NFkB2* translocation occurs in 2% of B and T cell lymphomas, and these lead to a C-terminal truncation of p100 and constitutive processing of p52 (Perkins, 2012). This translocation will also lead to a reduction in the IκB like function of p100, as a consequence of loss of the PID domain in rearranged p100 proteins (Xiao et al, 2001). Partial loss of the Ankyrin repeat in p100 due to this rearrangement also reduces its IκB activity although as long as 2 out of the 7 Ankyrin repeats are present, p100 is able to function as an IκB-like protein when transiently transfected into cells, leading to the conclusion that Ankyrin repeats are not the only mechanism for the IκB like function of p100 (Xiao et al, 2001). The C-terminal deletion of the *nfkb2* gene in mice results in aberrant production of p52. This leads to increased lymphocyte proliferation and gastric hyperplasia in mice (Ishikawa et al, 1997).

Viral proteins are involved in cancers such as Burkitts lymphoma or Hodgkins disease. For example, the EBV protein LMP-1 can cause cell transformation, cell proliferation and is required for lymphoblastoid cell line establishment (Atkinson et al, 2003). They can also induce p100 processing to p52 (Atkinson et al, 2003). The significance of *NFkB2* mutations found in lung cancer is not yet understood, although the location of one of them (V519F) in p100 is thought not to affect p52 activation, whereas the V281L mutation,

situated in the dimerization domain, could affect p52 dimerization affinity and specificity ((Perkins, 2012), Catalogue of Somatic Mutations in Cancer (COSMIC) Database).

In human breast cancer, the cyclin D1 gene is amplified in 15% of cases, while the protein is over-expressed in 30-50% of tumors, thereby increasing their proliferation (Fu et al, 2004). It is now well known, that NF-κB sub-units regulate cyclin D1 expression (Guttridge et al, 1999) and this regulation, in breast cancer, seems to go through the non-canonical pathway (Demicco et al, 2005). Moreover, immunostaining experiments showed that p52, the co-activator Bcl-3, and cyclin D1 but not RelA are over-expressed in breast tumour tissues (Cogswell et al, 2000). IKKα can also be activated in mammary epithelium (Cao et al, 2001), which leads to the processing of p100 to p52. Inactivation of the *Nfkb2* gene induces specific defects in B cell functions and in the architectural development of peripheric lymphoid organs (Xiao et al, 2001).

After stimulation by infectious agents such as bacterial or viral products, NF-κB is activated in inflammatory cells. This leads to the release of inflammatory cytokines, growth and survival signal, as well as angiogenic factors in the tumour micro-environment (Karin, 2006). This results in NF-κB activation in malignant cells, regulating the expression of cell cycle genes, anti-apoptotic genes and invasive protease (Karin, 2006), thus enabling the tumor progression.

1.4.3 NF-κB knockout mice

Due to the involvement of NF-κB in the immune response as well as cancer and inflammatory diseases, NF-κB mice knockout have been extensively studied (Gerondakis et al, 1999; Gerondakis et al, 2006; Hoffmann & Baltimore, 2006)) but will be briefly described here.

Interestingly, RelA is the only subunit required for embryonic development and post-natal survival. Indeed, *RelA -/-* mice died at the embryonic stage E14 of fatal liver apoptosis after sensitization to TNF-α (Gerondakis et al, 1999; Hoffmann & Baltimore, 2006). Mice lacking the RelB subunit are viable but present multiple pathological lesions and defects in immunity (Gerondakis et al, 1999). c-Rel knockout mice exhibit major defects in B and T cells development,

whereas *nfkb1 -/-* mice induce defects mainly in the B cell population (Gerondakis et al, 2006). *Nf-kb2 -/-* mice, the most relevant to my thesis, are able to develop normally but present disruptions in splenic and lymph node architecture (Caamano et al, 1998). They also display negative regulation of dendritic cell maturation (Speirs et al, 2004). NFkB2 has a role regulating naïve T cell function and limits nuclear translocation of RelA/p50 heterodimers (Gerondakis et al, 2006).

Double knockout *nfkb1/nfkb2* mice have also been studied and display a more severe phenotype that single subunit knockout. Although their embryogenesis occurs normally, these mice develop abnormalities after birth, such as growth retardation or craniofacial malformations. B cell development is also blocked in *nfkb1 -/- nfkb2 -/-* mice (Gerondakis et al, 2006).

1.5 Preliminary data

As described above, p52 is able to regulate the expression of genes having a role in cell proliferation, such as Cyclin D1 (Barre & Perkins, 2007; Schumm et al, 2006). Unpublished data by K.Schumm showed that p52 is also involved in the regulation of G2/M phase in U2OS osteosarcoma cells.

Transient depletion of p52 with siRNA oligos leads to an increase in G2/M phase cells and defects in mitosis ((Schumm et al, 2006) and unpublished observations). A significant lengthening of mitosis has also been detected visually after p52 siRNA treatment in U2OS cells expressing a YFG-H2B fusion protein, allowing chromosome separation in mitosis to be seen using a deconvolution microscope. Here, live cell imaging showed that cells lacking p52 could stay in metaphase for over 1h (whereas normal mitosis usually last 30 minutes to 1h in U2OS cells) before pursuing mitosis. Defects in chromosome segregation and alignment on the metaphasic plate were also observed, as chromosomes stayed attached to the outside of the cell and failed to line up on the metaphasic plate. When progression through anaphase and telophase finally happened, formation of micronuclei, which could come from chromosomes left behind, was detectable. Some cells were also seen appearing to go into apoptosis but managing to survive and progress though

mitosis (unpublished observation by K.Schumm, 2006). Moreover, p52 siRNA depletion resulted in an increase in multinucleate cells, as well as aberrant spindle formation.

An immunofluorescence study showed that p52 could be associated with the centrosome structure in U2OS cells in interphase and during mitosis. During metaphase, p52 was found in the center of the ring formed by α-tubulin, whereas during telophase, p52 localized in the MTOC. Colocalization between p52 and γ-tubulin was also observed during prophase, metaphase and telophase in U2OS cells, HT1080 cells (immortalized human fibrosarcoma) and HFF cells (immortalized human foreskin fibroblasts) (K.Schumm, unpublished observations).

U2OS cells stably expressing a shRNA against p100/p52 were created and immunofluorescence observation showed a significant increase in multinucleate cells compared to a control shRNA U2OS cell line. Electron microscopy revealed aberrant centrosome structures in the p100/p52 depleted stable cell lines. Moreover, these cells were sick, proliferated slowly and died after few passages compared to control cells (K.Schumm, unpublished observations).

Unpublished studies on the mechanism involved revealed that p52 could regulate the expression of genes involved in G2/M progression, such as Survivin. Survivin, a chromosome passenger protein, involved in the spindle checkpoint, is also a p53 target gene (Mirza et al, 2002). Furthermore, an increase in the mRNA levels encoding other proteins required for the spindle checkpoint, such as BubR1 or Mad2, was also seen after p52 depletion, although there is no evidence for direct regulation.

These data and unpublished observations by K.Schumm linked p52 with the centrosome structure and G2/M progression. However, further analyses were necessary to explain the role of the NF-κB p52 subunit in centrosome duplication.

1.6 Aims of the thesis

The aim of this thesis was to elucidate how p52 affects centrosome structure. To achieve this, I first studied the ability of p52 to regulate various genes involved in the cell cycle and the centrosome duplication. I found that some essential genes, such as PLK4 or its effector SAS6, were regulated by NF-κB subunits. I then showed that this regulation happens via a direct interaction of NF-κB subunits with the PLK4 promoter and that PLK4 promoter activity depends on NF-κB expression. I then investigated whether this regulation is cell cycle dependent.

2 Materials and Methods

2.1 Cell culture

2.1.1 Tissue culture cell lines and growth condition

U2OS are a human osteosarcoma cell line obtained from the Europan Collection of Cell cultures (ECACC). Cells of passage 6-28 were used. These cells are functionally null for $p16^{INK4A}$ and $p14^{ARF}$ due to hypermethylation of their promoter. They also contain a functional p53.

Hela are a human adenocarcinoma cell line obtained from the ATCC, and are functionally null for p53 due to the expression of the Human Papilloma Virus proteins E6 and E7.

H1299 cells, also obtained from the ATCC, are human non-small-cell lung carcinoma cells derived from a lymph node metastasis. H1299 cells do not express p53 due to a homozygous partial deletion.

Dr.Jorge Caamano (University of Birmingham) provided Mouse Embryonic Fibroblasts (MEF) wild-type (wt) cells. *nfκb2* null MEF cells were obtained from Dr. Ron Hay's laboratory in Dundee.

All cell lines were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% filtered fetal calf serum (FCS, Invitrogen), 1 U/mL penicillin, 1 μg/mL streptomycin (Lonza) and 2mM L-glutamine (Lonza), unless stated otherwise. All cells were subcultured when they reached ~80% confluency by washing with phosphate buffered saline (PBS, Lonza), treating with trypsin-EDTA and replating to ~20% of confluency.

2.1.2 Frozen cell storage

For long-term storage, cells of ~80% confluency were washed with PBS, detached using trypsin-EDTA and pelleted at 1000 revolutions per minute (rpm) for 5 minutes. The cell pellet was re-suspended in filtered FCS with 10% dimethyl sulphoxide (DMSO) and transferred to frozen storage vials. Cells were incubated at room temperature (RT) for 15 minutes before freezing slowly at -80°C and transferred to liquid nitrogen storage. Cells were recovered by

thawing a vial in a 37°C waterbath and plating on 10cm plates (Helena) in the required growth media.

2.1.3 RNAi transfection

Small Interfering RNA (siRNA) knockdown of specific mRNA was carried out as follows. Quantities are for a transfection of a 15cm plate and were scaled down relative to the size of the plate for smaller transfections. Cells were seeded at $4x10^6$ cells and transfected a few hours later. 5nM (final concentration) of siRNA were mixed with 1 mL of Opti-MEM media (Invitrogen) and 25 μL of Interferin reagent (Polyplus) according the manufacturer's instructions. The mix was left for 10-15 minutes at room temperature before adding drop-wise over the surface of the cells already covered by media. The cells were harvested 48h later as described in the corresponding experiment, unless stated otherwise. For some transfections, a pool of siRNAs was used with a final concentration of 5 nM of total siRNA added to the transfection mix. The sequences for the siRNAs used are shown below:

control = CAGUCGCGUUUGCGACUGG

p100/p52-A = CAGCCUAAGCAGAGAGGCU

p100/p52-B = CUACGAGGGACCAGCCAAG

p100/p52-C = GAUGAAGAUUGAGCGGCCU

RelA-1 = GCUGAUGUGCACCGACAAGUUTT

RelA-2 = GCCCUAUCCCUUUACGUCATT

RelB = UUGGAGAUCAUCGACGAGU

cRel = AUUGUGAAGGGCGAUCAGCA

p105/p50 = NNGGGGCUAUAAUCCUGGACU

pan-NF-κB = AAGGUGCAGAAAGAGGACA

PLK4-A = GGACCUUAUUCACCAGUUA

PLK4-B = UGAAGGACUUGGUCUUACA

2.1.4 DNA transfection

The cationic polymer polyethylenimine (PEI) was used for transfection of DNA into mammalian cell lines. This technique relies upon endocytosis of the DNA/PEI complexes (Durocher et al, 2002). Quantities shown below are for the transfection of a 15cm plate and were scaled down relative to the size of the plate for smaller transfections. Cell were seeded at $4x10^6$ cells and transfected more than 6 hours later. 1mL of Opti-MEM were mixed with 5 μg of DNA and 25 μL of PEI and the mix was left 15 minutes at RT before adding drop-wise over the surface of the cells already covered by media. The cells were harvested as described 48h later, unless stated otherwise.

2.1.5 CDK1 inhibitor

When required, cells were treated with the CDK1 inhibitor RO-3306 (Biomal) at 10 μM. The inhibitor was made up at a 1000X concentration in DMSO, and cells were simultaneously treated by DMSO alone as a control.

2.1.6 Cell synchronization

Various techniques were performed to synchronize U2OS cells:

Taxol (Sigma): cells were treated with 5 μM Taxol for 24 hours, washed 3 times in PBS and released in normal DMEM media.

Nocodazol (Sigma): cells were treated with 2 mM Nocodazol for 20 hours, washed 3 times in PBS and released in normal DMEM media.

Aphidicolin (Calbiochem): cells were treated with 1 nM Aphidicolin for 12 hours, washed 3 times in PBS and released in normal DMEM media.

Serum Starvation: cells were grown in DMEM medium with 0.5% FCS overnight, washed 3 times in PBS and released in normal DMEM media.

Hydroxy-urea (Sigma): cells were treated with 2 mM hydroxyl-urea (HU) overnight, washed 3 times in PBS and released in normal DMEM media.

Double thymidine block (Sigma): cells were treated with 2 mM thymidine for 18 hours, washed 3 times in PBS and released in normal DMEM media for 9

hours, treated a second time with 2 mM thymidine for 14 hours, washed 3 times in PBS and released in normal DMEM media.

Cells were then analyzed by FACS (Fluorescence Activated Cell Sorted) analysis to confirm synchronicity.

2.1.7 Cell proliferation assay

U2OS cells in 24 well plates were transfected in duplicate with siRNA, using the previously described conditions. 24 hours later, the cells were washed, detached with trypsin-EDTA and the duplicates were combined in the same well of a 6 well plate. The next day, cells were once again transfected with siRNA, using the previously described conditions. 24 hours later, the cells were washed, detached with trypsin-EDTA, counted and replated in 96 well plates at a concentration of 2, 000 cells per well. More than 6 hours later, the proliferation at day=0 (D0) was read using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) and a 96 well plate reader (POLARstar Omega, BMG Labtech). Following readings (D1, D2, etc.) were each taken 24 hours after the previous one.

2.1.8 Immunofluorescence

U2OS cells grown on cover slides were fixed for 30 minutes using 3.7% paraformaldehyde. After 3 washes in PBS, cells were permeabilised for 5 minutes at room temperature (RT) with PBS-Triton X100 (1%), washed 3 times in PBS and blocked in PBS – Tween (0.1%) – BSA (5%) for 1 hour at RT under agitation. Primary antibodies were diluted in PBS - Tween 0.05% - BSA 1% for 1 hour at RT (See Table 2.2 for antibodies dilutions). The slides were then washed 3 times in PBS – Tween (0.05%) before being incubated with secondary antibodies for 1h at RT in the dark. After 3 more washes in PBS-Tween 0.05%, slides were incubated with mounting medium containing DAPI (Vector Vectashield) for 5 minutes and then sealed and stored at -20°C. Slides were viewed on a Leica confocal imaging spectrophotometer system (TCS-SP2) and analyzed with Volocity imaging software at the University of Bristol.

2.1.9 Fluorescence Activated Cell Sorting (FACS)

Adherent and detached cells were harvested, pooled, washed once in PBS, and fixed in ice-cold 70% Ethanol at -20°C for at least 16 hours. The cells were then washed twice in PBS – Tween (0.05%) and resuspended in PBS containing 50 µg/ml of propidium iodide and 50 µg/ml of RNAse A. Cells were stained for 20 minutes at RT in the dark. Cells were then analyzed for cell cycle distribution with a BD FACSCanto II (6c) (Becton Dickinson) and FlowJo analyzing software. Red fluorescence (≥ 585 nm) was evaluated on a linear scale and pulse width analysis was used to exclude cell doublets and aggregates from the analysis. Cells with DNA content between 2N and 4N were designated as being in the G1, S, or G2/M phase of the cell cycle. Cells with a DNA content less than 2N were designated as Sub-G1. The number of cells in each stage of the cell cycle was expressed as a percentage of the total cumber of gated cells.

2.1.10 Reporter gene assay

Cells in 6 well plates were harvested by removing media and washing with PBS. 600 µL of Passive Lysis Buffer (PLB, Promega) were added to each well and left to incubate for 15 minutes at RT under agitation. Lysed cells were then removed to eppendorfs and centrifuged at 19, 350 g for 5 minutes. The supernatant was then assayed using the Luciferase Assay System (Promega) in a Lumat LB9507 luminometer (Bethold Technologies). Results were normalized to protein concentration and all experiments were performed a minimum of three times before calculating means and standard deviation as shown in the figures.

2.1.11 Chromatin ImmunoPrecipitation (ChIP)

At 70-80% confluency, cells were washed once in PBS and fixed for 10 minutes at RT with 1% formaldehyde in PBS. The crosslinking was blocked with a 5 minutes incubation in cold PBS- 0.125 M Glycine. The cells were washed twice in PBS, lysed in 500 mL of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL leupeptin, 1 mg/mL aprotinin) and incubated for 10 minutes at 4°C. Samples were sonicated 6 times (30 sec ON, 30 sec OFF, 5 Amp), followed by centrifugation at 14000 rpm for

10 minutes at 4°C. 20 μL of the supernatant was kept apart, diluted by 1:10 (Dilution buffer: 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 150 mM NaCl, 0.1% NP40, 0.05% deoxycholate) and stored at 4°C as "Input". The remaining supernatant was diluted three times and precleared for 2h at 4°C with 2mg of sheared salmon sperm DNA (ssDNA) and 30 μL of beads (1:1 mix of protein A- and protein G-sepharose, 50% slurry) per 500 μL. Immunoprecipitation was performed overnight with 1 μg of specific antibody at 4°C. The immune complexes were captured by addition of 30 μL of beads (1:1 mix of protein A- and protein G-sepharose, 50% slurry) and 2 μg of ssDNA for 1h at 4°C. Immunoprecipitates were washed sequentially for 5 minutes each in Wash Buffer 1 (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 150 mM NaCl), Wash Buffer 2 (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 500 mM NaCl), Wash Buffer 3 (0.25 M LiCl, 1% NP40, 1 deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH8.1) and TE Buffer (10 mM Tris-HCl and 1 mM EDTA). Complexes were eluted with 200 μL of Elution Buffer (1% SDS, 0.1 M $NaHCO_3$) for 1h at room temperature and pelleted at 2000 rpm for 5 minutes at RT. To reverse the crosslinks, the supernatants and the inputs (which were adjusted to 1% SDS, 0.1 M $NaHCO_3$) are incubated at 65°C for at least 6h. The proteins were removed with a phenol choloroform extraction; DNA was then extracted by ethanol precipitation and diluted in 100 μL of TE buffer. 5 μL was used for the PCR reactions.

PCR conditions were as follows: 95°C for 3 minutes and then 35 cycles of 95°C for 40 sec, 55°C for 40 sec, 72°C for 40 sec followed by an additional 7 minutes at 72°C. Quantitative PCR data were generated on a Rotor-Gene 3000 (Corbett Research) using the following experimental settings: Hold 95°C 7 min; Cycling (45 cycles of 95°C for 20 sec; 55°C for 20 sec; 72°C for 20 sec with fluorescence measurement); Melting Curve 55–99°C with a heating rate of 1°C every 5 sec. All values were calculated relative to Inputs levels and normalized to IgG levels using the Pfaffl method (Pfaffl, 2001). All experiments were performed a minimum of three times before calculating means and standard deviation as shown in figures. Human and mouse primers used for ChIP-PCR are as stated in Table 2.1

Table 2-1: Primers used for ChIP assays

Name	Sequence sense	Sequence antisense
Human primers		
PLK4-2634	GTC-CTA-TCA-AAA-CAG-CTA-GGT-TG	GAT-GAA-TTT-ATT-GGG-ATG-TAG-CC
PLK4-1947	GAG-GGG-TTT-GCT-GAG-GAG-CAG	CAC-GTG-ATA-TTG-TTT-GTC-TCT-C
PLK4-1256	TAC-GGT-ATG-TGA-TCC-TGT-ACT-AG	CAT-ACA-CTA-TGC-TCT-TAT-ACC-CC
Gapdh	CGG TGC GTG CCC AGT TG	GCG ACG CAA AAG AAG ATG
Mouse primers		
PLK4-21	GCT ACG GTC AGT CGT ACA CTG	GTG ACG TCA GCA CAC TCT CCA C
PLK4+135	GCC TCT AAG AAG TGG AGA GTG	CCA TCA GGA CGC CTT CTC TAC
PLK4+329	GGA GAG GAT CGA GGT GAG G	CCG CTT TAC TAG TGT CAC TG

2.2 Protein and RNA extracts

2.2.1 Protein extracts

The cells were harvested, pooled, washed once in PBS and lysed using a Total Lysis Buffer (25 mM HEPES, 300 mM KCl, 0.2 mM EDTA, 10% Glycerol, 0.1% NP-40, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 5 nM NaF, 500 μM Na_3VO_4). Cells were lysed by vortexing for a few seconds before incubating on ice for 15 minutes before centrifugation at 19 350 g for 15 minutes at 4°C.

2.2.2 Western Blotting

Proteins were separated by molecular weight using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were boiled for 5 minutes in an appropriate volume of 5x SDS buffer (5% SDS, 50% glycerol, 312.5 mM Tris, 25% β-mercapthoethanol, trace Bromophenol Blue, pH 6.8) and briefly centrifuged prior to loading on a gel. Samples were first run through a 5% w/v stacking gel (5% acrylamide, 0.2 M Tris pH 6.8, 0.1% SDS, 0.1% w/v ammonium persulfate (APS), and 0.1% v/v tetramethylethylenediamine (TEMED)). The resolving gel following contained 10% w/v acrylamide, 0.25 M

Tris pH 8.8, 0.1% SDS, 0.1% APS, 0.04% TEMED. Gels were run between 80 and 150 volts depending on the size of the gel and the level of separation required. The proteins were then semi-dry transferred to a polyvinylidine difloride (PVDF) membrane in PVDF transfer Buffer (20% methanol, 48 mM Tris, 39 mM Glycine, 0.02% SDS) at 21 volts for 30 minutes – 1.5 hours depending on the size of the protein being transferred. Membranes were blocked for one hour in tris buffered saline (TBS)-tween (20 mM Tris pH7.6, 150 mM NaCl, 0.1% tween) supplemented with 5% powered milk, washed once with TBS-Tween and incubated overnight at 4°C with primary antibodies (see table 2.2 for dilutions). Antibodies are diluted to the appropriate dilution in TBS – Tween 0.25% - BSA 2% - sodium azide (NaAz) 0.1%. The membrane was washed 3 x 10 minutes in TBS-Tween and incubated in secondary antibody, conjugated with the Horseradish Peroxidase (HRP) in TBS-Tween – 5% milk for 1h at RT before washing as before. Specific antibodies were visualized using enhanced-chemiluminescence (ECL) solution (Pierce) and exposed to autoradiography film. The membrane was probed for additional proteins by stripping away the first set of antibodies in Stripping Buffer (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol, pH 6.7) for 30 minutes at 55°C. The membrane was then washed in TBS-Tween, blocked and reprobed as described before.

2.2.3 Antibodies

Primary antibodies, as well as concentration or dilution, are used as stated in Table 2.2.

Table 2-2: Primary antibodies

ChIP stands for Chromatin Immuno-Precipitation, IF for Immuno- Fluorescence

Antibody	Company	Western Blot	ChIP	IF
p52 Monoclonal	Upstate	1/1000	1 µg	1/100
p52 Polyclonal (sc-848)	Santa Cruz	1/1000	1 µg	1/100
p52-pS222		1/400		
RelA polyclonal (sc-372)	Santa Cruz	1/1000	1 µg	
RelB polyclonal (sc-226)	Santa Cruz	1/1000	1 µg	
cRel polyclonal (sc-71)	Santa Cruz	1/1000	1 µg	
p50 (06-886)	Upstate	1/1000	1 µg	
PLK4	Cell Signalling	1/500		
CDK2	Santa Cruz	1/1000		
Cyclin B1	Santa Cruz	1/1000		
Cyclin A	Santa Cruz	1/1000		
Cyclin E	Santa Cruz	1/1000		
Cdc2	CRUK	1/1000		
β -actin	Sigma	1/10000		
RNApol II	Santa Cruz		1 µg	
Ac-Histone H3	Upstate		1 µg	
HA	CRUK		1 µg	
IgG	Sigma		1 µg	
Cyclin D1	Santa Cruz	1/1000		
γ -tubulin	Sigma			1/500
α -tubulin	Sigma			1/500
Centrin2	Abcam			1/1000
CDK1	CRUK	1/1000		
CDK4	Santa Cruz	1/1000		

Secondary antibodies used for Western Blotting were:

Anti-mouse IgG HRP-conjugate (Upstate, 12-349)

Anti-rabbit IgG HRP-conjugate (Sigma, AO545)

Secondary antibodies used for Immunofluorescence were as follow:

Rhodamine RedX-conjugated donkey Anti-rabbit IgG (Jackson Laboratories, 711295152)

FITC Anti-mouse IgG (BD Pharmingen, 554001)

2.2.4 RNA extracts

For reverse transcriptase PCR, mRNA was extracted with the Total RNA kit (Peqlab), according to manufacturers instructions. Briefly, DNA was first removed by binding to a column. After centrifugation, the filtrate from the column containing the RNA was diluted with an equivalent volume of 70% Ethanol before loading on a RNA binding column. After three washes with the provided buffers and drying the column, the RNA was eluted using 50 µL of TE buffer and quantified using a Thermo Scientific NanoDrop2000c spectrophotometer with the analysis software NanoDrop2000.

2.2.5 Reverse transcription

1 µg of RNA sample was reverse transcribed using the Quantitect Reverse Transcription Kit (QIAgen; 205313) according the manufacturer's instructions. Briefly, the genomic DNA was first removed by a 2 minutes incubation at 42°C using an appropriate buffer. The cDNA was then generated by adding Reverse transcriptase and its buffer, with a mix of primers to the RNA. This was incubated for 15 minutes at 42°C, followed by a 3 minutes incubation at 95°C to inhibit the enzyme. The cDNA stock was diluted by to a concentration of 5 ng/µL and 5µl (25ng) was used for PCR with GoTaq flexi DNA polymerase (Promega; M8305).

2.2.6 Quantitative Polymerase Chain Reaction (PCR)

Quantitative PCR was performed using GoTaq flexi DNA polymerase (Promega; M8305), with 3 nM of MgCl2, Sybr Green (Sigma) and 0.4 µM of each primer.

Quantitative PCR data were generated on a Rotor-Gene 3000 (Corbett Research) using the following experimental settings: Hold 95°C 7 min; Cycling (45 cycles of 95°C for 20 sec; 58°C for 20 sec; 72°C for 20 sec with fluorescence measurement); Melting Curve 50–99°C with a heating rate of 1°C every 5 sec. All values were calculated relative to untreated levels and normalized to GAPDH levels using the Pfaffl method (Pfaffl, 2001). All experiments were performed a minimum of three times before calculating means and standard deviation as shown in figures. Human and mouse primers used for PCR are as stated in Table 2.3:

Table 2-3: primers used for PCR

Gene	Sequence sense	Sequence antisense
Human primers		
Nf-κb2 (p100)	GGG CAG ACC AGT GTC ATT GAC	CCA TGC CGA TCC AGC AGA G
RelA	CTC-GGT-GGG-GAT-GAG-ATC-TTC	CCG-GTG-ACG-ATC-GTC-TGT-ATC
RelB	CAT-CGA-GCT-CCG-GGA-TTG-T	CTT-CAG-GGA-CCC-AGC-GTT-GTA
cRel	AGA GGG GAA TGC GTT TTA GAT ACA	CAG GGA GAA AAA CTT GAA AAC ACA
p50	TCC-CAT-GGT-GGA-CTA-CCT-GG	ATA-GGC-AAG-GTC-AGG-GTG-C
PLK4	GAT CAT TTG CTG GTG TCT A	ACC TCA TTT TGG ACT CTC TG
SAS6	GAA TGA GCA TTG AAC TAC A	GGT GAG TTA TCC AAA ATA GC
Cyclin B1	ATA-AGG-CGA-AGA-TCA-ACA-TGG-C	TTT-GTT-ACC-AAT-GTC-CCC-AAG-AG
Cyclin D1	CCA TTC CCT TGA CTG CCC GAG	GAC CAG CCT CTT CCT CCA C
CDK2	ATC TCT CGG ATG GCA GTA	GTT GTG TAC AAA GCC AGA AAC
GAPDH	GGT CGT ATT GGG CGC CTG GTC ACC	CAC ACC CAT GAC GAA CAT GGG GGC
Centrin2	GAA CTG GCA CCA TAG ATG T	TTT CCT GTC CCT TCC TTA T
CDK1	AAA CTA CAG GTC AAG TGG TAG CC	TCC TGC ATA AGC ACA TCC TGA
CDK4	AGA GTG TGA GAG TCC CCA ATG	CAA ACA CCA GGG TTA CCT TG
cyclin A	CGC TGG CGG TAC TGA AGT C	GAG GAA CGG TGA CAT GCT CAT
cyclin E	TTA CCC AAA CTC AAC GTG CAA	GCT CAA GAA AGT GCT GAT CCC
γ-tubulin	TAC AAC CCA GAG AAC ATC TA	CAT CTG CTT CTC GGT CTA T
Mouse primers		
Nf-κb2 (p100)	CTA-ATG-TGA-ATG-CCC-GGA-CC	GAG-CAG-CAT-TTA-GCA-GCA-GAG
PLK4	AGG-AGA-AAC-TAA-TGA-GCA-CCA-CA	TGG-CTC-TCG-TGT-CAG-TCC-AA
GAPDH	GCTACACTGAGGACCAGGTTG	GCCCCTCCTGTTATTATGGGG

2.3 Molecular Biology techniques

2.3.1 Escherichia coli growth conditions

Escherichia coli (E.coli) bacteria were grown in Luria-Bertani (LB) medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0) and LB agar plates (as for LB medium with 15 g of agar added prior to autoclaving.

2.3.2 Transformation of competent E. coli cells

Competent E. coli cells (Subcloning Efficiency DH5α competent cells, Invitrogen) were transformed with plasmid DNA using the heat shock method. Approximately 100 ng of plasmid DNA or 10 µL of ligation was added to 50 µL of competent cells and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds and immediately placed on ice for 2 minutes to recover. 500 µL of LB medium was then added and cells were shaken for 1 hour at 37°C to allow the bacteria to recover and produce the antibiotic resistance marker. The mixture was then spread onto the LB plates containing the required antibiotic (Ampicillin was used at 100 µg/mL, Kanamycin at 50 µg/mL) and grown overnight at 37°C.

2.3.3 Minipreparation of plasmid DNA

For small-scale preparation, plasmid DNA was extracted from a 3 mL overnight culture grown from a single colony in LB medium with the required antibiotic. DNA was extracted using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega) according to manufacturers instructions. Briefly, the overnight culture was centrifuged at 3272 g and the pellet containing the bacteria was lysed after resuspension. The bacterial lysate was precipitated and centrifuged, the supernatant being then loaded on a DNA binding column. After three washes with the appropriate buffers, the DNA was eluted in 100 µL TE buffer.

2.3.4 Maxipreparation of plasmid DNA

For large-scale experiments, plasmid DNA was extracted from a 300 mL overnight culture grown from a single colony in LB medium with the required antibiotic. Preparation of DNA was extracted using the Pureyield maxiprep kit (Invitrogen) according to manufacturers instructions. To describe the system briefly, the overnight culture was centrifuged at 3272 g and the pellet containing the bacteria is lysed. The bacterial lysate was precipitated and the whole suspension (containing DNA and bacterial lysate) was loaded on a DNA binding column, containing a filter that retains debris, previously correctly equilibrated. After three washes, the DNA was eluted with the elution buffer, precipitated with isopropanol, washed with 70% ethanol and then resuspended in 300 μL of TE buffer.

2.3.5 Quantification of DNA and RNA

The DNA or RNA concentration was quantified by measuring the optical absorbance at 260 nm with the Thermo Scientific NanoDrop2000c spectrophotometer with the analysis software NanoDrop2000. The concentration was calculated using the formula A260 1.0 = 50 μg/mL for double strand DNA or A260 1.0 = 40 μg/mL for RNA.

2.3.6 Agarose Gel electrophoresis

DNA products were resolved using 1-2% agarose gels (depending of the size of the fragment of interest) containing Tris-acetate Buffer (TAE, 40 mM Tris-acetate, 1 mM EDTA) and SYBR Safe DNA gel Stain (Invitrogen). Gel loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to each sample and the gel was run at 120V for up to 1 hour at RT. DNA was visualized using a luminescent Image Analyzer LAS-4000 (Fujifilm). To verify the size of the DNA, a 1 Kbp or 100 bp DNA ladder (Invitrogen) was run alongside. Fragments for use in subcloning were excised using a clean scalpel.

2.3.7 Purification of DNA fragments from agarose gels

DNA fragments were purified using the QIAquick gel extraction kit (Qiagen) according to manufacturers instructions. For this purpose, small sized agarose bands containing the DNA to extract were melted at 65°C in a buffer with beads which bind to the DNA. After few washes, beads were dried and DNA was eluted with 30 μL TE buffer.

2.3.8 Digestion of DNA with restriction endonucleases

When required for subcloning, DNA digestion was performed at required sites using recombinant enzymes and buffers supplied by New England Biolabs or Promega, according to manufacturers instructions. Digestion was typically performed in a 20 μL volume, using 0.5 μL of the required enzyme and incubating for 1 hour at 37°C.

2.3.9 Phosphatase treatment of linearized DNA

To prevent self-ligation of the DNA vector after digestion, the 5' phosphate groups were removed by the addition of Shrimp Alkaline Phosphatase (SAP, Promega), according to manufacturers instructions. This consisted of the addition of 1 μL of shrimp alkaline phosphatase enzyme and its buffer to the digested buffer and incubation for 15 minutes at 37°C.

2.3.10 Ligations

Ligations were performed using New England Biolabs T4 DNA ligase according to manufacturers instructions. A 1:5 ratio of vector:insert was used. Ligations were performed in a 20 μL volume using 0.5 μL enzyme and incubated at 16°C overnight. For each ligation, a control experiment without insert was set up to evaluate the level of self-ligation of the plasmid backbone.

2.3.11 DNA sequencing

DNA sequencing was performed by the sequencing service in Dundee (College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using ABI 3730 Capillary DNA Sequencers.

2.3.12 Plasmids

For all plasmids used in the study, see Table 2.4

Table 2-4: Plasmids used in the study

Name	Source	Description
RNAi plasmids		
pSilencer scramble	NDP lab	U6 plasmid containing a scramble RNAi oligo
pSilencer p100	NDP lab	U6 plasmid containing a p100/p52 RNAi oligo
pSilencer RelA	NDP lab	U6 plasmid containing a RelA RNAi oligo
pSilencer RelB	Rocha lab	U6 plasmid containing a RelB RNAi oligo
pSilencer cRel	Rocha lab	U6 plasmid containing a cRel RNAi oligo
pSilencer p50	Rocha lab	U6 plasmid containing a p105/p50 RNAi oligo
pSilencer panNF-κB	Rocha lab	U6 plasmid containing a RNAi oligo against all NF-κB subunits
RSV Expression plasmids		
pRcRSV-NRC/MS	NDP lab	pRcRSV basic plasmid (Invitrogen) with multiple cloning site insertion
pRcRSV-p52	NDP lab	p52 in pRcRSV-NRC/MS
pRcRSV-RelA	NDP lab	RelA in pRcRSV-NRC/MS
pRcRSV-RelB	NDP lab	RelB in pRcRSV-NRC/MS
pRcRSV-cRel	NDP lab	cRel in pRcRSV-NRC/MS
pRcRSV-p50	NDP lab	p50 in pRcRSV-NRC/MS

Table 2.4 (continued): Plasmids used in the study

HA Tagged Expression plasmids		
pCMV-HA	NDP lab	pCMV5 Eukaryotic Expression plasmid encoding an HA Tag
HA-p52	NDP lab	pCMV5 plasmid encoding HA tagged p52
HA-52-DBM	NDP lab	pCMV5 plasmid encoding HA tagged p52 with 2 aa substitutions (R52A/R54A)
HA-p52-S222A	NDP lab	pCMV5 plasmid encoding HA tagged p52 with 1 aa substitution (S222A)
HA-p52-S222D	NDP lab	pCMV5 plasmid encoding HA tagged p52 with 1 aa substitution (S222D)
Luciferase reporter plasmids		
prPLK4-2634-1256	Dundee Cell Products	smaller version of the prPLK4-wild type promoter, going from the |B site -2634 up to shortly after the transcription start.
prPLK4-full length	Dundee Cell Products	PLK4 promoter (4621 bp) inserted in pGL3 basic vector
prPLK4-Δ3537	This study	SacI deletion in prPLK4, deleting the |B site -3537, from 1 to 937 bp
prPLK4-Δ1256	This study	KpnI deletion in prPLK4, deleting the |B sites -3537 to -1256 from 1 to 3263 bp
prPLK4-+1	Dundee Cell Products	shortest version of the prPLK4 promoter, starting after the |B site -1256 and finishing shortly after the transcription start point, thus deleting all |B sites
prPLK4-2634-1256	Dundee Cell Products	smaller version of the prPLK4-wild type promoter, going from the |B site -2634 up to shortly after the transcription start.

