Function of polo-like kinase 3 in NF-κB-mediated proapoptotic response

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Summary

RelA, the p65 subunit of NF-κB transcription factors, plays a key role in regulation of antiapoptotic and proapoptotic responses. However, the downstream target genes regulated by RelA/NF-κB in initiation of proapoptotic signaling was not identified. We previously showed that RelA/NF-κB functioned as a proapoptotic factor by activating the p53-signaling pathway in response to doxycycline-induced superoxide. In the present study, we demonstrate that the ability of doxycycline/superoxide to induce expression of polo-like kinase 3 (Plk3) depends on NF-κB activity. We identified a κB binding site in the promoter of Plk3 and this κB site is directly involved in its induction by the RelA/NF-κB complex. Plk3 formed a complex with p53 and was involved in the phosphorylation of p53 on Ser-20 in response to superoxide. Inhibition of Plk3 expression by Plk3 small interfering RNA (siRNA) suppressed the doxycycline/superoxide-mediated apoptosis. Overexpression of wild-type Plk3 in HCT116 p53+/+ cells induced rapid apoptosis, whereas overexpression of wild-type Plk3 in HCT116 p53−/− cells and the kinase-defective mutant Plk3K91R in p53+/+ cells induced delayed onset of apoptosis. Furthermore, mutagenesis of Plk3 showed N-terminal domain (amino acid 1-26) is essential for induction of delay onset of apoptosis. These data show that Plk3 is a RelA/NF-κB-regulated gene that induces apoptosis in both p53-dependent and -independent signaling pathways, suggesting a possible mechanism for RelA/NF-κB regulated proapoptotic responses.
Introduction

RelA, the p65 subunit of the NF-κB transcription factor, plays a key role in protecting cells from proapoptotic stimuli (1-3). Many studies have shown that proapoptotic signals can induce NF-κB, which in turn induces the expression of the genes involved in suppressing apoptotic signals (4-6). However, a proapoptotic aspect of RelA activity has also been reported: recently, NF-κB was shown to induce cell death after T-cell receptor of the engagement or exposure to DNA-damaging agents (7-9). Other reports have shown that NF-κB activation is required for the onset of apoptosis induced by alphavirus or kainic acid (10,11). The opposing roles of NF-κB in regulating apoptosis have also been shown within individual cells. For example, in the same cells in which inhibition of NF-κB promotes the induction of apoptosis by glucocorticoids, NF-κB is required for the induction of apoptosis by stimulation of phorbol ester and ionomycin for mimicking T-cell activation (12). These findings suggest that whether the function of NF-κB is proapoptotic or antiapoptotic in a given cell depends on the cell type, extent of NF-κB activation, and nature of the apoptotic signals.

Doxycycline like chemotherapeutic agents, radiation, cytokines and other apoptosis inducers, causes structural and morphologic damage to the cell, such as cell shrinkage, chromatin condensation, and DNA fragmentation (13-15). Kroon and colleagues showed that tetracycline preferentially inhibited mitochondrial protein synthesis in cancer cells, including cytochrome C oxidase, the key component of the electron transport chain (16,17). Our previous report showed that doxycycline induces superoxide formation, suggesting that decreased synthesis of mitochondrial protein disrupts of electron transport function and results in electron leakage from the respiratory chain to O₂, thus elevating levels of superoxide radicals (18). However, the molecular mechanism by which doxycycline induces apoptosis is not yet completely understood.
Both NF-κB and p53 are activated in response to many stimuli, including DNA damage and oxidative stress (19,20). Reactive oxygen species-induced phosphorylation of p53 on Ser-20 is mediated in part by polo-like kinase-3 (Plk3) (21), a member of a conserved family of serine/threonine protein kinases that are important in cell cycle regulation and stress response signaling. Phosphorylation of p53 on Ser-15 and Ser-20 plays an important role in the p53-mediated apoptotic pathway (22). Our previous report showed that NF-κB-dependent phosphorylation of p53 on Ser-20 is an important event in initiating doxycycline-induced apoptosis (18). However, the proapoptotic downstream target genes induced by NF-κB have not been identified. We hypothesized that one of the NF-κB-regulated genes that encode the key proteins involved in the phosphorylation of p53 on Ser-20 initiates apoptosis in response to an increased level of superoxide. In this report, we show that the expression of (Plk3) is induced by doxycycline, and that this induction depends on the activation of NF-κB. Plk3 is involved in the phosphorylation of p53 on Ser-20 in response to doxycycline stimulation. Overexpression of wild-type Plk3, but not the N-terminus-truncated Plk3 (Δ1-141), induced rapid apoptosis in p53^{+/+} cells, whereas apoptosis was delayed in p53^{−/−} cells expressing wild-type Plk3 and in p53^{+/+} cells expressing the kinase-defective mutant Plk3^{K91R}. siRNA mediated inhibition of Plk3 expression suggests that Plk3 is essential for superoxide-induced cell death and deletion analysis of Plk3 showed N-terminal domain (amino acid 1-26) is essential for induction of delayed onset of apoptosis. Taken together, our results suggest that Plk3 is an NF-κB-regulated kinase that mediates p53 dependent and independent proapoptotic response in reaction to elevated levels of superoxide.
Materials and Methods.

