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NFκB regulates expression of Polo-like kinase 4

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Abbreviations: NFκB, nuclear factor κB; PLK4, Polo-like kinase 4; IκB, inhibitor of NFκB; TLR, toll-like receptor; IKK, IκB kinase; NEMO, NFκB essential modifier; CDK2, cyclin-dependent kinase; Skp2, S-phase kinase-associated protein 2; SAS6, spindle assembly 6 homolog; siRNA, short interfering RNA; FACs, fluorescence-activated cell sorting; MEFs, mouse embryonic fibroblasts; PCR, polymerase chain reaction; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco modified Eagle medium; FBS, fetal bovine serum; PBS, phosphate buffered saline

Activation of the NFκB signaling pathway allows the cell to respond to infection and stress and can affect many cellular processes. As a consequence, NFκB activity must be integrated with a wide variety of parallel signaling pathways. One mechanism through which NFκB can exert widespread effects is through controlling the expression of key regulatory kinases. Here we report that NFκB regulates the expression of genes required for centrosome duplication, and that Polo-like kinase 4 (PLK4) is a direct NFκB target gene. RNA interference, chromatin immunoprecipitation, and analysis of the PLK4 promoter in a luciferase reporter assay revealed that all NFκB subunits participate in its regulation. Moreover, we demonstrate that NFκB regulation of PLK4 expression is seen in multiple cell types. Significantly long-term deletion of the NFκB2 (p100/p52) subunit leads to defects in centrosome structure. This data reveals a new component of cell cycle regulation by NFκB and suggests a mechanism through which deregulated NFκB activity in cancer can lead to increased genomic instability and uncontrolled proliferation.

Introduction

In mammalian cells, the nuclear factor κB (NFκB) family of transcription factors contains 5 members: RelA (p65), RelB, c-Rel, NFκB1 (p105/p50), and NFκB2 (p100/p52), which can induce or repress the expression of target genes by binding DNA as homo- or hetero-dimers.[1,2] In unstimulated cells, the majority of NFκB is found localized in the cytoplasm in an inactive complex with proteins from the IκB (inhibitors of the NFκB proteins) family, which includes IκBα, IκBβ, IκBγ, IκBε, and Bcl-3.[1,2] Nuclear localization of NFκB complexes can be induced by various stimuli, including bacterial products, inflammatory cytokines, DNA damage, cell stress, viral proteins, and infection.[2]

In the classical (or canonical) NFκB pathway, stimuli such as inflammatory cytokines or toll-like receptor (TLR) ligands induce IκB kinase (IKK) complex activity.[2] The core IKK complex consists of 2 catalytic subunits, IKKα (IKK1), IKKβ (IKK2), and a regulatory subunit NEMO (IKKγ). In the classical pathway, IKKβ-dependent IκB phosphorylation results in IκB degradation by the proteasome, leading to the activation of RelA- and c-Rel-containing NFκB complexes.[2] The alternative (or non-canonical) NFκB pathway, induced by stimuli such as CD40 ligand and lymphotoxin β, involves activation of IKKα, which phosphorylates the p100 precursor, resulting in its proteasome-dependent processing to p52 and the nuclear localization of p52/RelB complexes.[2] Many cancer cell lines and primary tumors contain deregulated NFκB, which can result from mutation of upstream signaling components or oncogenic signaling, leading to an overactive IKK complex.[3]

The NFκB 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, and cell adhesion and proliferation.[1] NFκB activity and target genes are also linked to the cell cycle and proliferation. For example, NFκB can be required for the expression of the genes encoding Cyclin D1, Skp2, and c-Myc.[4-9] This laboratory previously reported that in some cell lines, such as U2OS osteosarcoma cells, NFκB is absolutely required for cell proliferation, and this is associated with regulation of these gene targets.[6,7] A common theme with these target genes is their ability to be regulated by p52 containing NFκB complexes. Cyclin D1 is one of the best-known NFκB target genes involved in cell cycle regulation during G_1 phase,[7] and its expression is regulated by p52 in co-operation with Bcl-3 and RelA.[6,7] Skp2, which can also

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be regulated by p52,[7,10] promotes the degradation of the CDK inhibitor p27, allowing cell cycle progression[5]. c-Myc promotes proliferation and can be regulated by the RelB/p52 heterodimer[4] and other p52 complexes.[7] However, many different NFκB complexes can participate in regulation of these genes, and our own data suggests a complex pattern of activation and repression, dependent upon cell cycle stage.[7]

These studies have focused on the role of NFκB in the transition through G_1 phase of the cell cycle, and relatively little is known about any potential role in inducing G_2 phase gene expression or regulation of mitosis. However, such a role was implied by analysis of cells following depletion of p100/p52 by siRNA, which in addition to a G_1 arrest also resulted in an increase of cells in G_2/M phase.[6] Furthermore, IKK activity has been directly linked with mitotic events. For example, IKKα can phosphorylate Aurora A,[11] while IKKβ has a role in bipolar spindle assembly.[12] In this manuscript we therefore investigated the ability of p52 and other NFκB subunits to regulate genes required for mitosis and have identified Polo-like kinase 4 (PLK4) as a bona fide NFκB target gene.

PLK4 is an atypical member of the Polo-like kinase family and a key regulator of centriolar duplication.[13] Overexpression of PLK4 induces over-amplification of centrosomes, while depletion reduces centriole number.[14-17] PLK4 works in concert with the cyclin-dependent kinase CDK2, CP110, and the PLK4 downstream regulator spindle assembly 6 homolog (SAS6) to ensure correct centrosome duplication in S-phase.[15] Although some studies link PLK4 expression to p53 activity,[18-21] the factors controlling transcriptional regulation of the PLK4 gene expression have largely not been identified. Here we reveal PLK4 as a new NFκB target gene, providing a direct link between NFκB activity and centrosome duplication, with implications for the role of these transcription factors in tumorigenesis.

Results

NFκB2 (p100/p52)-dependent regulation of cell cycle gene expression

Although U2OS cells require NFκB2 gene expression, which encodes the p100/p52 NFκB subunit, to proliferate in vitro,[6] our understanding of the gene targets involved in this process is limited. We therefore wished to extend these studies to determine any effects of this protein on target genes involved in different aspects of cell cycle progression. NFκB2 siRNA depletion was therefore performed in U2OS cells. Propidium iodide staining and subsequent FACs analysis confirmed reduced numbers of S phase cells (**Fig. 1A** and not shown), consistent with data described previously in this laboratory.[6] As expected, this was associated with downregulation of Cyclin D1 mRNA and protein levels (**Fig. 1B and C**). We also observed effects on other cell cycle regulators, including a strong depletion of CDK4 expression. This effect was confirmed in untransformed human dermal fibroblasts (Alessio Iannetti and Neil Perkins, unpublished observations), and could contribute to the increased numbers of G_1 phase cells (**Fig. 1A**). CDK1 and Cyclin B1 protein levels were largely unaffected by the NFκB2 siRNA (**Fig. 1C**). However, no significant effect on the number of G_2 phase cells was observed, whereas a decrease associated with an enhanced level of G_1 arrest would be expected if cells were able to progress unimpeded through this section of the cell cycle. This suggested that genes involved in other aspects of cell cycle progression might be regulated by this NFκB subunit in these cells.