3 Results Chapter 1:

Characterization of p52 regulation of the cell cycle

3.1 Introduction

Previous data in the lab showed that the p52 NF-κB subunit could localize to the centrosome during interphase and various stages of mitosis. Furthermore U2OS cells stably expressing p100/p52 shRNA displayed centrosome structure defects. p52 is also involved in G2/M progression in U2OS cells as p52 depletion leads to defects in mitosis. These are visualized by disruption of the microtubule network, defects in chromosome segregation, increase in multinucleate cells, aberrant chromosome structure, as well as poor chromosomal alignment on the metaphasic plate (K.Schumm, unpublished data). The first goal of this PhD project was to reproduce and confirm the centrosomal localization of p52. Using U2OS cells transiently transfected with control or p100/p52 siRNAs, any defects in mitotic spindle were investigated.

The p52 NF-κB subunit, by regulating the expression of genes involved in cell proliferation, can regulate cell growth and proliferation (Schumm et al, 2006). Effects of NF-κB in general and p52 in particular on the cyclin D1 promoter have been well described. Indeed, p52 co-operates with its co-activator Bcl-3 to regulate cyclin D1 expression (Schumm et al, 2006). This is illustrated, in many cell lines, by the down-regulation of cyclin D1 expression in the absence of p52, or its activation by p52/RelB recruitment upon TNF-α stimulation (Witzel et al, 2010). We decided to confirm these results using transient transfection of p100/p52 siRNA and to extend the analysis to the expression of other cyclins (cyclin A, cyclin B1, cyclin E) and CDKs (CDK1, CDK2, CDK4).

As defects in the centrosome structure were previously observed after p100/p52 depletion, I was also interested in investigating the regulation of genes involved in centrosome duplication. Indeed, some key effectors tightly regulate the centrosome duplication cycle. Alterations in their expression can inhibit centrosome duplication or promote over-amplification and thus lead to multinucleate cells. CP110 siRNA for example blocks reduplication (Chen et al,

2002), whereas p53 mutations induce centrosome amplification (Shinmura et al, 2007).

Among the list of proteins involved in centrosome duplication (Figure 1,7), we focused on PLK4, CDK2, SAS6, AuroraA and γ-tubulin, as the key regulators of this process. The expression of some of these genes was downregulated upon p52 depletion. PLK4 expression seemed the most affected; therefore its regulation was investigated upon depletion or over-expression of other NF-κB subunits.

3.2 Results

3.2.1 Co-localization of p100/p52 with the centrosome

The first experiment performed was to confirm p100/p52 localization to the centrosome. U2OS cells were fixed with paraformaldehyde and labeled with antibodies against p100/p52 and γ-tubulin to detect the centriole. DAPI was used to detect DNA and differentiate cells in interphase from cells in various mitotic phases (Figure 3.1). As shown with the three cell images presented in Figure 3.1, p100/p52 localized to the centrosome. Co-localization was also observed in the case of a multinucleate cell (middle panel). This data confirmed previous findings and created a solid basis for the project.

U2OS cells were then transiently transfected with a p100/p52 or control siRNA, to investigate any effect on the centrosome structure. The mitotic spindle was labeled with an α-tubulin antibody. Results showed that the centrosome structure was not affected by p52 depletion in this transient assay (Figure 3.2, top panel). The CDK1 inhibitor RO-3306 was also used to delay the progression through mitosis and to possibly enhance the p52 effect. However, no effect on the mitotic spindle was observed, whether p52 was present or deleted in cells treated with RO-3306 (Figure 3.2, bottom panel). Western blotting was performed to confirm the efficiency of siRNA transfection. These results demonstrated that p52 does not seem to affect the mitotic spindle in U2OS cells, in contrast to the previous results.

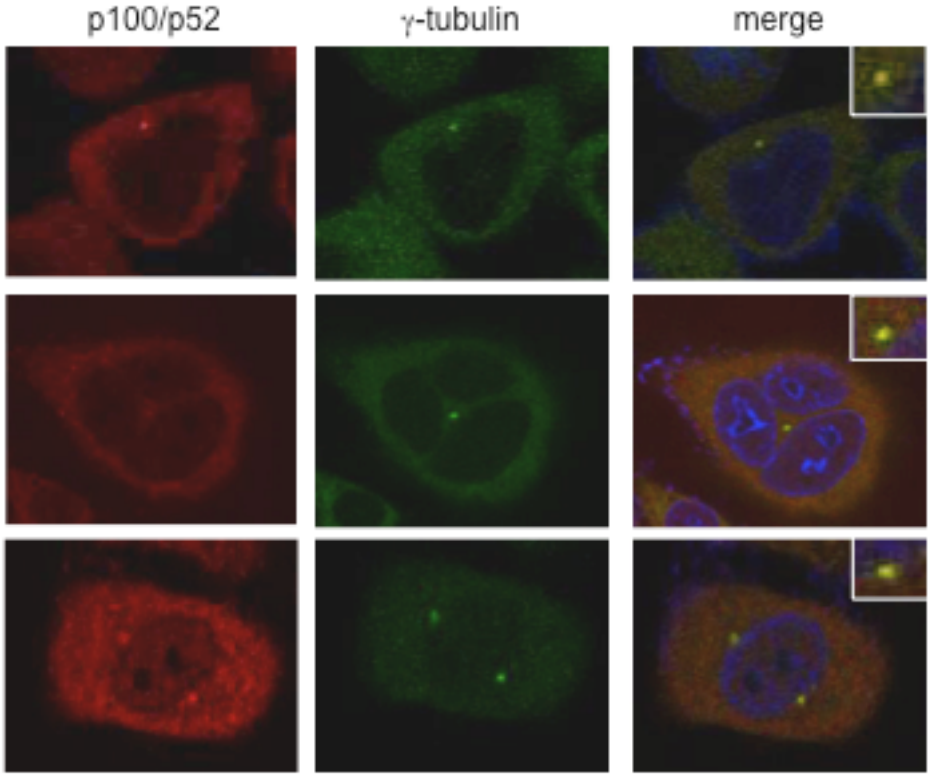


Figure 3-1: p52 co-localizes to the centrosome

U2OS cells were plated the night before imaging, fixed with paraformaldehyde the next day, and then labeled with antibodies as described in material and methods. Cells were labeled with antibodies for p100/p52 (red), γ-tubulin (green) and and stained with DAPI to reveal DNA (blue).

3 different cells, representative of the population, are shown. Insets show an enlargement of the centrosome area.

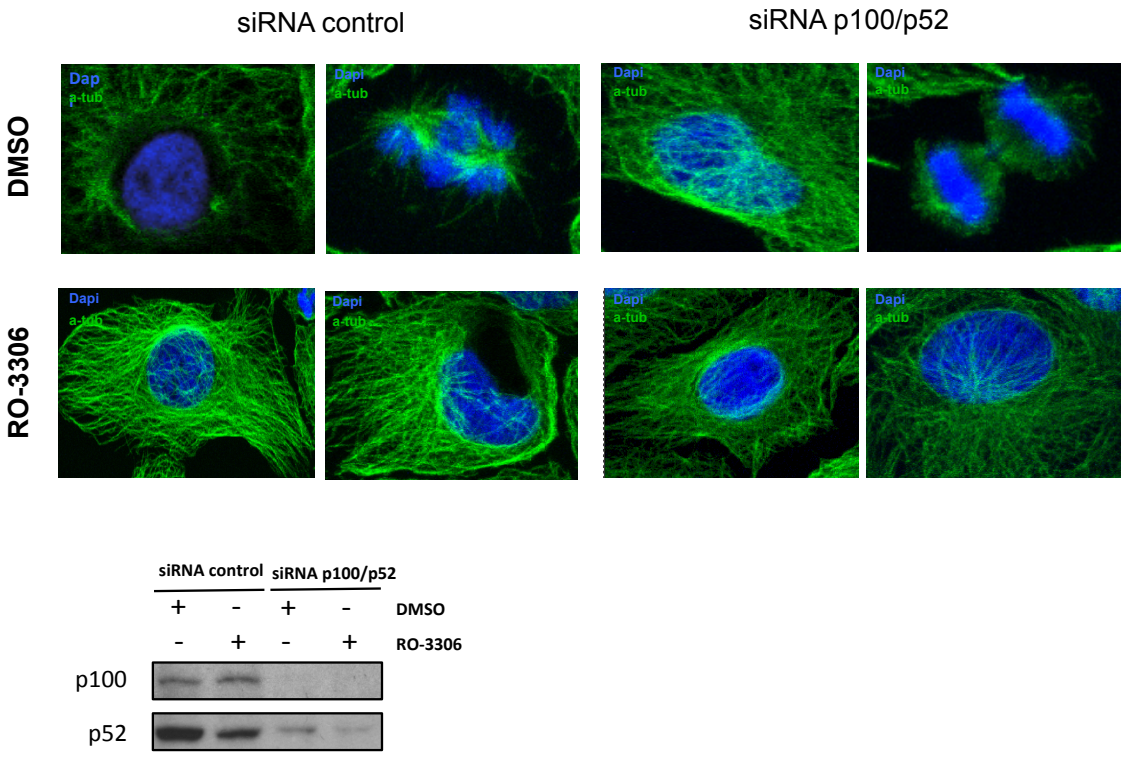


Figure 3-2: p52 transient depletion does not affect the mitotic spindle

U2OS cells were subjected to transient siRNA transfection. After 48h, cells were treated for 2h with the CDK1 inhibitor R0-3306 or DMSO, fixed with paraformaldehyde, and then labeled with α-tubulin antibody (green) and stained with DAPI to reveal the DNA as described in material and methods. Cells shown are representative of the population for each treatment. Protein expression is shown to demonstrate the efficiency of the siRNA transfection.

However, it is important to note that many previous effects were seen in stable cell lines expressing p100/p52 shRNA, where the cells overcame p52's other effects on the cell cycle. This will be discussed further in the discussion section of this capter.

3.2.2 p52 affects cyclin and CDK gene expression

It is well known that NF-κB is involved in the regulation of the cell cycle and the p52 subunit was previously reported to regulate cyclin D1 expression as well as participate in G1/S progression by our lab and others (Guttridge et al, 1999; Schumm et al, 2006). To more fully characterize this p52 effect, we decided to look at the expression of all cyclins and CDKs after p100/p52 siRNA depletion (Figure 3.3). A decrease in cyclin D1 expression following p100/p52 knockdown was detected with both mRNA (Figure 3.3.A) and protein (Figure 3.3.B), confirming previously published data. No significant effect was seen on cyclin A, cyclin E and cyclin B1 mRNA or protein expression, although cyclin E seemed to present a slight decrease in protein expression. In the same manner, CDK1 expression did not appear to be dependent of p52. By contrast, p52 depletion reduced the mRNA and protein expression of CDK4, involved in early G1, and CDK2, required during the G1 and S phases. FACS analysis of cellular DNA context in Figure 3.3.C demonstrated that the loss of CDK4 may not be due to a overall reduction of the number of cells in G1 phase, as the DNA profiles were similar for control cells and cells depleted of p52. This confirms the importance of p52 during the G1/S transition and S phase progression. Interestingly, as no effect was detected on the Cyclins and CDK responsible for G2/M transition, this would indicate that any effect seen on the mitotic spindle and chromosome arrangement by p52 depletion may not be linked to an incorrect G2/M progression, suggesting another type of p52 regulation for the mitotic spindle.

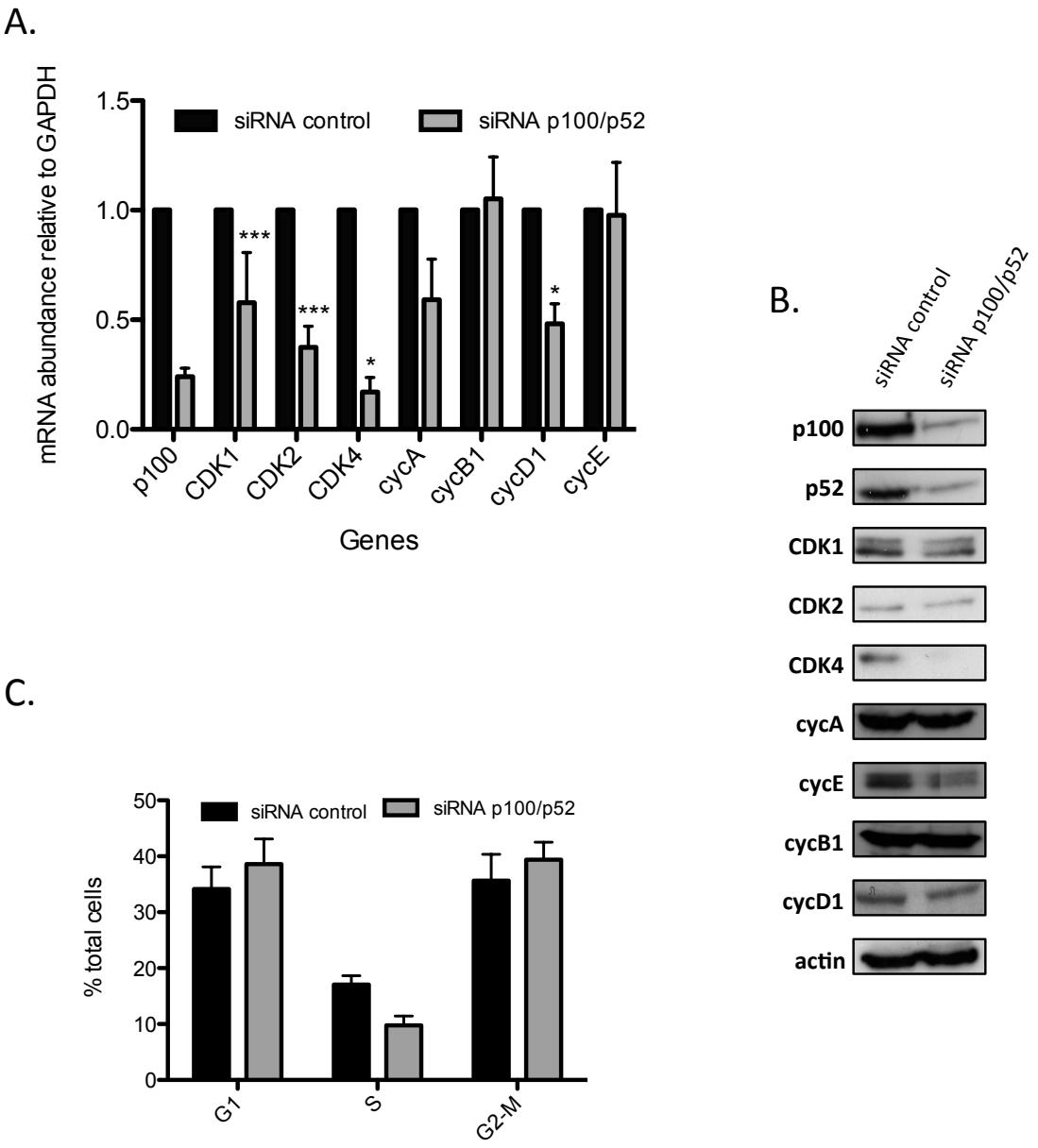


Figure 3-3: p52 depletion affects the expression of cyclin and CDK genes

U2OS cells were subjected to transient siRNA transfection and after 48h, cells were lysed. **A.** mRNA were analyzed by quantitative PCR, compared to a housekeeper gene and related to the expression with siRNA control. Errors bars show the standard error of at least 3 independent experiments **B**. Protein expression was detected by Western Blot. **C.** DNA profile after siRNA transfection was analyzed by flow cytometry

Error bars show the standard error of at least 3 independent experiments.

* P<0,05 ; *** P<0,001

3.2.3 Genes involved in the centrosome duplication

To further investigate any p52 effect on centrosome duplication and function, I decided to look at the expression of genes involved in centrosome duplication, such as PLK4, AuroraA, SAS6 and γ-tubulin following p100/p52 depletion. U2OS cells were transiently transfected with control or p100/p52 siRNAs and mRNA levels were determined by RT-PCR. Figure 3.4 demonstrates that, upon p52 depletion, γ-tubulin expression did not significantly vary, thus providing a negative control. However, CDK2 (as already detected in Figure 3.3), PLK4, AuroraA and the PLK4 effector SAS6 mRNA expression were significantly reduced in the absence of p52, however with different P-values. The loss of CDK2 upon p100/p52 depletion was observed previously in Figure 3.3. The loss of PLK4 expression was the strongest as the mRNA levels were reduced by 80% upon p100/p52 depletion. As SAS6 has been identified as one of the PLK4 effectors, the loss in SAS6 mRNA could be linked to the loss of PLK4. AuroraA expression was reduced by 50% after p100/p52 depletion. These effects provide a potential direct link between NF-κB and the regulation of the centrosome, as well as with the spindle checkpoint, which has not been shown previously.

Although PLK4 substrates have not all been identified, PLK4 can cooperate with CDK2 to enable correct centriole duplication (Habedanck et al, 2005). PLK4 causes the activation of down-stream regulators, such as SAS6, a coiled-coil protein recruited to the centriole (Rodrigues-Martins et al, 2007). Regulation of PLK4 gene expression has not been studied in much detail to date, and no link has been previously made with NF-κB. We therefore decided to focus the rest of the project on the regulation of PLK4 expression by NF-κB.

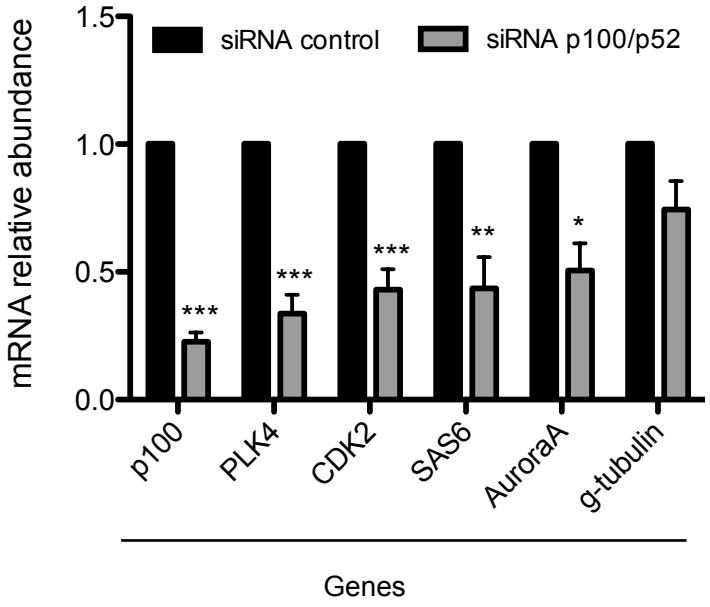


Figure 3-4: p52/p100 depletion affects the expression of various genes involved in centrosome duplication

U2OS cells were subjected to transient siRNA transfection. After 48h, cells were lysed and the RNA extracted. mRNA expression was analyzed by quantitative PCR, compared to a housekeeper gene and related to the expression with siRNA control. Errors bars show the standard error of at least 3 independent experiments. * P<0,05 ; ** P<0,001; *** P<0,0001

3.2.4 NF-κB affects PLK4 expression

NF-κB depletion reduces PLK4 expression

To begin studying the role of NF-κB in the regulation of PLK4 expression, a siRNA that targets all NF-κB subunits simultaneously (siRNA Pan-NFκB) (van Uden et al, 2011) was transfected into U2OS cells (Figure 3.5). Protein expression analysis showed that after simultaneous depletion of all NF-κB subunits, PLK4 protein levels were reduced (Figure 3.5, left panel). This was confirmed at the mRNA level by qPCR, where PLK4 mRNA decreased by more than 50% after Pan-NF-κB siRNA treatment (Figure 3.5, right panel). Western blot (left panel) and RT-PCR (data not shown) confirmed the transfection efficiency by showing that the levels of the different NF-κB subunits were all reduced.

Individual siRNAs were then used to determine the influence of each NF-κB subunit on PLK4. As can be seen in Figure 3.6, all NF-κB subunits independently reduced PLK4 levels, at both the protein (Figure 3.6, left panel) and mRNA (Figure 3.6, right panel) levels. However, this decrease did not happen with the same efficiency for all subunits. c-Rel depletion seemed to most affect PLK4 protein and mRNA levels, with PLK4 mRNA decreased by 80% after c-Rel knockdown. A significant decrease in PLK4 mRNA levels was also obtained after p100/p52 or RelB siRNA depletion. The effect on protein levels differed between subunits. Nevertheless an effect for all subunits was still seen. RelA depletion seemed to completely inhibit PLK4 protein expression, but the effect on mRNA was not as strong. Interestingly, RelA depletion also inhibits the expression of the other NF-κB subunits. In the absence of p50, protein levels remained unaffected but mRNA levels were reduced by 50%. The difference between mRNA and proteins levels might be due to the poor quality of commercial antibodies or to difficulties detecting low basal levels of PLK4 protein.

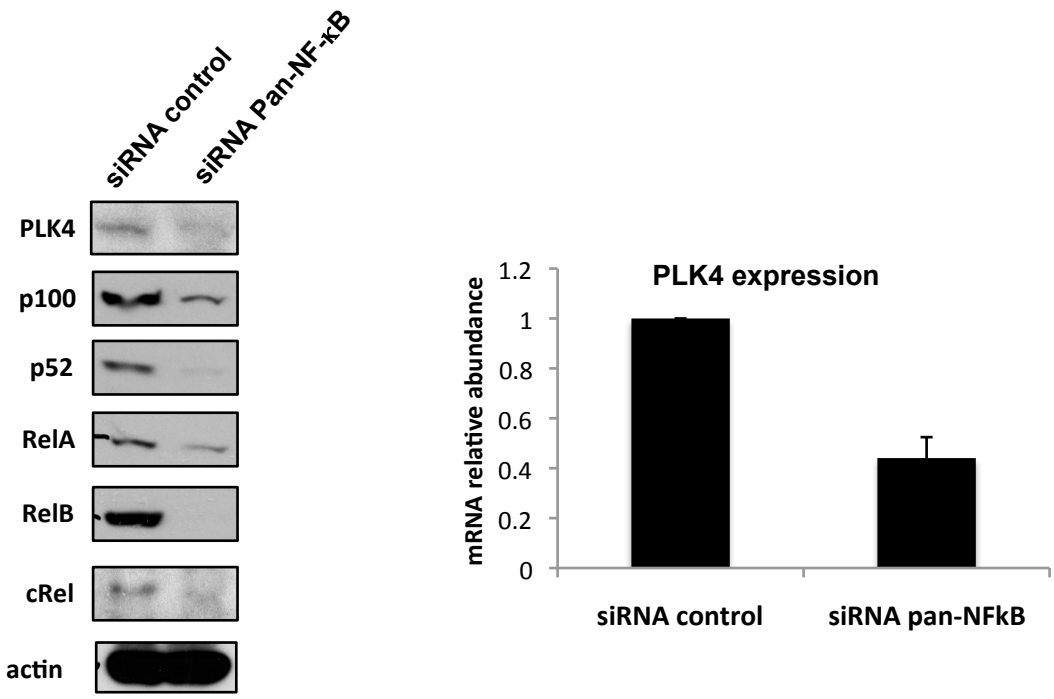


Figure 3-5: NF-κB depletion affects the expression of PLK4 in U2OS cells

U2OS cells were subjected to transient siRNA transfection against all NF-κB subunits. After 48h, cells were lysed and the proteins and RNA extracted. Protein levels were analyzed by Western Blot (left panel). mRNA expression (right panel) was analyzed by quantitative PCR, compared to a housekeeper gene (GAPDH) and related to the expression of the siRNA control. Results are the average of at least 3 independent experiments.

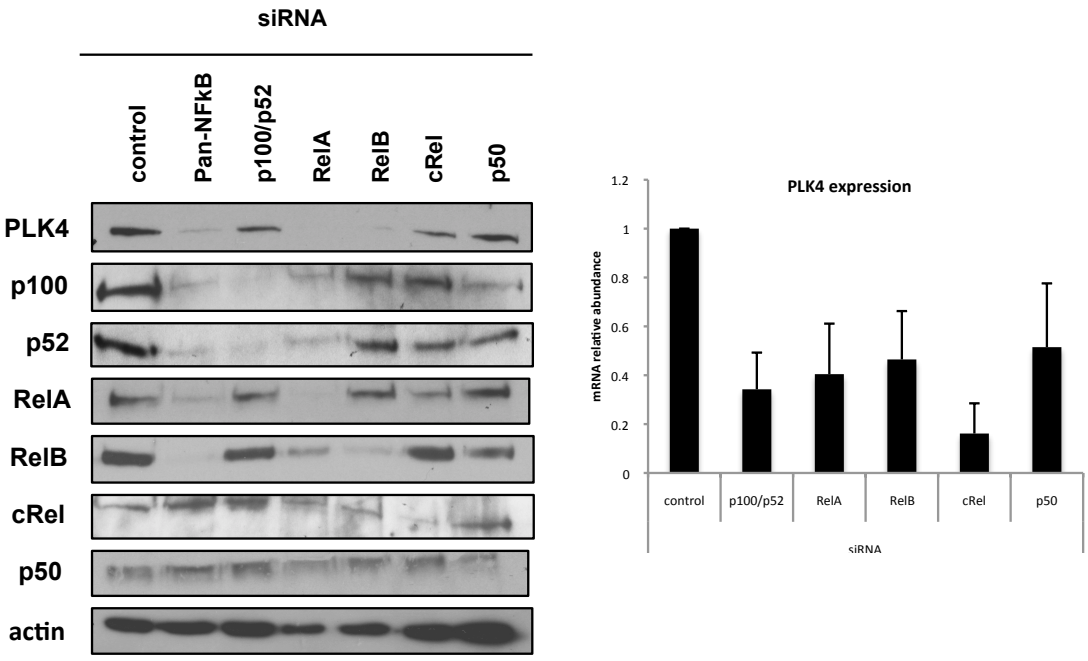


Figure 3-6: NF-κB subunit depletion affects the expression of PLK4 in U2OS cells

U2OS cells were subjected to transient siRNA transfection against various NF-κB subunits. After 48h, cells were lysed and the proteins and RNA extracted. Protein levels were analyzed by Western Blot (left panel). mRNA expression (right panel) was analyzed by quantitative PCR, compared to a housekeeper gene (GAPDH) and related to the expression of the siRNA control. Results are the average of at least 3 independent experiments.

Another hypothesis is that further post-transcriptional regulation could induce such differences between mRNA and protein levels. I have confirmed the overall effect of NF-κB subunit depletion on PLK4 mRNA and protein levels in other cell lines such as Hela cells and H1299 cells (data not shown).

Therefore, this data confirms that NF-κB depletion reduces PLK4 expression and that all subunits are involved in its regulation, although not all subunits seem to regulate PLK4 expression with the same efficiency.

NF-κB overexpression increases PLK4 expression

To confirm the NF-κB effect on PLK4 protein levels, Hela cells were transfected with plasmids to overexpress the different NF-κB subunits. As seen in Figure 3.7, subunits over-expression also induced an increase in PLK4 protein level. It is also notable that, although the overexpression efficiency did not seem significant for most NF-κB subunits, the effect on PLK4 protein levels was very strong. However, in the case of p52 overexpression, the results were not as convincing but the efficiency of the transfection was also less than in the case of other subunits. Nonetheless, this result confirms that PLK4 expression is regulated by NF-κB.

Depletion or overexpression of NF-κB subunits, individually or simultaneously, in various cell lines allowed me to determine that PLK4 is likely to be a NF-κB target gene. PLK4 could therefore provide the previously identified link between the p52 subunit and regulation of mitotic spindle formation.

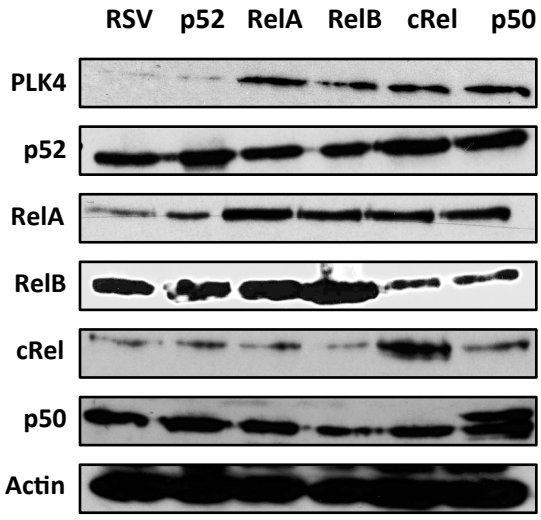


Figure 3-7: NF-κB subunit overexpression increases the expression of PLK4 in Hela cells

Hela cells were subjected to transient DNA transfection to overexpress NF-κB subunits. After 48h, cells were lysed and the proteins extracted. Protein levels were analyzed by Western Blot.

3.2.5 p52 and PLK4 affect cell proliferation

Our lab has previously shown that the p52 NF-κB subunit can reduce cell proliferation in cells transiently depleted of p100/p52 (Schumm et al, 2006). PLK4 is known to be essential for centrosome duplication and error free division. Therefore, to determine if loss of PLK4 could contribute to p52 effects on proliferation, U2OS cells were transiently transfected with siRNAs against p100/p52 or PLK4. MTT-MTS assays were performed to determine cell proliferation (Figure 3.8, left panel). Western blotting was used to verify the efficiency of transfection (Figure 3.8, right panel). As can be seen in the proliferation assay (Figure 3.8), the p100/p52 siRNA decreased the proliferation of U2OS cells, and the effect can be seen even at Day 1. PLK4 depletion also inhibited cell proliferation, significantly stronger than p52 alone.

These experiments confirm the importance of p52 and PLK4 to the correct progression through the cell cycle, at least in U2OS cells, and thus the necessity for tight regulation of both.

3.3 Discussion

Previous unpublished results in the Perkins lab showed that the p52 NF-κB subunit could associate with the centrosome in U2OS cells in interphase and during mitosis. To characterize the p52 effect on the cell cycle, my first experiment was to repeat and validate such data. To this extent, U2OS cells were observed by confocal microscopy, after immunostaining for p100/p52 and γ-tubulin (Figure 3.1). Observations showed that p100/p52 does indeed localize to the centrosome for cells in interphase. However, no co-localization of p100/p52 with the centrosome has been seen for cells in mitosis (data not shown). It is possible that incorrect experimental conditions were used, or p100/p52 localization to the centrosome in mitosis happened in only a low percentage of cells and therefore would have been missed in these conditions. Further experiments will be performed to validate the proposed p52 localization to the centrosome.

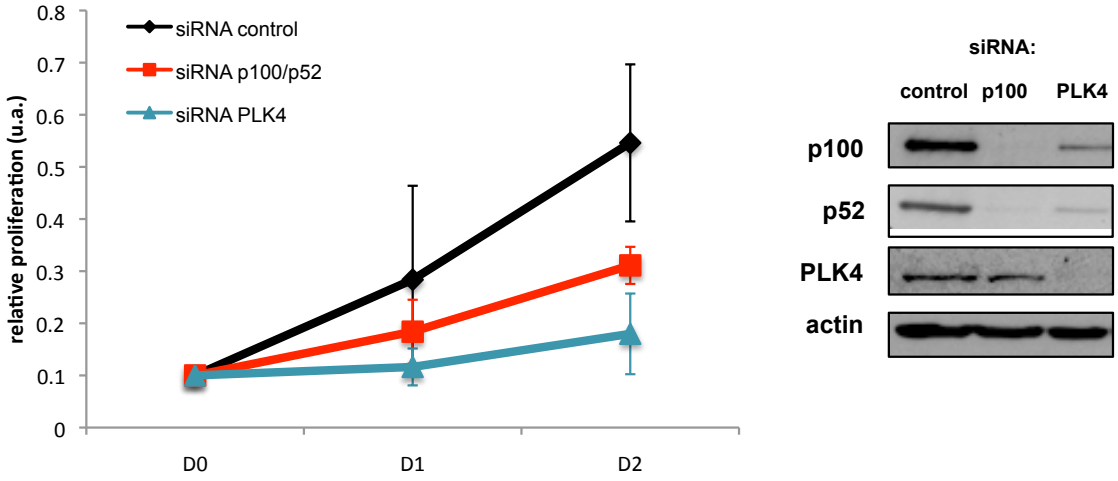


Figure 3-8: p52 and PLK4 affect cell proliferation

U2OS cells were transiently transfected with control, p100/p52 or PLK4 siRNAs and a MTT/MTS proliferation assay (left panel) was performed, as in Material and Methods. Western Blot (right panel) is shown for the siRNA transfection efficiency.

Error bars shows the standard deviation of at least 3 independent experiments.

Nonetheless, our experiments confirmed p100/p52 localization to the centrosome in cells in interphase. Further investigation will determine whether p100/p52 associates with centrosomal proteins. A technique recently developped in the lab allows the visualization by microscopy of the skeleton of a cell, as well as the internal structures but with no cytoplasm, therefore eliminating all cytoplasmic proteins (Kuznetsov et al, 2008). Optimization of this technique to specially envision the centrosome would allow us to more precisely observe the association of p100/p52 or other NF-κB subunits with the centrosome, by reducing the cytoplasmic NF-κB background.

The p52 NF-κB subunit has also been previously reported to regulate the expression of genes involved in the cell cycle, such as cyclin D1 (Barre & Perkins, 2007; Schumm et al, 2006). Analysis of various cyclins and CDKs showed regulation of their expression by p52 (Figure 3.3). Cyclin D1 was used as a control for this experiment, even though the decrease in mRNA and protein expression after p100/p52 siRNA depletion was not as strong as in already published results (Schumm et al, 2006). However, it is interesting to note that p52 regulates CDK4 and CDK2 expression, both of which are involved in the progression of G1 phase. It has been reported before that p52 depletion induces a decrease of cells in S phase and a small accumulation of cells in G1 (Schumm et al, 2006). So far, these results have been attributed to the regulation of cyclin D1 expression but it is possible that CDK2 and CDK4 also play a role in p52 regulation of the cell cycle. Appart from cyclin D1, my studies of cyclin expression did not show any effects of p100/p52 siRNA depletion, whether on the mRNA or protein levels. These results clarify p52 regulation of genes involved in the cell cycle and reveal more of its ability to regulate G1 phase.

Another way to determine the p52 effect on the centrosome structure and defects in the mitotic spindle is to study the expression of genes involved in centrosome duplication after p100/p52 depletion. For this PhD project, we decided to focus on some of them: PLK4, SAS6, CDK2, AuroraA and γ-tubulin. mRNA analysis showed that p100/p52 siRNA depletion induced a significant decreased in expression of those genes, with the exception of γ-tubulin. Interestingly, PLK4 and SAS6 down-regulation has been shown to inhibit

centriolar duplication (Bettencourt-Dias & Glover, 2007), whereas AuroraA siRNA treatment of Hela cells resulted in incorrect centriolar separation, incomplete cytokinesis and chromosome misalignement on the metaphasic plate (Marumoto et al, 2003). My data showing a decrease in the expression of those genes upon p100/p52 siRNA treatment directly links p52 to the regulation of centrosome duplication and suggests a possible mechanism to explain the defects in centrosome structure observed previously, which will be addressed later in the main discussion.