Reagents—Full coding region of human Plk3 (accession number BC013899) was cloned into pCMV-Tag2A (Stratagene, La Jolla, CA) expression vector by RT-PCR to generate Flag-tagged Plk3. The kinase-defective mutant Plk3$^{K91R}$ was generated by site-directed mutagenesis (23,24). The ATP-binding domain deletion mutant PLK3$^{\Delta N}$ (142-646 amino acid [aa]) was generated by Sca I restriction enzyme digestion. Plk3 N-terminal deletion mutant D52-107 was generated by Sfo-I and Bsg I digestion. Plk3 N-terminal deletion mutant D1-26 was generated by Srf I digestion. EGFP-fusion constructs were generated by cloning wild-type PLK3 or PLK3$^{\Delta N}$ into pEGFP-C1 (Clontech, Palo Alto, CA). The Plk3 promoter region was generated by PCR from BAC clone RP11-269F19 (Sanger Institute, Hinxton, U.K.) and cloned into pGL2-basic vector (Promega, San Luis Obispo, CA) for construction of the luciferase reporter construct Plk3p-Luc. The mutated κB site in the Plk3p-Luc-MT construct was generated by site-directed mutagenesis (Stratagene). Oligodeoxyribonucleotides were purchased from Sigma-Genosys (St. Louis, MO). Anti-NF-κB (p65), Plk3, Flag (M2), and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Affinity-purified p53 antibodies were obtained from Calbiochem (San Diego, CA). Antibodies specific for Ser-20-phosphorylated p53 were obtained from New England Biolabs (Beverly, MA). Purified p53 protein (1-342 aa) was obtained from Calbiochem. Plk3 oligonucleotides for RNA-mediated interference (RNAi) experiments were synthesized by Dharmacon (Lafayette, CO).

Cell cultures—The human tumor cell lines HEK293, HCT116, HCT116p53$^{-/-}$, MDAPanc-28/Puro, MDAPanc28/IκBαM, and WT and RelA$^{-/-}$ murine embryonic fibroblast (MEF) cell lines were previously described (5,25-27).
Electrophoretic Mobility Shift Assay (EMSA)—Preparation of nuclear extracts, EMSA, competition, and supershift assays were performed as described previously (28,29). The following $^{32}$P end-labeled DNA probes were used: HIVκB, 5'-AGTTGAGGGGACTTTCCCAGGC-3'; Plk3 WT κB, 5'-CCTGAGGTT $\text{GGAGTTCCAGACCAG}$-3'; Plk3 mutant κB, 5'-CCTGAGGTTAAAAGT TCCAGACCA G-3'; and Oct-1, 5'-TGTCAATGCAATACACTAG AA-3', for monitoring equal loading of nuclear extracts.

Northern Blot Analysis and RT-PCR—RNA isolation and Northern blot analysis were performed as previously described (30). The blots were hybridized with [α-$^{32}$P]-dCTP-labeled human Plk3 cDNA and GAPDH probes. RT-PCR analysis was performed with 28 PCR cycles using a pair of Plk3 primers (5'- CCTGCCGCCGGTTTCCTG-3', and 5'-AGCCTCAGGGCTTG GGTC-3') and separated on a 1% agarose gel.

Western Blot Analysis—Isolation of protein extracts and western blot analysis were performed as previously described using affinity-purified antibodies against p53, Ser-20-phosphorylated p53, Plk3, Flag (M2), and β-actin; with horseradish peroxidase-coupled goat anti-mouse IgG (Amersham) or rabbit anti-goat IgG (Dako, Copenhagen, Denmark) antibodies (18,27). The Lumi-Light western blot substrate (Roche) was used for detection.

Transient Transfection and Luciferase Assays—The Plk3 promoter firefly- and TK-Renilla-luciferase reporter gene plasmids were transfected into the HEK293, MDAPanc28, MDAPanc-28/IκBαM, and WT and RelA-null MEF cell lines by Fugene 6 (Roche) or HCT116 and HCT116p53−/− cells with the LipofectAmine methods (Invitrogen, Carlsbad, CA) as previously described (26). The activity of both the firefly and the Renilla luciferase, without or with
stimulation with doxycycline (50 µg/ml), was determined at 24 and 48 h; the experiments were performed in triplicate. The firefly luciferase activities were normalized to the activity of the Renilla luciferase, which served as an internal control.

siRNA Assay—The Plk3 small interfering RNA (siRNA) SMARTpool and nonspecific-pooled control (Dharmacon) were transfected into the indicated cells using TransIT-TKO transfection reagent according to the manufacturer’s protocol. Twenty-four hours after siRNA transfection, cells were treated with 50 µg/ml doxycycline for the indicated times and subjected to subsequent analyses.