NFκB2 (p100/p52)-dependent regulation of PLK4 expression

Based on the results in **Figure 1**, we therefore decided to examine other cell cycle-associated genes and, interestingly, discovered that NFκB2 depletion in U2OS cells resulted in downregulation of several genes involved in centrosome duplication: PLK4, AuroraA, the PLK4 downstream regulator SAS6, and CDK2, all showing significant reductions in mRNA levels (**Fig. 2A**, also **Fig. 1B**). Since it is a major effector of centrosome duplication with little being known about its transcriptional regulation, we decided to focus the rest of this study on PLK4. We next confirmed that these results were not restricted to U2OS cells and importantly found that NFκB2 depletion also resulted in downregulation of PLK4 expression in Hela cells as well as primary, untransformed, and non-immortalized human dermal fibroblasts (**Fig. 2B and C**). We independently observed loss of PLK4 expression in a microarray analysis of human dermal fibroblasts treated with an NFκB2 siRNA (Alessio Iannetti and Neil Perkins, unpublished observation). Depletion of PLK4 with a siRNA confirmed that it is required for the proliferation of U2OS cells, and that its loss likely contributes to the decrease in cell proliferation also seen upon treatment with siRNAs targeting NFκB2 (**Fig. 2D**). NFκB2 (p100/p52) regulation of PLK4 expression is therefore a widespread effect and can be seen in cells regardless of their transformed status. However, it is worth noting that we find that culture of cells in vitro frequently induces a basal level of alternative NFκB pathway activity and so these effects will likely be more restricted in vivo, where NFκB activity is more tightly controlled.

We were next interested in whether these effects were restricted to NFκB2 activity. Analysis of data from a genome-wide analysis of c-Rel-dependent gene expression in T cells revealed PLK4 as a potential target gene, although this was not validated in the study.[22] We used siRNAs targeting each NFκB subunit and found that all resulted in reduced PLK4 levels, with c-Rel depletion having the strongest effect (**Fig. 2E**). All siRNAs have been previously published and validated,[6,7,10] with western blot analysis confirming their effect on protein expression (not shown). In addition, a siRNA targeting all NFκB subunits simultaneously (siRNA Pan-NFκB)[23] was used to validate these effects. Targeting NFκB in this manner also reduced PLK4 mRNA and protein levels (**Fig. 2F and G**). Therefore this data confirmed that all NFκB subunits can potentially regulate PLK4 expression but did not demonstrate whether these effects were direct or indirect.

NFκB regulates PLK4 promoter activity

To address the question of whether NFκB regulation of PLK4 expression occurred through the PLK4 promoter, a luciferase reporter plasmid was created containing the genomic region from –3713 to +972 relative to the PLK4 transcription start site.

Consistent with the siRNA data in **Figure 2**, overexpression of all NFκB subunits was found to upregulate PLK4 promoter activity, with c-Rel and p50 having the stronger effects (**Fig. 3A–C**). Co-expression of NFκB subunits did not exhibit a strong combinatorial effect, but this probably reflects dimerization with endogenous NFκB.

NFκB directly binds to the PLK4 promoter

The luciferase assay data suggested that PLK4 could be a direct target of NFκB activity. To address this, we performed chromatin immunoprecipitation experiments, focusing initially on the binding of the p52 NFκB2 subunit. By sequence homology, we identified a number of putative NFκB binding sites upstream and downstream of the PLK4 transcription initiation site (**Fig. 4A**). For technical reasons with the PCR, likely caused by the high A/T content of much of the PLK4 promoter, we were not able to examine binding to all putative NFκB binding sites. However, analysis was possible for the kB elements at –2634, –1947, and –1256. Using PCR primers targeting these regions we were able to observe p52 binding in U2OS cells that was inhibited upon NFκB2 siRNA depletion, confirming the specificity of this signal (**Fig. 4B**). Overexpression of HA-tagged p52 in Hela cells followed by ChIP analysis with an anti-HA antibody, further validated p52 binding to the upstream region of the PLK4 promoter (**Fig. 4B**). Moreover, significantly reduced binding was observed when a DNA binding mutant of p52[6] was used in this assay (**Fig. 4C**).

We next used ChIP to analyze binding of all the NFκB subunits to the PLK4 promoter in U2OS cells. Consistent with our siRNA and luciferase assay data, this analysis revealed binding by all subunits to the PLK4 promoter but not to the GAPDH control promoter (**Fig. 5A–G**). Interestingly, the relative levels of binding varied depending on the region of the PLK4 promoter analyzed, indicating differential recruitment to different NFκB binding sites. It should be noted that due to differences with antibodies, relative levels of binding between different NFκB subunits cannot be accurately quantified. Binding of NFκB subunits to the PLK4 promoter was also confirmed in HeLa cells (not shown), validating PLK4 as a direct NFκB target gene in other cell models.

The PLK4 core promoter is sufficient to confer NFκB responsiveness

To understand more about the significance of NFκB regulation of PLK4 expression, we examined the putative promoter of the murine PLK4 gene. By contrast with the human promoter, we could only observe putative NFκB binding sites in the region of the core promoter and downstream of the transcription start site (**Fig. 6A**). Of these, only a few were conserved between mouse and human. Nonetheless, ChIP analysis in immortalized mouse embryonic fibroblasts (MEFs) confirmed NFκB binding to the mouse PLK4 promoter (**Fig. 6B**). Use of *nfkb2*$^{-/-}$ MEFs demonstrated the specificity of the p52 ChIP signal. Interestingly, in *nfkb2*$^{-/-}$ MEFS, the absence of p52 binding appears to be compensated for by increased levels of binding of other NFκB subunits, correlating with higher levels of PLK4 mRNA in these cells (**Fig. 6C**). This data implied that the core region of the PLK4 promoter might be sufficient to confer NFκB responsiveness. We therefore performed a deletion analysis of the human PLK4 promoter luciferase plasmid to produce a truncated form of the promoter from –330 to +259 relative to the transcription start site, thus removing many of the putative kB elements (**Fig. 6D**). Interestingly, with some minor differences, this plasmid was still responsive to overexpressed NFκB subunits (**Fig. 6E**). Although it is well established that transiently transfected reporter plasmids do not recapitulate all the elements of endogenous gene regulation, this data suggests a modulatory role for the distal NFκB sites and reveals that the PLK4 core promoter region alone is sufficient for NFκB regulation.