Due to the lack of published data concerning regulation of its expression, I decided to focus my research on NF-κB regulation of PLK4. siRNA depletion of all the NF-κB subunits simultaneously showed a decrease in PLK4 mRNA and protein levels (Figure 3.5). This result was confirmed after separate depletion of the NF-κB subunits in U2OS cells (Figure 3.6), Hela cells and H1299 cells (data not shown). Although all NF-κB subunits reduced PLK4 expression, c-Rel appeared to have the strongest effect on mRNA levels (Figure 3.6). However, this did not seem to be the case at the protein level, suggesting additional post-transcriptional regulation. Overexpression of the NF-κB subunits increased PLK4 protein levels in Hela cells (Figure 3.7), confirming NF-κB regulation of PLK4 expression. Although the increase in NF-κB subunit expression was low, a clear induction of PLK4 was detectable, with the exception of p52 overexpression. One hypothesis for such a strong effect on PLK4 expression after an apparently moderate increase in NF-κB subunit expression is that the low increase in NF-κB subunit expression may reflect transfection efficiency. If only 20 or 30% of the cells were transfected, only a small effect would be seen on NF-κB expression by western blot, as levels would remain high in the rest of untransfected cells. By contrast, as PLK4 basal levels are very low, an effect on its expression in the 20% of the cells that were transfected would be more easily detected and seen as a significant increase. These results will need to be confirmed by increasing the efficiency of transfection and quantification by q-PCR.

Previous results also revealed defects in the mitotic spindle after p100/p52 depletion in U2OS cells. My attempts to confirm these results were unsuccessful, as when U2OS cells were transiently depleted for p100/p52, no effects could be seen on the mitotic spindle (Figure 3.2). The use of a CDK1

inhibitor, which delays entry in mitosis and thus could possibly enhance a potential p52 influence, did not show any effect on the mitotic spindle. A possibility to explain such different results could arise from the fact that previous results were obtained using U2OS cells stably expressing p100/p52 shRNA, whereas my results were obtained after a transient depletion. p52, as a regulator of the cell cycle (Schumm et al, 2006), is required for correct cell cycle progression in U2OS cells and such stably depleted cells would have to overcome the absence of p52 to proliferate. The defects in mitotic spindle could then be coming from the cells adaptation to proliferating under these conditions. However, these results need to be treated carefully, as no kinase assay on CDK1 activity was performed to check the CDK1 inhibitor efficiency. Nonetheless, results obtained on proliferation assays support this hypothesis. Indeed, U2OS cells transiently depleted for p100/p52 or PLK4 showed a decrease in proliferation rate (Figure 3.8). The decrease in proliferation detected after p100/p52 siRNA is confirmed by previously published data (Schumm et al, 2006) but a stronger effect was detected after PLK4 depletion. A similar effect was previously found in PLK4 +/- MEFs (Swallow et al, 2005), emphasizing PLK4's influence on cell proliferation. As PLK4 depletion also induced defects in centrosome structure (Bettencourt-Dias & Glover, 2007), it is possible that PLK4 in involved in p52 regulation of the cell cycle and centrosome structure.

Together, my data suggest that PLK4 is a NF-κB target gene. This has been recently confirmed with another project in the laboratory. Human primary fibroblasts were transiently depleted for the p52 or RelB NF-κB subunit and subjected to a micro-array analysis (data not shown, personal communication with A.Iannetti). In these conditions, PLK4 expression was found down-regulated after both p52 and RelB siRNA depletion (Figure 3.9). Other genes involved in centrosome duplication and the cell cycle were also altered by p52 and RelB absence, such as CENPE, BubR1. These results confirm our data and highlight NF-κB's involvement in centrosome duplication regulation in multiples cell types. Although no further investigation has yet been pursued regarding regulation of centrosome duplication in fibroblasts, it will be interesting to confirm and quantify biochemically such results, as these cells validate my results in a "non-tumour" cell line.

Gene Name	Protein Name	Function	Regulation by NF-κB	Reference
CCNA2	Cyclin A2	Cell cycle		
CCNB1	Cyclin B1	Cell cycle		
CCNB2	Cyclin B2	Cell cycle		
CDK1	Cyclin-dependent kinase 1	Cell cycle		
CDK4	Cyclin-dependent kinase 4	Cell cycle		
CDK6	Cyclin-dependent kinase 6	Cell cycle	Yes	(Iwanaga et al, 2008)
CDK7	Cyclin-dependent kinase 7	Cell cycle		
PLK4	Polo-like kinase 4	Centrosome duplication		
CCP110	Centriolar coiled-coil protein 110kDa	Centrosome duplication		
BUB1	budding uninhibited by benzimidazoles 1 homolog (yeast)	Mitotic checkpoint		
CDC20	cell division cycle 20 homolog (S. cerevisiae)	Mitotic checkpoint		

Figure 3-9: Micro-array data after p100/p52 depletion in human fibroblasts

List of cell cycle associated genes seen to be significantly down-regulated (>1.5 fold) following a microarray analysis of human fibroblasts after p100/p52 siRNA depletion (Alessio Iannetti, unpublished data). Genes shown are involved in the cell cycle progression, centrosome duplication and the mitotic checkpoint. Most of these genes have not been previously described as NF-κB targets.

3.4 Conclusion

This results chapter focused on the characterization of p52's effects on the cell cycle regulation. These data showed p100/p52 colocalization with the centrosome and the regulation of the expression of various cyclins and CDKs. Moreover, the p52 NF-κB subunit regulates the expression of a number of genes involved in the regulation of centrosome duplication. One of these genes, PLK4, a key factor for centriolar duplication, was also regulated by the other NF-κB subunits.

Our next goal was then to determine whether the NF-κB regulation of PLK4 expression happens through a direct or indirect mechanism.

4 – Results Chapter 2: PLK4 regulation

4.1 Introduction

As seen in Chapter 3, NF-κB regulates the expression of various genes involved in centrosome duplication, including the polo kinase PLK4. However, it is still unclear whether this regulation is direct or required the activity of other transcription factors.

PLK4 belongs to the PLK family, sharing sequence homology and a kinase domain in its N terminus but has the characteristic of possessing only one polo box domain. PLK4 is a short-lived protein and contains 3 PEST sequences, one in the N-terminus and two in the C-terminus. Deletion and mutation experiments showed that only the first motif is responsible for PLK4 stability, by creating a SCF binding site, which can trigger PLK4 ubiquitinylation and degradation by the proteasome (Sillibourne & Bornens, 2010b), via the SCF subunit Cullin1 (Korzeniewski et al, 2009). PLK4 auto-phosphorylation also seems to be involved in protein stability and thus regulates its activity to prevent aberrant centrosome amplification (Holland et al, 2010).

PLK4, present in proliferative tissue, is thought to act as a tumor suppressor or as an oncogene, depending on the tumor context (Pellegrino et al, 2010). Furthermore, PLK4 is aberrantly amplified in colorectal cancer (Macmillan et al, 2001) but is down-regulated in human hepatocarcinoma and associated with poor survival prognosis (Liu et al, 2012). Recent data also identifies PLK4 as a key factor regulating centriolar duplication. Indeed, overexpression of PLK4, specifically the auto-phosphorylated form on Ser305 (Sillibourne et al, 2010), induces over-amplification of centrosomes (Rodrigues-Martins et al), while depletion reduces centriole number (Bettencourt-Dias et al, 2005). PLK4 works in concert with the cyclin-dependent kinase CDK2, CP110 and the PLK4 effector SAS6 to ensure correct centrosome duplication (Habedanck et al). Interestingly, PLK4 null mice die during gestation due to massive mitotic arrest cause by an accumulation of cyclin B1 in anaphase and telophase, suggesting that PLK4 is required for cyclin B1 degradation and mitotic exit (Hudson et al, 2001). PLK4 heterozygous knockout mice present

centrosome amplification, aneuploidy together with multipolar spindle formation and develop 15 times more spontaneous liver and lung cancer compared to wild-type mice (Ko et al, 2005).

Very few studies to date have looked at PLK4 transcriptional regulation. Results published in 2005 demonstrate that PLK4 is indirectly regulated by p53, likely through the recruitment of HDAC repressors. PLK4 overexpression also reduces apoptosis induced by p53 but no further study yet has been published to explain the mechanism involved (Li et al, 2005). It has also been demonstrated that Sak, the mouse homologue of PLK4, binds to p53 but the mechanism for the interaction is not known (Swallow et al, 2005).

In this chapter, I determined whether NF-κB directly or indirectly regulates PLK4 expression, by performing chromatin-immunoprecipitation (ChIP) analyses on the putative κB sites previously identified in the PLK4 promoter in a number of human cell lines, as well as in murine fibroblasts. In addition to investigate the mechanism of NF-κB regulation of the PLK4 promoter, luciferase reporter plasmids were created, carrying various deletions of the PLK4 promoter. PLK4 promoter activity was monitored with using luciferase assays, combined with the overexpression or downregulation of NF-κB subunits.

4.2 Results

4.2.1 The PLK4 promoter

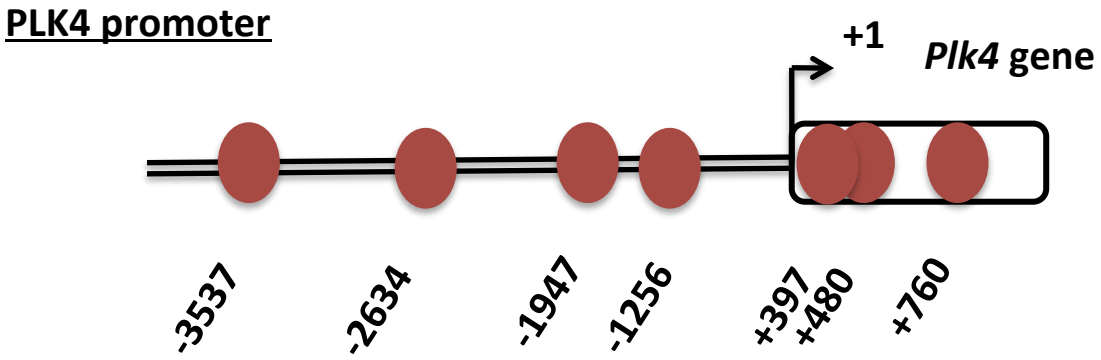
To determine the mechanism of regulation of PLK4 by NF-κB, it was essential to establish if NF-κB binds to the PLK4 promoter directly or if the regulation is indirect. To begin this analysis, the PLK4 promoter, between -4000 and +2000 bp from the initiation site, was subjected to a computational analysis using the MatInspector software to identify putative NF-κB sites. NF-κB sites usually follow the consensus GGG RNN YYC C (with R being a purine (adenosine or guanine) and Y a pyrimidine (cytosine or thymidine) nucleotide) (Natoli et al, 2005).

Analysis of the PLK4 promoter allowed the identification of seven potential NF-κB sites, as shown in Figure 4.1. Four of these sites were situated before the initiation site, at positions -3587, -2634, -1947, -1256 and three are placed shortly after the initiation site at positions +397, +480 and +760. These putative NF-κB sites were used to determine whether PLK4 is a direct or indirect NF-κB target gene and to investigate the mechanism behind NF-κB regulation of PLK4.

4.2.2 PLK4 is a direct NF-κB target gene

PCR primers were designed around each of the putative NF-κB sites identified above and chromatin-immunoprecipitation assays (ChIP) were performed to determine NF-κB binding to the PLK4 promoter. For the rest of this thesis, the primers around the putative sites prPLK4-2634, prPLK4-1947 and prPLK4-1256 will mainly be used, as these κB sites displayed the strongest and more reproducible results. ChIP assays in U2OS cells (Figure 4.2.A) and Hela cells (Figure 4.2.B) show that all NF-κB subunits bind to the PLK4 promoter. In U2OS cells, primers for GAPDH were used as a control to confirm the specificity of binding. The p50 subunit seemed to bind stronger than RelB however quantification would be necessary to confirm this observation. In Hela cells (Figure 4.2.B), all subunits were also bound to the three sites studied in the PLK4 promoter. p52, along with RelB and c-Rel, seemed to have stronger binding than RelA or p50. These differences in binding, which could correspond to the stronger effects seen on the mRNA level upon siRNA knockdown (Figure 3.6), suggested preferential involvement of some NF-κB subunits over others.

To quantify NF-κB binding to the PLK4 promoter, qPCR was performed after ChIP assays in U2OS cells (Figure 4.3) and in H1299 cells (Figure 4.4). In U2OS cells, as can be seen in Figure 4.3.A, p52 was bound to all three κB sites tested prPLK4-2634, prPLK4-1947 and prPLK4-1256. Significant binding was also detected for RelA and RelB for the prPLK4-2634 and prPLK4-1256 sites. p50 subunit binding was statistically significant only on the prPLK4-1256 site. Of all the NF-κB subunits RelB seemed to have the strongest binding for the prPLK4-1947 site, although this was not statistically significant.



NF-κB consensus binding site :

GGG RNN YYC C

-3587	GTG AAA TCC C
-2634	TGG GAA TCC C
-1947	AGG AAA TTC A
-1256	AAG AGG ATA CCC
+397	GGG AGG GTC CC
+480	AGG TGA GTC CC
+760	AGG TAT CCC C

R =purine, Y = pyrimidine

Figure 4-1: Potential NF-κB binding sites on the human PLK4 promoter

Schematic representation of the PLK4 promoter and its potential NF-κB binding sites, identified with a computational analysis using the Mat-Inspector software (the human PLK4 promoter analyzed correspond at -4000 bp before the transcription starting site, up to +2000 bp). Below are shown the sequence for the NF-κB consensus binding site, as well as the sequences for these potential NF-κB sites.

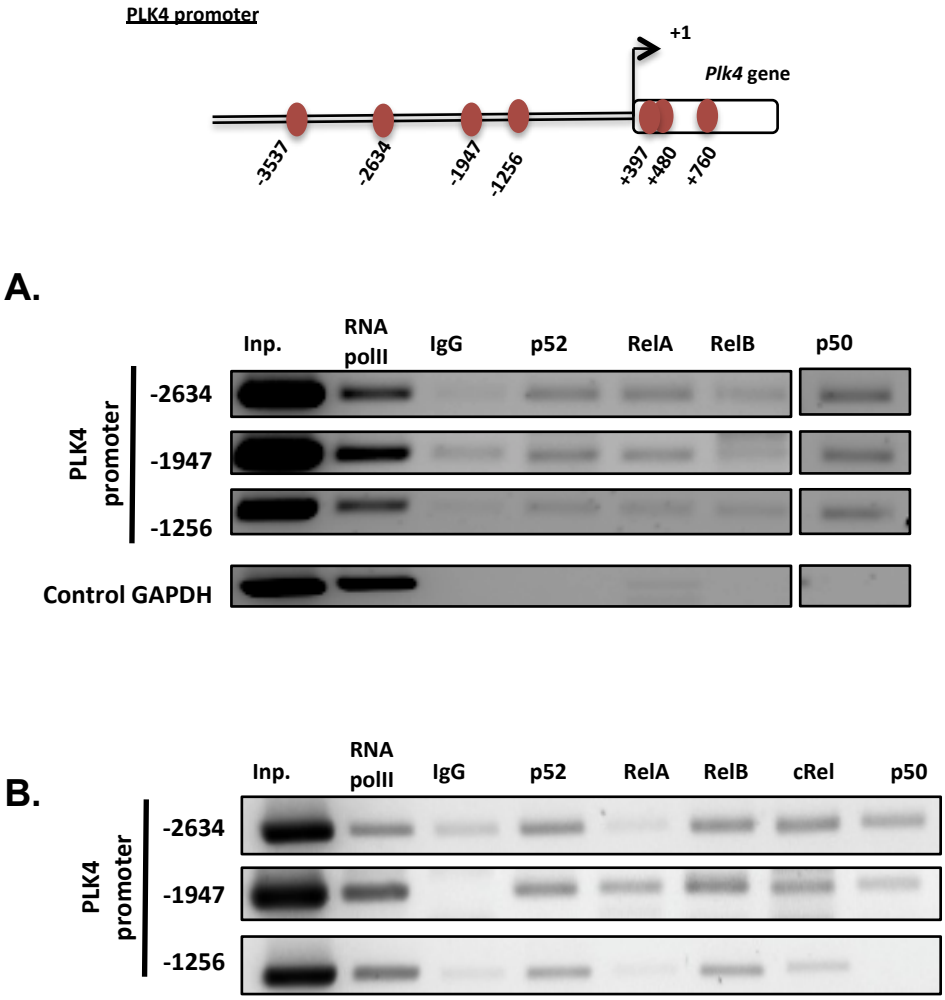


Figure 4-2: The PLK4 promoter is a NF-κB target

U2OS cells (**A**) or Hela cells (**B**) were fixed by parafornaldehyde and processed for a ChIP assay, using antibodies against NF-κB subunit, RNApolII as a positive and IgG as a negative control. PCR was performed with primers for the -2634, -1947 and -1256 NF-κB sites, and Gapdh as a negative control (**A**.).

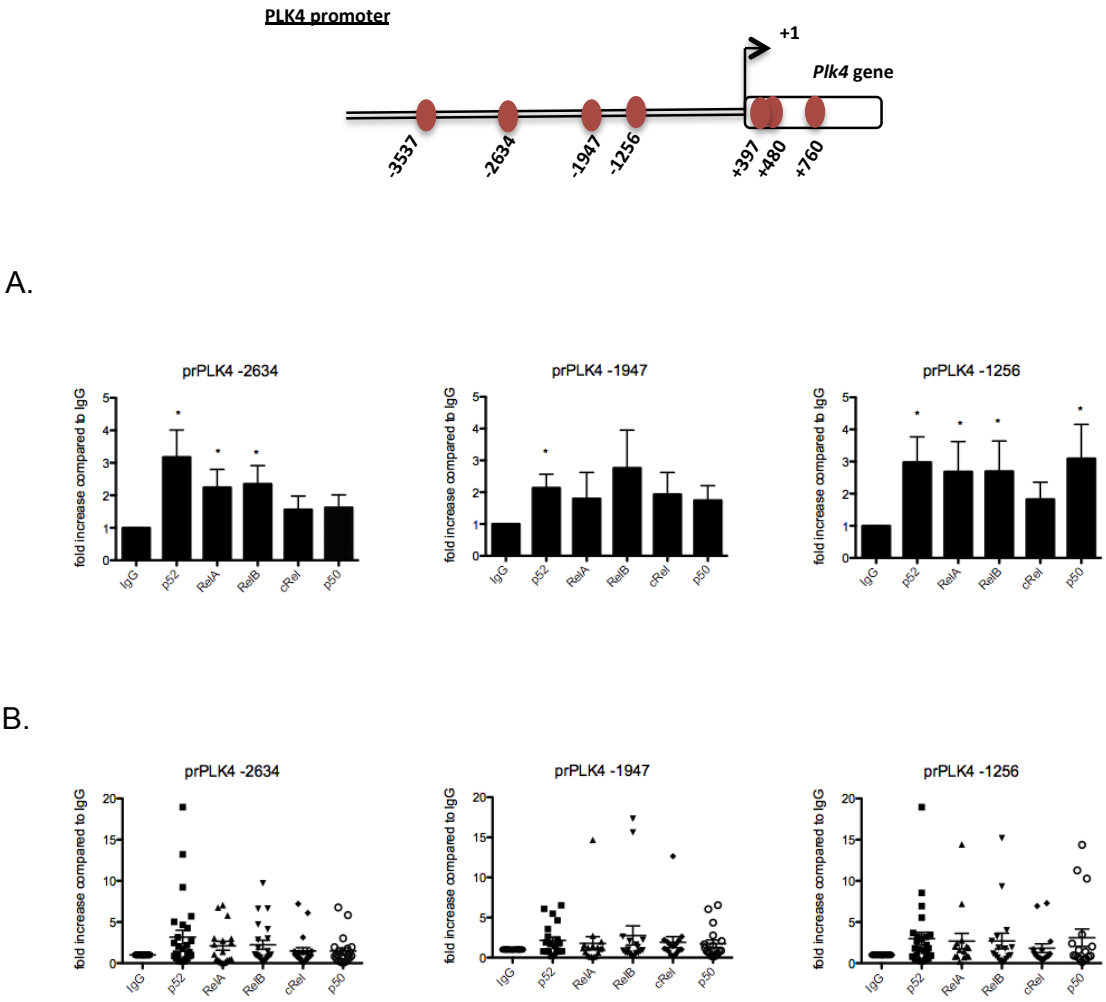


Figure 4-3: The PLK4 promoter is a NF-κB target

U2OS cells were fixed by paraformaldehyde and processed for a ChIP assay, using antibodies against NF-κB subunits and IgG as a negative control. qPCR was performed with primers for the PLK4 NF-κB binding sites -2634, -1947 and -1256. **A.** Graphic representation where the errors bars are representative of the Standard Error. * $P<0.05$. **B.** Scattered plot representation of results showing the diversity of binding observed.

As can be seen in Figure 4.3.B, high variability in the results was seen and despite multiple repeats, could not be reduced. Several populations could be found, some with strong binding and some with a lot milder binding, such as p52 binding on the prPLK4 -2634 site for example. This variability in U2OS cells was also found in H1299 cells to some extent. As seen in Figure 4.4.A and 4.4.B, NF-κB subunits also bind to the PLK4 promoter in H1299 cells, with the notable exception of p52. The strongest binding observed was for RelB and p50. However, not enough repeats were performed to apply statistical analysis to this data.

These results indicate that PLK4 is a direct NF-κB target gene and that all NF-κB subunits can bind to the PLK4 promoter. However, at this point, no definitive quantification of relative subunit binding was possible, as will be discussed later.

4.2.3 p52 binding on the PLK4 promoter is reversible

To verify the specificity of the p52 antibosy used to assess binding to the PLK4 promoter, U2OS cells were transiently transfected to deplete p52 expression with control or p100/p52 siRNAs. ChIP assays, quantified by qPCR were performed on the κB sites previously studied. Figure 4.5.A shows that indeed, the p52 binding to the PLK4 promoter detected is reversible, as it can be observed that in the case of the p100/p52 siRNA, p52 binding is lower than for the siRNA control with all prPLK4 κB sites. The reduction was approximately 50% compared to the control transfection, which roughly corresponded to the efficiency of the siRNA knockdown itself. This demonstrates the specificity of the p52 binding detected earlier in Figures 4.2, 4.3 and 4.4.

Further experiments also confirmed this p52 antibody specificity in Hela cells, as illustrated in Figure 4.5.B. Here, the same type of experiment was performed with transfection of control or p100/p52 siRNAs followed by ChIP assays quantified by qPCR. The same result was found with p52 binding to the various κB sites in the PLK4 promoter being reduced, between 50% and 70% compared to Hela cells treated with a control siRNA.

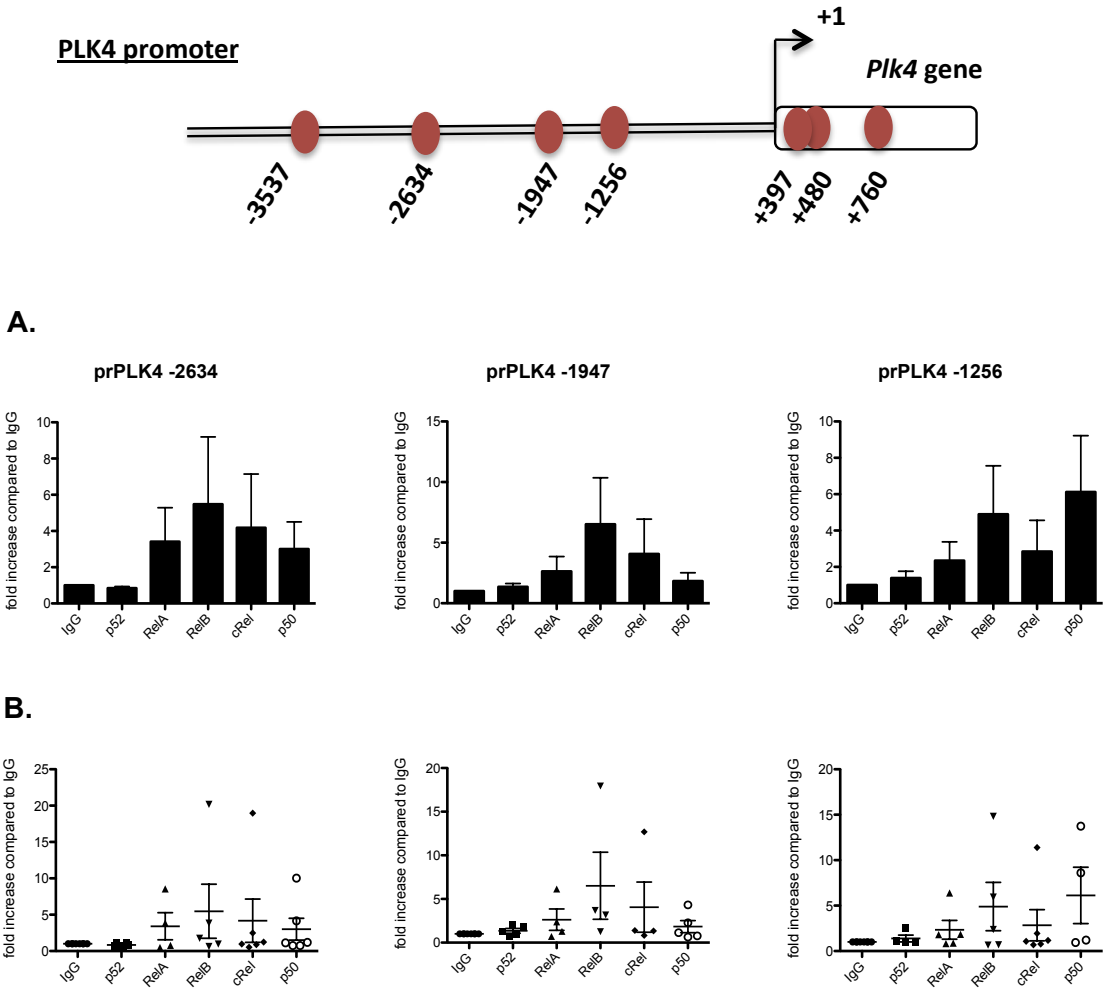
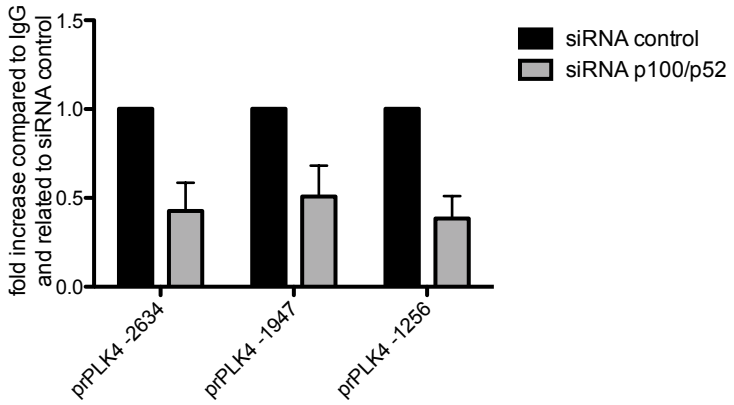


Figure 4-4: NF-κB binding on the PLK4 promoter in H1299 cells

H1299 growing cells were fixed by paraformaldehyde and processed for a ChIP assay, using antibodies against NF-κB subunits and IgG as a negative control. qPCR was performed with primers for the PLK4 NF-κB binding sites -2634, -1947 and -1256. **A.** Graphic representation where the errors bars are representative of the Standard Error. **B.** Scattered plot representation of results showing the diversity of binding observed.

A. U2OS cells



B. Hela cells

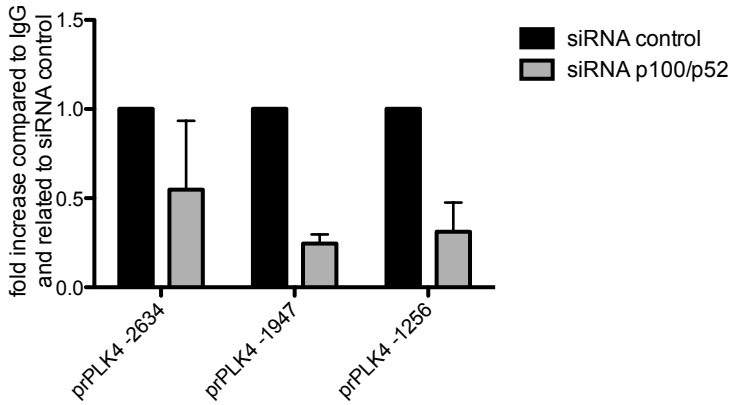


Figure 4-5: p52 binding on the PLK4 promoter is reversible

U2OS (**A.**) and Hela (**B.**) cells were subjected to transient siRNA transfection, with either control or p100/p52 siRNA. After 48h, the cells were processed for a ChIP assay, as described in Material and methods. qPCR was performed on the -2634, -1947 and -1256 binding sites. Error bars show the standard error of at least 3 independent experiments.

4.2.4 PLK4 is a target for exogenously expressed p52

Another way to check the specificity of p52 binding to the PLK4 promoter is to introduce a HA-tagged, exogenous form, of p52 within the cells, and to detect binding with an antibody against the tag. U2OS cells were thus transiently transfected to express either exogenous HA-tagged p52 or a DNA-binding mutant (DBM) (Schumm et al, 2006), alongside an empty HA control vector. ChIP assays using an HA antibody were then performed and quantified for binding to the -2634, -1947 and -1256 κB sites.

Exogenous p52 demonstrated a 7 fold higher binding compared to the background HA antibody control with the empty vector for the -2634 and -1947 κB sites; and by 3 fold for the -1256 κB site on the PLK4 promoter (Figure 4.6). Interestingly, when cells were transfected with the p52 DBM mutant, p52 binding on the promoter was not completely abolished but reduced by 50%. These results indicate that p52 clearly binds to the PLK4 promoter and this binding can be reduced by a DNA binding mutant. As the decrease in binding is not complete, this suggests that p52 recruitment can also result from interaction with other transcription factors through DNA-independent protein-protein interaction.

4.2.5 PLK4 is a NF-κB target gene in mouse

Previous data demontrated that PLK4 is a NF-κB target gene in multiple human cell lines: U2OS cells (Figures 4.2 to 4.6), Hela cells (Figures 4.2.B. and 4.5.B) and H1299 cells (Figure 4.4). However, to further validate the model, I investigated whether this was also the case in another mammalian model. I therefore examined the mouse sequence for PLK4 and its promoter. Analysis with the MatInspector software determined only three putative κB sites between region -4000 bp and +2000 bp of the mouse PLK4 promoter, with only one situated before the transcription start point at -21 bp (Figure 4.7.A). The last two sites were situated shortly after the transcription start, at position +135 and +329. Similarly to the human promoter, primers were designed around all of these putative κB sites and were used for ChIP assays.

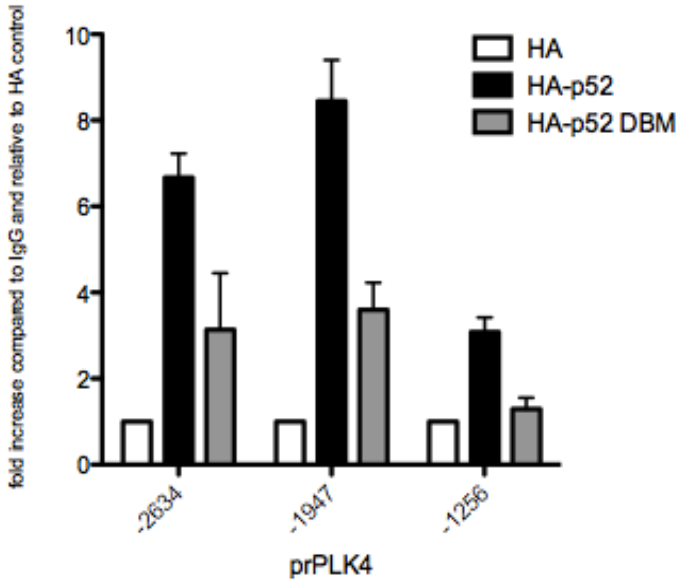


Figure 4-6: Exogenous p52 binds the PLK4 promoter

Hela cells were subjected to transient DNA transfection, with plasmids coding for exogenous p52 or the p52 binding mutant (DBM), both HA tagged. After 48h, the cells were processed for a ChIP assay, as described in Material and methods. qPCR was performed on the -2634, -1947 and -1256 binding sites .

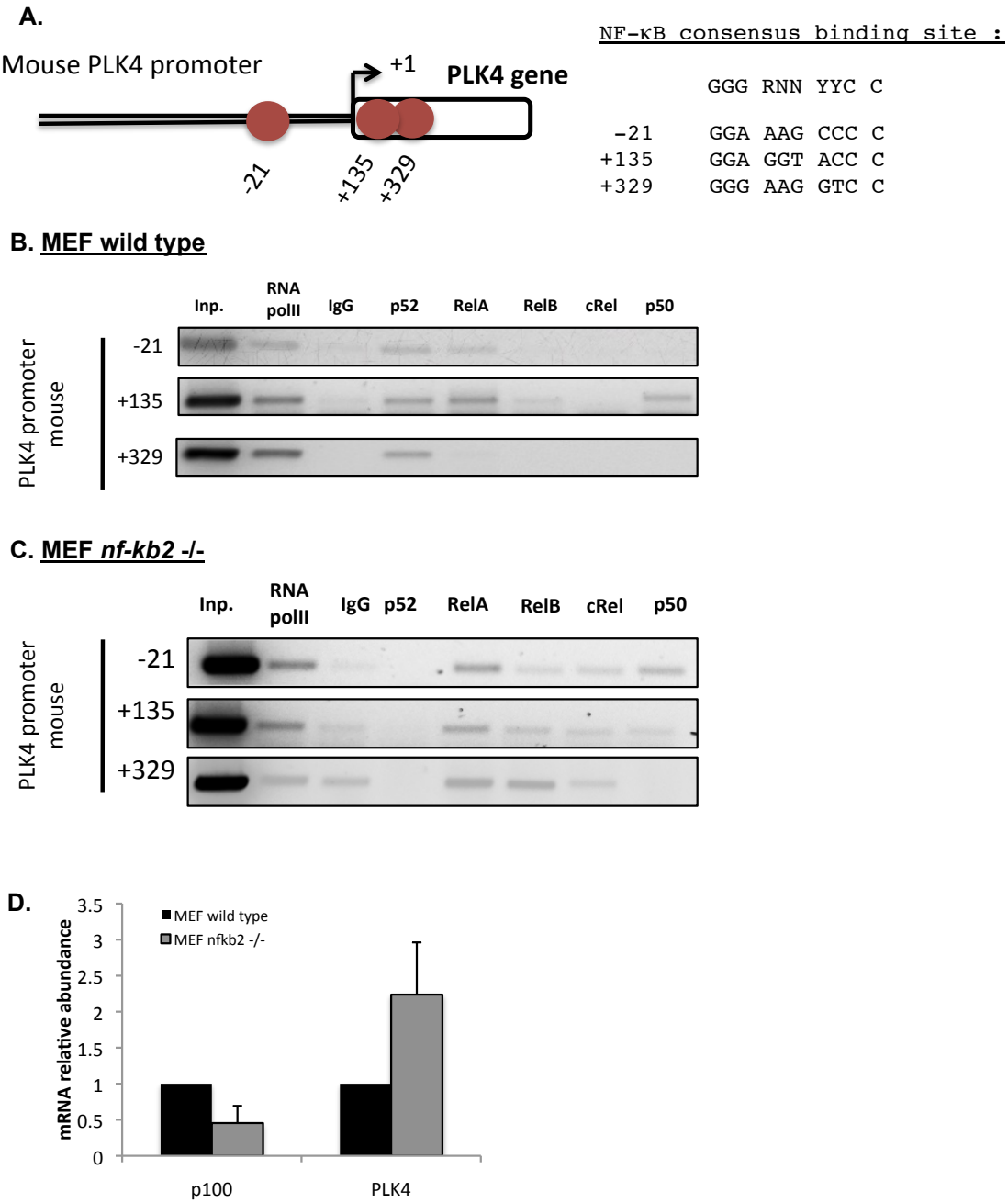


Figure 4-7: PLK4 is a NF-κB target gene in mouse

A. Schematic representation of the PLK4 promoter in the mouse. Putative NF-κB binding sites are shown with red circles, with the sequence for the putative κB sites written on the right. MEF wild type (**B.**) and *nfkb2* -/- (**C.**) growing cells were fixed with formaldehyde and processed for a ChIP assay, using antibodies against NF-κB subunits, RNApolII as a positive and IgG as a negative control. PCR was performed with primers for each potential NF-κB binding region. **D.** Growing cells (MEF wild type or *nfkb2* -/-) were lysed and the RNA extracted. mRNA expression was analyzed by quantitative PCR, compared to a housekeeper gene (GAPDH) and related to the expression with MEF wild type. Error bars show the standard error of at least 3 independent experiments.