Immunoprecipitation and in vitro Kinase Assay—The protein extracts (1 mg) were subjected to immunoprecipitation with 200 ng of anti-Flag (M2) antibody linked to agarose beads at 4°C overnight, followed by western blotting with anti-p53 and anti-Ser-20-phosphorylated p53 antibodies. For the kinase assay, the anti-Flag (M2) immunoprecipitates were incubated with purified p53 protein (1-342 aa) in 20 µl kinase buffer as previously described (27,31).

DNA Fragmentation—The level of DNA fragmentation in apoptotic cells was determined by gel electrophoresis. The cells were washed with PBS and incubated with DNA fragment isolation buffer (10 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100) for 30 min on ice. The extracts were centrifuged to remove the cell debris, and the low-molecular-weight DNA fragments were extracted from the supernatant with phenol/chloroform, precipitated with an equal volume of 2-propanol. The DNA pellets were washed with 75% ethanol, resuspended in TE buffer, and analyzed by separation on a 1.5% agarose gel.
**Immunocytochemistry and Fluorescence Microscopy**—HEK293 cells were seeded onto cover slips and transfected with EGFP-PLK3 or -PLK3ΔN fusion construct, or control vector using Fugene 6 (Roche). Twenty-four hours posttransfection, the cells were fixed with 4% paraformaldehyde in PBS followed by treatment with 0.2% Triton X-100 in PBS. Immunostaining of -γ-tubulin was performed using a Cy3-conjugate anti-γ-tubulin antibody (Sigma). The cover slips were mounted with the aqueous mounting medium ProLong Gold antifade reagent with DAPI (Molecular Probes) and analyzed by fluorescence microscopy. The images were captured on the Metamorph imaging system (Universal Imaging Corporation, Downingtown, PA).

**Colony formation assay**—Vector control plasmid and wild-type and mutated Plk3 expression plasmids were transfected into HEK293 cells by Fugene 6 (Roche), or HCT116 p53+/+ and HCT116p53−/− cells by lipofectamine 2000 (Invitrogen). After 24h transfection, cells were selected with 500µg/ml G418 for colony formation. Colonies were washed with PBS and stained with coomassie blue G-250. The number of colonies was directly counted. Alternatively, stained colonies were lysed with 1% SDS in PBS. Colony lysates were diluted in 0.1% SDS and measured the OD615 value.

**ChIP assay**—The chromatin immunoprecipitation (ChIP) assay was performed with a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, N.Y.) as previously described (18). Two PCR primers, 5′-TGCAATTCCCAGCCAGGCAAG-3′ and 5′-TGCAATTCCCAGCCAGGCAAG-3′, were used to amplify a 176-bp fragment, which corresponded to the predicted NF-κB enhancer region in the Plk3 promoter.
Results

*Doxycycline Induced-Plk3 Expression Is NF-κB-Dependent.* EMSAs, immunoblotting for IκBα degradation, RT-PCR, and northern blot analyses for Plk3 expression were performed using MDAPanc28/Puro and MDAPanc28/IκBαM cells stimulated with doxycycline (50 µg/ml) at the indicated times (Fig. 1). Within 24 h after doxycycline stimulation, both NF-κB DNA binding activity (Fig. 1a, lanes 1-10) and IκBα degradation (Fig. 1b, lanes 1-8) were detected in the MDAPanc28/Puro cells but not the MDAPanc28/IκBαM cells. The doxycycline-induced expression of Plk3 mRNA in MDAPanc28/Puro cells increased with the time of treatment and reached its maximum level by 48 h (Fig. 1c and d, lanes 1-4), whereas doxycycline-induced Plk3 expression in MDAPanc28/IκBαM cells was inhibited (Fig. 1c and d, lanes 5-8). Quantification of the induction of Plk3 expression is shown in Figure 1e. These results suggest that Plk3 is a target gene regulated by NF-κB.

*Plk3 expression is induced by various stimuli.* To determine whether Plk3 expression is induced by other NF-κB inducers in different cells, we stimulated HEK293/Puro and HEK293/IκBαM cells with doxycycline (50 µg/ml), MDAPanc-28/Puro and MDAPanc-28/IκBαM with TNF-α (10 ng/ml) and NIH3T3 cells with FGF-1 (20 ng/ml) and PMA (30 µg/ml) for various time point as indicated, and isolated total RNA for measuring the levels of Plk3 expression (Fig. 2 a-c). Within 12 hour of doxycycline stimulation of HEK293/Puro cells, the expression of Plk3 was induced and reached to maximal level at 48 hour of doxycycline stimulation (Fig. 2a, lanes 1-4). In contrast, the expression of Plk3 was not induced in the doxycycline-stimulated HEK293/IκBαM cells (Fig. 2a). These results are consistent with the doxycycline-induced Plk3
expression in MDAPanc-28 cells in Figure 1 c and d. In MDAPanc-28/Puro, but not in MDAPanc-28/IκBαM cells, the expression of Plk3 was induced by TNF-α stimulation. Interestingly, the TNF-α-induced Plk3 expression appears to be biphasic, which occurred at 1 and 24 hour of TNF-α-stimulation (Fig. 2 b). This is consistent with the previous reports demonstrating biphasic NF-κB activation (27,32,33). Figure 2 c shows that the expression of Plk3 is also induced by FGF-1 and PMA. These results suggest that the expression of Plk3 is induced by various NF-κB inducers in different cell lines.