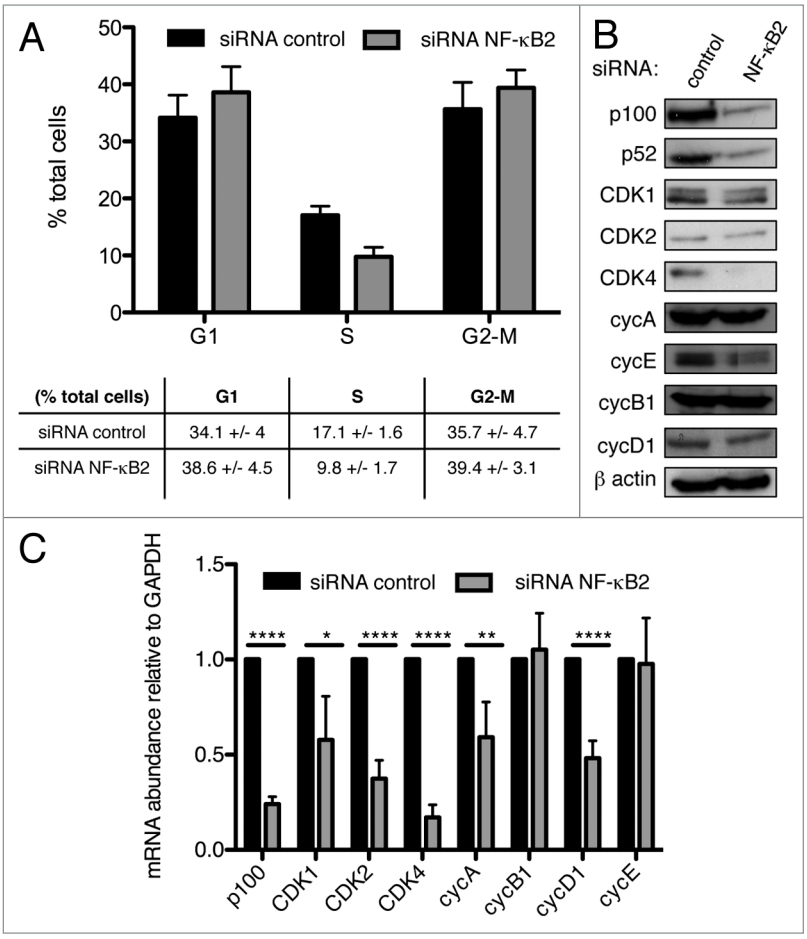

Figure 1. Regulation of cyclin/CDK expression by NFκB2 (p52/p100). (**A**) p52/p100 depletion results in reduced cells in S phase. U2OS cells were subjected to transient transfection using a control or NFκB2 siRNA for 48 h. DNA profile after siRNA transfection in U2OS cells was analyzed by flow cytometry. (**B and C**) p52/p100 depletion affects the expression of cyclin and CDK genes. U2OS cells were subjected to transient transfection for 48h using a control or NFκB2 siRNA. The expression of genes involved cell cycle progression was analyzed for mRNA expression by qPCR (**B**) or for protein levels by western blot (**C**) Results are the average of at least 3 independent experiments, and bars show the standard error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Long-term depletion of NFκB2 results in defects in centrosome structure

We were unable to visualize effects on centrosome duplication and structure resulting from short-term depletion of NFκB subunits using siRNA (not shown). At least in part this may result from other effects on the cell cycle and proliferation, resulting in a failure to progress through S-phase, where centrosome duplication is performed. However, we have found that long-term depletion of NFκB2 in U2OS cells, through the creation of cells lines stably expressing an shRNA, results in cells that overcome

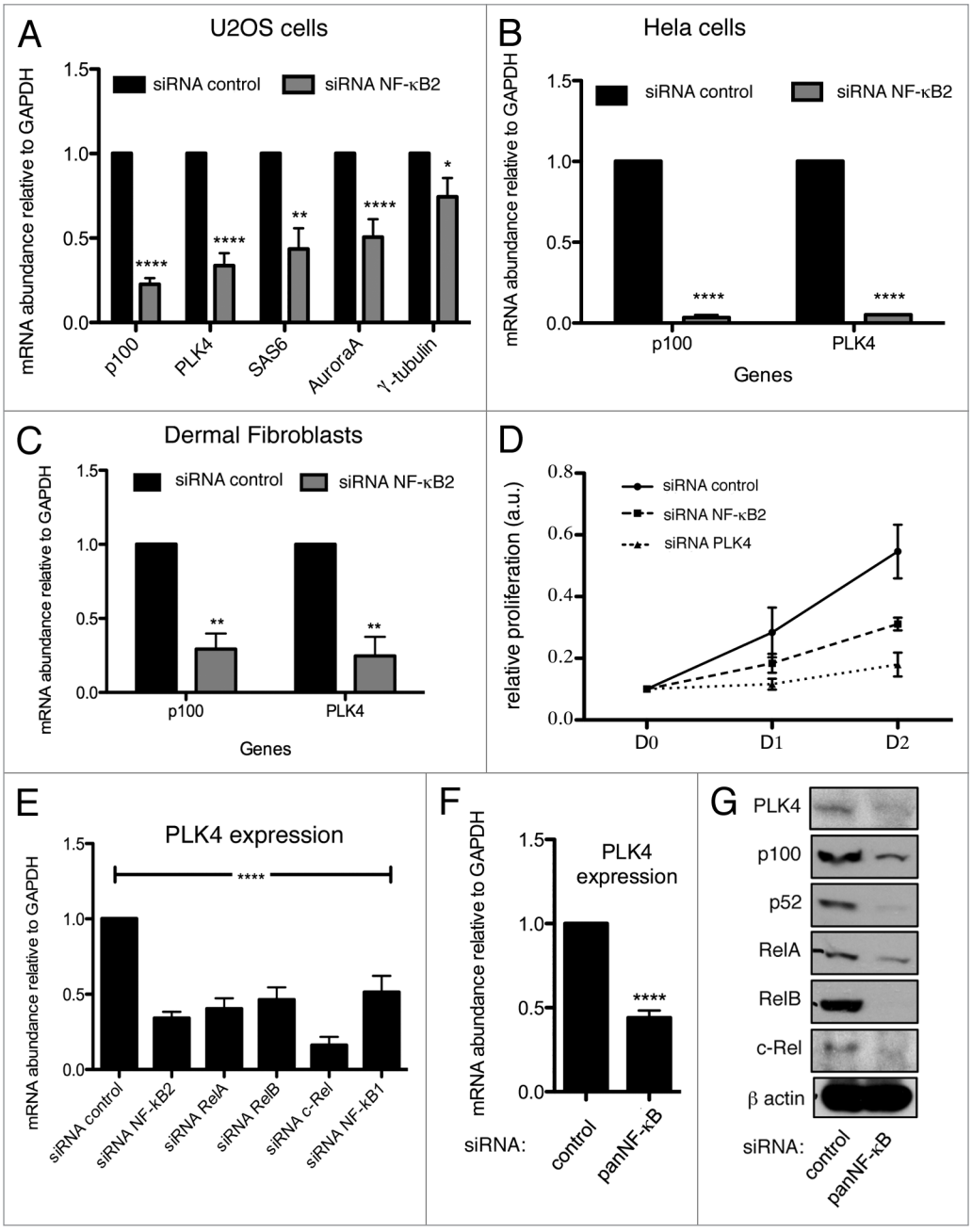

Figure 2. NFκB regulates PLK4 expression. (**A**) p52/p100 depletion regulates the expression of various genes involved in centrosome duplication. U2OS cells were subjected to transient transfection for 48 h using a control or NFκB2 siRNA. The mRNA expression of various genes involved in centrosome duplication was analyzed by qPCR. (**B and C**). p52/p100 depletion leads to reduced PLK4 expression. Hela cells (**B**) or dermal fibroblasts (**C**) were subjected to transient transfection using a control, or NFκB2 siRNA and PLK4 mRNA expression was analyzed by qPCR. (**D**) p52/p100 and PLK4 depletion inhibits cell proliferation. U2OS cells were subjected to transient transfection using a control, NFκB2, or PLK4 siRNAs as indicated. Cell proliferation was then analyzed using the MTT assay 54 h after initial transfection (D0) and then 24, 48, and 72 h after this (D1, D2 and D3). (**E–G**). Depletion of all NFκB subunits regulates the expression of PLK4 in U2OS cells. U2OS cells were subjected to transient transfection for 48 h using a control or siRNAs targeting all NFκB subunits either singly (**D**) or together using a pan-NFκB siRNA (**E and F**). PLK4 mRNA expression was analyzed by qPCR (**D and E**) or western blot (**F**). mRNA expression (**A–E**) was analyzed by quantitative PCR, compared with a housekeeper gene (GAPDH) and related to the expression with siRNA control. Results are the average of at least 3 independent experiments and bars show the standard error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