NF-κB binding to these various sites was first studied in wild-type mouse embryonic fibroblasts (MEF) with ChIP assays using antibodies against each NF-κB subunit, as well as RNA pol II as a positive control and IgG as a negative control (Figure 4.7.B). It can be seen that p52 seemed to be the only NF-κB subunit to bind all three putative sites, while RelA only bound to -21 and +329; and p50 only to +135. RelB and c-Rel did not appear to bind the PLK4 promoter in wild type MEF, which is interesting considering RelB had the strongest binder in human cancer cells.

To confirm the specificity of binding, untreated *nf-kb2 -/-* MEFs were used. Figure 4.7.C shows that in *nf-kb2 -/-* MEFs, p52 binding was completely abolished, validating the previous assay and confirming the specificity of the signal. Interestingly, in the absence of p52, RelB and c-Rel seem to be able to bind the PLK4 promoter. Further experiments will be required to quantify this NF-κB binding and determine if p52 has a negative effect on c-Rel and RelB binding. During cell cultivation, it became clear that the *nf-kb2 -/-* MEFs were growing faster than the wild type MEFs (data not shown). As p52 is required for cell cycle progression of U2OS cells and p100/p52 depletion reduces cells in S phase, this suggests that *nf-kb2 -/-* MEFs had to overcome the absence of p52 to allow proliferation.

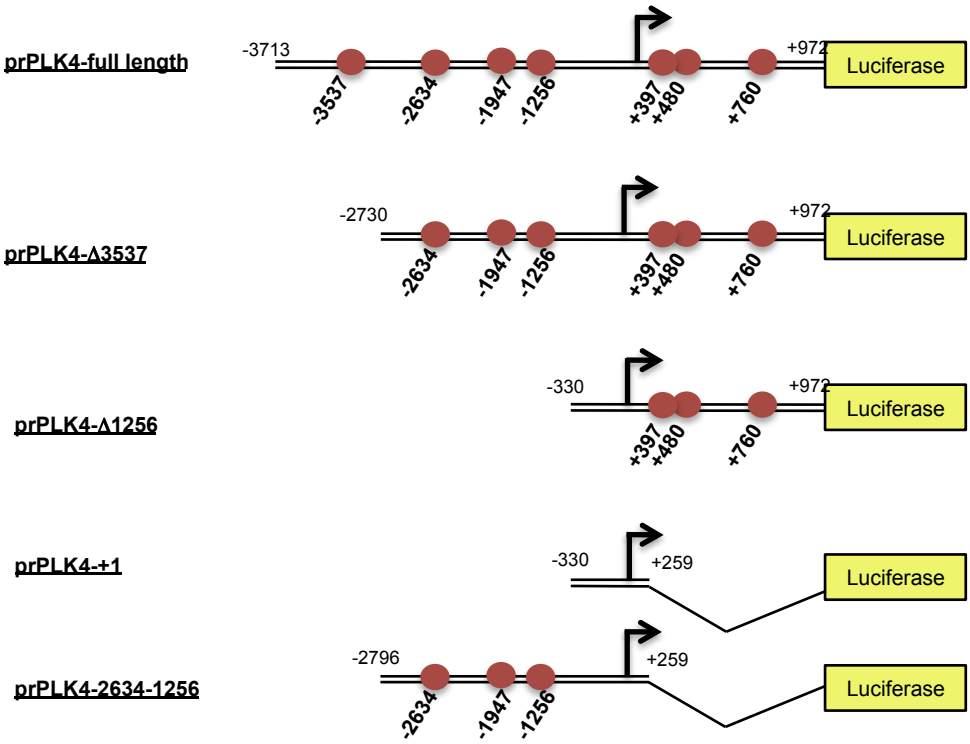
Given the previous effects seen with p52 on PLK4 expression, I investigated PLK4 expression in *nf-kb2 -/-* MEFs compared to wild type MEFs. Both cell types were cultured and analyzed for mRNA expression by qPCR (Figure 4.7.D). Q-PCR analysis showed that in *nf-kb2 -/-* MEFs, p100 expression is indeed reduced compared to wild type. It is notable that the basal level is lower than 50% for the control but not completely absent. This might be due the mouse knockout being created by deletions of crucial exons but still allowing mRNA transcription of the rest of the gene. Interestingly, in contrast to human cells, where siRNA depletion of p100/p52 reduced PLK4 expression (Figure 3.4), in *nf-kb2 -/-* MEFs, the PLK4 levels were twice as high than in wild type MEFs. This is consistent both with the observed increase in proliferation, as a decrease in PLK4 reduces proliferation (Figure 3.8) and also with the fact that p52 is required for proper PLK4 regulation. Moreover, this implies that p52 can also be a negative regulator of PLK4 expression, and that other NF-κB subunits can compensate for its absence.

4.2.6 Luciferase constructs of the PLK4 promoter

The data above demonstrates that PLK4 is a NF-κB target gene but so far, the mechanism behind this was unclear and it was difficult to predict which homo- or heterodimers were involved. To begin to address this issue, five constructs of the PLK4 promoter, driving expression of a luciferase reporter gene, were designed (Figure 4.8.A). The first construct consisted of the full length PLK4 promoter, containing all seven putative κB sites identified earlier. prPLK4-Δ3587 has a deletion of the most distal κB site, -3587, while prPLK4-Δ1256 has all sites up to the initiation start site removed and contains a minimal promoter starting 300 bp before the transcription initiation start (Figure 4.8.A). In the construct prPLK4-+1, the only promoter region remaining flanked the transcription initiation point (see Material and Methods), whereas the prPLK4-2634-1256 was designed to contain only the three main κB sites (-2634, -1947 and -1256) including the transcription initiation start. These various constructs were used to clarify the importance of the various κB sites and NF-κB binding preferences.

Before looking at the NF-κB effect on these plasmids, it was necessary to check the basal activation of the various constructs. U2OS cells were transiently transfected with the different plasmids and luciferase assays were performed to check the activity of the promoter. Normalizing the results to harvested protein level, Figure 4.8.B shows that, although the deletion of the -3587 κB site did not have any effect on the basal activation of the PLK4 promoter, other mutations decreased the activity by approximately 40%. This means that the -3587 site is probably not involved in the regulation of basal PLK4 activity but all other κB sites are, although no single deletion is enough to completely shut down PLK4 activity.

A.



B.

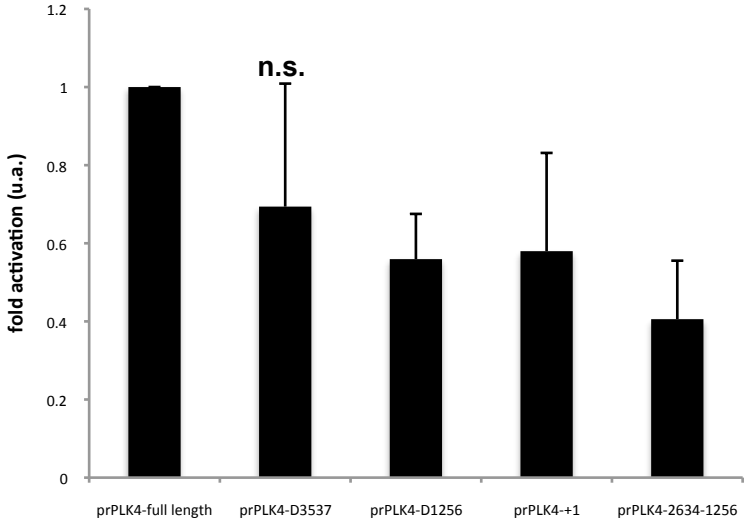


Figure 4-8: Luciferase constructs for the PLK4 promoter

A. Schematic overview of the various PLK4 promoter constructs used for the luciferase assays. Constructs prPLK4-full length, prPLK4-+1 and prPLK4-2634-1256 were made by Dundee Cell Products. Constructs prPLK4-D3537 and prPLK4-D1256 were generated by restriction enzyme digestion (SacI and KpnI respectively) and ligation, using the prPLK4-full length as template, as described in Material and Methods. **B.** Basal activation for the various luciferase constructs, relative to prPLK4-full length. Error bars show the standard error of at least 3 independent experiments.

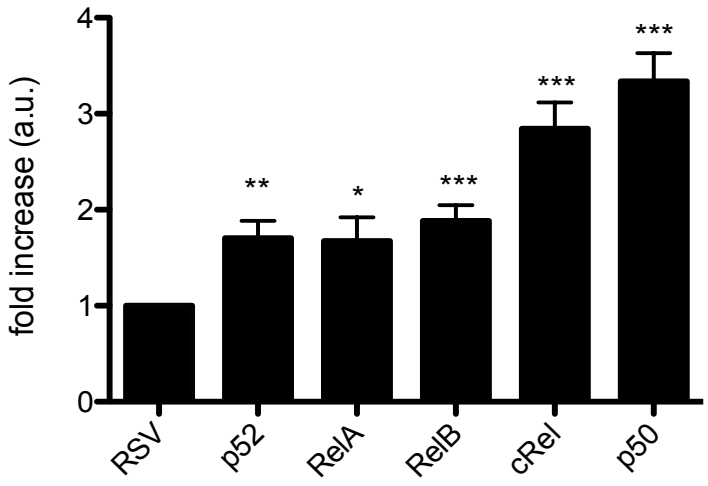
4.2.7 NF-κB subunit regulation of the PLK4 promoter

To look at the effect of NF-κB on the PLK4 promoter, U2OS cells were transfected with plasmids overexpressing (Figure 4.9.A) or down-regulating (Figure 4.9.B) the various NF-κB subunits and luciferase assays were performed. With the exception of the p100/p52 shRNA, published previously (Schumm et al, 2006), all NF-κB subunits shRNAs have been validated by our collaborator's lab (Rocha lab, Dundee, personal communication). Overexpression of the NF-κB subunits showed that all of them increased PLK4 promoter activity to varying levels (Figure 4.9.A). p52, RelA and RelB doubled the activity, whereas c-Rel and p50 increased the activity three fold. Curiously, down-regulating the NF-κB subunits did not have the opposite effect. Deletion of p100/p52, c-Rel or p50 did not affect the average activity of the PLK4 promoter, while RelA deletion slightly reduced its activity. Despite being small, this decrease was significantly different to the control. Interestingly, RelB depletion by shRNA had a similar effect to its overexpression: full length PLK4 promoter activity increased by two fold whether RelB was overexpressed (Figure 4.9.A) or absent (Figure 4.9.B).

The increase in promoter activity after RelB depletion contradicts the effect seen on PLK4 mRNA expression, when RelB siRNA depletion decreased PLK4 mRNA levels (Figure 3.6). The cause of duality of this effect by the RelB subunit is not known.

U2OS cells were also depleted for all NF-κB subunits simultaneously using the pan-NF-κB shRNA, along with the luciferase reporter plasmid and luciferase assays were performed. However, no effect could be detected compared to the control (data not shown), suggesting that the effects of independent NF-κB subunits could neutralize each other.

A.



B.

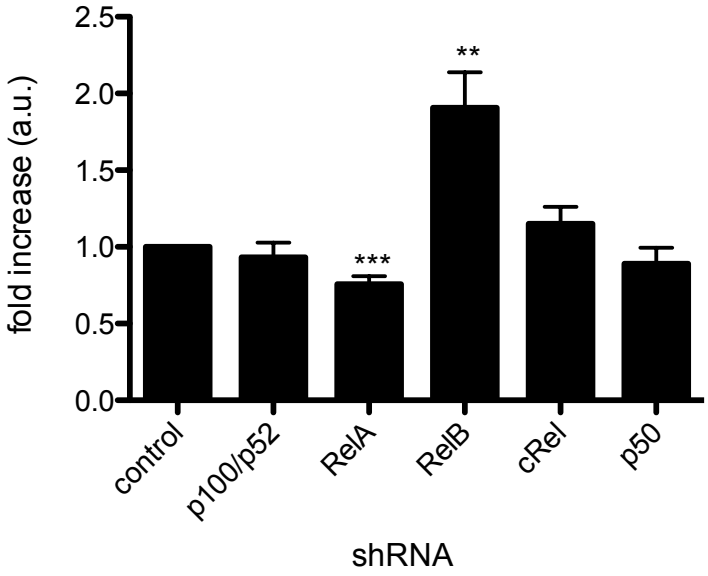


Figure 4-9: NF-κB subunits effects on the PLK4 promoter

A. Overexpression of NF-κB subunits. U2OS cells were subjected to transient DNA transfection to overexpress NF-κB subunits, together with a plasmid encoding the luciferase reporter gene, under the control of the full length PLK4 promoter. **B. NF-κB depletion**. U2OS cells were subjected to transient DNA transfection to down-regulate NF-κB subunits, along with a plasmid encoding the luciferase reporter gene, under the control of the full length PLK4 promoter. 48h later, cells were processed for a luciferase assay. Results were normalized to protein yield and then shown relative to a plasmid control. Error bars show the standard error of at least 3 independent experiments. * P<0,05; ** P<0,001; *** P<0,0001

4.2.8 Cooperation between NF-κB subunits

In order to identify the composition of possible the NF-κB dimers most actively regulating the PLK4 promoter, U2OS cells were transfected with the PLK4 promoter luciferase reporter and a combination of NF-κB subunit expression plasmids. Figure 4.10.A shows that the strongest effect on luciferase assays was seen with c-Rel and p52 co-expression. When p52 was present in combination with RelB and p50, the effect was larger than when p52 was in the presence of RelA. RelA had a more potent effect with p50 (Figure 4.10.B), but RelA effects were generally weaker than with other subunits. Indeed, the strongest activation of the PLK4 promoter was obtained after co-expression of c-Rel with RelB (Figure 4.10.C) or with p50 (Figure 4.10.D). It is interesting to note that p52 usually increased PLK4 promoter activity by only two fold, whereas any involvement of c-Rel or RelB usually increased activity by at least three fold at least (with the exception of RelA) (Figure 4.10).

This data suggest that c-Rel and RelB could be the main components of the NF-κB dependent regulation of PLK4 promoter activity.

4.2.9 Depletion of multiple NF-κB subunits

As RelA and RelB were the only subunits giving a significant effect on PLK4 promoter activity after depletion by shRNA, to identify the influence of the other subunits on this effect, U2OS cells were co-transfected with different shRNA expressing plasmids in order to down-regulate two NF-κB subunits simultaneously. Luciferase assays were then performed to determine PLK4 promoter activity. However, no matter which combination of NF-κB subunits was tried with RelA or RelB depletion, no further effect was seen on full length PLK4 promoter activity (Figure 4.11.A. and 4.11.B.). The strongest cooperative effect was seen with simultaneous RelB and p50 depletion, although this was not statistically significant and would need further validation. By contrast, despite the fact that single depletion of c-Rel or p50 did not affect PLK4 promoter activity (Figure 4.9.B. and Figure 4.11.C), a simultaneous depletion of these subunits increased PLK4 promoter activity (Figure 4.11.C). Once again, it is interesting to note that depletion increased PLK4 promoter activity, in the same way as the over-expression.

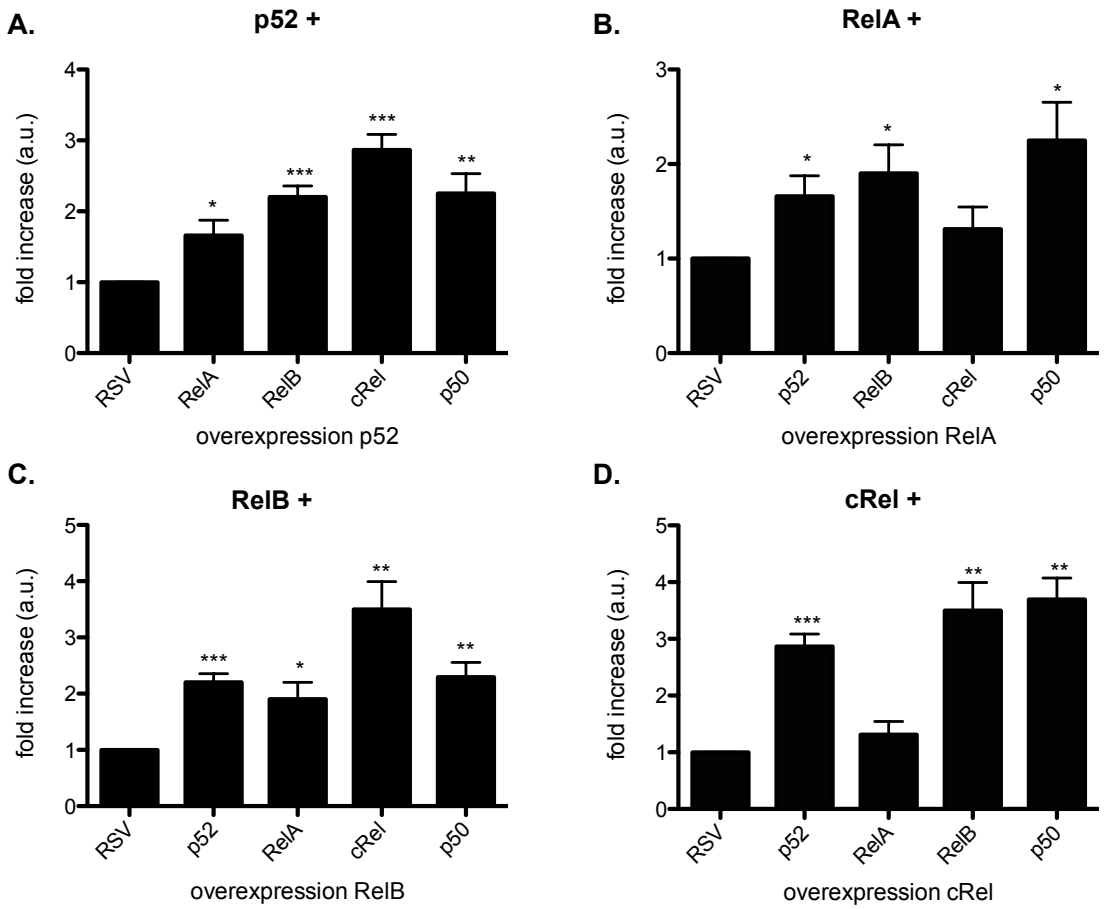


Figure 4-10: NF-κB subunits cooperatively induce the PLK4 promoter

U2OS cells were subjected to transient DNA transfection to overexpress various combination of NF-κB subunits, along with a plasmid encoding the luciferase reporter gene under the control of the full length PLK4 promoter. **A**. overexpression of p52, with the other NF-κB subunits. **B**. overexpression of RelA, with the other NF-κB subunits. **C**. overexpression of RelB, with the other NF-κB subunits. **D**. overexpression of cRel, with the other NF-κB subunits. Error bars show the standard error of at least 3 independent experiments. * P<0,05; ** P<0,001; *** P<0,0001

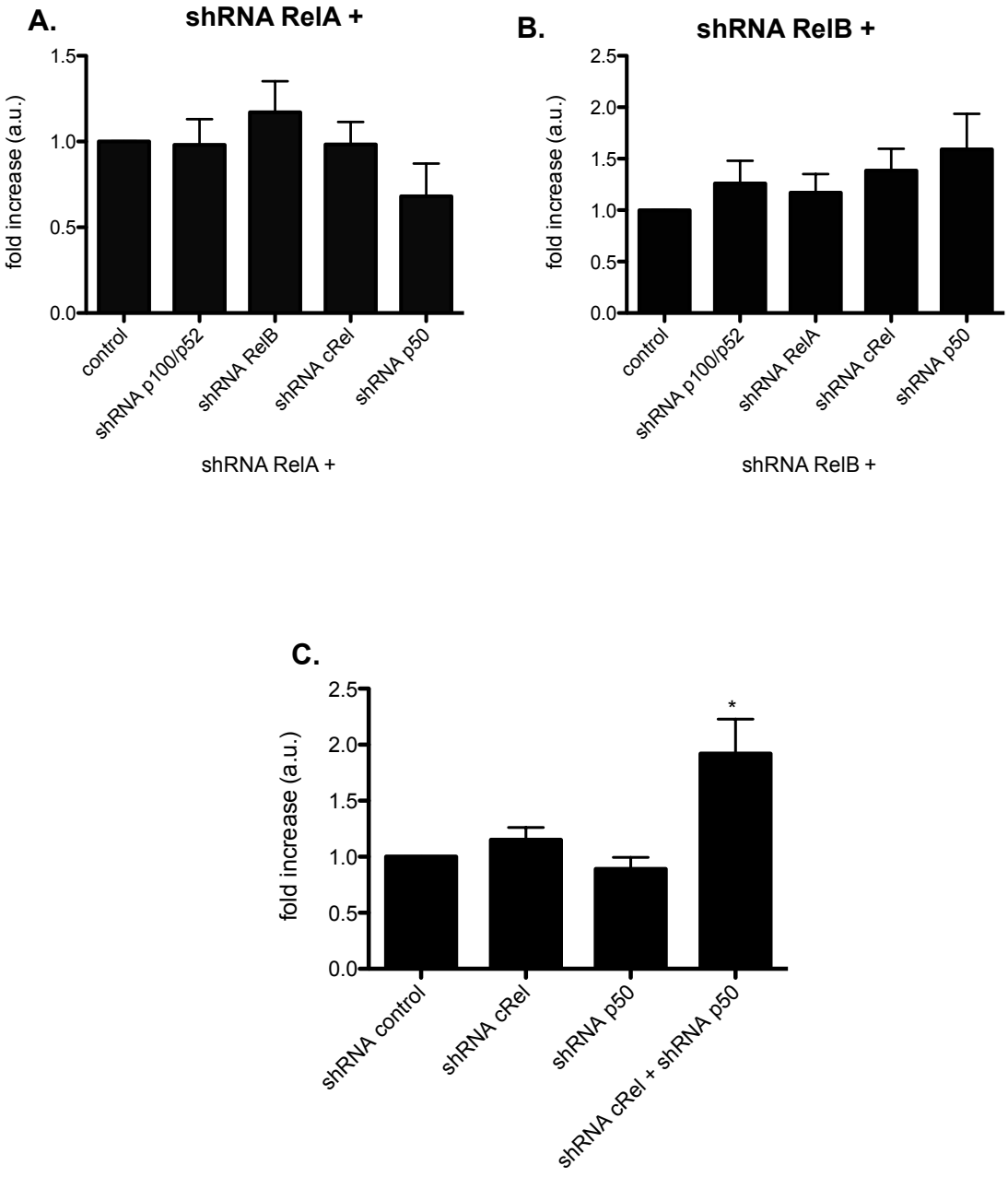


Figure 4-11: shRNA depletion of multiple NF-κB subunits

U2OS cells were subjected to transient DNA transfection to down-regulate various combination of NF-κB subunits, along with a plasmid encoding the luciferase reporter gene, under the control of the full length PLK4 promoter. 48h later, cells were processed for a luciferase assay. Results were normalized to protein yield and then shown relative to a plasmid control. **A.** and **B.** RelA (**A**.) or RelB (**B**.) depletion, combined with the depletion of the other NF-κB subunits. **C.** cRel and p50 depletion, separately or combined. Error bars show the standard error of at least 3 independent experiments. * P<0,05

4.2.10 Differential effect of RelB depletion with PLK4 promoter deletions

As the RelB shRNA was the only one to affect PLK4 promoter activity in a luciferase assay (Figure 4.9.B), I investigated the effect of depleting this subunit on the various PLK4 promoter deletions constructs (Figure 4.8). As observed in Figure 4.12, RelB depletion affected all PLK4 constructs, increasing their activity. However, the effect was the strongest for the construct prPLK4+1, which contained only the region flanking the transcription initiation start. However, when the PLK4 promoter was deleted of all κB sites 5' to the transcription initiation start, but leaving the κB sites downstream intact, RelB depletion had only a minimal effect (Figure 4.12).

4.2.11 NF-κB subunit effects on the PLK4 promoter deletion constructs

As overexpression of all NF-κB subunits increased PLK4 promoter activity with varying intensity (Figure 4.9.A), I next investigated their effect on the PLK4 promoter deletion constructs. U2OS cells were transfected with plasmids overexpressing the various NF-κB subunits, along with the PLK4 promoter deletion luciferase reporter plasmids. The most interesting effect was once again seen with c-Rel overexpression (Figure 4.13). Here, c-Rel overexpression increased full length promoter activity by three fold but all the deletion constructs exhibited a 4 fold increase in activity. Similar effects were seen with p50 and RelB overexpression, where promoter deletions had little effect on their ability to induce PLK4 promoter activity.

By contrast, the effects of p52 and RelA overexpression did show some selectivity. Here, the full length promoter and the prPLK4-Δ3587 and prPLK4-Δ1256 mutants were induced but the effect on the two other constructs (prPLK4+1 and prPLK4-2634-1256) was significantly reduced compared to the full length promoter. This suggests that with these subunits, some contribution to regulation of PLK4 promoter activity derives from both the upstream and downstream κB sites.

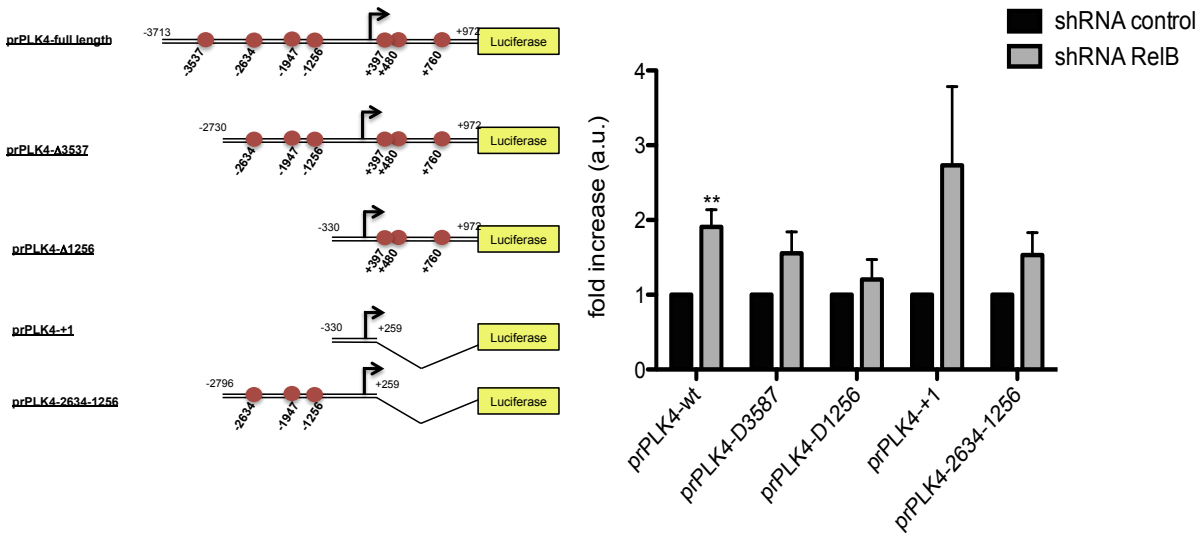


Figure 4-12: RelB depletion regulates the different PLK4 promoter constructs

U2OS cells were subjected to transient DNA transfection to down-regulate the RelB NF-κB subunit, along with a plasmid encoding the luciferase reporter gene, under the control of the various PLK4 promoter constructs. 48h later, cells were processed for a luciferase assay. Results were normalized to protein yield and then shown relative to a plasmid control. Error bars show the standard error of at least 3 independent experiments. ** P<0,001

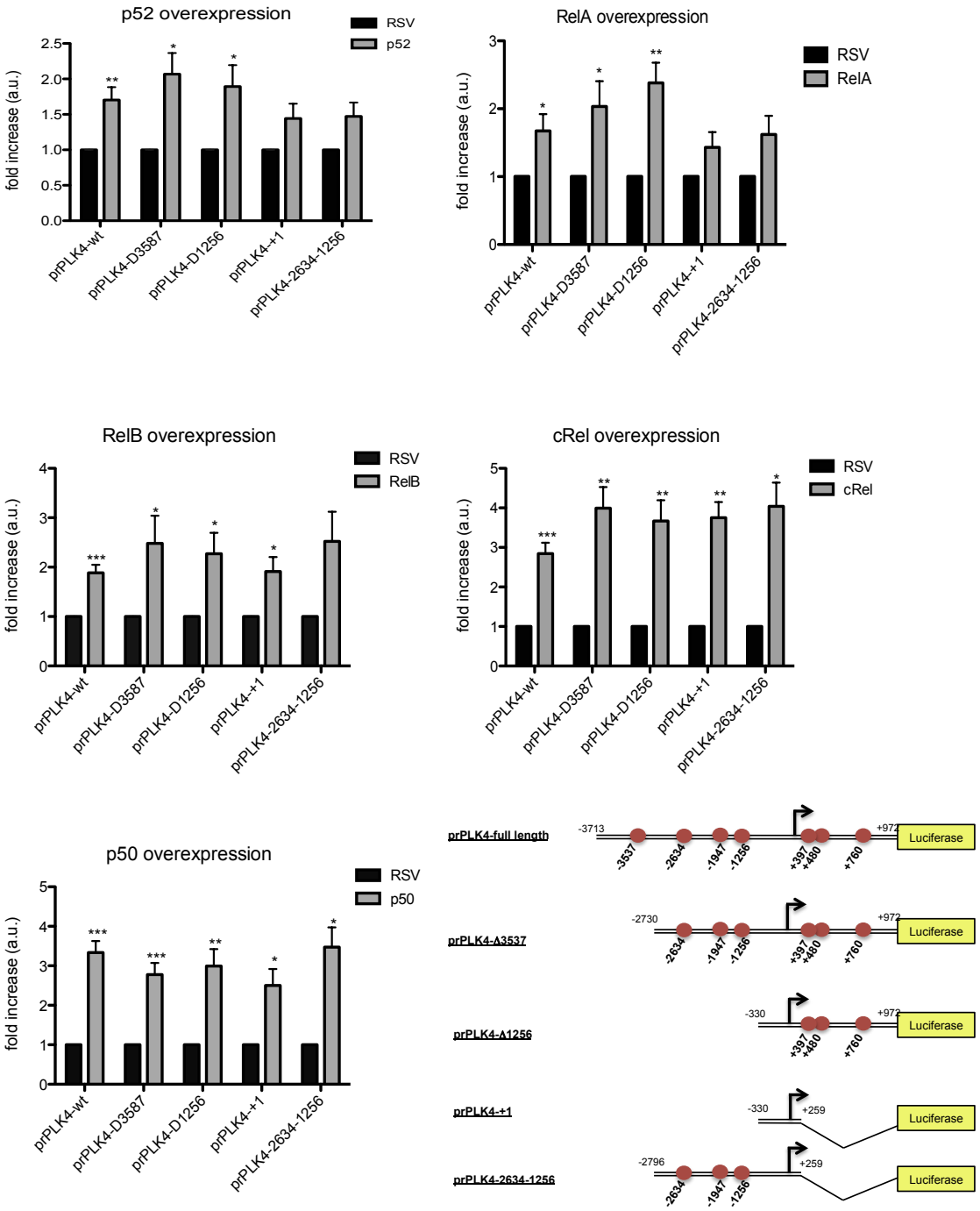


Figure 4-13: Effects of NF-κB subunits on the different PLK4 promoter constructs

U2OS cells were subjected to transient DNA transfection to overexpress NF-κB subunits, along with a plasmid encoding the luciferase reporter gene, under the control of the various PLK4 promoter constructs. 48h later, cells were processed for a luciferase assay. Results were normalized to protein yield and then shown relative to a plasmid control. Error bars show the standard error of at least 3 independent experiments. * P<0,05; ** P<0,001; *** P<0,0001

4.3 Discussion

Previous results in chapter 3 showed that the p52 NF-κB subunit regulates the expression of genes involved in centrosome duplication. Further studies demonstrated that one of these genes, PLK4, is a target gene for all NF-κB subunits. The next investigation was to determine whether this regulation is direct, through NF-κB binding to the PLK4 promoter, or indirect, through association with other transcription factors.

Consensus κB sites have been previously described in the literature (Natoli et al, 2005) and analysis of the PLK4 promoter revealed seven such potential κB sites. ChIP analysis in human cell lines (U2OS cells and Hela cells) demonstrated that NF-κB subunits bind to the PLK4 promoter, mainly on three of these potential κB sites, the -2634, -1947 and -1256 sites (Figure 4.2). Quantification showed that the NF-κB subunits bind to the PLK4 promoter with various intensities (Figure 4.3.A).

One of the main issues encountered during this thesis was experimental variability. As seen in Figure 4.3.B, final quantification of NF-κB binding to the PLK4 promoter was hard to determine, as two sets of data seems to be available for each subunit. For some experiments, NF-κB binding to the κB sites in the PLK4 promoter is very strong, for other experiments, binding is just slightly above the IgG negative control. Interestingly, for the prPLK4-2634 κB site, p52 displayed very strong binding, whereas for the prPLK4-1947 κB site, RelA, RelB and c-Rel have stronger binding in U2OS cells (Figure 4.3.B). The prPLK4-1256 κB site does not show any preference for a particular NF-κB subunit. However, binding was different in H1299 cells, where RelB seemed the more important subunit as it was associated with all three κB sites in the PLK4 promoter. However, these results in H1299 cells are preliminary, as more repeats are needed, so no final conclusion can be drawn from them. Despite multiple repeats, changes in experimental conditions or modifications of the ChIP protocol, the variability observed could not been resolved. Other modifications of the ChIP protocol will be performed with more repeats in order to have definitive and publishable results. However, despite the reproductibility

issue in the quantification of NF-κB binding to the PLK4 promoter, the results demonstrate that PLK4 is a direct NF-κB target gene.

Specificity of binding for the p52 subunit was confirmed using siRNA depletion in U2OS cells and Hela cells (Figure 4.5). After p100/p52 siRNA treatment, p52 binding was reduced by half in both cell lines for all κB sites tested. Similar experiments are also ongoing to validate the binding specificity of the other NF-κB subunits. Moreover, the binding of the various NF-κB subunits will be quantified after deletion of one or several of the subunits, to underline the influence of NF-κB subunits on others' binding to the PLK4 promoter. Moreover, exogenous HA-tagged p52 transfection in Hela cells allowed p52 binding to be validated using an HA antibody (Figure 4.6). Overexpression of a DNA binding mutant of p52 unable to bind DNA but still able to dimerize and associate with other transcription factors (Schumm et al, 2006), showed a decrease in p52 binding but not a complete depletion. This suggests that association with another NF-κB subunit or interaction with heterologous transcription factor seems to be sufficient for p52 binding. Thus it will be necessary to determine exactly which complexes are involved in NF-κB regulation of PLK4. To facilitate this, ChIP reverse (ReChIP) analysis will be performed in various cell lines. Along with siRNA depletion experiments, ReChIP analysis should allow us to determine the main complexes involved in NF-κB regulation of PLK4 expression.

In order to validate PLK4 as a NF-κB target gene, the orthologous mouse promoter was studied and three putative κB sites were identified. Interestingly, the first thing to note is the placement of these κB sites (Figure 4.7). None of them are situated in a region analogous to the human promoter. Instead, they can be found closer to the transcription initiation start. ChIP analysis in wild type MEFs demonstrated p52 binding to the three putative κB sites, whereas the p50 subunit was only present in the mprPLK4+135 site and RelA bound to the mprPLK4-21 and mprPLK4+135 PLK4 κB sites (Figure 4.7.B). RelB and cRel did not appear to bind to the PLK4 promoter in these conditions. By contrast, it is interesting to note that when p52 was absent (obtained with *nfkb2 -/-* MEFs, Figure 4.7.C), RelB and cRel were able to bind to the PLK4 promoter. Further experiments will be required to quantify this NF-κB binding and determine if p52 has a negative effect on c-Rel and RelB binding. Nonetheless, these

experiments validated PLK4 as a NF-κB target gene, whether in human cancer cells or in murine fibroblasts.