**Plk3 Promoter Is Inducible With NF-κB.** To further investigate how NF-κB mediates Plk3 induction by doxycycline stimulation in MDAPanc28 cells, we next cloned the promoter (1.8 kb) of the human Plk3 gene from the BAC clone RP11-269F19 (accession number AL592166) from human chromosome 1, which contains the whole genomic sequence of Plk3. Sequence analysis revealed that the Plk3 promoter has a GC-rich region located from –78 bp to –328 bp upstream of the translation initiation site. Of all the 9 reported transcripts found in the nucleotide and EST sequence databases at NCBI, there are three mRNAs (accession numbers: BC013899, AJ293866, and NM_004073) and six ESTs (accession number: BM921223, BQ065567, BQ066297, BG437825, BF205939, and CD109602), with different length in the 5’ untranslated sequence initiated from the Plk3 promoter region (-63bp to –239 bp). These transcripts were possibly due to lack of definite transcription start site, a characteristic of the GC-rich promoter. A κB enhancer element (TGGGAGTTCC) from –1130 bp to –1121 bp was identified and other previously identified cis-regulatory elements, such as binding site for basal transcription factor Sp-1, are also present (Fig. 3 a). To confirm that the predicted κB motif in the Plk3 promoter is a bona fide NF-κB binding site, we performed EMSA for NF-κB DNA binding activity using 25-
bp oligonucleotides with a WT κB motif (5’-CCTGAGGTGGGAGTTCCA GACCAG-3’) and mutant motif (5’-CCTGAGGTAAAAGT TCCAGACCAG-3’). As shown in competition and anti-p65 antibody super-shift assays (Fig. 3 b), the predicted WT NF-κB binding motif, but not the mutant, bound to p65/p50 and p50/p50 NF-κB complexes. These data suggest that this is indeed an NF-κB DNA-binding site.

To determine whether p65/NF-κB binds to the κB site identified in the Plk3 promoter in vivo, we performed ChIP assays. As shown in Figure 3 c, the Plk3 promoter DNA sequence was amplified from the anti-p65/NF-κB chromatin immunoprecipitates in the PCR reaction using the specific PCR primers flanking the κB site in the Plk3 promoter region. The results suggest that p65/NF-κB directly interacted with the κB site in the Plk3 promoter region in MDAPanc-28/Puro cells, but this interaction was inhibited in MDAPanc-28/IκBαM cells. To determine whether the κB site identified in the Plk3 promoter is functional, we cloned the 1.8-kb promoter region of Plk3 into pGL2-basic luciferase reporter vector (Plk3pLucWT) and generated the mutated κB site in Plk3 promoter luciferase reporter vector (Plk3pLucMT). The luciferase reporter activity was significantly stimulated by doxycycline in MEF cells transfected with the Plk3pLucWT but not with the Plk3pLucMT reporter gene construct (Fig. 3 d). Stimulation with doxycycline induced high levels of luciferase activity in MDAPanc28/Puro and MEF cells, but not in MDAPanc28/IκBαM or RelA-null MEF cells, transfected with the Plk3pLucWT reporter gene construct (Fig. 3 e and f). These data indicate that the induction of Plk3 gene expression by doxycycline is likely mediated directly by NF-κB and the κB site present in the Plk3 promoter.

Doxycycline-Induced Phosphorylation of p53 on Ser-20 and Expression of p21waf1 Are Plk3-Dependent. We previously showed that the doxycycline-induced phosphorylation of p53 on Ser-
20 in MDAPanc28 cells is NF-κB dependent and doxycycline-induced apoptosis was detected at 48 h in HCT116 p53^{+/+} cells, but significantly delayed in HCT116 p53^{-/-} cells (18). To determine whether Plk3 is involved in the phosphorylation of p53 on Ser-20 after doxycycline stimulation, we inhibited Plk3 expression using Plk3 siRNA (Fig. 4a). Doxycycline-induced phosphorylation of p53 on Ser-20 and p21^{waf1} expression was completely inhibited (Fig. 4a and b). To confirm the involvement of Plk3 in the phosphorylation of p53, we performed immunoprecipitation assays using Flag-Plk3-transfected MDAPanc28/Puro cells without and with doxycycline stimulation. As shown in Figure 4c, doxycycline induced complex formation between Plk3 and p53. The in vitro kinase assay using Flag-Plk3 and Flag-Plk3^{K91R} immunoprecipitated from doxycycline-stimulated cells, with purified p53 protein (1-342 aa) as a substrate, suggest that Plk3 phosphorylated p53 on Ser-20 (Fig. 4d). Taken together, these results suggest that Plk3 is directly involved in the phosphorylation of p53 on Ser-20 response to doxycycline stimulation.