the proliferative block, although they only survive for a limited number of passages. Using electron microscopy, we analyzed centrosome structure in these cells. Very few centrosomes could be identified in these cells, and those that could be observed did not have the normal appearance seen with cells expressing a control shRNA (**Fig.** 7). This outcome probably results from a combination of effects resulting from depletion of NFκB2 in addition to PLK4. Nonetheless, it establishes a functional link between NFκB activity and the correct duplication and structure of centrosomes.

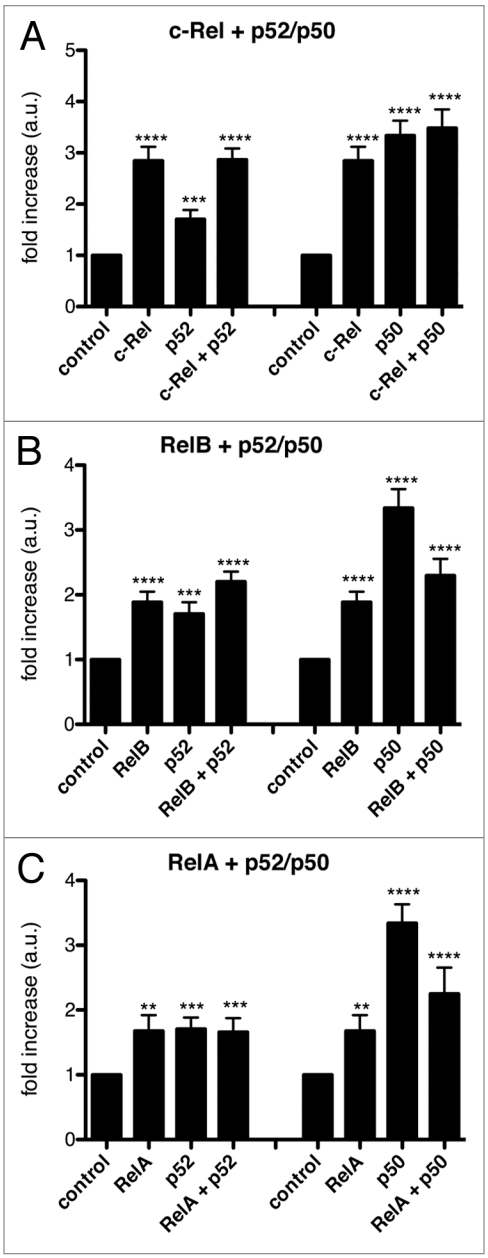

Figure 3. NFκB regulates the PLK4 promoter activity. (**A–C**) Overexpression of NFκB subunits increases PLK4 promoter activity. U2OS cells were transiently transfected with 1 μg of the full-length PLK4 promoter luciferase plasmid and 1 μg of either individual RSV NFκB subunit expression plasmids or the indicated combinations. Results are the average of at least 3 independent experiments, and bars show the standard error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

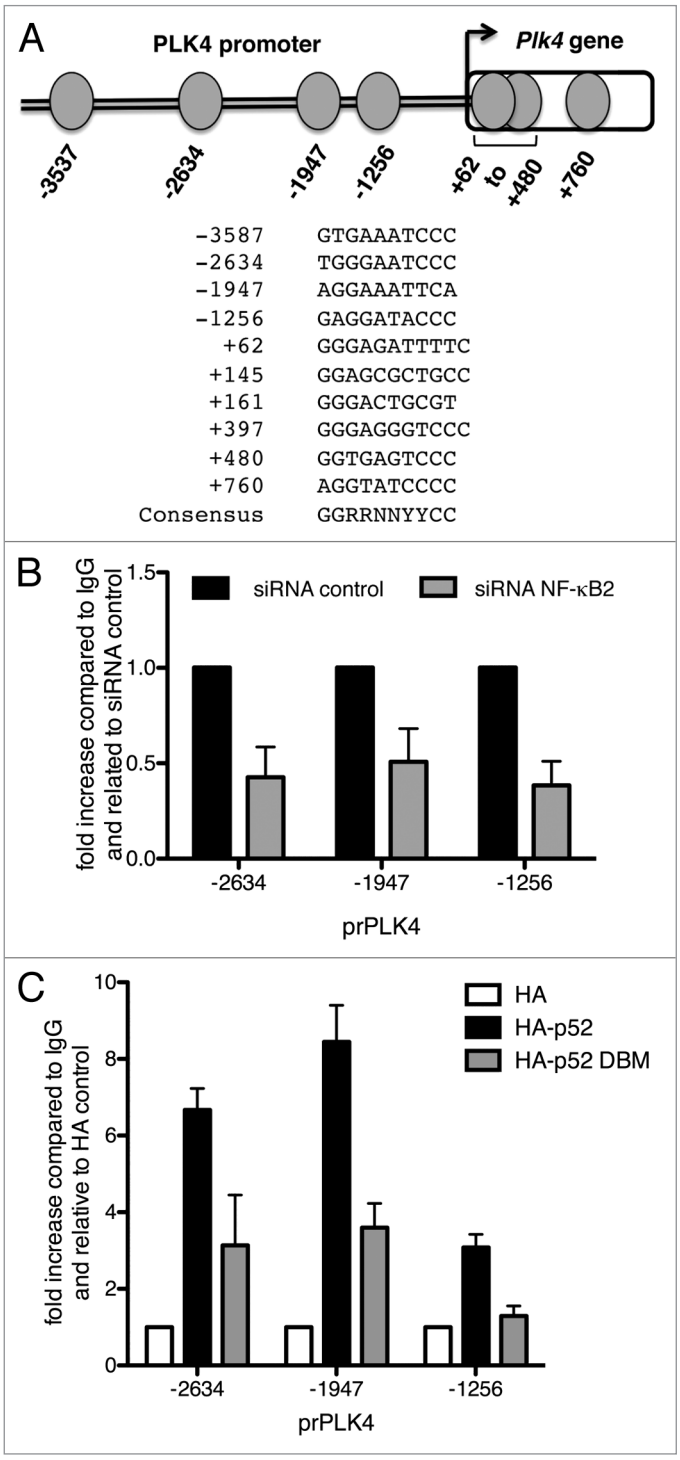

Figure 4. p52 specifically binds to multiple sites on the PLK4 promoter. (**A**) Schematic representation of the various putative κB sites identified in the human PLK4 promoter, with their sequences below. (**B**) p52 binding on the PLK4 promoter is reversible. U2OS cells were subjected to transient siRNA transfection, with either control or NFκB2 siRNA. After 48 h, the cells were processed for ChIP analysis. (**C**) Exogenous p52 binds the PLK4 promoter. Hela cells were subjected to transient DNA transfection, with plasmids coding for exogenous Ha tagged p52 or the p52 binding mutant (DBM). After 48 h, the cells were processed for ChIP analysis. Results are the average of at least 3 independent experiments, and bars show the standard error.