As *nfkb2 -/-* MEFs were at our disposal, it was then interesting to study basal mRNA levels for PLK4. In *nfkb2 -/-* MEFs, PLK4 mRNA levels increased two fold compared to wild type MEFs. This implies that p52 can also be a negative regulator of PLK4 expression, in addition to the seemingly positive regulatory role found in human cell lines (Figures 3.4 and 3.6). Unquantified observations showed that *nfkb2 -/-* MEFs proliferated faster than wild type MEFs (data not shown), meaning that *nfkb2 -/-* MEFs overcame the absence of p52 for proliferation. This increased PLK4 mRNA levels in *nfkb2 -/-* MEFs correlate with this, suggesting other NF-κB subunits can compensate for p52 absence in regulating PLK4 expression. The finding that other subunits also bind to the PLK4 promoter in the absence of p52 is consistent with this. However, the exact mechanism still needs to be identified.

To help understand the mechanism of NF-κB regulation of the PLK4 gene, various deletions of the human promoter were created in luciferase reporter plasmids. These deletions removed one or more of the κB sites previously identified. Luciferase assays on the basal PLK4 promoter activity showed that suppressing the upstream κB site did not have an effect on PLK4 promoter activity, but keeping only a minimal promoter without any κB sites decreased the basal activity by two (Figure 4.8).

Overexpression of the NF-κB subunits increased the full length PLK4 promoter activity to various degrees, with the p50 subunit having the strongest effect. This corroborates results seen at the protein levels (Figure 3.7), where overexpression of the NF-κB subunits increased endogenous PLK4 protein levels, p50 overexpression having a strong effect on endogenous PLK4 levels. Furthermore, overexpression of the NF-κB subunits with the various PLK4 deletions constructs did not reveal a specific NF-κB responsive region (Figure 4.13). This suggests that despite NF-κB binding these regions (shown in Figures 4.2 and 4.3), the critical sites for regulation happen somewhere else, the luciferase reporter plasmids do not accurately reflect the occupation of the endogenous genes.

Experiments to look at NF-κB subunit cooperation demonstrated that induction of the full length PLK4 promoter activity is stronger in case of overexpression of multiple NF-κB subunits than the overexpression of only one subunit (Figure 4.10). Higher induction of the PLK4 promoter activity was obtained after co-overexpression of c-Rel with p52, RelB or p50. Interestingly, c-Rel/RelA overexpression did not have a different effect than overexpression of RelA or c-Rel separately, suggesting that this hetero-dimer does not regulate PLK4 promoter activity. These experiments illustrate the complexity of NF-κB regulation of the PLK4 promoter, as not one hetero-dimer appeared to be more important than another. However, it seems that whenever c-Rel consistently was a strong inducer of promoter activity when co-expressed with another NF-κB subunit.

Surprising results were obtained on the PLK4 promoter activity after NF-κB shRNA depletion. Indeed, the only subunits showing an effect were RelA, with a small decrease in PLK4 promoter activity, and RelB, where depletion induced an increase in PLK4 promoter activity (Figure 4.9.B). These results are the opposite to the effect seen after siRNA depletion, which decreased endogenous PLK4 mRNA and protein levels (Figure 3.6). Other NF-κB subunit depletion did not have an effect on the PLK4 promoter, as did depletion of all NF-κB subunits at once with the pan-NF-κB shRNA (data not shown). Depletion of combinations of NF-κB subunits also did not show any significant effect for potential RelA or RelB hetero-dimers (Figure 4.11). However, it is interesting to note that, although single c-Rel or p50 shRNA depletion did not affect PLK4 promoter activity, dual depletion of these two subunits increased full length promoter activity (Figure 4.11.C). c-Rel/p50 co-depletion also had the same effect on the PLK4 promoter activity as overexpressing the c-Rel/p50 heterodimer, suggesting a major influence of this dimer on the PLK4 promoter activity regulation. However, these results suggest that the luciferase reporter plasmid does not accurately mimic the activity of the endogenous PLK4 promoter.

RelB depletion also increased the activity of all PLK4 constructs (Figure 4.12), with the strongest activation being seen for the minimal promoter, and a reduced effect when the PLK4 promoter lacked the κB sites upstream of the transcription start. This data suggests that RelB repression of the PLK4

promoter happens through the core promoter region and results either from the presence of a cryptic unidentified κB site or occurs through an indirect mechanism. Previous results support both hypotheses. Indeed, the trouble with ChIP reproductibiliy, showing either very high binding of NF-κB subunits (including RelB, Figure 4.3) or reduced binding could be explained by the presence of an unknown κB site. Unpredictable sonication during the ChIP protocol could sometimes produce DNA fragments containing this unknown κB site but recognized with the primers designed to the other κB sites, while at other times these sites were excluded. Alternatively, ChIP signals could result from DNA looping between this distal κB site and the promoter regions detected using PCR. However, the hypothesis of regulation luciferase assays not accurately reporting endogenous activity is supported by the opposing effects seen between PLK4 promoter activity (Figure 4.9) and PLK4 expression (Figure 3.6) following RelB depletion. Indeed, RelB depletion increased PLK4 promoter activity but reduced endogenous PLK4 mRNA and protein expression. To date, experiments do not allow us to completely discard any of these hypotheses so more investigation will be necessary to either identify this unknown κB site or to explain the indirect mechanism. However, luciferase assays support the hypothesis that, so far, I have been looking at a wrong κB sites and that NF-κB regulation probably happens somewhere else within the PLK4 gene. Unfortunately, these assays were performed at the end of the study, not allowing me the time to re-analyze the PLK4 promoter and identify potential new κB sites responsible for NF-κB binding to the promoter. Another observation to support the hypothesis of investigating the wrong κB sites is the study of the mouse promoter. The human and mouse promoters share a high sequence homology, especially close to the transcription initiation start and close to the identified κB sites in mice. Therefore, it is possible that the human PLK4 promoter posseses cryptic NF-κB binding sites closer to the initiation transcription start analogous to the sites in the mouse promoter, which are involved in its regulation. Investigating this will be a priority to complete this study.

The absence of an effect after depletion of other NF-κB subunits on PLK4 promoter activity in the luciferase assays has various explanations. Even though the shRNA plasmids have been validated, it is possible that the level of

NF-κB depletion encountered is not enough to affect PLK4 promoter activity, with the exception of RelB. Indeed, RelB is the subunit seen to bind more strongly the PLK4 promoter (Figures 4.3. and 4.4), so even a small change in the expression of RelB could affect promoter activity. Alternatively, as discussed above, it is possible that the luciferase reporter plasmid does not correctly mimic PLK4 promoter activity. This could results either from the exclusion of a distal enhancer region that mediates NF-κB effects with the endogenous gene. Alternatively, NF-κB may mediate effects on chromatin structure that have little effect with a transiently transfected reporter plasmid, as luciferase reporter plasmids are not properly chromatinized.

4.4 Conclusion

This chapter allowed us to determine that PLK4 is a direct target gene for NF-κB regulation and that NF-κB subunits bind to various κB sites on the promoter. However, PLK4 promoter activity analysis, due to a variety of sometimes opposing effects, raises the possibility of either an unidentified κB sites or an indirect mechanism being involved in NF-κB regulation of PLK4 expression. Since PLK4 is a tightly cell cycle regulated gene, the next chapter will describe attempts to determine the NF-κB's regulation during the cell cycle and potentially identify opposite effects during various cell cycle phases.

5 – Results Chapter 3: Cell cycle regulation

5.1 Introduction

As seen previously, PLK4 is a direct target for NF-κB regulation. Indeed, all NF-κB subunits bind to various κB sites on the PLK4 promoter. Moreover, NF-κB overexpression or depletion affects PLK4 expression, as well as PLK4 promoter activity. However, so far, it has not been possible to determine a regulatory mechanism for NF-κB on the PLK4 promoter.

A possible explanation arises from the tight regulation of PLK4 during the cell cycle. Experiments in murine fibroblasts showed that SAK (PLK4 murine orthologous form) mRNA levels were minimal in G1 phase, increased in late G1 to be maximal through S phase and mitosis, before decreasing in early G1 (Fode et al, 1996). This was demonstrated using a nocodazole-induced mitotic arrest or restart of cell growth after quiescence. However, regulation of protein levels or kinase activity during the cell cycle has not yet been studied (Winkles & Alberts, 2005).

To investigate whether NF-κB subunits have potentially different effects on PLK4 regulation during the cell cycle, cell cycle synchronization was performed. Various agents were tested to optimize cell synchronization. DNA profile analysis allowed me to determine the various phases of the cell cycle in order to study PLK4 and p100/p52 mRNA and proteins level variability.

ChIP analysis for each phase of the cell cycle was then performed, to determine variation in the binding of the NF-κB subunits to the PLK4 promoter. Two synchronization agents were used to validate the results. Different NF-κB binding to the PLK4 promoter during the phases of the cell cycle could explain the interesting effects seen on the PLK4 promoter activity after NF-κB depletion, which were opposite to the effects seen at mRNA levels.

5.2 Results

5.2.1 Cell synchronization

In order to study the effect of the cell cycle on NF-κB regulation of PLK4 and consequent variations in mRNA expression, several agents were used to synchronize the cells. U2OS cells were treated with different drugs, as stated in Material and Methods, washed and released in fresh media. Different time-points were then harvested, as indicated in Figure 5.2, and the DNA profile was checked by flow cytometry. Three of the agents did not give successful synchronization, while two others gave good synchronization and progression through the cell cycle after release. These are described below.

The first trial used aphidicolin, which stops the cells in late G1 by inhibiting DNA polymerase α, thus blocking DNA synthesis (Figure 5.1). Before washing and release, 60% of the cells were blocked in G1, which was not sufficient for a robust synchronization (Figure 5.2.A). Moreover, after release the cells did not show correct progression through S and G2/M phases.

Serum starvation, which blocks the cells in G0/G1 by forcing the cells to go into G0 quiescence due to nutritional deprivation (Figure 5.1), induced a 60% accumulation of the U2OS cells in G0/G1 phase but they did not re-enter the cell cycle after washing and release, even after 24h (Figure 5.2.B).

The last unsuccessful agent was nocodazol. It blocks the polymerization of microtubules, and as a consequence, cells that enter into mitosis fail to form a correct mitotic spindle and therefore remain in G2/M (Figure 5.1). U2OS cells treated in this way were indeed blocked in G2/M but did not progress into the cell cycle after release (Figure 5.2.C).

Synchronization was more efficient using two agents that block in late G1 – early S phase. Hydroxy-urea decreases the production of dNTPs by inhibition of ribonucleotide reductase and thymidine induces an imbalance of dNTPs and thus blocks DNA synthesis (Figure 5.1).

Drugs used	Mode of Action	Block
Hydroxy-urea	Decreases the production of dNTPs by inhibition of ribonucleotide reductase (Ma & Poon, 2011)	Late G1/ early S phase
Thymidine	Inhibition of DNA synthesis by creation of an imbalance of dNTPs (Ma & Poon, 2011)	early S phase
Serum starvation	Nutritional deprivation, forcing the cells to go into G0 quiescence phase (Kues et al, 2000)	G0/G1
Aphidicolin	Inhibitor of the DNA polymerase a: blocks DNA synthesis (Ma & Poon, 2011)	Late G1
Nocodazole	Blocks the polymerization of microtubules: cells enter into mitosis but can not form the mitotic spindle (Jordan et al, 1992)	G2/M (prophase)

Table 5-1: Overview of drugs tested for synchronization

Table summarizing the drugs tested for synchronization, as well as their mechanism of action and blocking point during the cell cycle.

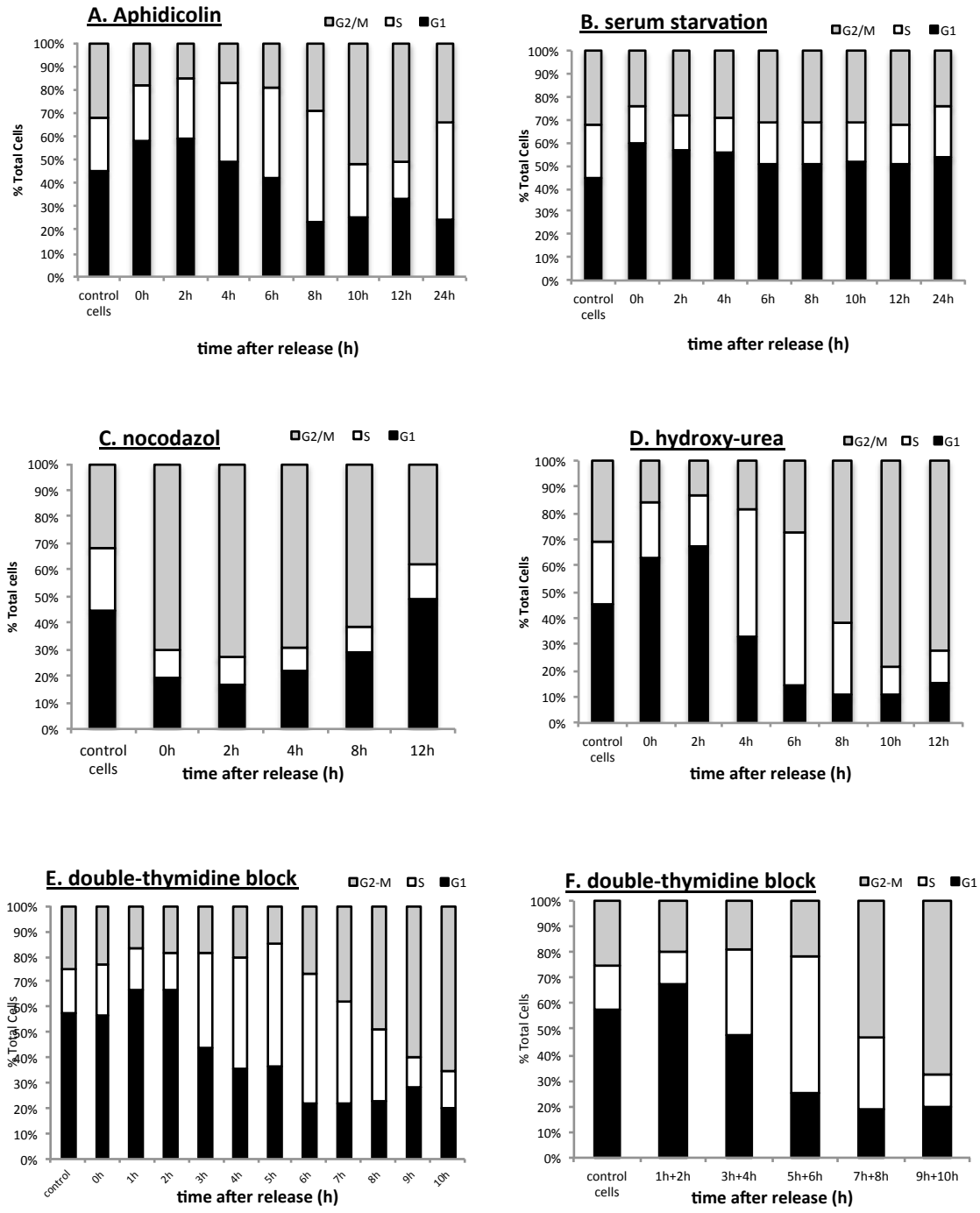


Figure 5-2: cell cycle analysis after synchronization using the various drugs tested

Cells were treated by the indicated synchronization agents: **A** aphidicolin (1 nM for 12h), **B.** serum starvation (growth overnight in 0.5%FBS), **C.** nocodazol (2 mM for 20h), **D.** hydroxy-urea (2 mM overnight), **E.** and **F**. double-thymidine block (2 mM for 18h, release for 9h, 2 mM for 14h) as described in Material and Methods and the DNA profile was analyzed by flow cytometry. Data presented shows the DNA profile at different times after release in comparison to untreated cells (control cells)

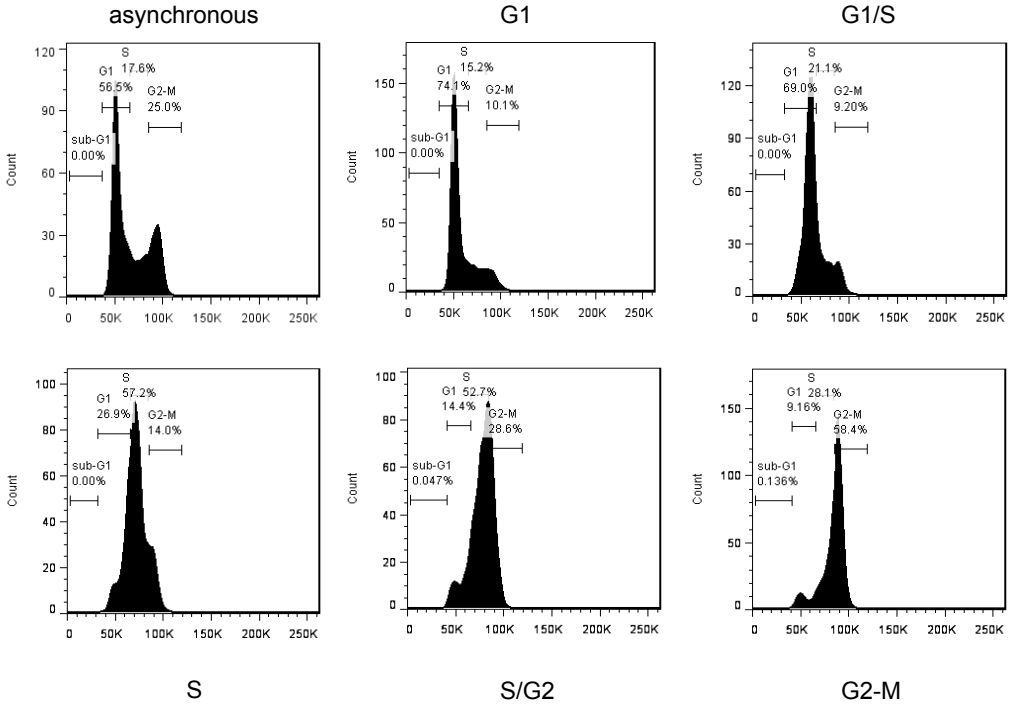
After hydroxy-urea treatment, cells were blocked between 65% and 74% in G1 phase. After release, the cells then progressed in a synchronized fashion through the cell cycle, to reach between 60% and 75% in G2/M phase, 10 hours after release (Figure 5.2.D and Figure 5.3.A). Good results were also obtained using a double-thymidine block. Cells were harvested every hour after release from the double-thymidine block, and cell cycle analysis demonstrated a 60% block in G1 phase that progressed to 70% of the cells ending up in G2/M, 10 hours after release (Figure 5.2.E). Best results were obtained by pooling cells from two time-points, such as mixing cells from 1h and 2h after release, or 3h and 4h after release (Figure 5.2.F). This allowed for an improved homogenization of the cells and better DNA profile (Figure 5.3.B). Thus this technique will be used later.

5.2.2 PLK4 and p52 levels during the cell cycle

As PLK4 is a tightly regulated gene during the cell cycle (Pearson & Winey, 2010; Winkles & Alberts, 2005), and the p52 NF-κB subunit is involved in this regulation, U2OS cells were synchronized using hydroxy-urea or a double-thymidine block and mRNA and protein levels were investigated at each phase of the cell cycle. As seen in Figure 5.4.A, p100 mRNA levels increased to reach a maximum in G1/S and S phases, before slowly decreasing to go back to the initial level. By contrast, PLK4 levels decreased to reach a minimum in S phase and then quickly rose to be at a maximum in S/G2 and G2/M phases. Treatment of the cells by hydroxy-urea (Figure 5.4.A, left panel) gave clearer results than the double-thymidine block (Figure 5.4.B, right panel), but this is probably only due to the fact that more results were available for the first technique, and mRNA levels have the same profile with both types of synchronization, confirming my results.

Protein levels were then studied using the double-thymidine block. Generally p100 and p52 protein levels did not vary much during the cell cycle (Figure 5.4.B). However, PLK4 protein levels were almost undetectable prior to S phase, where it slightly increased and then reached a maximum in S/G2 phase, before once again decreasing slightly.

A. Hydroxy-urea



B. Double Thymidine block

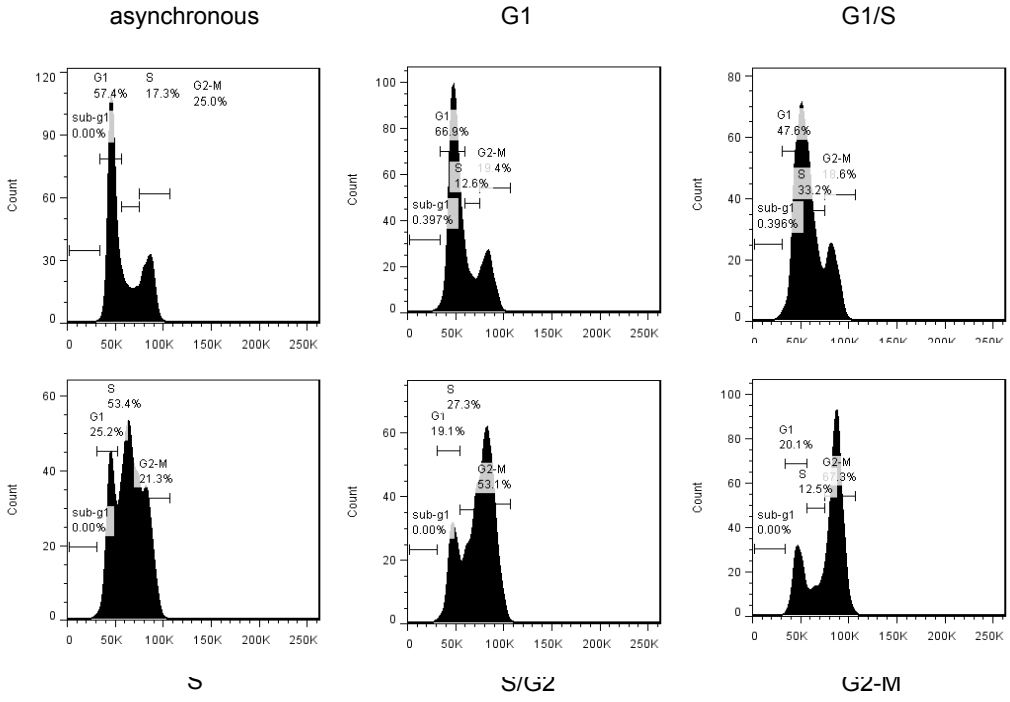


Figure 5-3: DNA profiles after synchronization with hydroxy-urea or double-thymidine block

Cells were treated with hydroxy-urea (**A.)** or double-thymidine block (**B.**) as described in Material and Methods and the DNA profile was analyzed by flow cytometry. Data show the DNA profile at different times after release and is representative of 3 independent experiments.

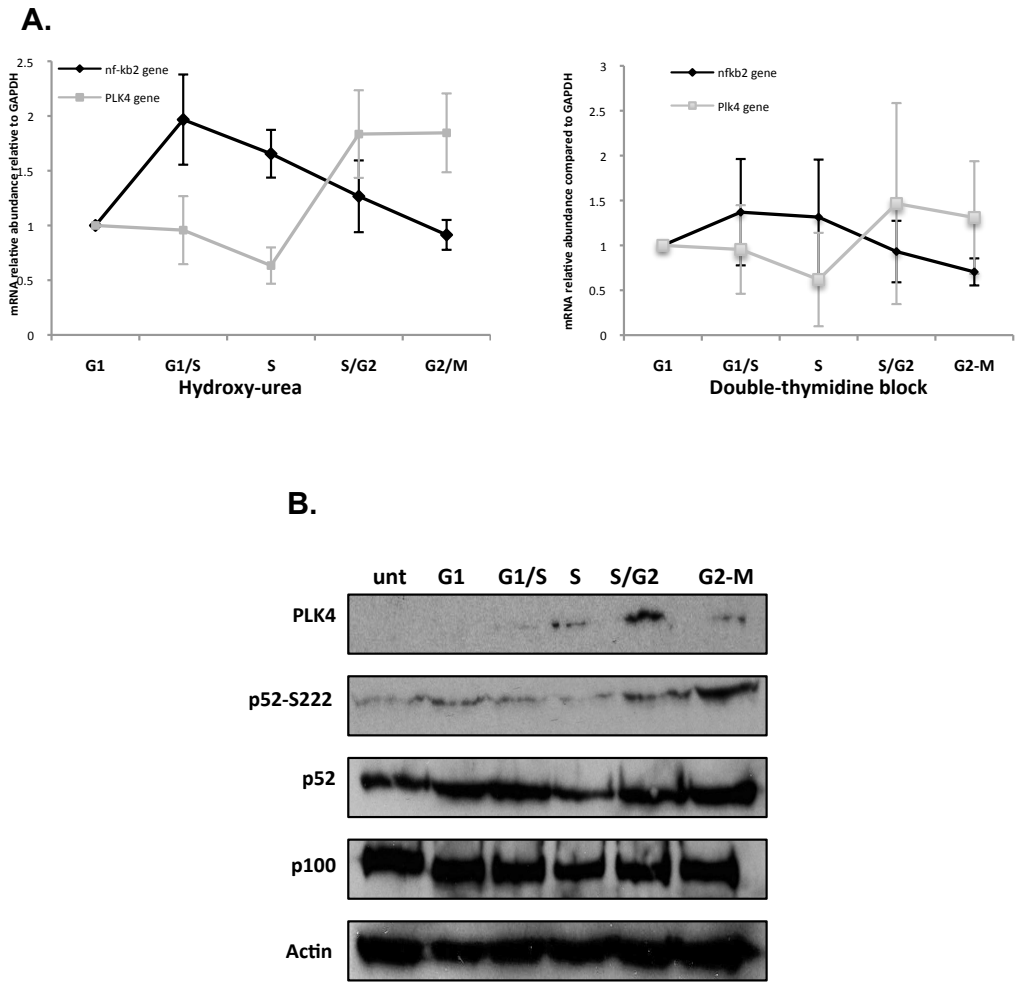


Figure 5-4: PLK4 and p52 levels during the cell cycle

A. Cells were synchronized with hydroxy-urea (left panel) or double-thymidine block (right panel), released as described in Material and Methods and. mRNA expression was analyzed by quantitative PCR, compared to the housekeeper gene GAPDH and related to the expression at the G1 phase of the cell cycle. Error bars represent the standard deviation for at least 3 independent experiments. **B.** Cells were synchronized with a double-thymidine block, released as described in Material and Methods and analyzed by Western Blot for PLK4 expression and S222-p52 protein phosphorylation.

Similar results were found after hydroxy-urea treatment (data not shown). These results, together with the mRNA profile, indicate that post-transcriptional modification is likely to be involved after the regulation by transcription factors.

A phosphoproteomic study demonstrated, in 2008, that p52 can be phosphorylated at Ser222 and this phosphorylation is specific for mitosis (Dephoure et al, 2008). Being interested in post-transcriptional modifications on the NF-κB subunits, our laboratory produced an antibody for the phosphorylated form of Ser222-p52. Looking at the various phases of the cell cycle, I confirmed that phosphorylation at Ser222 of p52 slowly increases and reaches its maximal level in G2/M phase (Figure 5.4.B).

5.2.3 Cyclin and CDKs levels during the cell cycle

A part of my project was to characterize the effect of p52 on cell proliferation, I thus decided to look at cyclin and CDK mRNA levels during the different phases of the cell cycle following cell synchronization. CDK1, CDK2 and CDK4 mRNA levels did not vary much during the cell cycle (Figure 5.5). The strongest result can be seen for cyclin B1 expression, where a clear increase was detected during the cell cycle with a maximum being seen in G2/M phase, compared to the basal level at G1. Cyclin E mRNA peaked at the G1/S transition, where its protein is needed and then decreased slowly. Cyclin D1 levels did not vary much during the cell cycle and cyclin A mRNA had a similar profile as cyclin B1, with an increase through the cell cycle to reach the highest level in S/G2 and G2/M. This is to be expected because cyclin A forms a complex with CDK1 to promote entry into mitosis (Deshpande et al, 2005).

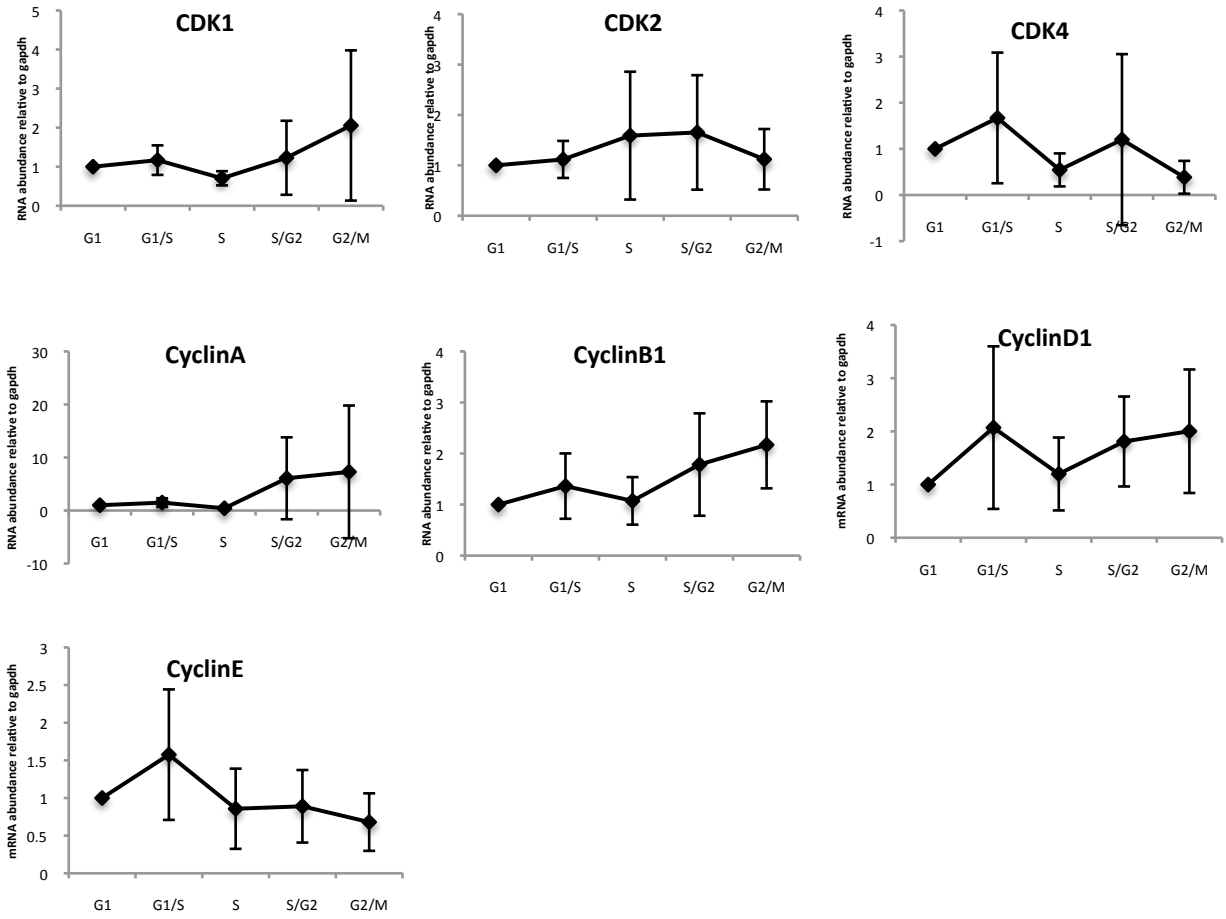


Figure 5-5: cyclin and CDK mRNA expression during the cell cycle

U2OS cells were synchronized using a double-thymidine block and released as described in Material and Methods. mRNA expression was analyzed by quantitative PCR, compared to the housekeeper gene GAPDH and related to the expression at the G1 phase of the cell cycle. Errors bars represent the standard deviation for at least 3 independent experiments.

5.2.4 NF-κB binding to the PLK4 promoter during the cell cycle

Earlier I determined that PLK4 was a NF-κB target gene but that NF-κB binding on the PLK4 promoter was difficult to quantify due to high variability. I also showed, in agreement with other published data, that PLK4 expression is tightly regulated during the cell cycle. A possible explanation of these effects is that NF-κB binding to the PLK4 promoter is cell cycle dependent, which could explain the problems with variability, as the cells studied were asynchronous.

To investigate this hypothesis, U2OS cells were synchronized in G1 phase using hydroxy-urea and released as described in Material and Methods. ChIP assays were performed for each phase of the cell cycle, with antibodies against each NF-κB subunit and using IgG as a negative control. Quantification was performed using qPCR with primers against the PLK4 promoter κB sites previously studied (Figure 5.6). The first observation of the overall data is, with few exceptions (the p52 and p50 subunits for example), NF-κB subunits behave similarly for the various κB sites tested at each phase of the cell cycle (Figure 5.6). After hydroxy-urea synchronization, p52 binding was higher in G1/S for the prPLK4-2634 κB site, whereas it was higher in S phase for the prPLK4-1947 κB site and at the same level for the prPLK4-1256 site (Figure 5.6). RelA seemed to mainly bind the three κB sites in S/G2 phase. The binding of the RelB subunit to the PLK4 promoter is higher in G2/M, but RelB's presence was also detected at the prPLK4-1947 site in S phase and at the prPLK4-1256 site in G1/S phase. c-Rel showed preferential binding at the end of the cell cycle (from S phase to G2/M phase), with the exception of the prPLK4-1256 κB site. The p50 subunit mainly bound in S phase, except for the prPLK4-2634 site where maximal occupancy was seen in G2/M phase (Figure 5.6). However, despite numerous, reproducibility was still an issue during the experiment, so another type of cell synchronization was investigated, with the hope of decreasing the variability of the results.

Results were clearer with synchronization with the double-thymidine block, but this other type of synchronization did not solve all the issues with reproducibility. In Figure 5.7, it can be seen that p52 was bound primarily in G1 phase to both the prPLK4-1947 and prPLK4-1256 sites, and in G1 and G1/S phases at the prPLK4-2634 site (Figure 5.7).