**Plk3 Induces p53-dependent and –independent Apoptosis.** Previously, we showed that NF-κB functioned as a proapoptotic factor by activating the p53-signaling pathway in response to superoxide (18). Superoxide-induced apoptosis was significantly delayed in p53-null HCT116 and MDAPanc-28/IκBαM cells, suggesting that NF-κB and p53 are involved in superoxide-induced apoptosis in these cells (18). To determine the role of Plk3, a downstream target gene regulated by NF-κB, in proapoptotic signaling cascade, we transfected Plk3 wild-type and mutants into HCT116p53^{+/+} or HCT116p53^{-/-} cells and measured the DNA fragmentation. Consistent with our previous finding (18), superoxide-induced apoptosis was delayed in the absence of p53 function in HCT116p53^{-/-} cells (Fig. 5a). Similarly, Plk3-mediated apoptosis was delayed in HCT116p53^{-/-} cells, but not delayed in HCT116p53^{+/+} cells (Fig. 5b). The
apoptosis induced by overexpression of a kinase defective Plk3 mutant (Plk3K91R) (mutated ATP-binding site in the Plk3 catalytic domain) in HCT116p53+/+ cells was also delayed, whereas an N-terminus deletion mutant (amino acid 1-141) of Plk3 (Plk3ΔN) failed to induce apoptosis in HCT116p53+/+ cells (Fig. 5c). These results suggest that Plk3- induced rapid onset of apoptosis is p53-dependent and Plk3-mediated delayed onset of apoptosis is p53-independent, and that Plk3 kinase activity may be partially involved in induction of apoptosis.

Plk3-Induced delayed onset of apoptosis is Independent of p53. To further determine the effects of Plk3 on p53-independent cell death, we performed colony formation assays. Overexpression of Plk3 and Plk3K91R, but not Plk3ΔN, in HEK293 cells completely inhibited colony formation (Fig. 6a, b). Overexpression of Plk3 greatly inhibited colony formation in both HCT116 p53+/+ and HCT116 p53−/− cells (Fig. 6c, d). The quantification of colony formation was summarized in Figure 6e and f. Overexpression of Plk3 in HEK293 cells significantly inhibited cell growth by inducing apoptosis and overexpression of Plk3K91R decreased but did not abolish this effect, whereas Plk3ΔN totally abolished this effect (Fig. 6h and i). These results are consistent with those of the colony formation assays (Fig. 6a-f). Taken together, these results further suggest that overexpression of Plk3 can mediate p53-independent apoptosis.

N-terminal domain of Plk3 is required for inducing apoptosis. To determine the domain in Plk3 that play an essential role in the delayed proapoptotic signaling cascade, we carried out mutagenesis of Plk3. Since the N-terminus deletion mutant (amino acid 1-141) of Plk3 (Plk3ΔN) failed to induce apoptosis in HCT116p53+/+ cells (Fig. 5c), we focused our analysis on the N-terminus of Plk3. The Plk3 deletion mutants generated were summarized in Figure 7a. Overexpression of wild-type Plk3 and Plk3 deletion mutant D52-107 inhibited colony formation...
(Fig. 7 c and d). On the other hand, overexpression of Plk3 deletion mutant D1-26 failed to inhibit colony formation (Fig. 7 b and e). Quantification of the colony formation is shown in Figure 7 f. Taken together, these results suggest that the N-terminal domain of 1-26 amino acid in Plk3 is required for its apoptotic function. Deletion of these 26 amino acids destroys three SH3 domains on Plk3, which in turn may inhibit crucial interactions between Plk3 and other proapoptotic signaling molecules.

Plk3 Is Essential in Doxycycline-Induced Apoptosis. To determine whether Plk3 play an essential role in doxycycline-induced apoptosis, we inhibited Plk3 expression in doxycycline-stimulated cells using Plk3 siRNA. In both HEK293 and MDAPanc-28/Puro cells, doxycycline-induced-apoptosis was inhibited by the transfection of Plk3 siRNA but not a control siRNA (Fig. 8 a and b). However, no apoptosis was detectable in TNF-α (50 ng/ml) treated MDAPanc-28/Puro cells (Fig. 8 c), even though Plk3 expression is induced by TNF-α (Fig. 2 b). Taken together, these data suggest that Plk3 plays a key role in doxycycline-induced apoptosis and ectopic expression of Plk3 induces apoptosis. Furthermore, the findings imply that whether the function of NF-κB is proapoptotic or antiapoptotic may depends on nature of stimuli and outcome of the expression of genes in response to such stimuli.

To determine whether the subcellular localization of Plk3 was involved in induction of apoptosis. Both EGFP -tagged wild-type Plk3 and -N-terminus deletion mutant of Plk3 (Plk3\textsuperscript{\textDelta N}) were transfected into HEK293 cells and analyzed by fluorescence microscopy. As shown in Figure 8 b, Plk3 mainly localized to cell membrane and a condensed spot in the transfected cell and much weaker diffuse distribution in the cytoplasm, whereas a very diffused distribution was found in the cells expressing EGFP- Plk3\textsuperscript{\textDelta N}. The control EGFP vector generated a diffused pattern. Double-labeling experiments using an anti-γ-tubulin antibody showed that Plk3 is
localized around centrosomes in a condensed spot. These subcellular localization patterns suggest that Plk3 may be involved in regulating the microtubule dynamics and dysregulation of Plk3 may induce mitotic catastrophe.