Discussion

Appropriate PLK4 expression is critical for normal centrosome duplication and correct cell cycle progression. Too much PLK4 results in amplification of centrosomes,[17] while its depletion reduces centriole number.[16] NFκB regulation of PLK4 expression thereby provides a mechanism to link inflammatory and immune signaling to centrosome duplication and mitosis. Moreover, we also observed NFκB regulation of the SAS6, CDK2, and Aurora A genes, although in these cases we did not determine by ChIP analysis if these effects were direct. SAS6 downregulation has been also shown to inhibit centriolar duplication,[24] whereas AuroraA siRNA treatment of Hela cells resulted in incorrect centriolar separation, incomplete cytokinesis, and chromosome misalignment on the metaphasic plate.[25] PLK4 works in concert with the cyclin-dependent kinase CDK2 and SAS6 to ensure correct centrosome duplication.[15] These data suggest a coordinated regulatory pathway linking NFκB function to mitotic regulation that is consistent with previous data linking IKKα and IKKβ to this process.[11,12] However, it is interesting to note that PLK4 has also been shown to be required for a TLR induced anti-viral response, involving the TNFAIP2 gene,[26] which has previously been shown to be an NFκB target.[27] This pathway may therefore have a distinct role facilitating NFκB-driven innate immune responses.

Regulation of effector kinases provides a powerful mechanism through which signaling pathways can crosstalk and have widespread cellular effects. Under normal physiological conditions, these effects are balanced to ensure appropriate cell proliferation. These effects were observed in cultured cells in vitro, where a basal level of NFκB activity is typically observed. In vivo, it is likely that regulation of centrosomal duplication by NFκB occurs only in specific circumstances. Similar to NFκB regulation of other genes regulating cell proliferation such as Cyclin D1 and c-Myc, it is probable that other transcription factors and signaling pathways regulate PLK4 expression in different contexts.

NFκB regulation of PLK4 also provides a potential route through which aberrant activation of NFκB in cancer, either through inflammation or oncogene activation, could lead to genomic instability. We, and others, have previously documented crosstalk between the NFκB and the p53 tumor suppressor pathways.[3] It is interesting to note that p53 has recently been shown to downregulate PLK4 expression.[18,19] Moreover, p53 has previously been shown to repress expression of PLK1,[28] a kinase related to PLK4 that is also involved in G_2/M transition, and that can also be an NFκB target gene.[29] These data suggest that regulation of these kinases provides another route of potentially antagonistic crosstalk between the NFκB and p53 pathways.

In analyzing the PLK4 promoter, we identified a number of putative NFκB binding sites. ChIP analysis suggested that NFκB complexes may differentially occupy both the distal and proximal promoter binding sites. However, this type of analysis can also produce a positive signal if DNA looping is occurring between different promoter regions. Indeed, when we examined the mouse promoter, the only sites conserved were in the region of the core promoter and immediately downstream of the transcription start site. Furthermore, deletion analysis of the promoter in luciferase assays suggested that the core promoter is sufficient to confer NFκB regulation. Therefore, it is plausible that the core promoter represents the site of direct NFκB interaction, and the signals seen with primers to upstream regions result from DNA looping effects. Alternatively, regulation of the human gene may be more complex than that of the mouse, and this could be reflected by the presence of multiple NFκB binding sites.

We have previously observed differential NFκB subunit occupancy at target gene promoters at different cell cycle stages.[7] Furthermore, this is associated with differential recruitment of coactivators and co-repressors. For example, regulation of Cyclin D1 expression in early passage U2OS cells involves p52-dependent recruitment of coactivators in G_1 phase followed by a switch to p52-dependent recruitment of co-repressors in G_2 phase.[7] It is very likely that similar regulation is occurring with PLK4, and that the recruitment of all NFκB family members to the promoter may reflect a more complex, cell cycle-dependent regulation with differential effects being exerted in G_1, S, and G_2 phases. Assessing the contribution of the different κB sites will be technically challenging. It is unlikely that mutation of these sites within the PLK4 promoter luciferase plasmid will be informative, as such reporters frequently fail to recapitulate complex mechanisms of gene regulation requiring regulation of chromatin structure and DNA looping. However, informative clues might be obtained through ChIP-Seq analysis of cells at different cell cycle stages to examine differential recruitment of NFκB subunits (and their modified forms) to different κB elements.

NFκB responds to an incredibly diverse set of stimuli in all cell types. It is a key component of the ability of an organism to adapt to and survive infection and stress. Taken together with previous results from this and other laboratories, this study adds to the data supporting cell cycle regulation as being a vital component of the NFκB signaling. Integrating cell division and proliferation with NFκB function will be required for an appropriately coordinated cellular response. However, when NFκB becomes aberrantly regulated in disease, particularly cancer, these cell cycle target genes provide a pathway that can lead to increased genomic instability and uncontrolled proliferation.

Materials and Methods

Cell growth, siRNA, and DNA transfection

U2OS cells, Hela cells, H1299 cells, wild-type, and *nfkb2*$^{-/-}$ MEF were maintained at 37 °C, 5% CO_2 in DMEM (Lonza), with 10% FBS (Life Technologies), and antibiotics.

RNAi transfection was performed in cells at 30% confluency, mixing 5 nM siRNA with Interferin reagent (Polyplus) according manufacturer's conditions. Forty-eight hours post-transfection, cells were harvested and processed as required. DNA transfection for luciferase assays were performed using 2 μg DNA with PEI using manufacturer's conditions and left to rest for 48 h.

Flow cytometry

DNA profile was analyzed by flow cytometry. Forty-eight hours after siRNA transfection, cells were detached, PBS washed, and pelleted. Cells were then resuspended in PBS containing 50 μg/ml of propidium iodide and 50 μg/ml of RNase A. Cells