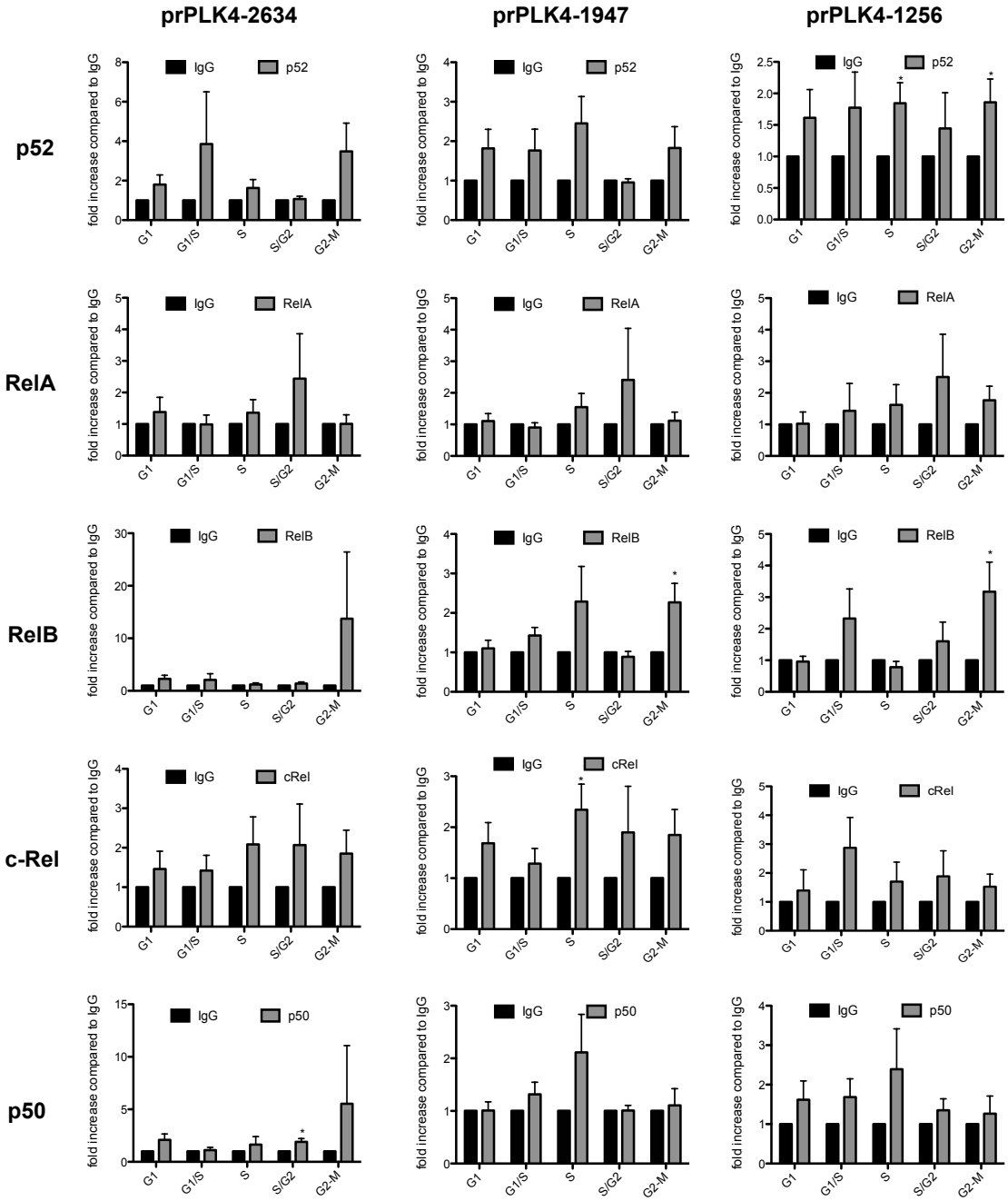


Figure 5-6: NF-κB binding on the PLK4 promoter during the cell cycle after hydroxy-urea treatment

U2OS cells were synchronized with hydroxy-urea, released as described in Material and Methods, fixed by paraformaldehyde and processed for a ChIP assay for each phase of the cell cycle, using antibodies against the various NF-κB subunit and IgG as a negative control. qPCR was performed with primers for the NF-κB binding sites, -1947 and -1256. Error bars show the standard error of at least 3 independent experiments. * P<0,05

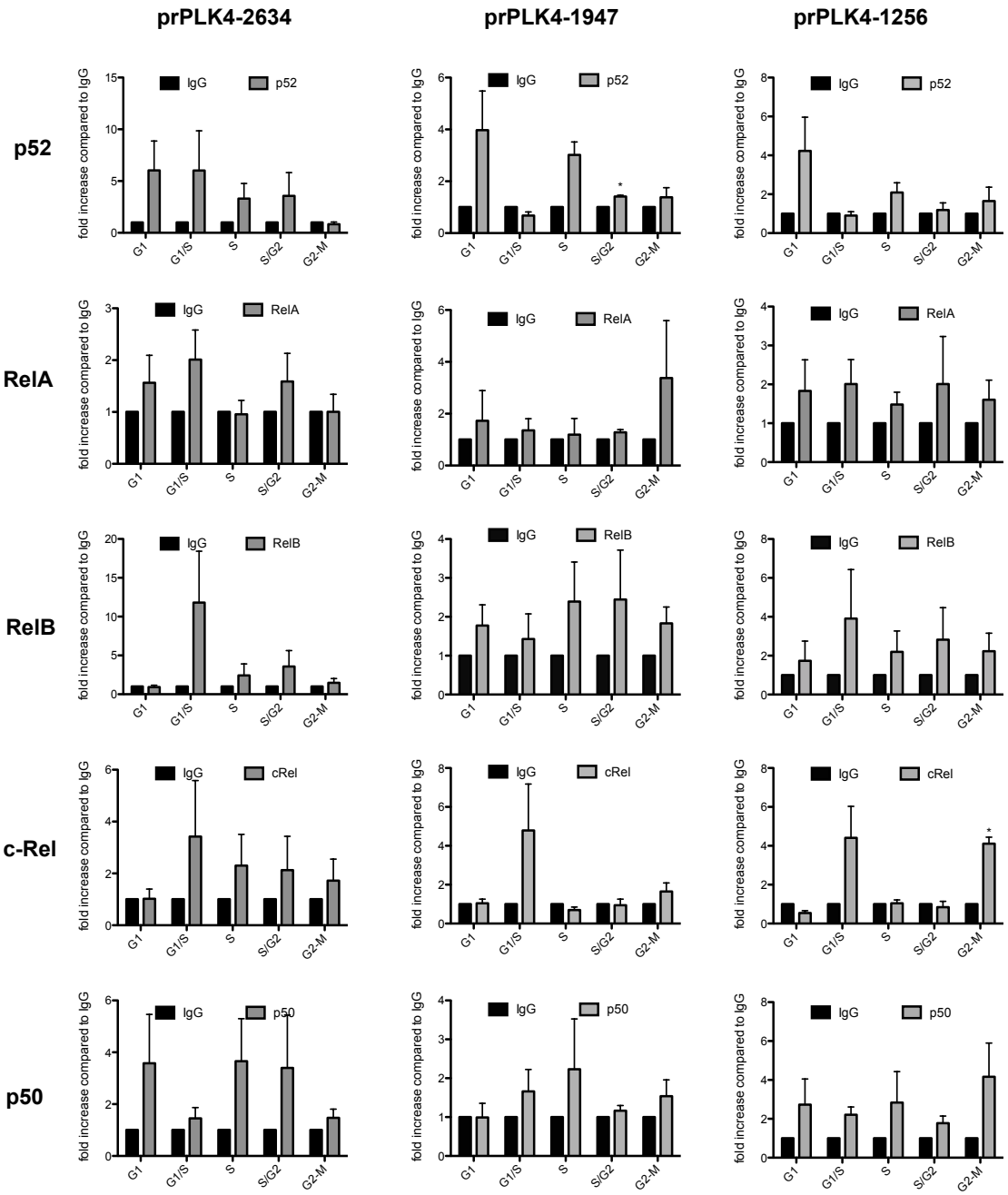


Figure 5-7: NF-κB binding on the PLK4 promoter during the cell cycle after double-thymidine block

U2OS cells were synchronized with a double-thymidine block, released as described in Material and Methods, fixed by paraformaldehyde and processed for a ChIP assay for each phase of the cell cycle, using antibodies against the various NF-κB subunit and IgG as a negative control. qPCR was performed with primers for the NF-κB binding sites, -1947 and -1256. Error bars show the standard error of at least 3 independent experiments. * P<0,05

The RelA subunit showed the same pattern of binding for the prPLK4-2634 and prPLK4-1256 sites, with main binding being early in the cell cycle, whereas RelA was present at the prPLK4-1947 site during G2/M. RelB mainly associated with the prPLK4-2634 site in G1/S phase, to the prPLK4-1947 site in S phase and S/G2, while binding was slightly earlier in G1/S for the prPLK4-1256 site. c-Rel binding mainly occurred in G1/S phase for all three κB sites, whereas p50 seemed more important in S phase (Figure 5.7).

However, as it can been seen in Figures 5.6 and 5.7, error bars for each experiment were very large. Therefore, whit this thesis, I have included two independent, representative, experiments for the cell synchronization using double-thymidine block (Appendix A to E). These experiments illustrate the problems seen with variability between experiments. The p52 subunit, in one experiment, mainly bound to the three κB sites in G1 and S phase, whereas in the second experiment, the binding occurred in G1/S and S/G2, almost as if the timing for cell synchronization was altered (Appendix A). RelA showed a different profile between the two experiments (Appendix B), with the main binding for the three κB sites in G2/M for the exp2, whereas the first experiment demonstrated strong binding in S/G2 for the prPLK4-2634 and prPLK-1256 sites and in S phase for the prPLK4-1947 site. RelB binding occurred either in S phase or S/G2 phase (Appendix C). Binding of c-Rel was strongly detected in G2/M for the three κB sites in one experiment, and in G1/S phase in the other (Appendix D). The p50 subunit was the one with the most variation between experiments. In one experiment, very strong binding occurred in G2/M phase for the prPLK4-1947 site (Appendix E, experiment 2), whereas in the other experiment, no significant binding could be detected (Appendix E, experiment 1). p50 either bound, to the same extent, the prPLK4-2634 site in G1 phase (Appendix E, Experiment 1) or in S/G2 (Appendix E, Experiment 2). Regarding the prPLK4-1256 site, the two independent experiments showed p50 binding either in G1 phase or in S/G2 and G2/M phases. These variations of results were not reduced, regardless of the changes in experimental conditions that were tried.

Figure 5.8 summarizes the percentage of experiments in which binding of NF-κB subunits to the various PLK4 promoter κB sites was higher than the IgG control, for each phase of the cell cycle. In red are those with a percentage

higher than 50%. It is interesting to note that the prPLK4-2634 and prPLK4-1256 sites have a very similar pattern. p52 was the main subunit binding during the G1 and S phases but the other subunits were more involved in the rest of the cell cycle, specifically p50 during the transition phase (83.3% and 100% of binding on both sites during G1/S and 66.7 and 83.3% during S/G2) (Figure 5.8). At the prPLK4-1256 site, c-Rel and RelB were always bound in G2/M (and half the time for prPLK4-2634). This finding indicates that those proteins are likely to form a complex with p50, which was also detected in this time point. The prPLK4-1947 site had a different pattern early in the cell cycle, but the G2/M phase was similar (binding of RelB and c-Rel) (Figure 5.8).

Based on the correlations between the binding patterns of individual NF-κB subunits, some potential dimers regulating PLK4 expression during the cell cycle can be hypothesized. In S phase a dimer of p52-RelB seems to be forming, whereas in S/G2 there appears to be a hetero-dimer of p52-RelA (p52 always binds in S and S/G2 phases, and c-Rel seems mainly in G2/M phase). ReChIP analysis needs to be performed to confirm these observations.

5.3 Discussion

As seen previously, NF-κB binds to the PLK4 promoter and affects its activity. However, these results suggest that the mechanism involved is more complex than expected. As PLK4 is a tightly cell cycle regulated gene, it was investigated whether the cell cycle affects NF-κB regulation of the PLK4 expression. To this extend, cell synchronization was optimized in U2OS cells and several agents were tested.

Nocodazole treatment, which induces a mitotic arrest by blocking microtubule polymerization (Figure 5.1), resulted in more than 70% of cells becoming arrested in G2/M phase. However, it could not be use for our experiments, as the cells did not re-enter the cell cycle when nocodazole was removed (Figure 5.2). Similarly, it has been published that nocodazole is not suitable for synchronizing Hela cells (Cooper et al, 2006).

	prPLK-2634					
	G1	G1/S	S	S/G2	G2/M	
p52	80	66.7	66.7	83.3	16.7	% positive binding
RelA	60	83.3	50	66.7	33.3	
RelB	40	83.3	66.7	83.3	50	
cRel	16.7	60	50	50	50	
p50	50	83.3	66.7	66.7	50	

	prPLK4-1947					
	G1	G1/S	S	S/G2	G2/M	
p52	50	20	100	100	33.3	% positive binding
RelA	33.3	50	66.7	100	50	
RelB	50	33.3	80	66.7	83.3	
cRel	50	50	50	33.3	66.7	
p50	33.3	66.7	40	83.3	50	

	prPLK4-1256					
	G1	G1/S	S	S/G2	G2/M	
p52	60	40	80	33.3	50	% positive binding
RelA	40	80	60	50	50	
RelB	60	80	60	83.3	100	
cRel	20	80	60	33.3	100	
p50	50	100	80	83.3	83.3	

Figure 5-8: percentage of positive NF-κB binding on the PLK4 promoter

Tables summarizing the percentage of positive binding for the NF-κB subunits on the PLK4 promoter during the cell cycle. 5 or more experiments were done for each NF-κB subunit and percentage was calculated for each binding higher than IgG control related to n total of experiments. U2OS cells were synchronized double-thymidine block, released as described in Material and Methods, fixed by parafornaldehyde and processed for a ChIP assay for each phase of the cell cycle. In red, the case where a positive binding was detected in more than 50% of the experiments performed.

Serum starvation, which forces the cell to go into G0 quiescence phase due to lack of nutriments, also did not work in our conditions (Figure 5.2). This result was surprising, as serum starvation was previously used in the lab to synchronize U2OS cells (Barre & Perkins, 2007; Roche et al, 2004). It does illustrate, however, how experimental conditions can affect the outcome of experiments. The use of a new batch of cells or different culture conditions such as different serum can affect results. Cell passage number can also have an effect, as demonstrated with regulation of oxygen consumption by RelA in U2OS cells, where different results were obtained depending on whether the cells were from an early or late passage (Johnson et al, 2011). Cell synchronization using aphidicolin, thereby blocking the cells in late G1 phase by inhibition of DNA polymerase α, thus stopping DNA synthesis (Figure 5.1). Aphidicolin was known to be efficient for cell synchronization (Pedrali-Noy et al, 1980). Treatment of U2OS cells resulted in 60% of cells synchronized in G1 phase, while release did lead to progression through the cell cycle (Figure 5.2). However, the proportions of cells in each phase were too low for our purpose, as we did not obtain more than 50% of cells in G2/M phase. Therefore, this technique was also discarded for later experiments.

Two techniques were optimized successfully, both blocking cells in late G1 or early S phase. Double-thymidine block inhibits DNA synthesis by creation of an imbalance of dNTPs, whereas hydroxy-urea decreases the production of dNTPs available for DNA synthesis by inhibition of ribonucleotide reductase (Figure 5.1). Both techniques allowed good synchronization of cells in G1 phase, as well as progression through the cell cycle (Figures 5.2 and 5.3), corresponding to what has been published for these agents (Ma & Poon, 2011). However, it is important to note that all these synchronization techniques used chemical agents that can induce stress responses and possibly DNA damage responses in the cells. Consequently, they are not as efficient or as reproducible as elutriation. Elutriation is a centrifugation technique that separates the cells depending on their size and had been used previously with success on U2OS cells to enrich cells at different cell cycle stages (Barre & Perkins, 2007). Nonetheless, despite its attractiveness, synchronization by elutriation was not possible due to the lack of equipment available. Therefore, the results obtained with hydroxy-urea or double-thymidine block must be considered with some caution.

Analysis of the *nfkb2* and PLK4 genes reveal that they display opposing patterns of expression during the cell cycle (Figure 5.4). Indeed, *nfkb2* is maximal in G1/S, which is expected due to the involvement of p52 at the G1/S transition (Hinz et al, 1999; Rocha et al, 2003b; Schumm et al, 2006). By contrast, PLK4 levels started to rise in S phase to be maximal in G2/M, in accordance with what has been previously published (Pearson & Winey, 2010). These results were detected by both means of synchronization, hydroxy-urea and double-thymidine block. Protein levels were slightly different (Figure 5.4.B), with maximal levels being reached in S/G2 phase followed by a rapid decrease in G2/M, suggesting post-transcriptional regulation. This result would be in accordance with the fact that PLK4 is a short-live protein (Sillibourne & Bornens, 2010b). The absence of PLK4 protein detected in G1 and G1/S phases is contradicted by literature stating that maximal PLK4 kinase activity is found in G1/S phase at the duplicating mother centriole (Pearson & Winey, 2010). Further analysis would need to be required to explain this result but it is possible that, PLK4 being located at the centriole, the level of proteins was too low to be detected by western blot.

In 2008, a phospho-proteomic study identified some phospho sites in p100/p52 specifically phosphorylated during G2/M phase (Dephoure et al, 2008). Our lab is particularly interested in the p52 Ser222 phosphorylation; therefore it was investigated in the synchronized U2OS cells. Ser222 phosphorylation has also been shown to be carried out by GSK3β and be responsible for the repression of the Skp2 promoter by p52 (Barre & Perkins, 2010). My results showed an increase of Ser222 phosphorylation during the cell cycle, with a maximum level in G2/M phase (Figure 5.4), concurring with published data (Dephoure et al, 2008). Besides GSK3β, little is known about kinases responsible for Ser222-p52 phosphorylation, and it will be interesting to identify the kinases involved during mitosis. I also investigated the effect of a few G2/M kinase inhibitors, such as a PLK inhibitor, to block PLK1 and PLK3 kinase activity; roscovitin, a CDK inhibitor and RO-3306, an inhibitor specific to CDK1, under the conditions recommended by the manufacturers. However, no apparent effect was detected on Ser222 p52 phosphorylation after these treatments, possible indication that these kinases are not involved in p52 modification (data not shown). However, these results have to be treated

carefully, as no positive control was performed to confirm the experimental conditions.

Our laboratory will soon be in possession of transgenic mice mutated at Ser222 in the *nfkb2* gene: S222A mice which will lack the ability of being phosphorylated at Ser222, and S222D mice, which are phospho-mimetic mutants of the wild type mice. It will be interesting to determine PLK4 levels in these mice, and determine whether Ser222 p52 phosphorylation is involved in PLK4 regulation of centrosome duplication or affects p52's functions in G2/M phase.

ChIP analyses were performed on each phase of the cell cycle for the three κB sites previously identified (Figures 5.6 to 5.9 and Appendix A to E), either after hydroxy-urea or double-thymidine block. Both types of synchronization did not show the same results and furthermore, all results demonstrated a high variability between experiments (Figures 5.6. and 5.7). Detailed analysis of two independent experiments (Appendix A to E) showed a shift of the phase of binding in many of the cases, with, for example, the p52 subunit binding to the prPLK4-2634 site mainly in G1 and S phases for experiment 1 and in G1/S and S/G2 phases for experiment 2 (Appendix A). This shift was also found for the RelB subunit at the prPLK4-2634 site, with binding in S or S/G2 phase (Appendix C).

A possible explanation for this shift in binding in different phases of the cell cycle is that despite our careful analysis of the cell DNA profile to separate the various phases, it is possible that cell cycle progression varied between experiments and FACS analysis was not sensitive enough to detect this. A possible way to overcome this would be to base the separation between phases not on the DNA profile but on a biochemical analysis. We could, for example, use one of the cyclins, such as cyclin B1, as a reference with the highest mRNA/protein expression being used as a marker for G2/M phase, whereas the lowest would mean G1 phase. Now that the PLK4 levels have been described, they could also be used as reference. This could lead to reduced variation between experiments. Another possibility is to perform cell synchronization based on cell size by using elutriation. Other factors can also be involved to explain these results. For example, although experiments were repeated as closely as possible, external factors such as cell confluency within the plate or

the passage number could affect the cell culture conditions and thus the response to the synchronization agent. Chapter 4 also raised also some issues of reproducibility and it was suggested that potential unknown κB sites were still to be identified, situated closer to the initiation transcription start. If this was the case, it could also explain the variation between the results obtained with independent experiments. More careful analysis will be performed in the future to remedy these issues.

Importantly, this shift between phases of the cell cycle did not always happen and some results were more reproducible, such as c-Rel binding in G1/S phase to the prPLK4-1947 and prPLK4-1256 sites (Appendix D). Consequently, it was possible to determine the percentage of experiments in which NF-κB binding was higher than IgG control (Figure 5.8). The general pattern showed that, assuming the κB sites were correct, NF-κB binds to the prPLK4-2634 site mainly during the early cell cycle (G1 to S), whereas for the prPLK4-1947 site, later binding, from S to G2/M phases can be detected. However, for the prPLK4-1256 κB site, no conclusion could be made, as various NF-κB subunits bind at different times of the cell cycle. This suggests that NF-κB regulation of the PLK4 promoter is more complex than expected, as potentially the same subunit could bind at different κB sites during various stages of the cell cycle, and possibly induce different response of the promoter. This hypothesis could also explain the effect seen on the PLK4 promoter activity in luciferase assays, where NF-κB depletion had very little effect, whereas it had a strong effect on expression of the endogenous gene.

5.4 Conclusion

In this chapter, I studied the expression of the *nfkb2* and PLK4 genes during each phase of the cell cycle and I determined that they have opposing mRNA patterns. I also studied NF-κB binding to the various κB sites in the PLK4 promoter during the cell cycle. Although this generated some hypotheses about the potential mechanism involved, the data is still too preliminary to fully understand the NF-κB regulation of the PLK4 promoter and further experiments will be required.

6 Discussion

6.1 Introduction

The aim of this thesis was to investigate the regulation of cancer cell proliferation and mitosis by NF-κB. I was especially interested in the characterization of the effect of the p52 subunit on the cell cycle and to explore its regulation of centrosome duplication. To support this purpose, I based my research on previous data obtained in the lab, including both published and unpublished observations. These data demonstrated that p52 was involved in cell proliferation through the regulation of cyclin D1 expression. The p52 NF-κB subunit also appeared to be involved in regulation of the centrosome, as its depletion induced major defects in centrosome structure in the cell but no further analysis had been performed to determine the mechanism of regulation. Therefore, it was interesting to try to resolve how p52 might regulate both centrosome structure and duplication. The results presented in this thesis link p52 to the centrosome and suggest that this NF-κB subunit regulates the expression of genes involved in centrosome duplication. More importantly, one of these genes, PLK4, is identified in this thesis as a new NF-κB target gene that could provide a link between NF-κB and the mitotic events. These implications will be discussed in this section and possible future experiments will be highlighted.

6.2 The p52 NF-κB subunit and the cell cycle

The link between NF-κB and regulation of the cell cycle is now well known. Indeed, in 1998, Grumont et al described that *c-Rel* -/- or *nfkb1* -/- B cells failed to proliferate in response to mitogen activation and induced an accumulation of cells in G1 phase (Grumont et al, 1998). A few months later, Guttridge et al made the link with the cyclin D1 regulation by NF-κB (Guttridge et al, 1999). From then on, a lot of research was invested in this area and soon,

various links were found. For example, c-Rel induces an increase in cyclin D1 expression and promotes mammalian tumorigenesis (Romieu-Mourez et al, 2003), while the p50 subunit could have an opposite effect. Formation of c-Rel/p50 heterodimers induces cyclin D1 expression (Romieu-Mourez et al, 2003), whereas in TNF-α stimulated cells, p50 could associate to the cyclin D1 promoter and repress its transcription (Witzel et al, 2010). In the cell lines investigated in the Perkins lab, the main NF-κB subunit involved in cyclin D1 regulation is p52. As described before, p52 depletion by siRNA treatment induces G1/S arrest through the loss of cyclin D1 expression (Schumm et al, 2006). My data confirmed p52 regulation of cyclin D1 protein and mRNA expression (Figure 3.3). The expression of other cyclin genes was not affected by the p100/p52 siRNA depletion; although, CDK2 and CDK4 expression were seen to be reduced. This was supported by recent results in the Perkins lab showing the down-regulation of CDK4 expression upon p100/p52 siRNA depletion in human fibroblast (Figure 3.9) (A. Iannetti, unpublished micro-array data). Therefore, future experiments will try to determine, using ChIP analysis, whether CDK4 is a direct NF-κB target and what are the dimers involved in its regulation. To date, no connection has been made between p52 and CDK2 but RelA has been shown to regulate CDK2 expression in laryngeal squamous cancer cell (Liu et al, 2011). It is probable that CDK2 and CDK4 regulation is involved in the p52 regulation of the G1/S transition, but nothing more have been described yet. It would be interested to test whether CDK2 or CDK4 downregulation blocks the cells in G1/S phase and in what extent this can be linked solely to regulation by p52. Ultimately, this would help to clarify the oncogene and tumor suppressor roles of NF-κB.

6.3 NF-κB and centrosomes

Centrosomal abnormalities are frequently found in cancer, especially in the early events of tumorigenesis and these usually lead to chromosome missegregation. They usually trigger multinucleate cells and chromosomal instability and can be used as prognostic indicator for tumor progression (Gustafson et al, 2000; Kuo et al, 2000; Pihan et al, 2001). An important but

hypothetical link between centrosome abnormalities and NF-κB was made when these were commonly found in hematologic malignancies, tumours often overexpressing p52 (Rayet & Gelinas, 1999). However, some of our preliminary data (K.Schumm, unpublished observations) suggested that p52 down-regulation also induces such centrosome abnormalities, as it was observed by immunofluorescence and electron microscopy. Although I was not able to repeat these observations, I was able to localize p100/p52 to the centrosome. As U2OS cells undergo cell cycle arrest upon p100/p52 depletion, an effect on centrosome is unlikely to be seen since a consequence of this is also the arrest of the centrosome duplication cycle. Further experiments, using stable cell lines depleted for p100/p52 that have overcome the arrest in cell cycle, will be used to confirmed these effects

6.4 Regulation of the centrosome duplication by NF-κB

CDK2 and CDK4 have been described as regulators of centrosome duplication. Indeed, Adon et al demonstrated that in case of p53 -/- MEFs, CDK2 and CDK4 were hyperactive and, through hyperphosphorylation of Rb and uncontrolled E2F-dependent transcription, lead to the aberrant expression of molecules involved in centrosome duplication (Adon et al, 2010). However, in case of these *p53-/-* MEFS, when either CDK2 or CDK4, or both, were depleted, normal centrosome duplication was restored (Adon et al, 2010). These results confirmed the importance of CDK proteins for normal centrosome regulation. My data showing that p52 (and possibly other NF-κB subunits, but it will need to be confirmed) participates in the regulation of CDK2 and CDK4 expression could then suggest NF-κB involvement in the regulation of centrosome duplication in the case of the absence of p53. This idea will be further investigated using Hela cells, which express a non functional p53 protein, or H1299 cells, which are p53 depleted. H1299 cells are a particularly interesting system for investigation, as my laboratory possesses a p53 inducible variation of this cell line. Using this cell line, I would be able to determine if p52 depletion is involved in CDK2 and CDK4 regulation of centrosome duplication and any p53 involvement in this process. This hypothesis is supported by a recent study demonstrating that CDK2 is a RelA target gene and that inducing

RelA upregulates CDK2 expression and promotes centrosome amplification (Liu et al, 2011). Overepressing the p52 subunit will be an important experiment to perform, in order to determine possible aberrant centrosome number.

PLK4, whose down-regulation reduces centriole number and thus induces aneuploidy, was recently described to be associated with a poor survival prognosis in hepatocellular carcinoma (Liu et al, 2012). My results showed that NF-κB is essential for PLK4 expression regulation and its promoter activity in some cell types. Among the defects in mitosis previously detected after p100/p52 depletion in U2OS cells, a failure in cytokinesis and multinucleate cells could be seen. These results can be linked to my regulation of PLK4 expression by p52. This hypothesis is supported by the fact that *PLK4+/-* MEFs exhibit defective cytokinesis, an increase in centrosome number and multinucleate cells (Rosario et al, 2010). So PLK4 could be the link between the p52 subunit and the phenotypes of aberrant centrosome structure, mitotic spindle, multinucleate cells and chromosomal instability previously observed.

This will be investigated further, with the help of wild type or *nfkb2*-/- MEFs. Observations in these cells will determine whether they have increased centrosome number, which is the expected result due to the upregulation of PLK4 detected in these cells (Chapter 4). Previous unpublished data suggested that p52 siRNA depletion did not increase centrosome number. This is consistent with my data, shown either by immunofluorescence (Figure 3.2) or with the p52 regulation of PLK4. Indeed, p100/p52 siRNA depletion decreases PLK4 expression (Figure 3.4), and PLK4 reduction has been shown to block the centrosome duplication (Bettencourt-Dias & Glover, 2007). It has also been shown that PLK4 overexpression induces centriole overamplification (Kleylein-Sohn et al, 2007), therefore the effect of overexpressing p52 and other NF-κB subunit will be studied.

It is interesting to note that CDK2 and CDK4 down-regulation, induced by p100/p52 depletion in my model, is thought to amplify centrosome duplication (Adon et al, 2010), whereas centrosome overamplification will be inhibited by reduced PLK4 expression (Bettencourt-Dias & Glover, 2007), also regulated by NF-κB. My own observations determined that p100/p52 depletion did not increase the number of centrosome (Figure 3.2 and K.Schumm unpublished observation), therefore supporting PLK4 regulation effect. It is possible that

centrosome overamplification expected after CDK2 and CDK4 down-regulation is overcome by the p52 effect on PLK4, key factor for centriole duplication. However, further analysis will be required to confirm this hypothesis.

6.5 Cell cycle effect

My data suggests that p52's main effect on the cell cycle is regulation of the G1/S transition, through the regulation of CDKs and cyclin D1. It is also the start of the centrosome duplication cycle, which supports my hypothesis of p52 involvement in this process. To emphasize this, PLK4 is mainly active in late G1/early S phase, to initiate centriole duplication. However, my experiments demonstrated that PLK4 mRNA was maximal towards the end of the cell cycle, from late S phase to G2/M, which was confirmed by published data (Winkles & Alberts, 2005). I then investigated whether p52 and NF-κB regulation vary during the cell cycle. To achieve this, I used synchronized cells and studied the binding of NF-κB to the PLK4 promoter. Although unfortunately no definitive mechanism could be concluded, due to the in the binding seen, my analysis showed that NF-κB appears to mainly bind to one κB site, the prPLK4-2634 site, during the early cell cycle (G1 to S phases) and to another, prPLK4-1947, during the late cell cycle (S to G2/M phases). It is possible that this change of binding sites induces different responses in PLK4 promoter activity, but this will need to be investigated further, possibly using reporter gene assays on synchronized cells. This altered effect of p52 during the cell cycle has already been described for the regulation of c-myc, cyclin D1 and Skp2 expression (Barre & Perkins, 2007) and is linked to the phosphorylation of RelA inducing the formation of different activator or repressor heterodimers. It is a mechanism that could possibly be applied to PLK4, a tightly cell cycle regulated gene, especially as p52 is known to possess cell cycle-dependent phosphorylation sites, such as Ser222.

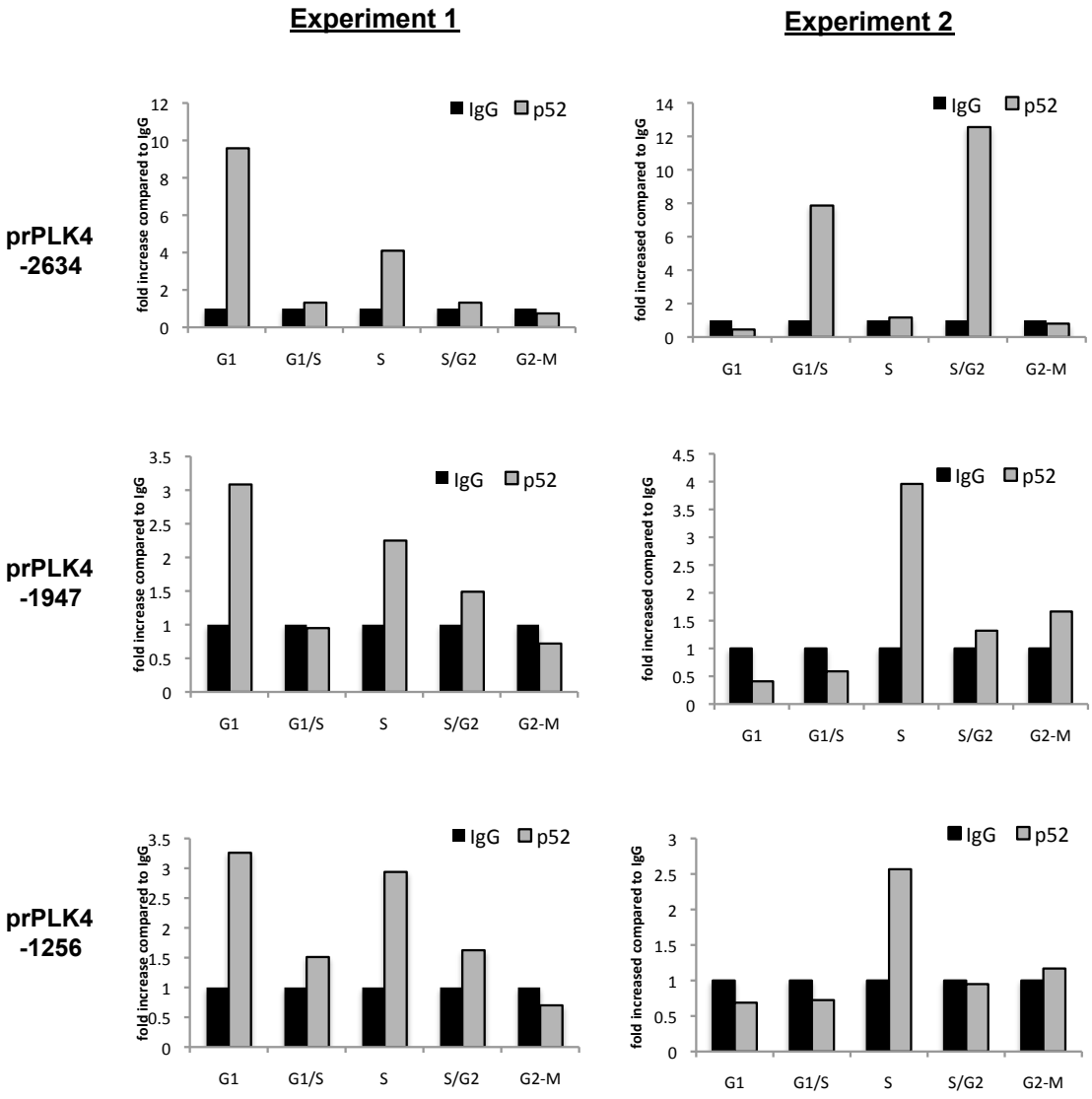
6.6 NF-κB phosphorylation

The Ser222 phosphorylation of the p52 subunit has been described previously as a specific event of mitosis (Dephoure et al, 2008), which was confirmed in this study (Chapter 5). However, no mitotic kinase has yet been identified as being responsible for this phosphorylation. A phospho-proteomic study identified the p52 subunit as a potential target for PLK1 phosphorylation (Grosstessner-Hain et al, 2011) and sequence analysis of the p100 sequence found consensus sites for the Aurora A, CDK and PLK kinases. Nonetheless, my analysis suggests that in my conditions, the PLK1 and CDKs kinases were not responsible for Ser222 phosphorylation of p52 (Chapter 5), as inhibiting their kinase activity had no effect (data not shown). It will be interesting as to investigate whether PLK4 depletion reduces Ser222 p52 phosphorylation and also to determine if Ser222 phosphorylation affects p52 regulation of PLK4. This will be helped by the creation of Ser222 p52 mutant mice, soon available in the lab. These mice, containing mutations at the Ser222 residue that will render Ser222 either non phosphorylable (S222A mice) or phospho mimicking but insensible to Ser/Thr kinases (S222D mice), will aid in the determination the mechanism behind p52-dependent regulation of G2/M phase. Other data suggests that NF-κB's phosphorylation state may be important in the regulation of PLK4. For example, the RelA subunit is phosphorylated at the Thr505 in S phase (Barre & Perkins, 2007), inhibiting its transcriptional activity. An ongoing project in the lab found that transgenic T505A mice displayed multiple mitotic defects in the liver and that PLK4 mRNA levels were higher than in wild-type mice (personal communication), suggesting that Thr505 RelA phosphorylation is involved in RelA regulation of PLK4. This mouse model reinforce our hypothesis of differential effects due to the phosphorylation state of NF-κB and will be used for further experiments, in parallel with the S222 mouse models.

