A role for I κ B kinase 2 in bipolar spindle assembly

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I κ B kinase 2 (IKK2 or IKK β) is a component of the IKK complex that coordinates the cellular response to a diverse set of extracellular stimuli, including cytokines, microbial infection, and stress. In response to an external stimulus, the complex is activated, resulting in the phosphorylation and subsequent proteasome-mediated degradation of I κ B proteins. This event triggers the nuclear import of the NF- κ B transcription factor, which activates the transcription of genes that regulate a variety of fundamental biological processes, including immune response, cell survival, and development. Here, we define an essential role for IKK2 in normal mitotic progression and the maintenance of spindle bipolarity. Chemical and genetic perturbation of IKK2 promotes the formation of multipolar spindles and chromosome missegregation. Depletion of IKK2 results in the deregulation of Aurora A protein stability and coincident hyperactivation of a putative Aurora A substrate, the mitotic motor KIF11. These data support a function for IKK2 as an antagonist of Aurora A signaling during mitosis. Additionally, our results indicate a direct role for IKK2 in the maintenance of genome stability and underscore the potential for oncogenic consequences in targeting this kinase for therapeutic intervention.

Aurora A | mitosis | NF- κ B | spindle polarity

The protein kinase I κ B kinase 2 (IKK2) is associated with a 700- to 900-kDa multiprotein complex in the cytoplasm, which contains additional subunits, including the highly homologous IKK1/ α (IKK1) and a nonenzymatic regulatory protein IKK γ (NEMO)(1-3). In response to a number of external stimuli, including TNF- α and LPS, the complex is activated, resulting in the phosphorylation of I κ B proteins. The phosphorylated form of I κ B is recognized and ubiquitinated by the SKP1/Cul1/F-box protein (SCF)- β -transducin repeat-containing protein (β TRCP) complex, targeting it for degradation by the proteasome. This event triggers the nuclear import of the NF- κ B transcription factor, which activates the transcription of genes that regulate a variety of important physiological events (4, 5). A number of noncanonical activities for NF- κ B pathway members have also been described. For example, NEMO coordinates a cellular response to genotoxic stress with the ataxia telangiectasia mutated (ATM) kinase independent of the IKK complex (6). Additionally, IKK1 has been shown to phosphorylate histone H3 and DNA-bound RelA, resulting in enhancement and repression of transcription, respectively (7-9). NF- κ B-independent activities of IKK include the regulation of keratinocyte differentiation and tumor promotion through inhibition of the oncogenic transcription factor FOXO3a (10, 11).

Control of mitosis is achieved through the integration of complex signaling pathways, which ensures strict temporal and spatial regulation (12, 13). Aurora kinase A is a key regulator of several steps in mitosis, including centrosome maturation and bipolar spindle assembly (14). Activation of Aurora A triggers the phosphorylation of a number of substrates that regulate mitotic progression, including the kinesin-like motor protein KIF11/Eg5, an important component in generating poleward forces for the maintenance of bipolar spindles (15-17). In a reciprocal regulatory circuit, Aurora A protein is rapidly degraded in an anaphase-promoting complex (APC/C)-dependent manner at the conclusion of mitosis (18).

Here, we demonstrate that reduction of IKK2 activity results in the induction of multipolar spindles, accompanied by increased chromosomal segregation defects and cellular transformation. We further demonstrate that these spindle abnormalities result from IKK2-dependent misregulation of Aurora A protein stability and concomitant hyperactivation of KIF11/Eg5. These results define a role for IKK2 in regulation of the metaphase-to-anaphase transition and suggest that inhibition of this kinase promotes genetic instability and oncogenic transformation.