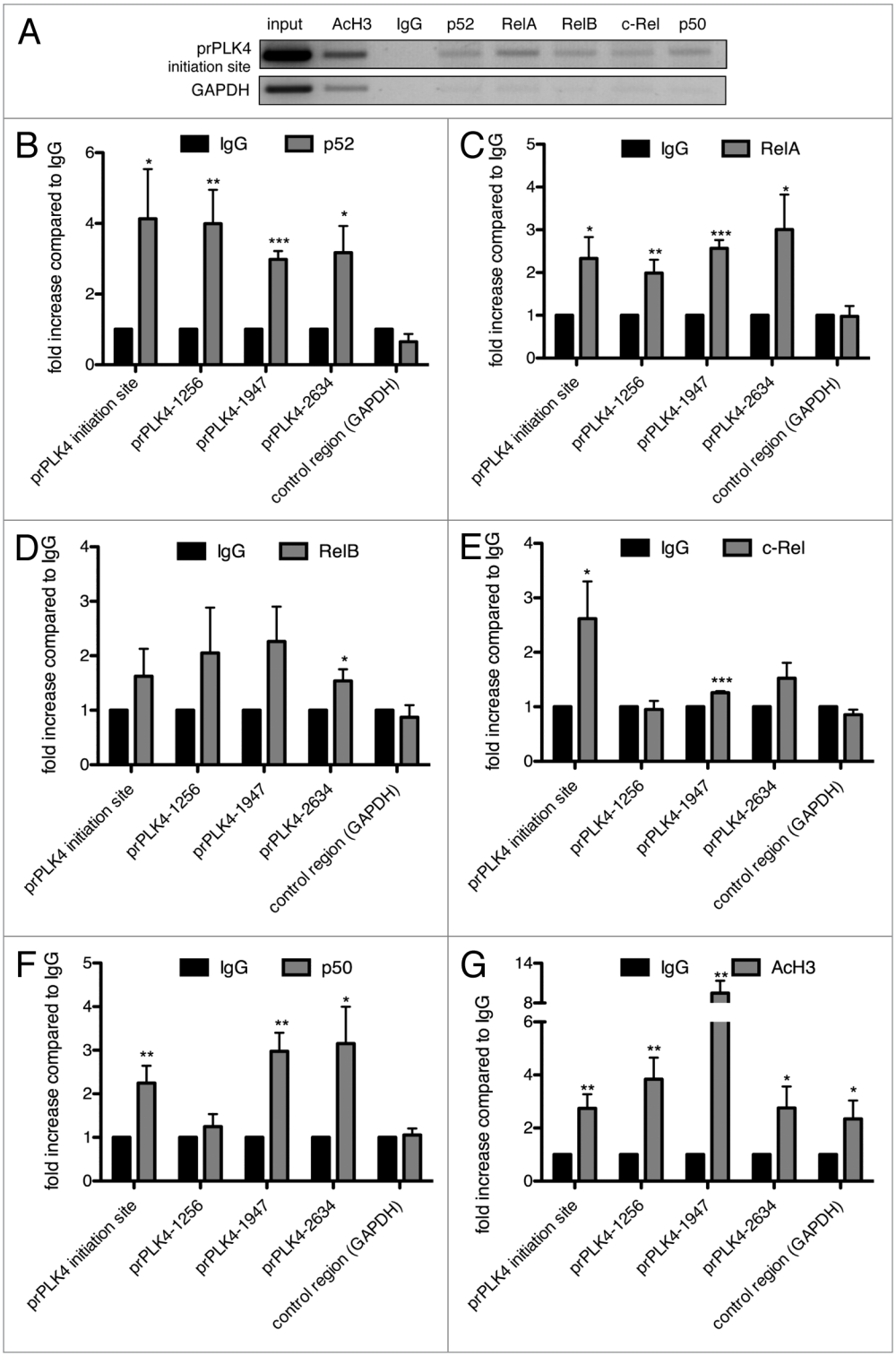

Figure 5. The PLK4 promoter is a NFκB target. U2OS cells were fixed by paraformaldehyde and processed for a ChIP assay, using antibodies against different NFκB subunits, using acetylated histone H3 (AcH3) as a positive and IgG as a negative control. (**A**) Recruitment of the NFκB subunits to the PLK4 promoter. PCR was performed with primers for the PLK4 promoter transcription initiation site. (**B–G**) Quantification of NFκB recruitment to the PLK4 promoter. qPCR were performed after ChIP analysis on the indicated NFκB subunits and binding sites (initiation site, prPLk4-2634, prPLK4-1947, and prPLK4-1256) together with control promoter region (GAPDH). Results are expressed as binding relative to the IgG control (which is normalized to 1) and is the average of at least 3 independent experiments. Bars show the standard error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

were then processed for analysis for cell cycle distribution with a BD FACSCanto II (6c) (Becton Dickinson) and FlowJo analyzing software. Five thousand cells were analyzed to determine the DNA profile for each condition. Results shown are averages derived from 3 separate experiments, and error bars indicate the standard error.

MTS proliferation assay

U2OS cells in 24-well plates were transfected in duplicate with siRNAs, using the previously described conditions. Twenty-four 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. Twenty-four hours later, the cells were washed, detached with trypsin-EDTA, counted, and replated in 96-well plates at a concentration of 2000 cells per well. Six hours later, the proliferation at day = 0 (D0) was determined by MTS assay (Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay) and a 96-well plate reader (POLARstar Omega, BMG Labtech). Readings were then taken over 3 d sequentially 24 h after the previous one (D1, D2, D3). Results shown are averages derived from 3 separate experiments, and error bars indicate the standard error.

Luciferase assays

The PLK4 promoter luciferase reporter plasmid was created by Dundee Cell Products. After PBS washes, cells were lysed with Passive Lysis Buffer (PLB, Promega) and centrifuged at 19350 g for 5 min. 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 3 times before calculating means and standard error as shown in the figures.

Chromatin immunoprecipitation (ChIP)

All cell lines used for ChiP assay, either grown to 70% confluency or analyzed 48 h after transfection, were cross-linked with 1% formaldehyde at room temperature for 10 min. Media was removed, and cells were incubated with cold glycine 0.125 M for 3 min at room temperature and washed twice with cold PBS, before being scraped into 0.5 mL of RIPA buffer (0.1% SDS, 1% Triton, 0.5% deoxycholate, 0.5% NP40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 µg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 50 µg/ml Na_3VO4, 50 µg/ml NaF) and left on ice for 10 min. Samples were then sonicated on ice 7 times. Each sonication was for 30 s with a 30 s gap between each sonication. Supernatants