**Discussion**

Transcription factor NF-κB can regulate both proapoptotic and antiapoptotic signaling pathways; however, less is known about the mechanism, by which NF-κB induces apoptosis. Our results, summarized in Figure 8c, suggest a possible mechanism by which NF-κB regulates proapoptotic-signaling cascades by inducing Plk3 expression, which in turn activates p53-dependent and -independent apoptotic pathways in doxycycline/superoxide-induced cell death.

We previously described a possible role of NF-κB signaling cascades in doxycycline/superoxide-induced apoptosis and showed that doxycycline/superoxide-induced apoptosis is inhibited in cells lacking functional NF-κB activity and is partly dependent on NF-κB-mediated p53 activation (18). In that study, p53 was stabilized primarily by reducing Hdm2 full-length protein and by inducing a kinase to phosphorylate p53 protein at Ser-20, which in turn induced the expression of its downstream target genes, such as PUMA, for initiating proapoptotic signaling (18). These results suggest that NF-κB regulates a kinase that functions as a proapoptotic factor to activate the p53 signaling pathways. Previous studies have shown that Plk3 functionally links DNA damage to cell cycle arrest and apoptosis via the p53 pathway by interacting directly with p53 and phosphorylating p53 on Ser-20 in response to reactive oxygen species and irradiation (21,24,34). Our studies demonstrate the following findings: first, Plk3 is an NF-κB downstream target gene in response to doxycycline/superoxide stimulation; second, the promoter of the Plk3 gene contains a κB site that is directly involved in its induction by the
NF-κB complexes; third, after induction by doxycycline/superoxide, Plk3 forms a complex with p53 and may be directly involved in the phosphorylation of p53 on Ser-20; fourth, Plk3 plays a key role in doxycycline-induced apoptosis; and fifth, Plk3 induces cell death, possibly by inducing p53-dependent and -independent pathways.

Similar to growth-factor withdrawal, chemotherapeutic agents, radiation, cytokines and other apoptosis inducers, doxycycline induces apoptotic cell death with structural and morphologic features such as cell shrinkage, mitochondrial swelling, chromatin condensation, and DNA fragmentation (13-15). These agents have been evaluated in preclinical cancer models and have entered recent clinical trials in patients with malignant diseases (35-37). Our previous study showed that doxycycline induced the formation of superoxide, which may in turn induce NF-κB activation (18). In this report, we have shown that doxycycline/superoxide-induced apoptosis is partly mediated by phosphorylation of p53 on Ser-20 by Plk3.

A previous study showed that Plk3 acts downstream of both ATM and Chk2 in this important pathway of DNA damage-dependent activation of p53 and that Chk2 in fact stimulates Plk3 activity in response to DNA damage (38). Plk3 plays an important role in the regulation of microtubule dynamics and centrosomal function in the cell, and the deregulated expression of Plk3 results in cell cycle arrest and apoptosis (39). Plk3 has been shown to localize to the cellular cortex and to the cell midbody during exit from mitosis (40), a finding that is consistent with the notion that Plk3 plays a role in cytokinesis, and that suggests that altered expression of Plk3 interferes with cellular proliferation by impeding cytokinesis.

The expression of Plk3 is transiently induced by growth factors and cytokines and does not require protein synthesis for transcriptional activation (23). Three AU-rich elements (AREs), the most common RNA-destabilizing elements known in mammalian cells, are present in the 3'-untranslated regions of Plk3, and are also present in many labile mRNAs (41,42). These findings
suggest that the activity of Plk3 is transcriptionally and post-transcriptionally regulated. Our results further show that overexpression of Plk3 induces apoptosis, suggesting that transcriptional and post-transcriptional regulation of Plk3 plays a key role in the control of Plk3 function. However, the posttranslational regulation of Plk3 and the mechanisms by which Plk3 induces apoptosis still remain unclear.

In summary, our results suggest a mechanism by which NF-κB functions as a proapoptotic factor. In response to high levels of superoxide induced by doxycycline, NF-κB, a key regulator of the antiapoptotic pathway, is required to induce Plk3 to initiate apoptosis independent of p53 activity, possibly involving the SH3 domain in the N-terminus of Plk3, and to phosphorylate p53 protein at Ser-20, which in turn induces the expression of its downstream target genes, such as PUMA, to initiate proapoptotic signaling.