Results

We have previously conducted genomewide siRNA screens to identify proteins required for the proper regulation of cell-cycle progression (unpublished work and ref. 20). Interestingly, these analyses revealed that a significant number of genes associated with TNF- α /NF- κ B signaling, including IKK2, may participate in the transition through the mitotic phase of the cell cycle [supporting information (SI) Figs. 6 and 7]. Specifically, reduction in IKK2 mRNA levels in HeLa cells resulted in a 50% increase of their mitotic index (unpublished work). To further investigate this observation, we transfected siRNA oligonucleotides targeting IKK2 into synchronized HeLa cells. FACS analysis demonstrated that depletion of IKK2 induced an accumulation of cells with 4N DNA content, confirming that the absence of this kinase results in a delayed transition through the cell cycle (Fig. 1A and SI Fig. 8A). Although the thymidine block/release procedure triggered a slight activation of the IKK complex, this induction was resolved before the cells transitioned through G₂/M (SI Fig. 8A). Because these data indicated that IKK2 reduction may affect the metaphase-to-anaphase transition, we sought to determine whether defects in spindle architecture might underlie the observed phenotype. Mitotic spindles were visualized through immunofluorescent labeling of microtubules. Whereas 90-95% of control siRNA-treated cells displayed normal bipolar spindle structures, between 20% and 25% of cells with reduced IKK2 protein levels contained defective spindle morphology (Fig. 1B and C). These structures primarily contained supernumerary spindle poles; however, monopolar spindles were occasionally observed. Additional human cell lines, including U2OS (osteosarcoma) and HCT116 (colorectal carcinoma), also displayed multipolar spindles when transfected with siRNA directed against IKK2 (SI Fig. 8B). To understand the

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Abbreviations: IKK, I κ B kinase; β TRCP, β -transducin repeat-containing protein; MEF, mouse embryonic fibroblast.

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Fig. 3. Biochemical and functional interactions between members of the IKK complex and Aurora A. (A) Coimmunoprecipitation of members of the IKK signalsome, but not downstream pathway members, with Aurora A and TPX2 from mitotic HeLa cells. (B) Addition of importin α/β disrupts interaction between IKK2 and Aurora A. Aurora A was immunoprecipitated from mitotic HeLa cell extracts and incubated with BSA (–) or the indicated importin proteins. IKK2 interaction was assessed by Western blot analysis. (C) Bacterially expressed, constitutively active IKK2 and/or kinase-dead Aurora A were incubated with γ - 32 P ATP and subjected to gel electrophoresis and autoradiography. Phosphorylation and autophosphorylation species of Aurora A and IKK2 (*), respectively, are shown. (D) HEK293T cells were transiently transfected with the indicated constructs, and equivalent quantities of Aurora A complexes purified by immunoprecipitation were subjected to Western blotting with the indicated antibodies. (E) HEK293T cells were transiently transfected with the indicated constructs, and equivalent quantities of total protein were subjected to Western blotting with the indicated antibodies.

Fig. 4. IKK2 is an antagonist of AuroraA-KIF11 signaling. (A) HeLa cells treated with the indicated siRNAs were synchronized by a double thymidine block and released, and mitotic extracts were assessed for Aurora A levels by Western blot. Cytochrome c serves as a loading control. (B) Suppression of the spindle defect in IKK2-depleted cells by Aurora A depletion is shown. HeLa cells were treated with the indicated siRNA combinations and scored for multipolar spindle defects. (C) Suppression of the spindle defect in IKK2-deficient cells by Aurora A inhibition is shown. HeLa cells treated with the indicated siRNAs and the Aurora A-specific inhibitor VX-680 were scored for multipolar spindles. (D) siIKK2-treated cells contain elevated levels of phosphorylated KIF11. Protein extracts from cells prepared as described in A were probed with antibodies that detect phosphorylated and total KIF11. (E) Treatment of IKK2-depleted cells with the KIF11 inhibitor monastrol (25 μ M) reduces multipolar spindles. Monastrol was added 48 h after transfection with the indicated siRNAs, and cells were fixed for spindle staining after a 4-h incubation period. Data points are indicated as mean values ($\pm$ 1 SD). *P* values were determined by using Student's *t* test.

Furthermore, cotransfection of an activated form of IKK2 (SE) and DN β -TRCP resulted in a significant stabilization of the Aurora A protein (Fig. 3E). Taken together, these results suggest that IKK2 phosphorylation of Aurora A targets it for β -TRCP-mediated proteasomal degradation, a mechanism that parallels the IKK-directed targeting of I κ B during canonical NF- κ B signaling.