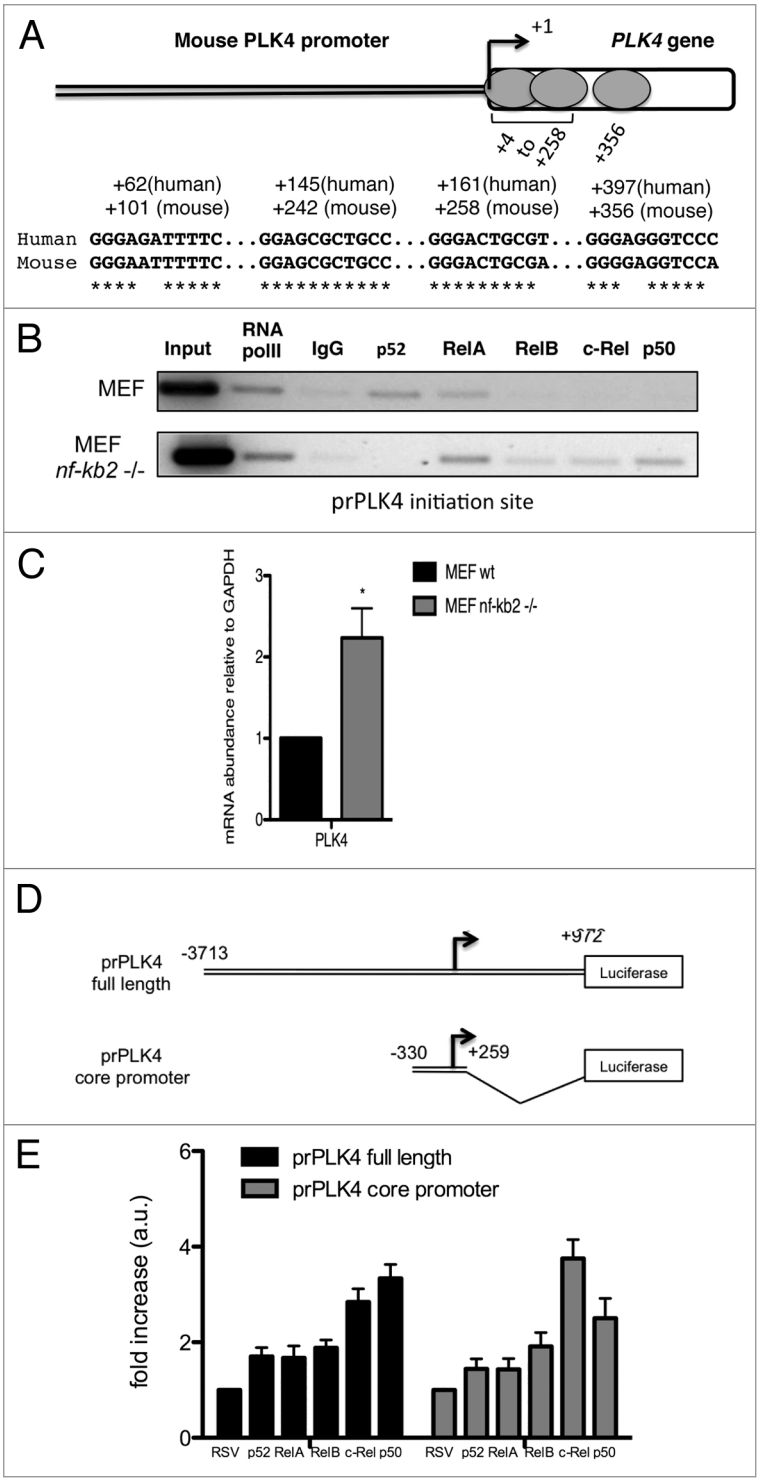

Figure 6. NFκB regulation of PLK4 is conserved in mouse cells. (**A**) Schematic representation of the various putative κB sites identified in the mouse PLK4 promoter. Sequences below show the conservation of sequences between the mouse and human genes. (**B**) Recruitment of NFκB subunits to the mouse PLK4 promoter. Wild-type and *nfkb2* $^{-/-}$ MEFs were fixed with formaldehyde and processed for ChIP analysis, using antibodies against the indicated NFκB subunits, using RNApolII as a positive and IgG as a negative control. PCR was performed with primers for the initiation site. (**C**) *nfkb2* $^{-/-}$ MEFs show elevated levels of PLK4 mRNA. Wild-type and *nfkb2* $^{-/-}$ MEFs were analyzed by qPCR to determine the basal levels for PLK4 expression. (**D**) Schematic representation of the full-length human PLK4 promoter luciferase reporter and its core promoter mutant. (**E**) The PLK4 core promoter is sufficient to confer NFκB responsiveness. U2OS cells were transiently transfected with 1 µg of the full-length or core PLK4 promoter luciferase plasmids and 1 µg of either individual RSV NFκB subunit expression plasmids. Results are the average of at least 3 independent experiments, and bars show the standard error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

were recovered by centrifugation at 12 000 rpm in an eppendorf microfuge for 10 min at 4 °C before being diluted 1:1 in dilution buffer (1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl supplemented with 0.1% NP40, protease and phosphatase inhibitors). Samples were then precleared for 2 h at 4 °C with sheared salmon sperm DNA (1 μg/ml) and 20 μl of protein A and G-agarose beads. At this stage, 20 μl of the material was kept as input material. Immunoprecipitations were performed overnight with specific antibodies (2 μg). The immune complexes were captured by incubation with 20 μl of protein A and G-agarose beads and salmon sperm DNA (1 μg/ml) for 1 h at 4 °C. The immunoprecipitates were washed sequentially for 5 min each at 4 °C in TSE 1 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH 8.1,150 mM NaCl), TSE 2 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH 8.1500 mM NaCl), Buffer 3 (250 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and TE buffer (1 mM EDTA, 10 mM Tris-HCl pH = 8.1). Beads were then eluted with 500 μl of Elution buffer (1% SDS,100 mM NaHCO3).

To reverse the crosslinks, samples, including "Input", were incubated at 65 °C overnight in a waterbath with 0.2 M NaCl. DNA was ethanol precipitated following Phenol-Chloroform extraction. For PCR, 5 μl of DNA was used from an 80 μl DNA preparation and subjected to 40 cycles of PCR amplification.

For all ChIP results shown are averages derived from 3 separate experiments, and error bars indicate the standard error.

Quantitative RNA analysis

Total RNA was extracted with PeqLab gold total RNA extraction kit according to the manufacturer's directions. For reverse transcriptase PCR (RT-PCR), 1 μg RNA sample were transcribed with Quantitect Reverse Transcription Kit (QIAgen). The cDNA stock was diluted by 200 and 5 μl was used for PCR with GoTaq flexi DNA-polymerase (Promega).

Quantitative PCR data was generated on a Rotor-Gene Q (Qiagen) using the following experimental settings: hold, 95 °C for 5 min; cycling, (95 °C for 20 s; 58 °C for 20 s; 72 °C for 20 s with fluorescence measurement) × 40; melting curve, 50–99 °C with a heating rate of 1 °C every 5 s. All values were calculated relative to untreated levels and normalized to GAPDH levels using the Pfaffl method.[30] Each RNA sample was assayed in triplicate, and the results shown are averages derived from 3 separate experiments with error bars indicating the standard error.

Creation of NFκB2 shRNA stable cell lines

NFκB2 (p100/p52) shRNA and control plasmids used to generate stable U2OS cell lines were created using the pSilencer plasmid (Ambion) using a 5′ BamH1 site and a 3′ HindIII site. U2OS cells were seeded at 8 × 10^5 cells/ml in a 6-well dish the night before transfection. The next day cells were transfected with 1.5 μg of pooled NFκB2 (p100/p52) shRNA expressing plasmids or the control plasmid using Genejuice transfection reagent (Novagen), according to the manufacturer's instructions. Forty-eight hours post-transfection, cells were selected with Hygromycin B. Cells were from a pool of stable clones that emerged in the weeks after selection. NFκB2 (p100/p52) shRNA and control shRNA sequences have been published previously.[6]

Electron microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacocylate buffer. Post-fixation was performed in 1% osmium tetroxide in 0.1 M cacodylate. Cells were washed in buffer before being dehydrated in graded ethanols to absolute alcohol and then overnight in 1% uranyl acetate in absolute ethanol. This was followed by propylene oxide treatment for 2 × 15 min followed by 50:50 propylene oxide:durcupan resin treatment overnight (Durcupan, Sigma). Propylene was evaporated for 2 h before addition of fresh resin for 3 to 4 h. Cells were placed into molds in fresh resin and polymerized at 60 °C for about 24 h. Sections were cut on a Leica Ultracut UCT ultramicrotome and collected on Pioloform coated 100 mesh copper grids. Sections were stained with uranyl acetate and Reynolds lead citrate and examined using a TecnaI 12