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Footnote

Abbreviations: Plk3, polo-like kinase 3; aa, amino acid; RNAi, RNA-mediated interference; MEF, murine embryonic fibroblast; EMSA, electrophoretic mobility shift assay; SiRNA, small interfering RNA. FGF-1, fibroblast growth factor-1; PMA, phorbol 12-myristate 13-acetate; Dox, doxycycline.
Figure Legends

**Fig. 1.** Doxycycline-induced Plk3 expression is preceded by increased NF-κB DNA-binding activity and degradation of IκBα.  
(a) Electrophoretic mobility shift assay (EMSA) of NF-κB DNA binding activity in the nuclei of MDAPanc28/Puro and MDAPanc28/IκBαM cells stimulated with doxycycline (50 µg/ml) for the indicated times.  p65/p50 and p50/p50 complexes are indicated.  Ten micrograms of nuclear extracts was used in this analysis using an HIV-κB probe.  Oct-1 probe was used as a loading control.  F.P., free probe.  
(b) Western blot analysis of IκBα degradation in MDAPanc28/Puro and MDAPanc28/IκBαM cells stimulated with doxycycline (50 µg/ml) for the indicated times.  Fifty micrograms of cytoplasmic protein was probed with anti-IκBα antibody.  Relative protein loading was shown using anti-β-actin antibody.  
(c) RT-PCR and (d) Northern blot analyses for Plk3 expression in MDAPanc28/Puro and MDAPanc28/IκBαM cells stimulated with doxycycline (50 µg/ml) for the indicated times.  Twenty micrograms of total RNA at the indicated time points was analyzed using a human Plk3 cDNA probe, and GAPDH probe was used as an internal control for RNA loading.  
(e) Fold induction of Plk3 expression by doxycycline as determined by phosphoimaging.

**Fig. 2.** Plk3 expression is induced by various stimuli.  
(a) Northern blot analyses for Plk3 expression in HEK293/Puro and HEK293/IκBαM cells stimulated with doxycycline (50 µg/ml) for the indicated times.  
(b) Northern blot analyses for Plk3 expression in MDAPanc28/Puro and MDAPanc28/IκBαM cells stimulated with TNF-α (10 ng/ml) for the indicated times.  Twenty micrograms of total RNA isolated from these cell lines at the indicated time points were analyzed using a human Plk3 cDNA probe and the levels of 28S and 18S ribosomal RNA were used as controls for RNA loading.  
(c) Western blot analyses for Plk3 expression in NIH3T3
cells stimulated with FGF-1 (20 ng/ml) and PMA (50 µg/ml) for the indicated times. Fifty micrograms of protein extracts were probed with anti-Plk3 antibody. The control for protein loading was determined by using anti-β-actin antibody.

**Fig. 3. Plk3 promoter structure and reporter gene assay.** (a) Schematic diagram of the Plk3 promoter, indicating the NF-κB DNA binding motif and GC-rich region. (b) Results of EMSA for the computer program-predicted NF-κB DNA motif. The nuclear extract of doxycycline-stimulated MDAPanc28/Puro cells (48 h) was used for the binding, binding competition, and supershift assays as indicated. S.S., supershift; N.S., nonspecific; F.P., free probe. (c) ChIP assays were performed using MDAPanc-28/Puro and MDAPanc-28/κBαM cells with and without anti-p65 (NF-κB) antibody and doxycycline stimulation as indicated. Luciferase reporter assays for the Plk3 promoter with WT and mutant κB binding motifs were performed in (d) WT-MEFs, (e) MDAPanc28/Puro and MDAPanc28/κBαM cells, (f) WT and RelA⁻/⁻ MEFs, using transient transfection of Plk3pLucWT or Plk3pLucMT without or with stimulation with doxycycline (50 µg/ml). Dox, doxycycline.

**Fig. 4. Doxycycline-induced phosphorylation of p53 on Ser-20 and the expression of p21waf1 are Plk3-dependent.** In MDAPanc28/Puro cells, Plk3 siRNA inhibited (a) doxycycline-induced Plk3 expression and phosphorylation of p53 on Ser-20 and (b) doxycycline-induced p21waf1 expression. MDAPanc28/κBαM cells were used as a control, and β-actin levels were analyzed as a control for protein loading. (c) Doxycycline stimulation-induced complex formation between Plk3 and p53. Flag-tagged Plk3 was immunoprecipitated from MDAPanc28/Puro cells without and with doxycycline stimulation using anti-Flag (M2) antibody linked to agarose beads.
The immunoprecipitates, eluted with Flag peptide, were resolved by SDS-PAGE and analyzed with anti-p53 antibody. (d) Involvement of Plk3 in the phosphorylation of p53 on ser-20. Phosphorylation of the purified p53 protein (1-342 aa) was analyzed in an *in vitro* kinase assay using immunoprecipitated Flag-tagged WT Plk3 and kinase-defective Plk3 (Plk3^K91R\(^{\text{\textregistered}}\)).