6.7 Final conclusion

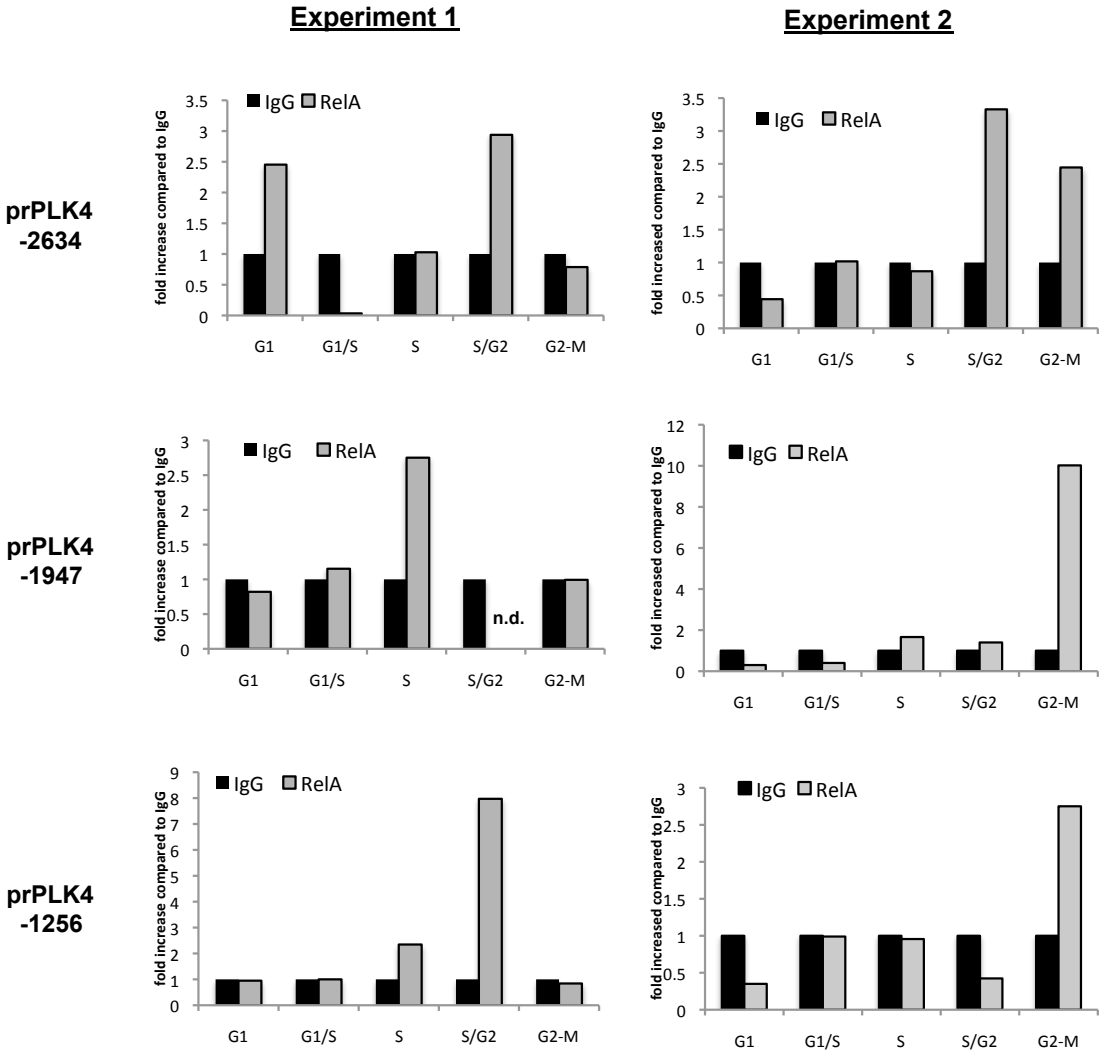
This thesis aimed to explain the role of the p52 NF-κB subunit in cell proliferation and centrosome duplication. Although no final explanation was found, some interesting leads were discovered. First, I determined that p52 regulates other genes involved in the cell cycle in addition to cyclin D1. It also became apparent that p52 is involved in the regulation of centrosome duplication genes and that one of these genes, PLK4, is a direct NF-κB target gene. I demonstrated that multiple κB sites mediate NF-κB binding to the PLK4 promoter and that these sites can differentially regulate NF-κB binding during the cell cycle, resulting in various effects on promoter activity. Further analysis will be required to fully identify these effects, to complete the characterization of NF-κB regulation of the PLK4 promoter and ultimately, NF-κB regulation of centrosome duplication.

7 Appendixes



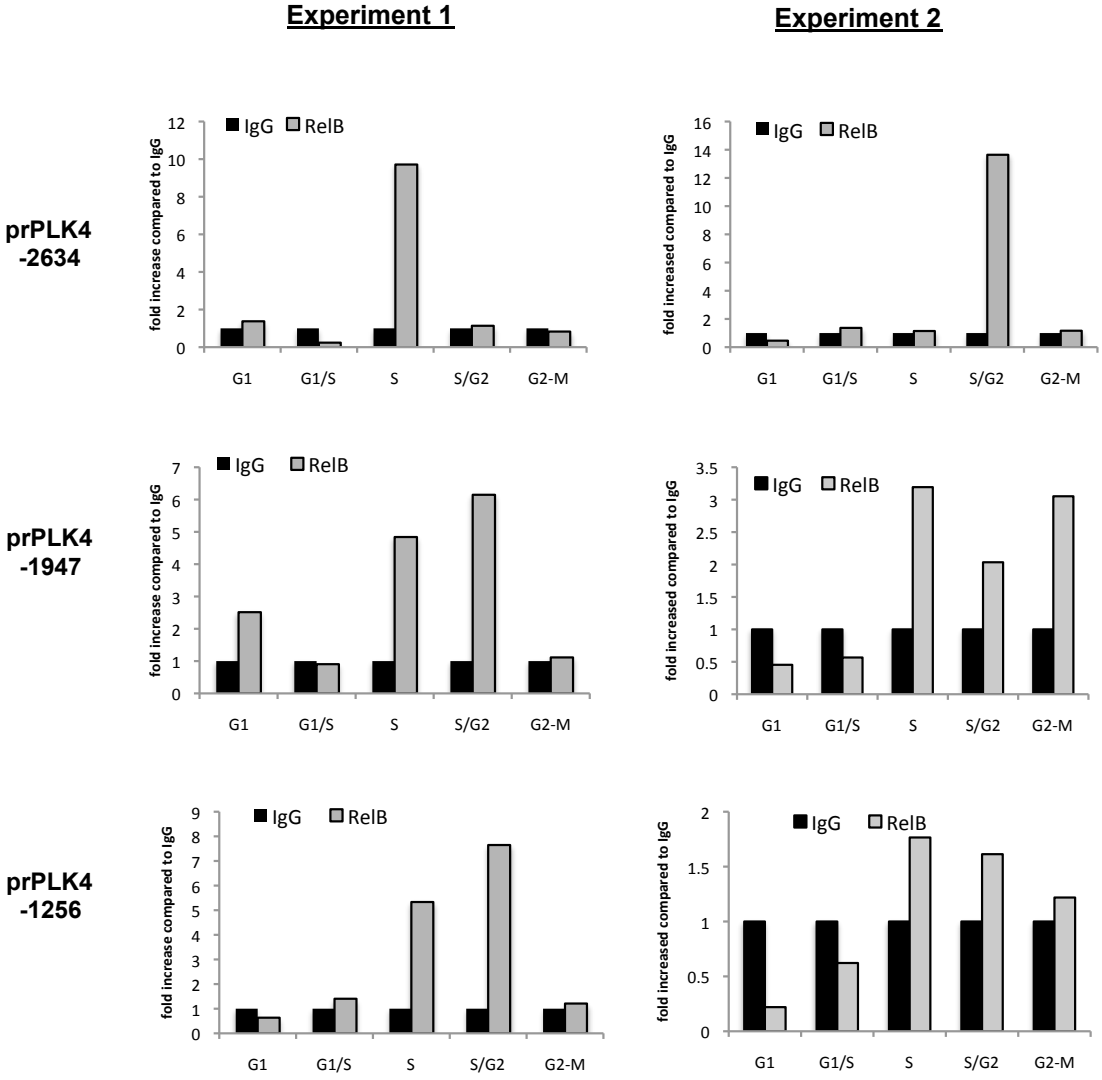
Appendix 7-1: the p52 subunit binds the PLK4 promoter during the cell cycle

U2OS cells were synchronized double-thymidine block, released as described in Material and Methods, fixed by parafornaldehyde and processed for a ChIP assay for each phase of the cell cycle, using antibodies against the p52 subunit and IgG as a negative control. qPCR was performed with primers for the NF-κB binding sites -2634, -1947 and -1256. Data show a representation of 2 independent experiments, demonstrating the variation of results obtained



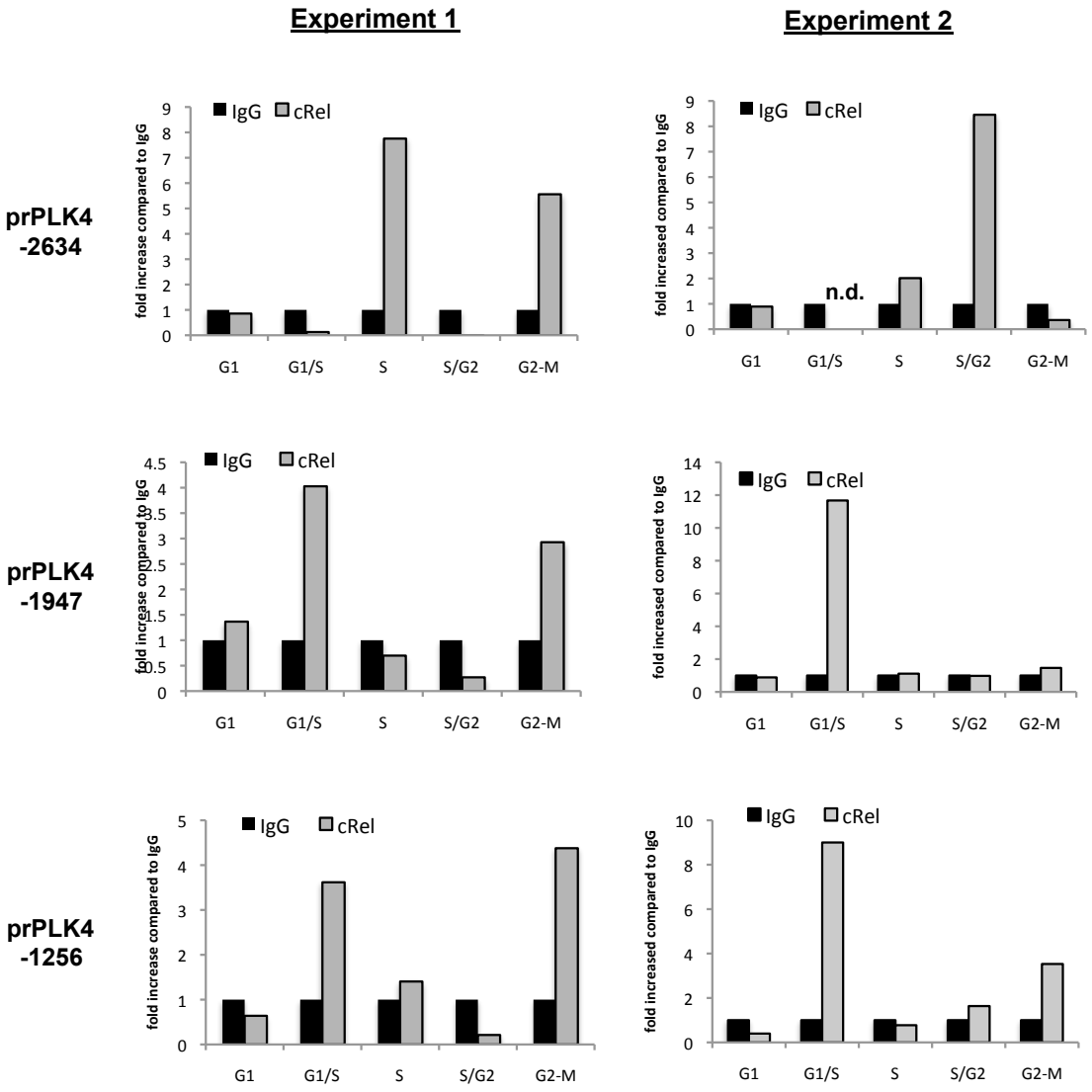
Appendix 7-2: the RelA subunit binds the PLK4 promoter during the cell cycle

U2OS cells were synchronized double-thymidine block, released as described in Material and Methods, fixed by parafornaldehyde and processed for a ChIP assay for each phase of the cell cycle, using antibodies against the RelA subunit and IgG as a negative control. qPCR was performed with primers for the NF-κB binding sites -2634, -1947 and -1256. Data show a representation of 2 independent experiments, demonstrating the variation of results obtained



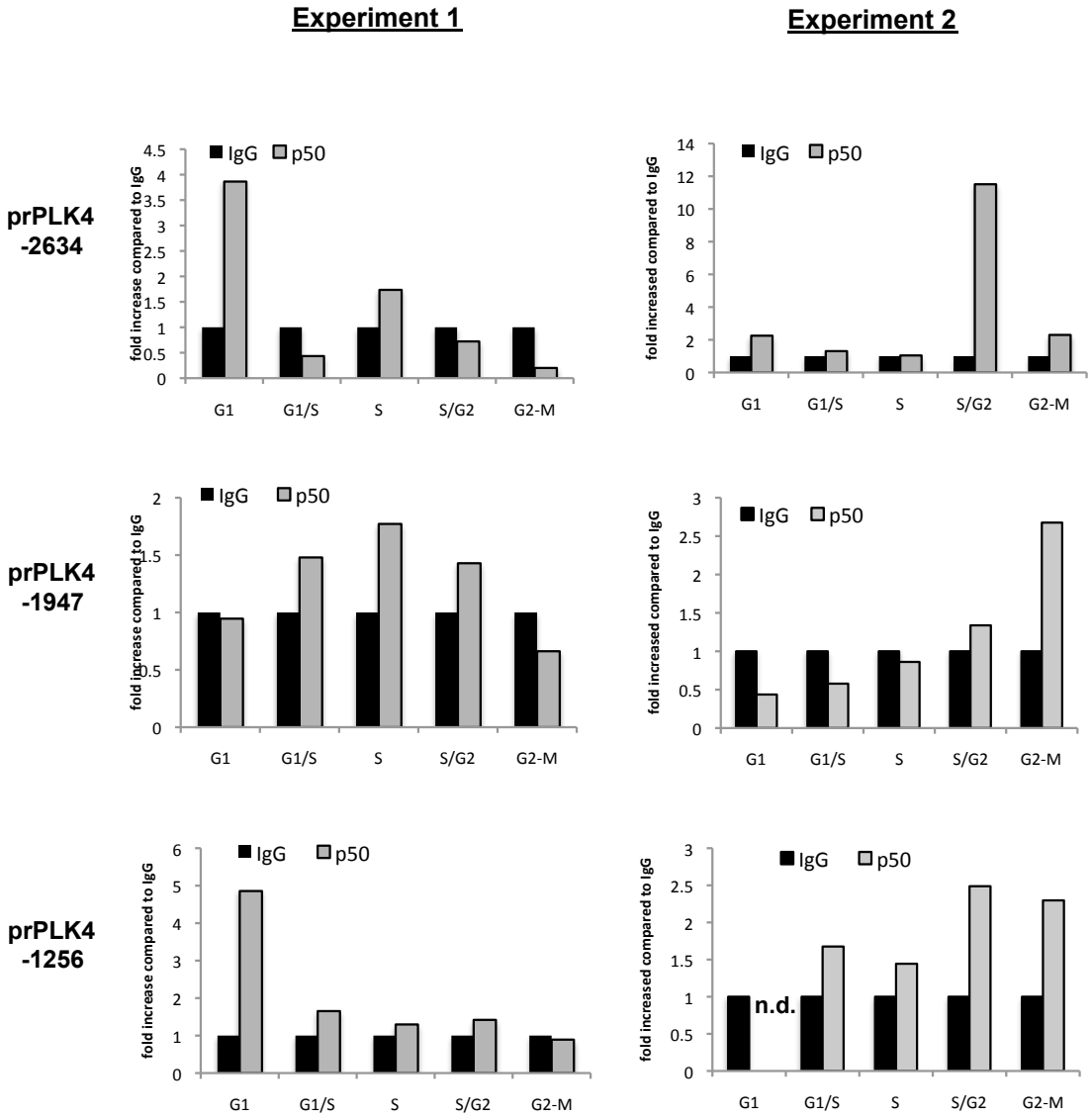
Appendix 7-3: the RelB subunit binds the PLK4 promoter during the cell cycle

U2OS cells were synchronized double-thymidine block, released as described in Material and Methods, fixed by parafornaldehyde and processed for a ChIP assay for each phase of the cell cycle, using antibodies against the RelB subunit and IgG as a negative control. qPCR was performed with primers for the NF-κB binding sites -2634, -1947 and -1256. Data show a representation of 2 independent experiments, demonstrating the variation of results obtained



Appendix 7-4: the cRel subunit binds the PLK4 promoter during the cell cycle

U2OS cells were synchronized double-thymidine block, released as described in Material and Methods, fixed by parafornaldehyde and processed for a ChIP assay for each phase of the cell cycle, using antibodies against the cRel subunit and IgG as a negative control. qPCR was performed with primers for the NF-κB binding sites -2634, -1947 and -1256. Data show a representation of 2 independent experiments, demonstrating the variation of results obtained



Appendix 7-5: the p50 subunit binds the PLK4 promoter during the cell cycle

U2OS cells were synchronized double-thymidine block, released as described in Material and Methods, fixed by parafornaldehyde and processed for a ChIP assay for each phase of the cell cycle, using antibodies against the p50 subunit and IgG as a negative control. qPCR was performed with primers for the NF-κB binding sites -2634, -1947 and -1256. Data show a representation of 2 independent experiments, demonstrating the variation of results obtained.

8 References

Abu-Amer Y, Ross FP, McHugh KP, Livolsi A, Peyron JF, Teitelbaum SL (1998) Tumor necrosis factor-α activation of nuclear transcription factor-κB in marrow macrophages is mediated by c-Src tyrosine phosphorylation of IκBα. *J Biol Chem* **273**(45)**:** 29417-29423

Adon AM, Zeng X, Harrison MK, Sannem S, Kiyokawa H, Kaldis P, Saavedra HI (2010) Cdk2 and Cdk4 regulate the centrosome cycle and are critical mediators of centrosome amplification in p53-null cells. *Mol Cell Biol* **30**(3)**:** 694-710

Amir RE, Haecker H, Karin M, Ciechanover A (2004) Mechanism of processing of the NF-κB2 p100 precursor: identification of the specific polyubiquitin chain-anchoring lysine residue and analysis of the role of NEDD8-modification on the SCF(β-TrCP) ubiquitin ligase. *Oncogene* **23**(14)**:** 2540-2547

Arnosti DN, Kulkarni MM (2005) Transcriptional enhancers: Intelligent enhanceosomes or flexible billboards? *J Cell Biochem* **94**(5)**:** 890-898

Atkinson PGP, Coope HJ, Rowe M, Ley SC (2003) Latent Membrane Protein 1 of Epstein-Barr Virus Stimulates Processing of NF-κB2 p100 to p52. *J Biol Chem* **278**(51)**:** 51134-51142

Badano JL, Teslovich TM, Katsanis N (2005) The centrosome in human genetic disease. *Nat Rev Genet* **6**(3)**:** 194-205

Bakal CJ, Finan D, LaRose J, Wells CD, Gish G, Kulkarni S, DeSepulveda P, Wilde A, Rottapel R (2005) The Rho GTP exchange factor Lfc promotes spindle assembly in early mitosis. *Proc Natl Acad Sci U S A* **102**(27)**:** 9529-9534

Baldwin AS (2012) Regulation of cell death and autophagy by IKK and NF-κB: critical mechanisms in immune function and cancer. *Immunol Rev* **246**(1)**:** 327-345

Barre B, Coqueret O, Perkins ND (2010c) Regulation of activity and function of the p52 NF-κB subunit following DNA damage. *Cell Cycle* **9**(24)**:** 4795-4804

Barre B, Perkins ND (2007) A cell cycle regulatory network controlling NF-κB subunit activity and function. *EMBO J* **26**(23)**:** 4841-4855

Barre B, Perkins ND (2010) The Skp2 promoter integrates signaling through the NF-κB, p53, and Akt/GSK3β pathways to regulate autophagy and apoptosis. *Mol Cell* **38**(4)**:** 524-538

Barre B, Perkins ND (2010b) Phosphorylation of the p52 NF-κB subunit. *Cell Cycle* **9**(24)**:** 4774-4775

Basak S, Kim H, Kearns JD, Tergaonkar V, O'Dea E, Werner SL, Benedict CA, Ware CF, Ghosh G, Verma IM, Hoffmann A (2007) A fourth IκB protein within the NF-κB signaling module. *Cell* **128**(2)**:** 369-381

Bash J, Zong WX, Gelinas C (1997) c-Rel arrests the proliferation of HeLa cells and affects critical regulators of the G1/S-phase transition. *Mol Cell Biol* **17**(11)**:** 6526-6536

Bettencourt-Dias M, Glover DM (2007) Centrosome biogenesis and function: centrosomics brings new understanding. *Nat Rev Mol Cell Biol* **8**(6)**:** 451-463

Bettencourt-Dias M, Rodrigues-Martins A, Carpenter L, Riparbelli M, Lehmann L, Gatt MK, Carmo N, Balloux F, Callaini G, Glover DM (2005) SAK/PLK4 is required for centriole duplication and flagella development. *Curr Biol* **15**(24)**:** 2199-2207

Betts JC, Nabel GJ (1996) Differential regulation of NF-κB2 (p100) processing and control by amino-terminal sequences. *Mol Cell Biol* **16**(11)**:** 6363-6371

Bharadwaj R, Yu H (2004) The spindle checkpoint, aneuploidy, and cancer. *Oncogene* **23**(11)**:** 2016-2027

Bharti AC, Takada Y, Shishodia S, Aggarwal BB (2004) Evidence That Receptor Activator of Nuclear Factor NF-κB Ligand Can Suppress Cell Proliferation and Induce Apoptosis through Activation of a NF-κB-independent and TRAF6-dependent Mechanism. *J Biol Chem* **279**(7)**:** 6065-6076

Bista P, Zeng W, Ryan S, Bailly V, Browning JL, Lukashev ME (2010) TRAF3 controls activation of the canonical and alternative NF-κB by the lymphotoxin β receptor. *J Biol Chem* **285**(17)**:** 12971-12978

Blazkova H, von Schubert C, Mikule K, Schwab R, Angliker N, Schmuckli-Maurer J, Fernandez PC, Doxsey S, Dobbelaere DA (2007) The IKK inhibitor BMS-345541 affects multiple mitotic cell cycle transitions. *Cell Cycle* **6**(20)**:** 2531-2540

Bours V, Franzoso G, Azarenko V, Park S, Kanno T, Brown K, Siebenlist U (1993) The oncoprotein Bcl-3 directly transactivates through κ B motifs via association with DNA-binding p50B homodimers. *Cell* **72**(5)**:** 729-739

Bren GD, Solan NJ, Miyoshi H, Pennington KN, Pobst LJ, Paya CV (2001) Transcription of the RelB gene is regulated by NF-κ B. *Oncogene* **20**(53)**:** 7722-7733

Buss H, Dorrie A, Schmitz ML, Hoffmann E, Resch K, Kracht M (2004) Constitutive and interleukin-1-inducible phosphorylation of p65 NF-κB at serine 536 is mediated by multiple protein kinases including IκB kinase IKK-α, IKKβ, IKKε, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. *J Biol Chem* **279**(53)**:** 55633-55643

Caamano JH, Rizzo CA, Durham SK, Barton DS, Raventos-Suarez C, Snapper CM, Bravo R (1998) Nuclear factor NF-κB2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J Exp Med* **187**(2)**:** 185-196

Campbell KJ, Perkins ND (2004b) Reprogramming RelA. *Cell Cycle* **3**(7)**:** 869-872

Campbell KJ, Rocha S, Perkins ND (2004) Active repression of antiapoptotic gene expression by RelA(p65) NF-κB. *Mol Cell* **13**(6)**:** 853-865

Cao Y, Bonizzi G, Seagroves TN, Greten FR, Johnson R, Schmidt EV, Karin M (2001) IKKα Provides an Essential Link between RANK Signaling and Cyclin D1 Expression during Mammary Gland Development. *Cell* **107**(6)**:** 763-775

Carmody RJ, Ruan Q, Palmer S, Hilliard B, Chen YH (2007) Negative regulation of toll-like receptor signaling by NF-κB p50 ubiquitination blockade. *Science* **317**(5838)**:** 675-678

Catz SD, Johnson JL (2001) Transcriptional regulation of bcl-2 by nuclear factor κ B and its significance in prostate cancer. *Oncogene* **20**(50)**:** 7342-7351

Chen D, Li Z, Yang Q, Zhang J, Zhai Z, Shu HB (2003) Identification of a nuclear protein that promotes NF-κB activation. *Biochem Biophys Res Commun* **310**(3)**:** 720-724

Chen F, Demers LM, Vallyathan V, Lu Y, Castranova V, Shi X (1999) Involvement of 5'-flanking κB-like sites within bcl-x gene in silica-induced Bcl-x expression. *J Biol Chem* **274**(50)**:** 35591-35595

Chen Z, Indjeian VB, McManus M, Wang L, Dynlacht BD (2002) CP110, a cell cycle-dependent CDK substrate, regulates centrosome duplication in human cells. *Dev Cell* **3**(3)**:** 339-350

Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS, Jr. (2000) Selective activation of NF-κB subunits in human breast cancer: potential roles for NF-κ B2/p52 and for Bcl-3. *Oncogene* **19**(9)**:** 1123-1131

Coope HJ, Atkinson PGP, Huhse B, Belich M, Janzen J, Holman MJ, Klaus GGB, Johnston LH, Ley SC (2002) CD40 regulates the processing of NF-κ B2 p100 to p52. *Embo Journal* **21**(20)**:** 5375-5385

Cooper S, Iyer G, Tarquini M, Bissett P (2006) Nocodazole does not synchronize cells: implications for cell-cycle control and whole-culture synchronization. *Cell Tissue Res* **324**(2)**:** 237-242

Correa RG, Tergaonkar V, Ng JK, Dubova I, Izpisua-Belmonte JC, Verma IM (2004) Characterization of NF-κB/IκB proteins in zebra fish and their involvement in notochord development. *Mol Cell Biol* **24**(12)**:** 5257-5268

Cude K, Wang Y, Choi H-J, Hsuan S-L, Zhang H, Wang C-Y, Xia Z (2007) Regulation of the G2-M cell cycle progression by the ERK5-NF-κB signaling pathway. *J Cell Biol* **177**(2)**:** 253-264

Cuschieri L, Nguyen T, Vogel J (2007) Control at the cell center - The role of spindle poles in cytoskeletal organization and cell cycle regulation. *Cell Cycle* **6**(22)**:** 2788-2794

Dai Y, Chen S, Wang L, Pei XY, Funk VL, Kramer LB, Dent P, Grant S (2011) Disruption of IκB kinase (IKK)-mediated RelA serine 536 phosphorylation sensitizes human multiple myeloma cells to histone deacetylase (HDAC) inhibitors. *J Biol Chem* **286**(39)**:** 34036-34050

Dammermann A, Muller-Reichert T, Pelletier L, Habermann B, Desai A, Oegema K (2004) Centriole assembly requires both centriolar and pericentriolar material proteins. *Dev Cell* **7**(6)**:** 815-829

Das KC, White CW (1997) Activation of NF-κB by Antineoplastic Agents. ROLE OF PROTEIN KINASE C. *J Biol Chem* **272**(23)**:** 14914-14920

de Carcer G, Escobar B, Higuero AM, Garcia L, Anson A, Perez G, Mollejo M, Manning G, Melendez B, Abad-Rodriguez J, Malumbres M (2011b) Plk5, a polo

box domain-only protein with specific roles in neuron differentiation and glioblastoma suppression. *Mol Cell Biol* **31**(6)**:** 1225-1239

de Carcer G, Manning G, Malumbres M (2011) From Plk1 to Plk5: functional evolution of polo-like kinases. *Cell Cycle* **10**(14)**:** 2255-2262

Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, Li Z-W, Karin M, Ware CF, Green DR (2002) The Lymphotoxin-β Receptor Induces Different Patterns of Gene Expression via Two NF-κB Pathways. *Immunity* **17**(4)**:** 525-535

Demicco EG, Kavanagh KT, Romieu-Mourez R, Wang X, Shin SR, Landesman-Bollag E, Seldin DC, Sonenshein GE (2005) RelB/p52 NF-κB Complexes Rescue an Early Delay in Mammary Gland Development in Transgenic Mice with Targeted Superrepressor IκBα Expression and Promote Carcinogenesis of the Mammary Gland. *Mol Cell Biol* **25**(22)**:** 10136-10147

Denicourt C, Dowdy SF (2004) Cip/Kip proteins: more than just CDKs inhibitors. *Genes & Development* **18**(8)**:** 851-855

Dephoure N, Zhou C, Villén J, Beausoleil SA, Bakalarski CE, Elledge SJ, Gygi SP (2008) A quantitative atlas of mitotic phosphorylation. *Proceedings of the National Academy of Sciences* **105**(31)**:** 10762-10767

Deshpande A, Sicinski P, Hinds PW (2005) Cyclins and cdks in development and cancer: a perspective. *Oncogene* **24**(17)**:** 2909-2915

Doxsey S (2001) Re-evaluating centrosome function. *Nat Rev Mol Cell Biol* **2**(9)**:** 688-698

Duensing A, Liu Y, Tseng M, Malumbres M, Barbacid M, Duensing S (2006) Cyclin-dependent kinase 2 is dispensable for normal centrosome duplication but required for oncogene-induced centrosome overduplication. *Oncogene* **25**(20)**:** 2943-2949

Durocher Y, Perret S, Kamen A (2002) High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* **30**(2)**:** E9

Elowe S, Hummer S, Uldschmid A, Li X, Nigg EA (2007) Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions. *Genes Dev* **21**(17)**:** 2205-2219

Eto A, Muta T, Yamazaki S, Takeshige K (2003) Essential roles for NF-κB and a Toll/IL-1 receptor domain-specific signal(s) in the induction of IκB-ζ. *Biochem Biophys Res Commun* **301**(2)**:** 495-501

Fagerlund R, Kinnunen L, Kohler M, Julkunen I, Melen K (2005) NF-κB is transported into the nucleus by importin α3 and importin α4. *J Biol Chem* **280**(16)**:** 15942-15951

Fiorini E, Schmitz I, Marissen WE, Osborn SL, Touma M, Sasada T, Reche PA, Tibaldi EV, Hussey RE, Kruisbeek AM, Reinherz EL, Clayton LK (2002) Peptide-induced negative selection of thymocytes activates transcription of an NF-κB inhibitor. *Mol Cell* **9**(3)**:** 637-648

Fode C, Binkert C, Dennis JW (1996) Constitutive expression of murine Sak-a suppresses cell growth and induces multinucleation. *Mol Cell Biol* **16**(9)**:** 4665-4672

Fong A, Zhang M, Neely J, Sun SC (2002b) S9, a 19 S proteasome subunit interacting with ubiquitinated NF-κB2/p100. *J Biol Chem* **277**(43)**:** 40697-40702

Frankland-Searby S, Bhaumik SR (2012) The 26S proteasome complex: an attractive target for cancer therapy. *Biochim Biophys Acta* **1825**(1)**:** 64-76

Fu M, Wang C, Li Z, Sakamaki T, Pestell RG (2004) Minireview: Cyclin D1: Normal and Abnormal Functions. *Endocrinology* **145**(12)**:** 5439-5447

Fujioka S, Schmidt C, Sclabas GM, Li Z, Pelicano H, Peng B, Yao A, Niu J, Zhang W, Evans DB, Abbruzzese JL, Huang P, Chiao PJ (2004) Stabilization of p53 is a novel mechanism for proapoptotic function of NF-κB. *J Biol Chem* **279**(26)**:** 27549-27559

Fukasawa K (2005) Centrosome amplification, chromosome instability and cancer development. *Cancer Lett* **230**(1)**:** 6-19

Funderburg NT, Jadlowsky JK, Lederman MM, Feng Z, Weinberg A, Sieg SF (2011) The Toll-like receptor 1/2 agonists Pam(3) CSK(4) and human β-defensin-3 differentially induce interleukin-10 and nuclear factor-κB signalling patterns in human monocytes. *Immunology* **134**(2)**:** 151-160

Fusco AJ, Savinova OV, Talwar R, Kearns JD, Hoffmann A, Ghosh G (2008) Stabilization of RelB Requires Multidomain Interactions with p100/p52. *J Biol Chem* **283**(18)**:** 12324-12332

Gerondakis S, Grossmann M, Nakamura Y, Pohl T, Grumont R (1999) Genetic approaches in mice to understand Rel/NF-κB and IκB function: transgenics and knockouts. *Oncogene* **18**(49)**:** 6888-6895

Gerondakis S, Grumont R, Gugasyan R, Wong L, Isomura I, Ho W, Banerjee A (2006) Unravelling the complexities of the NF-κB signalling pathway using mouse knockout and transgenic models. *Oncogene* **25**(51)**:** 6781-6799

Ghosh S, Hayden MS (2008) New regulators of NF-κB in inflammation. *Nat Rev Immunol* **8**(11)**:** 837-848

Gilmore TD (1999) Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. *Oncogene* **18**(49)**:** 6925-6937

Gisselsson D (2005) Mitotic instability in cancer - Is there method in the madness? *Cell Cycle* **4**(8)**:** 1007-1010

Glotzer M (2005) The Molecular Requirements for Cytokinesis. *Science* **307**(5716)**:** 1735-1739

Goto H, Kiyono T, Tomono Y, Kawajiri A, Urano T, Furukawa K, Nigg EA, Inagaki M (2006) Complex formation of Plk1 and INCENP required for metaphase-anaphase transition. *Nat Cell Biol* **8**(2)**:** 180-187

Goudeau B, Huetz F, Samson S, Di Santo JP, Cumano A, Beg A, Israel A, Memet S (2003) IκBα/IκBε deficiency reveals that a critical NF-κB dosage is required for lymphocyte survival. *Proc Natl Acad Sci U S A* **100**(26)**:** 15800-15805

Govind S (1999) Control of development and immunity by rel transcription factors in Drosophila. *Oncogene* **18**(49)**:** 6875-6887

Grosstessner-Hain K, Hegemann B, Novatchkova M, Rameseder J, Joughin BA, Hudecz O, Roitinger E, Pichler P, Kraut N, Yaffe MB, Peters JM, Mechtler K (2011) Quantitative phospho-proteomics to investigate the polo-like kinase 1-dependent phospho-proteome. *Mol Cell Proteomics* **10**(11)**:** M111 008540

Grumont RJ, Rourke IJ, O'Reilly LA, Strasser A, Miyake K, Sha W, Gerondakis S (1998) B lymphocytes differentially use the Rel and nuclear factor κB1 (NF-κB1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. *J Exp Med* **187**(5)**:** 663-674

Gustafson LM, Gleich LL, Fukasawa K, Chadwell J, Miller MA, Stambrook PJ, Gluckman JL (2000) Centrosome hyperamplification in head and neck

squamous cell carcinoma: a potential phenotypic marker of tumor aggressiveness. *Laryngoscope* **110**(11)**:** 1798-1801

Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS, Jr. (1999) NF-κB Controls Cell Growth and Differentiation through Transcriptional Regulation of Cyclin D1. *Mol Cell Biol* **19**(8)**:** 5785-5799

Habedanck R, Stierhof YD, Wilkinson CJ, Nigg EA (2005) The Polo kinase Plk4 functions in centriole duplication. *Nat Cell Biol* **7**(11)**:** 1140-1146

Hanahan D, Weinberg RA (2000) The Hallmarks of Cancer. *Cell* **100**(1)**:** 57-70

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* **144**(5)**:** 646-674

Hayden MS, Ghosh S (2012) NF-κB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev* **26**(3)**:** 203-234

Herrup K, Yang Y (2007) Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? *Nat Rev Neurosci* **8**(5)**:** 368-378

Heusch M, Lin L, Geleziunas R, Greene WC (1999) The generation of nfkb2 p52: mechanism and efficiency. *Oncogene* **18**(46)**:** 6201-6208

Higashimoto T, Chan N, Lee YK, Zandi E (2008) Regulation of IκB kinase complex by phosphorylation of γ-binding domain of IκB kinase β by Polo-like kinase 1. *J Biol Chem* **283**(51)**:** 35354-35367

Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M (1999) NF-κB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. *Mol Cell Biol* **19**(4)**:** 2690-2698

Hoffmann A, Baltimore D (2006) Circuitry of nuclear factor κB signaling. *Immunol Rev* **210:** 171-186

Hoffmann A, Leung TH, Baltimore D (2003) Genetic analysis of NF-κ B/Rel transcription factors defines functional specificities. *Embo Journal* **22**(20)**:** 5530-5539

Hoffmann A, Levchenko A, Scott ML, Baltimore D (2002) The IκB-NF-κB signaling module: temporal control and selective gene activation. *Science* **298**(5596)**:** 1241-1245