Consistent with this model, Western blot analysis of extracts derived from synchronized HeLa cells revealed that the reduction of IKK2 expression induced a sustained elevation in endogenous Aurora A protein levels throughout mitosis (Fig. 4A), and analysis of Aurora A mRNA levels suggests that this regulation occurs at the posttranscriptional level (SI Fig. 11). Furthermore, these cells also showed a commensurate increase of phosphorylated Aurora A (SI Fig. 10B). These results demonstrate that depletion of IKK2 results in an overall increase of activated Aurora A in mitosis and indicate that IKK2 acts as an antagonist of Aurora A activity.

To determine whether misregulation of Aurora A was sufficient to account for the spindle defects caused by the inhibition of IKK2, we cotransfected siRNAs targeting both IKK2 and Aurora A into cells. Quantification of the multipolar spindle phenotype revealed that depletion of Aurora A measurably reversed the mitotic defects caused by the IKK2 siRNAs (Fig. 4B). We also blocked Aurora kinase activity by using the small molecule inhibitor VX-680 and assessed spindle abnormalities in IKK2 siRNA-transfected cells (Fig. 4C and ref. 30). This Aurora kinase inhibitor shows 30- and 8-fold selectivity for Aurora A over Aurora B and Aurora C, respectively. We found that low concentrations (1 nM) of the inhibitor also reduced the occurrence of multipolar spindles in IKK2-deficient cells (Fig. 4C and ref. 30). Taken together, these results indicate that the role of IKK2 in bipolar spindle maintenance is, at least partially, mediated by regulation of Aurora A activity.

KIF11/Eg5/KSP is a putative target of Aurora A kinase and has a microtubule plus-end directed motor activity that is required for

the assembly of bipolar spindles (15–17). We therefore tested whether the absence of IKK2 altered the phosphorylation state of KIF11. Consistent with a role downstream of Aurora and IKK2 mitotic activity, phosphorylation of KIF11 is substantially increased in cells with IKK2 siRNA-induced elevation of Aurora A protein levels (Fig. 4D). Additionally, we used a small molecule inhibitor of KIF11, monastrol, to determine whether KIF11 activity is required for multipolar spindle formation in cells lacking IKK2. Forty-eight hours after transfection with a siRNA directed against IKK2, cells were treated with low concentrations (25 μ M) of the KIF11 inhibitor, and then stained to determine spindle architecture. Antagonism of KIF11 in IKK2-depleted cells also reduced the fraction of cells with multipolar spindles to levels comparable to the negative control (Fig. 4E). These data indicate that KIF11 function is required for multipolar spindle formation in the absence of IKK2 and supports a function for IKK2 as an antagonist of an Aurora A-mediated pathway that regulates bipolar spindle assembly.

Spindle abnormalities caused by inappropriate Aurora A activation are often associated with chromosome segregation defects and aneuploidy (22, 31). Therefore, we tested whether IKK2 depletion induced chromosomal aberrations in human cell lines. Reduction of IKK2 consistently resulted in a 3-fold increase in the number of micronuclei, an indicator of chromosomal instability, in multiple cell lines (Fig. 5A and ref. 32). Additionally, treatment of HeLa cells with a small molecule inhibitor to IKK2 also resulted in a significant accumulation of micronuclei, suggesting that spindle defects caused by IKK2 inhibition may lead to chromosomal missegregation events (Fig. 5B). To ascertain whether sustained reduction of IKK2 promotes a transformed phenotype, we assessed the ability of IKK2-deficient MEFs to induce tumors in nude mice. As expected, s.c. injection of wild-type (IKK2^{+/+}) cells did not manifest detectable tumor masses (4/4). However, the introduction of IKK2^{-/-} MEFs into these immunocompromised animals promoted the

Fig. 5. Depletion of IKK2 results in aneuploidy and increased transformation potential. (A) Cells of the indicated type were transfected with control and IKK2 siRNAs, stained with Hoechst dye, and assessed for micronuclei, indicated as mean values (± 1 SD). (B) HeLa cells treated with the IKK2 inhibitor [5-(*p*-fluorophenyl)-2-ureido]thiophene-3-carboxamide were scored as in A. (C) IKK2^{-/-} and wild-type littermate control MEFs were expanded in culture for six passages after isolation, then cell suspensions were s.c.-injected into nude mice and assessed for tumor formation after 4 weeks.

growth of large tumor nodes (4/4; Fig. 5C). These data are consistent with a recent report (33) that concludes that IKK2 deficiency promotes cellular migration and growth.