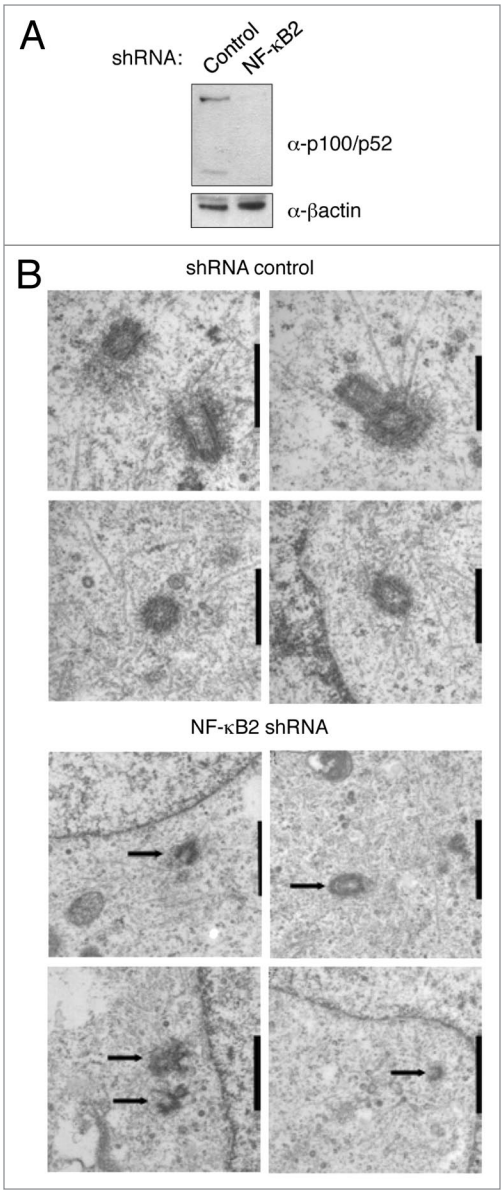

Figure 7. p52/p100 depletion affects centrosome structure. (**A**) Western blot analysis of stable shRNA-expressing cells. (**B**) Centrosome structure was analyzed by electron microscopy in U2OS cells stably expressing NFκB2 (p52/p100) shRNA or a control shRNA.

(FEI) electron microscope. Images recorded on Fuji digital plates and processed in a Ditabis plate scanner.

Antibodies

Antibodies used were: anti-p52/p100 (05-361 Millipore), anti-RelB (4954S Cell Signaling), anti-β-Actin (A5441, Sigma), anti-RelA (sc-372 Santa Cruz), anti-c-Rel (sc-71 Santa Cruz), anti-p50 (06–886 Upstate), anti-PLK4 (3258S Cell Signaling), cyclin D1 (556977 BD pharmingen), cyclin A (CC02 Calbiochem), cyclin E (sc-481 Santa Cruz), cyclin B1 (sc-752 Santa Cruz), CDK2 (sc-6248 Santa Cruz), CDK4 (sc-260 Santa Cruz), CDK1 (CRUK antibodies).

Antibodies used in ChIP assay were: anti-p52/p100 (sc-848 Santa Cruz), anti-RelB (sc-226 Santa Cruz), anti-RelA (sc-372 Santa Cruz), anti-c-Rel (sc-71 Santa Cruz), anti-p50 (sc-114 Santa Cruz), anti-AcHistone 3 (06-599 Millipore).

siRNAs

siRNA control, siRNA NFκB2, siRNA RelB, siRNA RelA, siRNA c-Rel, siRNA NFκB1, siRNA pan-NFκB described[6,10,23]

Oligonucleotides

RT-PCR (human)

NFκB2 For-GGGCAGACCAGTGTCATTGAG
Rev-CCATGCCGATCCAGCAGAG
RelB For-CATCGAGCTCCGGGATTGT
Rev-CTTCAGGGACCCAGCGTTGTA
RelA For – CTC-GGT-GGG-GAT-GAG-ATC-TTC
Rev- CCG-GTG-ACG-ATC-GTC-TGT-ATC
NFκB1 For – TCC-CAT-GGT-GGA-CTA-CCT-GG
Rev- ATA-GGC-AAG-GTC-AGG-GTG-C
c-Rel For – AGA GGG GAA TGC GTT TTA GAT ACA
Rev- CAG GGA GAA AAA CTT GAA AAC ACA
PLK4 For – GAT CAT TTG CTG GTG TCT A
Rev – ACC TCA TTT TGG ACT CTC TG
SAS6 For – GAA TGA GCA TTG AAC TAC A
Rev – GGT GAG TTA TCC AAA ATA GC
Cyclin A For – CGC TGG CGG TAC TGA AGT C
Rev – GAG GAA CGG TGA CAT GCT CAT
Cyclin B1 For – ATA-AGG-CGA-AGA-TCA-ACA-TGG-C
Rev – TTT-GTT-ACC-AAT-GTC-CCC-AAG-AG
Cyclin D1 For – CCA TTC CCT TGA CTG CCC GAG
Rev – GAC CAG CCT CTT CCT CCA C
Cyclin E For – TTA CCC AAA CTC AAC GTG CAA
Rev – GCT CAA GAA AGT GCT GAT CCC
CDK1 For – AAA CTA CAG GTC AAG TGG TAG CC
Rev – TCC TGC ATA AGC ACA TCC TGA
CDK2 For – ATC TCT CGG ATG GCA GTA
Rev – GTT GTG TAC AAA GCC AGA AAC
CDK4 For – AGA GTG TGA GAG TCC CCA ATG
Rev - CAA ACA CCA GGG TTA CCT TG
Centrin2 For – GAA CTG GCA CCA TAG ATG T
Rev – TTT CCT GTC CCT TCC TTA T
g-tubulin For – TAC AAC CCA GAG AAC ATC TA
Rev – CAT CTG CTT CTC GGT CTA T
AuroraA For – CACCACTTGGAACAGTCTATA
Rev - AATAACTCTCTTCGAATGACA
GAPDH For - GGTCGTATTGGGCGCCTGGTCACC
Rev - CACACCCATGACGAACATGGGGGC

RT-PCR (mouse)

NFκB2 For- CTA-ATG-TGA-ATG-CCC-GGA-CC
Rev - GAG-CAG-CAT-TTA-GCA-GCA-GAG
PLK4 For – AGG-AGA-AAC-TAA-TGA-GCA-CCA-CA
Rev – TGG-CTC-TCG-TGT-CAG-TCC-AA
GAPDH For – GCTACACTGAGGACCAGGTTG
Rev – GCCCCTCCTGTTATTATGGGG

ChIP (human)

prPLK4–2634 For – GCT-AGG-TTG-AAA-GGA-GCT-CTC-AC
Rev – AGC-CCC-CTC-ATA-ATT-CAA-GG
prPLK4–1947 For – TTG-TGA-GAG-AGG-GGT-TTG-CT
Rev – ACA-TTT-GGA-GGC-ACG-TGA-TA
prPLK4–1256 For – GGA-ACA-ATT-GAC-AAA-TCG-GAA-T
Rev – AAC-ATT-TTG-CCA-CAC-TTG-TTT-TT
prPLK4 initiation site For – TGG-CCC-CGA-AGT-CTA-GAA-CC
Rev – CAG-GCT-CGG-CTC-TCT-AAA-CC
Primers for Cyclin D1 and GAPDH have been described[6]

ChIP (mouse)

mprPLK4 initiation site For - GCT ACG GTC AGT CGT ACA CTG
Rev - GTG ACG TCA GCA CAC TCT CCA C

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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