Holland AJ, Lan W, Niessen S, Hoover H, Cleveland DW (2010) Polo-like kinase 4 kinase activity limits centrosome overduplication by autoregulating its own stability. *J Cell Biol* **188**(2)**:** 191-198

Hu MCT, Hung MC (2005) Role of IκB kinase in tumorigenesis. *Future Oncology* **1**(1)**:** 67-78

Hu Y, Baud V, Oga T, Kim KI, Yoshida K, Karin M (2001) IKKα controls formation of the epidermis independently of NF-κB. *Nature* **410**(6829)**:** 710-714

Hudson JW, Kozarova A, Cheung P, Macmillan JC, Swallow CJ, Cross JC, Dennis JW (2001) Late mitotic failure in mice lacking Sak, a polo-like kinase. *Curr Biol* **11**(6)**:** 441-446

Irelan JT, Murphy TJ, DeJesus PD, Teo H, Xu D, Gomez-Ferreria MA, Zhou Y, Miraglia LJ, Rines DR, Verma IM, Sharp DJ, Tergaonkar V, Chanda SK (2007) A role for IκB kinase 2 in bipolar spindle assembly. *Proc Natl Acad Sci U S A* **104**(43)**:** 16940-16945

Ishikawa H, Carrasco D, Claudio E, Ryseck RP, Bravo R (1997) Gastric hyperplasia and increased proliferative responses of lymphocytes in mice lacking the COOH-terminal ankyrin domain of NF-κB2. *J Exp Med* **186**(7)**:** 999-1014

Iwanaga R, Ozono E, Fujisawa J, Ikeda MA, Okamura N, Huang Y, Ohtani K (2008) Activation of the cyclin D2 and cdk6 genes through NF-κB is critical for cell-cycle progression induced by HTLV-I Tax. *Oncogene* **27**(42)**:** 5635-5642

Jackson JR, Patrick DR, Dar MM, Huang PS (2007) Targeted anti-mitotic therapies: can we improve on tubulin agents? *Nat Rev Cancer* **7**(2)**:** 107-117

Johnson RF, Witzel, II, Perkins ND (2011) p53-dependent regulation of mitochondrial energy production by the RelA subunit of NF-κB. *Cancer Res* **71**(16)**:** 5588-5597

Jordan MA, Thrower D, Wilson L (1992) Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *J Cell Sci* **102 (Pt 3):** 401-416

Jost PJ, Ruland Jr (2007) Aberrant NF-κB signaling in lymphoma: mechanisms, consequences, and therapeutic implications. *Blood* **109**(7)**:** 2700-2707

Joyce D, Bouzahzah B, Fu M, Albanese C, D'Amico M, Steer J, Klein JU, Lee RJ, Segall JE, Westwick JK, Der CJ, Pestell RG (1999) Integration of Rac-

dependent Regulation of Cyclin D1 Transcription through a Nuclear Factor-κB-dependent Pathway. *J Biol Chem* **274**(36)**:** 25245-25249

Joyce D, Chris A, Jay S, Maofu F, Boumediene B, Richard GP (2001) NF-κB and cell-cycle regulation: the cyclin connection. *Cytokine & growth factor reviews* **12**(1)**:** 73-90

Karin M (2006) Nuclear factor-κB in cancer development and progression. *Nature* **441**(7092)**:** 431-436

Karin M, Cao Y, Greten FR, Li Z-W (2002) NF-κB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2**(4)**:** 301-310

Kashatus D, Cogswell P, Baldwin AS (2006) Expression of the Bcl-3 proto-oncogene suppresses p53 activation. *Genes Dev* **20**(2)**:** 225-235

Kato T, Jr., Delhase M, Hoffmann A, Karin M (2003) CK2 Is a C-Terminal IκB Kinase Responsible for NF-κB Activation during the UV Response. *Mol Cell* **12**(4)**:** 829-839

Kawai T, Akira S (2006) TLR signaling. *Cell Death Differ* **13**(5)**:** 816-825

Kim JY, Morgan M, Kim DG, Lee JY, Bai L, Lin Y, Liu ZG, Kim YS (2011) TNFα induced noncanonical NF-κB activation is attenuated by RIP1 through stabilization of TRAF2. *J Cell Sci* **124**(Pt 4)**:** 647-656

Kitamura H, Kanehira K, Okita K, Morimatsu M, Saito M (2000) MAIL, a novel nuclear IκB protein that potentiates LPS-induced IL-6 production. *FEBS Lett* **485**(1)**:** 53-56

Klement JF, Rice NR, Car BD, Abbondanzo SJ, Powers GD, Bhatt PH, Chen CH, Rosen CA, Stewart CL (1996) IκBα deficiency results in a sustained NF-κB response and severe widespread dermatitis in mice. *Mol Cell Biol* **16:** 2341 - 2349

Kleylein-Sohn J, Westendorf J, Le Clech M, Habedanck R, Stierhof YD, Nigg EA (2007) Plk4-induced centriole biogenesis in human cells. *Dev Cell* **13**(2)**:** 190-202

Ko MA, Rosario CO, Hudson JW, Kulkarni S, Pollett A, Dennis JW, Swallow CJ (2005) Plk4 haploinsufficiency causes mitotic infidelity and carcinogenesis. *Nat Genet* **37**(8)**:** 883-888

Korzeniewski N, Zheng L, Cuevas R, Parry J, Chatterjee P, Anderton B, Duensing A, Munger K, Duensing S (2009) Cullin 1 functions as a centrosomal suppressor of centriole multiplication by regulating polo-like kinase 4 protein levels. *Cancer Res* **69**(16)**:** 6668-6675

Kotani S, Tugendreich S, Fujii M, Jorgensen PM, Watanabe N, Hoog C, Hieter P, Todokoro K (1998) PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. *Mol Cell* **1**(3)**:** 371-380

Koyanagi M, Hijikata M, Watashi K, Masui O, Shimotohno K (2005) Centrosomal P4.1-associated Protein Is a New Member of Transcriptional Coactivators for Nuclear Factor-κB. *J Biol Chem* **280**(13)**:** 12430-12437

Krikos A, Laherty CD, Dixit VM (1992) Transcriptional activation of the tumor necrosis factor α-inducible zinc finger protein, A20, is mediated by κ B elements. *J Biol Chem* **267**(25)**:** 17971-17976

Kues WA, Anger M, Carnwath JW, Paul D, Motlik J, Niemann H (2000) Cell cycle synchronization of porcine fetal fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. *Biol Reprod* **62**(2)**:** 412-419

Kumar A, Takada Y, Boriek AM, Aggarwal BB (2004) Nuclear factor-κB: its role in health and disease. *Journal of Molecular Medicine* **82**(7)**:** 434-448

Kuo KK, Sato N, Mizumoto K, Maehara N, Yonemasu H, Ker CG, Sheen PC, Tanaka M (2000) Centrosome abnormalities in human carcinomas of the gallbladder and intrahepatic and extrahepatic bile ducts. *Hepatology* **31**(1)**:** 59-64

Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS (2008) Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat Protoc* **3**(6)**:** 965-976

Leidel S, Delattre M, Cerutti L, Baumer K, Gonczy P (2005) SAS-6 defines a protein family required for centrosome duplication in C. elegans and in human cells. *Nat Cell Biol* **7**(2)**:** 115-125

Leidner J, Palkowitsch L, Marienfeld U, Fischer D, Marienfeld R (2008) Identification of lysine residues critical for the transcriptional activity and polyubiquitination of the NF-κB family member RelB. *Biochem J* **416**(1)**:** 117-127

Lewander A, Gao J, Carstensen J, Arbman G, Zhang H, Sun XF (2012) NF-κB p65 phosphorylated at serine-536 is an independent prognostic factor in Swedish colorectal cancer patients. *Int J Colorectal Dis* **27**(4)**:** 447-452

Li C, Lin M, Liu J (2004) Identification of PRC1 as the p53 target gene uncovers a novel function of p53 in the regulation of cytokinesis. *Oncogene* **23**(58)**:** 9336-9347

Li J, Tan M, Li L, Pamarthy D, Lawrence TS, Sun Y (2005) SAK, a new polo-like kinase, is transcriptionally repressed by p53 and induces apoptosis upon RNAi silencing. *Neoplasia* **7**(4)**:** 312-323

Li Z, Niu J, Uwagawa T, Peng B, Chiao PJ (2005b) Function of polo-like kinase 3 in NF-κB-mediated proapoptotic response. *J Biol Chem* **280**(17)**:** 16843-16850

Liao G, Sun S-C (2003) Regulation of NF-κB2//p100 processing by its nuclear shuttling. *Oncogene* **22**(31)**:** 4868-4874

Libermann TA, Baltimore D (1990) Activation of interleukin-6 gene expression through the NF-κB transcription factor. *Mol Cell Biol* **10**(5)**:** 2327-2334

Lim R, Winteringham LN, Williams JH, McCulloch RK, Ingley E, Tiao JY, Lalonde JP, Tsai S, Tilbrook PA, Sun Y, Wu X, Morris SW, Klinken SP (2002) MADM, a novel adaptor protein that mediates phosphorylation of the 14-3-3 binding site of myeloid leukemia factor 1. *J Biol Chem* **277**(43)**:** 40997-41008

Lin DC, Zhang Y, Pan QJ, Yang H, Shi ZZ, Xie ZH, Wang BS, Hao JJ, Zhang TT, Xu X, Zhan QM, Wang MR (2011) PLK1 Is transcriptionally activated by NF-κB during cell detachment and enhances anoikis resistance through inhibiting β-catenin degradation in esophageal squamous cell carcinoma. *Clin Cancer Res* **17**(13)**:** 4285-4295

Lin L, Ghosh S (1996) A glycine-rich region in NF-κB p105 functions as a processing signal for the generation of the p50 subunit. *Mol Cell Biol* **16**(5)**:** 2248-2254

Liu JL, Ma HP, Lu XL, Sun SH, Guo X, Li FC (2011) NF-κB induces abnormal centrosome amplification by upregulation of CDK2 in laryngeal squamous cell cancer. *Int J Oncol* **39**(4)**:** 915-924

Liu L, Zhang CZ, Cai M, Fu J, Chen GG, Yun J (2012) Downregulation of polo-like kinase 4 in hepatocellular carcinoma associates with poor prognosis. *PLoS One* **7**(7)**:** e41293

Lombardi L, Ciana P, Cappellini C, Trecca D, Guerrini L, Migliazza A, Maiolo AT, Neri A (1995) Structural and functional characterization of the promoter regions of the NFKB2 gene. *Nucleic Acids Res* **23**(12)**:** 2328-2336

Luo J, Liu X (2012) Polo-like kinase 1, on the rise from cell cycle regulation to prostate cancer development. *Protein Cell* **3**(3)**:** 182-197

Ma HT, Poon RY (2011) Synchronization of HeLa cells. *Methods Mol Biol* **761:** 151-161

Macmillan JC, Hudson JW, Bull S, Dennis JW, Swallow CJ (2001) Comparative expression of the mitotic regulators SAK and PLK in colorectal cancer. *Ann Surg Oncol* **8**(9)**:** 729-740

Madge LA, May MJ (2011) The NF-κB paradox: RelB induces and inhibits gene expression. *Cell Cycle* **10**(1)**:** 6-7

Maier HJ, Marienfeld R, Wirth T, Baumann B (2003) Critical Role of RelB Serine 368 for Dimerization and p100 Stabilization. *Journal of Biological Chemistry* **278**(40)**:** 39242-39250

Malmanche N, Maia A, Sunkel CE (2006) The spindle assembly checkpoint: Preventing chromosome mis-segregation during mitosis and meiosis. *FEBS Letters* **580**(12)**:** 2888-2895

Malumbres M, Barbacid M (2001) Milestones in cell division : To cycle or not to cycle: a critical decision in cancer. *Nat Rev Cancer* **1**(3)**:** 222-231

Marienfeld R, Berberich-Siebelt F, Berberich I, Denk A, Serfling E, Neumann M (2001) Signal-specific and phosphorylation-dependent RelB degradation: a potential mechanism of NF-κB control. *Oncogene* **20**(56)**:** 8142-8147

Marumoto T, Honda S, Hara T, Nitta M, Hirota T, Kohmura E, Saya H (2003) Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. *J Biol Chem* **278**(51)**:** 51786-51795

Massoumi R, Chmielarska K, Hennecke K, Pfeifer A, Fassler R (2006) Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-κB signaling. *Cell* **125**(4)**:** 665-677

Matta H, Chaudhary PM (2004) Activation of alternative NF-κB pathway by human herpes virus 8-encoded Fas-associated death domain-like IL-1 β-

converting enzyme inhibitory protein (vFLIP). *Proc Natl Acad Sci U S A* **101**(25)**:** 9399-9404

May MJ, Ghosh S (1997) Rel/NF-κB and IκB proteins: an overview. *Seminars in Cancer Biology* **8**(2)**:** 63-73

Meraldi P, Lukas J, Fry AM, Bartek J, Nigg EA (1999) Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. *Nat Cell Biol* **1**(2)**:** 88-93

Minakhina S, Steward R (2006) Nuclear factor-κB pathways in Drosophila. *Oncogene* **25**(51)**:** 6749-6757

Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, Wen SF, Wang L, Kirschmeier P, Bishop WR, Nielsen LL, Pickett CB, Liu S (2002) Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. *Oncogene* **21**(17)**:** 2613-2622

Mistry P, Deacon K, Mistry S, Blank J, Patel R (2004) NF-κB Promotes Survival during Mitotic Cell Cycle Arrest. *J Biol Chem* **279**(2)**:** 1482-1490

Moorthy AK, Savinova OV, Ho JQ, Wang VY, Vu D, Ghosh G (2006) The 20S proteasome processes NF-κB1 p105 into p50 in a translation-independent manner. *EMBO J* **25**(9)**:** 1945-1956

Mordmuller B, Krappmann D, Esen M, Wegener E, Scheidereit C (2003) Lymphotoxin and lipopolysaccharide induce NF-κB-p52 generation by a co-translational mechanism. *EMBO Rep* **4**(1)**:** 82-87

Moreno R, Sobotzik JM, Schultz C, Schmitz ML (2010) Specification of the NF-κB transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IKK ε. *Nucleic Acids Res* **38**(18)**:** 6029-6044

Motoyama M, Yamazaki S, Eto-Kimura A, Takeshige K, Muta T (2005) Positive and negative regulation of nuclear factor-κB-mediated transcription by IκBζ, an inducible nuclear protein. *J Biol Chem* **280**(9)**:** 7444-7451

Nadiminty N, Lou W, Lee SO, Lin X, Trump DL, Gao AC (2006) Stat3 activation of NF-κB p100 processing involves CBP/p300-mediated acetylation. *Proc Natl Acad Sci U S A* **103**(19)**:** 7264-7269

Natoli G, Saccani S, Bosisio D, Marazzi I (2005) Interactions of NF-κB with chromatin: the art of being at the right place at the right time. *Nat Immunol* **6**(5)**:** 439-445

Neri A, Chang CC, Lombardi L, Salina M, Corradini P, Maiolo AT, Chaganti RS, Dalla-Favera R (1991) B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. *Cell* **67**(6)**:** 1075-1087

Nigg EA (1998) Polo-like kinases: positive regulators of cell division from start to finish. *Curr Opin Cell Biol* **10**(6)**:** 776-783

Nigg EA (2002) Centrosome aberrations: cause or consequence of cancer progression? *Nat Rev Cancer* **2**(11)**:** 815-825

Niida H, Nakanishi M (2006) DNA damage checkpoints in mammals. *Mutagenesis* **21**(1)**:** 3-9

Nishikori M, Ohno H, Haga H, Uchiyama T (2005) Stimulation of CD30 in anaplastic large cell lymphoma leads to production of nuclear factor-κB p52, which is associated with hyperphosphorylated Bcl-3. *Cancer Sci* **96**(8)**:** 487-497

Novack DV, Yin L, Hagen-Stapleton A, Schreiber RD, Goeddel DV, Ross FP, Teitelbaum SL (2003) The IκB function of NF-κB2 p100 controls stimulated osteoclastogenesis. *J Exp Med* **198**(5)**:** 771-781

O'Connell CB, Khodjakov AL (2007) Cooperative mechanisms of mitotic spindle formation. *J Cell Sci* **120**(10)**:** 1717-1722

Ohazama A, Hu Y, Schmidt-Ullrich R, Cao Y, Scheidereit C, Karin M, Sharpe PT (2004) A dual role for IKKα in tooth development. *Dev Cell* **6**(2)**:** 219-227

Ohta T, Essner R, Ryu JH, Palazzo RE, Uetake Y, Kuriyama R (2002) Characterization of Cep135, a novel coiled-coil centrosomal protein involved in microtubule organization in mammalian cells. *J Cell Biol* **156**(1)**:** 87-99

Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, Jensen LJ, Gnad F, Cox J, Jensen TS, Nigg EA, Brunak S, Mann M (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci Signal* **3**(104)**:** ra3

Pacifico F, Leonardi A (2006) NF-κB in solid tumors. *Biochem Pharmacol* **72**(9)**:** 1142-1152

Park K-J, Krishnan V, O'Malley BW, Yamamoto Y, Gaynor RB (2005) Formation of an IKKα-Dependent Transcription Complex Is Required for Estrogen Receptor-Mediated Gene Activation. *Molecular Cell* **18**(1)**:** 71-82

Pearson CG, Winey M (2010) Plk4/SAK/ZYG-1 in the regulation of centriole duplication. *F1000 Biol Rep* **2:** 58

Pedrali-Noy G, Spadari S, Miller-Faures A, Miller AO, Kruppa J, Koch G (1980) Synchronization of HeLa cell cultures by inhibition of DNA polymerase α with aphidicolin. *Nucleic Acids Res* **8**(2)**:** 377-387

Pellegrino R, Calvisi DF, Ladu S, Ehemann V, Staniscia T, Evert M, Dombrowski F, Schirmacher P, Longerich T (2010) Oncogenic and tumor suppressive roles of polo-like kinases in human hepatocellular carcinoma. *Hepatology* **51**(3)**:** 857-868

Perkins ND (1997) Achieving transcriptional specificity with NF-κB. *The International Journal of Biochemistry & Cell Biology* **29**(12)**:** 1433-1448

Perkins ND (2004b) NF-κB: Tumour promoter or tumour suppressor? *British Journal of Cancer* **91:** S7-S7

Perkins ND (2006) Post-translational modifications regulating the activity and function of the nuclear factor κ B pathway. *Oncogene* **25**(51)**:** 6717-6730

Perkins ND (2007) Integrating cell-signalling pathways with NF-κB and IKK function. *Nat Rev Mol Cell Biol* **8**(1)**:** 49-62

Perkins ND (2012) The diverse and complex roles of NF-κB subunits in cancer. *Nat Rev Cancer* **12**(2)**:** 121-132

Perkins ND, Gilmore TD (2006b) Good cop, bad cop: the different faces of NF-κB. *Cell Death Differ* **13**(5)**:** 759-772

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**(9)**:** e45

Pham LV, Fu L, Tamayo AT, Bueso-Ramos C, Drakos E, Vega F, Medeiros LJ, Ford RJ (2011) Constitutive BR3 receptor signaling in diffuse, large B-cell lymphomas stabilizes nuclear factor-κB-inducing kinase while activating both canonical and alternative nuclear factor-κB pathways. *Blood* **117**(1)**:** 200-210

Pianetti S, Arsura M, Romieu-Mourez R, Coffey RJ, Sonenshein GE (2001) Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκBα that can be inhibited by the tumor suppressor PTEN. *Oncogene* **20**(11)**:** 1287-1299

Pihan GA, Purohit A, Wallace J, Malhotra R, Liotta L, Doxsey SJ (2001) Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res* **61**(5)**:** 2212-2219

Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y (2004) NF-κB functions as a tumour promoter in inflammation-associated cancer. *Nature* **431**(7007)**:** 461-466

Poehlmann A, Roessner A (2010) Importance of DNA damage checkpoints in the pathogenesis of human cancers. *Pathol Res Pract* **206**(9)**:** 591-601

Prajapati S, Tu Z, Yamamoto Y, Gaynor RB (2006) IKKα regulates the mitotic phase of the cell cycle by modulating Aurora A phosphorylation. *Cell Cycle* **5**(20)**:** 2371-2380

Prasad S, Ravindran J, Aggarwal BB (2010) NF-κB and cancer: how intimate is this relationship. *Mol Cell Biochem* **336**(1-2)**:** 25-37

Ramakrishnan P, Wang W, Wallach D (2004) Receptor-specific signaling for both the alternative and the canonical NF-κB activation pathways by NF-κB-inducing kinase. *Immunity* **21**(4)**:** 477-489

Rauert H, Wicovsky A, Muller N, Siegmund D, Spindler V, Waschke J, Kneitz C, Wajant H (2010) Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). *J Biol Chem* **285**(10)**:** 7394-7404

Rayet B, Gelinas C (1999) Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* **18**(49)**:** 6938-6947

Raynaud-Messina B, Mazzolini L, Moisand A, Cirinesi AM, Wright M (2004) Elongation of centriolar microtubule triplets contributes to the formation of the mitotic spindle in γ-tubulin-depleted cells. *J Cell Sci* **117**(Pt 23)**:** 5497-5507

Rice NR, MacKichan ML, Israel A (1992) The precursor of NF-κB p50 has IκB-like functions. *Cell* **71**(2)**:** 243-253

Rocha S, Campbell KJ, Perkins ND (2003) p53- and Mdm2-independent repression of NF-κB transactivation by the ARF tumor suppressor. *Mol Cell* **12:** 15 - 25

Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND (2005b) Regulation of NF-κB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. *EMBO J* **24**(6)**:** 1157-1169

Rocha S, Martin AM, Meek DW, Perkins ND (2003b) p53 Represses Cyclin D1 Transcription through Down Regulation of Bcl-3 and Inducing Increased Association of the p52 NF-κB Subunit with Histone Deacetylase 1. *Mol Cell Biol* **23**(13)**:** 4713-4727

Roche KC, Wiechens N, Owen-Hughes T, Perkins ND (2004) The FHA domain protein SNIP1 is a regulator of the cell cycle and cyclin D1 expression. *Oncogene* **23**(50)**:** 8185-8195

Rodrigues-Martins A, Riparbelli M, Callaini G, Glover DM, Bettencourt-Dias M (2007) Revisiting the role of the mother centriole in centriole biogenesis. *Science* **316**(5827)**:** 1046-1050

Romieu-Mourez R, Kim DW, Shin SM, Demicco EG, Landesman-Bollag E, Seldin DC, Cardiff RD, Sonenshein GE (2003) Mouse mammary tumor virus c-rel transgenic mice develop mammary tumors. *Mol Cell Biol* **23**(16)**:** 5738-5754

Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Sonenshein GE (2002) Protein kinase CK2 promotes aberrant activation of nuclear factor-κB, transformed phenotype, and survival of breast cancer cells. *Cancer Res* **62**(22)**:** 6770-6778

Rosario CO, Ko MA, Haffani YZ, Gladdy RA, Paderova J, Pollett A, Squire JA, Dennis JW, Swallow CJ (2010) Plk4 is required for cytokinesis and maintenance of chromosomal stability. *Proc Natl Acad Sci U S A* **107**(15)**:** 6888-6893

Rosette C, Karin M (1995) Cytoskeletal control of gene expression: depolymerization of microtubules activates NF-κB. *J Cell Biol* **128**(6)**:** 1111-1119

Rothwarf DM, Karin M (1999) The NF-κB activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci STKE* **1999**(5)**:** RE1

Ryu HJ, Kim JE, Yeo SI, Kim MJ, Jo SM, Kang TC (2011) ReLA/P65-serine 536 nuclear factor-κB phosphorylation is related to vulnerability to status epilepticus in the rat hippocampus. *Neuroscience* **187:** 93-102

Saccani S, Pantano S, Natoli G (2003) Modulation of NF-κB activity by exchange of dimers. *Mol Cell* **11**(6)**:** 1563-1574

Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S (2003) TWEAK induces NF-κB2 p100 processing and long lasting NF-κB activation. *J Biol Chem* **278**(38)**:** 36005-36012

Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W (1999) IκB kinases phosphorylate NF-κB p65 subunit on serine 536 in the transactivation domain. *J Biol Chem* **274**(43)**:** 30353-30356

Salisbury JL, Suino KM, Busby R, Springett M (2002) Centrin-2 is required for centriole duplication in mammalian cells. *Curr Biol* **12**(15)**:** 1287-1292

Samson SI, Memet S, Vosshenrich CA, Colucci F, Richard O, Ndiaye D, Israel A, Di Santo JP (2004) Combined deficiency in IκBα and IκBε reveals a critical window of NF-κB activity in natural killer cell differentiation. *Blood* **103**(12)**:** 4573-4580

Sasaki CY, Barberi TJ, Ghosh P, Longo DL (2005) Phosphorylation of RelA/p65 on serine 536 defines an IκBα-independent NF-κB pathway. *J Biol Chem* **280**(41)**:** 34538-34547

Savinova OV, Hoffmann A, Ghosh G (2009) The Nfkb1 and Nfkb2 proteins p105 and p100 function as the core of high-molecular-weight heterogeneous complexes. *Mol Cell* **34**(5)**:** 591-602

Scheibel M, Klein B, Merkle H, Schulz M, Fritsch R, Greten FR, Arkan MC, Schneider G, Schmid RM (2010) IκBβ is an essential co-activator for LPS-induced IL-1β transcription in vivo. *J Exp Med* **207**(12)**:** 2621-2630

Schneider G, Saur D, Siveke JT, Fritsch R, Greten FR, Schmid RM (2006) IKKα controls p52/RelB at the skp2 gene promoter to regulate G1- to S-phase progression. *Embo Journal* **25**(16)**:** 3801-3812

Schumm K, Rocha S, Caamano J, Perkins ND (2006) Regulation of p53 tumour suppressor target gene expression by the p52 NF-κB subunit. *EMBO J* **25**(20)**:** 4820-4832

Schwarzer R, Dorken B, Jundt F (2012) Notch is an essential upstream regulator of NF-κB and is relevant for survival of Hodgkin and Reed-Sternberg cells. *Leukemia* **26**(4)**:** 806-813

Seitz CS, Deng H, Hinata K, Lin Q, Khavari PA (2000) Nuclear Factor κB Subunits Induce Epithelial Cell Growth Arrest. *Cancer Res* **60**(15)**:** 4085-4092

Sekine S, Tagami S, Yokoyama S (2012) Structural basis of transcription by bacterial and eukaryotic RNA polymerases. *Curr Opin Struct Biol* **22**(1)**:** 110-118

Sen R, Baltimore D (1986) Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. *Cell* **47**(6)**:** 921-928

Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun S-C, Karin M (2001) Activation by IKKα of a Second, Evolutionary Conserved, NF-κB Signaling Pathway. *Science* **293**(5534)**:** 1495-1499

Seo SI, Song SY, Kang MR, Kim MS, Oh JE, Kim YR, Lee JY, Yoo NJ, Lee SH (2009) Immunohistochemical analysis of NF-κB signaling proteins IKKε, p50/p105, p52/p100 and RelA in prostate cancers. *APMIS* **117**(8)**:** 623-628

Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV (1990) κ B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. *J Exp Med* **171**(1)**:** 35-47

Sheehy AM, Schlissel MS (1999) Overexpression of RelA Causes G1 Arrest and Apoptosis in a Pro-B Cell Line. *J Biol Chem* **274**(13)**:** 8708-8716

Sheikh MS, Huang Y (2003) Death receptor activation complexes: it takes two to activate TNF receptor 1. *Cell Cycle* **2**(6)**:** 550-552

Shembade N, Harhaj EW (2012) Regulation of NF-κB signaling by the A20 deubiquitinase. *Cell Mol Immunol* **9**(2)**:** 123-130

Shih VF, Kearns JD, Basak S, Savinova OV, Ghosh G, Hoffmann A (2009) Kinetic control of negative feedback regulators of NF-κB/RelA determines their pathogen- and cytokine-receptor signaling specificity. *Proc Natl Acad Sci U S A* **106**(24)**:** 9619-9624

Shiloh Y (2001) ATM and ATR: networking cellular responses to DNA damage. *Curr Opin Genet Dev* **11**(1)**:** 71-77

Shinmura K, Bennett RA, Tarapore P, Fukasawa K (2007) Direct evidence for the role of centrosomally localized p53 in the regulation of centrosome duplication. *Oncogene* **26**(20)**:** 2939-2944

Sillibourne JE, Bornens M (2010b) Polo-like kinase 4: the odd one out of the family. *Cell Div* **5:** 25

Sillibourne JE, Tack F, Vloemans N, Boeckx A, Thambirajah S, Bonnet P, Ramaekers FC, Bornens M, Grand-Perret T (2010) Autophosphorylation of polo-like kinase 4 and its role in centriole duplication. *Mol Biol Cell* **21**(4)**:** 547-561

Solan NJ, Miyoshi H, Carmona EM, Bren GD, Paya CV (2002) RelB Cellular Regulation and Transcriptional Activity Are Regulated by p100. *J Biol Chem* **277**(2)**:** 1405-1418

Song B, Liu XS, Davis K, Liu X (2011) Plk1 phosphorylation of Orc2 promotes DNA replication under conditions of stress. *Mol Cell Biol* **31**(23)**:** 4844-4856

Song L, Dong W, Gao M, Li J, Hu M, Guo N, Huang C (2010) A novel role of IKKα in the mediation of UVB-induced G0/G1 cell cycle arrest response by suppressing Cyclin D1 expression. *Biochim Biophys Acta* **1803**(2)**:** 323-332

Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE (1997) Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. *J Clin Invest* **100**(12)**:** 2952-2960

Speirs K, Lieberman L, Caamano J, Hunter CA, Scott P (2004) Cutting edge: NF-κB2 is a negative regulator of dendritic cell function. *J Immunol* **172**(2)**:** 752-756

Strnad P, Leidel S, Vinogradova T, Euteneuer U, Khodjakov A, Gonczy P (2007) Regulated HsSAS-6 levels ensure formation of a single procentriole per centriole during the centrosome duplication cycle. *Dev Cell* **13**(2)**:** 203-213

Strozyk E, Poppelmann B, Schwarz T, Kulms D (2006) Differential effects of NF-κB on apoptosis induced by DNA-damaging agents: the type of DNA damage determines the final outcome. *Oncogene* **25**(47)**:** 6239-6251

Sun SC (2011) Non-canonical NF-κB signaling pathway. *Cell Res* **21**(1)**:** 71-85

Sun SC, Ganchi PA, Ballard DW, Greene WC (1993) NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. *Science* **259**(5103)**:** 1912-1915

Swallow CJ, Ko MA, Siddiqui NU, Hudson JW, Dennis JW (2005) Sak/Plk4 and mitotic fidelity. *Oncogene* **24**(2)**:** 306-312

Takeda K, Takeuchi O, Tsujimura T, Itami S, Adachi O, Kawai T, Sanjo H, Yoshikawa K, Terada N, Akira S (1999) Limb and skin abnormalities in mice lacking IKKα. *Science* **284**(5412)**:** 313-316

Tang G, Yang J, Minemoto Y, Lin A (2001) Blocking caspase-3-mediated proteolysis of IKKβ suppresses TNF-α-induced apoptosis. *Mol Cell* **8**(5)**:** 1005-1016

Ten RM, Paya CV, Israel N, Le Bail O, Mattei MG, Virelizier JL, Kourilsky P, Israel A (1992) The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. *EMBO J* **11**(1)**:** 195-203

Tergaonkar V, Bottero V, Ikawa M, Li Q, Verma IM (2003) IκB kinase-independent IκBα degradation pathway: functional NF-κB activity and implications for cancer therapy. *Mol Cell Biol* **23**(22)**:** 8070-8083

Tezil T, Bodur C, Kutuk O, Basaga H (2012) IKKβ mediates chemoresistance by sequestering FOXO3; a critical factor for cell survival and death. *Cell Signal* **24**(6)**:** 1361-1368

Toyoshima-Morimoto F, Taniguchi E, Nishida E (2002) Plk1 promotes nuclear translocation of human Cdc25C during prophase. *EMBO Rep* **3**(4)**:** 341-348

Toyoshima-Morimoto F, Taniguchi E, Shinya N, Iwamatsu A, Nishida E (2001) Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. *Nature* **410**(6825)**:** 215-220

Trecca D, Guerrini L, Fracchiolla NS, Pomati M, Baldini L, Maiolo AT, Neri A (1997) Identification of a tumor-associated mutant form of the NF-κB RelA gene with reduced DNA-binding and transactivating activities. *Oncogene* **14**(7)**:** 791-799

Tsou M-FB, Stearns T (2006) Controlling centrosome number: licenses and blocks. *Current Opinion in Cell Biology* **18**(1)**:** 74-78

Tsuchiya Y, Asano T, Nakayama K, Kato T, Jr., Karin M, Kamata H (2010) Nuclear IKKβ is an adaptor protein for IκBα ubiquitination and degradation in UV-induced NF-κB activation. *Mol Cell* **39**(4)**:** 570-582

van Uden P, Kenneth NS, Webster R, Muller HA, Mudie S, Rocha S (2011) Evolutionary conserved regulation of HIF-1β by NF-κB. *PLoS Genet* **7**(1)**:** e1001285

Vatsyayan J, Qing G, Xiao G, Hu J (2008) SUMO1 modification of NF-κB2/p100 is essential for stimuli-induced p100 phosphorylation and processing. *EMBO Rep* **9**(9)**:** 885-890

Weiss RH (2003) p21Waf1/Cip1 as a therapeutic target in breast and other cancers. *Cancer Cell* **4**(6)**:** 425-429

Whiteside ST, Epinat JC, Rice NR, Israel A (1997) IκBε, a novel member of the IκB family, controls RelA and cRel NF-κB activity. *EMBO J* **16**(6)**:** 1413-1426

Widera D, Mikenberg I, Elvers M, Kaltschmidt C, Kaltschmidt B (2006) Tumor necrosis factor α triggers proliferation of adult neural stem cells via IKK/NF-κB signaling. *BMC Neurosci* **7:** 64

Winkles JA, Alberts GF (2005) Differential regulation of polo-like kinase 1, 2, 3, and 4 gene expression in mammalian cells and tissues. *Oncogene* **24**(2)**:** 260-266

Witzel, II, Koh LF, Perkins ND (2010) Regulation of cyclin D1 gene expression. *Biochem Soc Trans* **38**(Pt 1)**:** 217-222

Wu K, Jiang S-W, Thangaraju M, Wu G, Couch FJ (2000) Induction of the BRCA2 Promoter by Nuclear Factor-κB. *J Biol Chem* **275**(45)**:** 35548-35556

Wu ZH, Miyamoto S (2008b) Induction of a pro-apoptotic ATM-NF-κB pathway and its repression by ATR in response to replication stress. *EMBO J* **27**(14)**:** 1963-1973

Wu ZQ, Liu X (2008) Role for Plk1 phosphorylation of Hbo1 in regulation of replication licensing. *Proc Natl Acad Sci U S A* **105**(6)**:** 1919-1924

Xiao G, Harhaj EW, Sun S-C (2001) NF-κB-Inducing Kinase Regulates the Processing of NF-κB2 p100. *Molecular Cell* **7**(2)**:** 401-409

Yang CH, Murti A, Pfeffer LM (2005) Interferon induces NF-κB-inducing kinase/tumor necrosis factor receptor-associated factor-dependent NF-κB activation to promote cell survival. *J Biol Chem* **280**(36)**:** 31530-31536

Zamora R, Espinosa M, Ceballos-Cancino G, Segura B, Maldonado V, Melendez-Zajgla J (2010) Depletion of the oncoprotein Bcl-3 induces centrosome amplification and aneuploidy in cancer cells. *Mol Cancer* **9:** 223

Zhang W, Wang J, Zhang Y, Yuan Y, Guan W, Jin C, Chen H, Wang X, Yang X, He F (2010) The scaffold protein TANK/I-TRAF inhibits NF-κB activation by recruiting polo-like kinase 1. *Mol Biol Cell* **21**(14)**:** 2500-2513

Zhong H, May MJ, Jimi E, Ghosh S (2002) The Phosphorylation Status of Nuclear NF-κB Determines Its Association with CBP/p300 or HDAC-1. *Molecular Cell* **9**(3)**:** 625-636