Discussion

A number of studies have putatively identified several other components of the NF- κ B pathway that are essential for mitotic progression, including IKK ϵ , IRAK1, RIPK1, NFKBIA, and MEKK1 (SI Fig. 6 and refs. 34 and 35). In addition, we and others have observed that IKK1, IKK2, and NEMO individually associate with protein complexes containing Aurora A, but further analysis is required to determine whether the 700- to 900-kDa IKK signalosome complex is directly regulating bipolar spindle assembly (36). However, these findings implicate a potential intersection between a significant number of NF- κ B signaling molecules and critical mitotic events.

Our data specifically indicate an essential role for IKK2 in mitotic spindle function. Mitotic cells depleted of IKK2 activity maintain increased Aurora A levels, and spindle abnormalities in these cells are partially reversed by inhibition of the mitotic kinase. Thus, these results support a model in which IKK2 acts as an antagonist of Aurora A during mitosis. Phosphorylation of Aurora A kinase by IKK2 likely targets it for β -TRCP-mediated degradation and serves to maintain appropriate levels of Aurora A activity to assure proper bipolar spindle assembly and mitotic progression. Recent work has suggested that members of the NF- κ B pathway may serve both upstream and downstream of the mitotic regulator Aurora kinase A (36, 37). In the case of upstream regulation, it has been suggested that other members of the IKK complex may activate Aurora A activity, whereas our results indicate that IKK2 plays an antagonistic role to the Aurora A–KIF11 signal transduction pathway to maintain mitotic spindle integrity (Fig. 4 and ref. 37). With respect to a potential downstream regulation of the mitotic kinase by IKKs, it may be possible that IKK and Aurora A kinases play reciprocal regulatory roles in the complex that we and others describe (Fig. 34 and ref. 36).

The NF- κ B pathway plays both pivotal and apparently paradoxical roles in tumorigenesis. Activating mutations in genes that encode NF- κ B pathway members have previously been associated with various cancers, including colon cancer, liver cancer, melanoma, leukemias, and lymphomas (38). These genetic abnormalities include the amplification of *c-rel* in non-Hodgkin's B cell lymphomas, homozygous deletion of the *I κ B α* locus in Hodgkin's lymphoma, and the constitutive activation of IKK activity in melanomas and breast cancer (39). Additionally, the NF- κ B complex transcriptionally activates tumor-promoting proinflammatory cytokines, which has been shown to play a critical role in inflammation-associated cancers (39). Deletion of IKK2 in both intestinal epithelial and myeloid tissues reduced tumor formation in a mouse model for colitis-associated tumorigenesis, which can be attributed to the antiapoptotic or proinflammatory roles for the IKK/NF κ B pathway in each respective cell type (40). Conversely, IKK signaling can also induce the expression of proapoptotic genes, and inhibition of the pathway has been shown to promote cancer in certain cell types (41, 42). Selective ablation of the regulatory subunit, NEMO, in mouse hepatocytes leads to increased tumorigenesis through modulation of the apoptotic and oxidative stress responses, most likely caused by chronic induction of cell death, inflammatory response, and compensatory cell proliferation (43). Furthermore, MEFs derived from IKK2 knockout mice are known to exhibit increased proliferation and migration properties, consistent with a tumor suppressive role (33). Thus, it is likely this complex relationship between NF- κ B pathway components and tumor suppression or promotion significantly depends on cellular context and microenvironments.

The present study reveals that the depletion of IKK2 activity in both tumor cell lines and MEFs leads to spindle anomalies that promote chromosome segregation defects, aneuploidy, and transformation. Previous work has shown that immortalized NF- κ B *relA*^{-/-} MEFs exhibit a transformed phenotype, but transforming ability varied in a cell line-dependent manner (44, 45). Although we did not detect p53 pathway abnormalities in the IKK2^{-/-} MEFs used for the *in vivo* tumorigenesis studies described here (Fig. 5C