**Fig. 5. Plk3 induces p53-dependent and -independent apoptosis.** (a) Doxycycline-induced early onset of apoptosis is dependent on p53. HCT116p53^+/+ (lanes 1-4) and HCT116p53^-/- (lanes 5-8) cells were treated with 50 µg/ml of doxycycline for the indicated times. (b) Overexpression of Plk3 induced early onset of apoptosis in HCT116p53^+/+ cells (lanes 1-4) and delayed (lanes 5-8) onset of apoptosis in HCT116p53^-/- cells. (c) Overexpression of Plk3^K91R induced delayed onset of apoptosis in HCT116p53^+/+ cells (lanes 1-4) and overexpression of Plk3^ΔN in HCT116p53^+/+ cells (lanes 5-8) failed to induce apoptosis. HCT116p53^+/+ or HCT116p53^-/- cells were transfected with wild-type Plk3, Plk3^K91R, or Plk3^ΔN expression vector for the indicated times. DNA fragmentation assays were performed and DNA marker is shown.

**Fig. 6. Plk3-induced delayed onset of apoptosis is p53-independent.** (a and b) Colony formation of HEK293 cells transfected with control expression vector (Vec-CTL), Plk3 N-terminus deletion mutant (Plk3^ΔN\(^{\text{\textregistered}}\)), and kinase-defective Plk3 (Plk3^K91R\(^{\text{\textregistered}}\)). (c and d) HCT116 and HCT116p53^-/- cells transfected with control expression vector and WT Plk3. Colonies were stained and counted. (e) Summary of the colony formation counts in HEK293 cells transfected with control, WT, and mutant Plk3 expression vectors. (f) Summary of the colony formation counts in HCT116 and HCT116p53^-/- cells transfected with vector control (Vec-CTL) and WT Plk3 expression vectors. (g) HEK293 cells were transfected with control, WT and mutant Plk3 expression vectors as indicated. Forty-eight hours after transfection, the cells were harvested,
and the numbers of living cells were calculated using trypan blue exclusion staining. (i) Analysis of the effect of Plk3 on apoptosis by DNA fragmentation assay. HEK293 cells were transfected with WT or mutant Plk3 expression vectors as indicated (lanes 1-3). Forty-eight hours after transfection or stimulation, cells were harvested and DNA fragmentation assays performed.

**Fig. 7. N-terminal domain of Plk3 is required for inducing delayed onset of apoptosis.** (a) Schematic representations of the Plk3 wild-type and deletion mutants. The full-length of wild-type Plk3 protein and the locations of the deletions in the Plk3 mutants are indicated and numbered. Colony formation of HEK293 cells transfected with (b) control expression vector (Vec-CTL), (c) wild-type Plk3, and Plk3 N-terminus deletion mutants (d) D53-107 and (e) D1-26. (f) Quantification of the colony formation in HEK293 cells transfected with control, WT, and mutant Plk3 expression vectors in b-e.

**Fig. 8. Plk3 is essential in doxycycline-induced apoptosis.** (a-c) Analysis of the effect of Plk3 expression on apoptosis by siRNA using DNA fragmentation assay. HEK293 cells (a, lanes 1-4) and MDAPanc-28/Puro cells (b, lanes 1-4) were treated with 50 µg/ml doxycycline in the presence of control (CTL) and Plk3 siRNA. MDAPanc-28/Puro cells (c, lanes 1-4) were treated with TNF-α (50 ng/ml) in the presence of control (CTL) and Plk3 siRNA. Forty-eight hours after transfection or stimulation, cells were harvested and DNA fragmentation assays performed. (d) Colocalization of overexpressed Plk3 and centrosomes. EGFP control vector, EGFP-wt-PLK3, and EGFP-Plk3 N-terminus deletion mutant (Plk3ΔN) were transfected into HEK293 cells. Twenty-four hours after transfection, these cells were fixed, stained with anti-tubulin-γ antibody and DAPI, and visualized with fluorescence microscopy. (e) Proposed working model of the NF-κB-regulated proapoptotic signaling cascade. In response to doxycycline or other
stimulations, NF-κB induces Plk3 expression, which in turn activates p53-independent apoptotic signaling and p53-dependent apoptotic signaling by phosphorylation of p53 on Ser-20.
Figure 1. Li et al

| a | MDAPanc28/Puro | MDAPanc28/IkBαM |
|---|---|---|
| Dox | p65/p50 - p50/p50 - Oct-1 - | p65/p50 - p50/p50 - Oct-1 - |
| 0  | 12 | 24 | 48 | 72 | 0 | 12 | 24 | 48 | 72(h) |
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |

| b | MDAPanc28/Puro | MDAPanc28/IkBαM |
|---|---|---|
| Dox | IkBαM - IkBαM - | IkBαM - IkBαM - |
| 0  | 12 | 24 | 48 | 0 | 12 | 24 | 48 | 48(h) |
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |

| c | MDAPanc28/Puro | MDAPanc28/IkBαM |
|---|---|---|
| Dox | Plk3 | | |
| 0  | 12 | 24 | 48 | 0 | 12 | 24 | 48 | 48(h) |
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |

| d | MDAPanc28/Puro | MDAPanc28/IkBαM |
|---|---|---|
| Plk3 | GAPDH | | |
| 0  | 12 | 24 | 48 | 0 | 12 | 24 | 48 | 48(h) |
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |

| e | MDAPanc28/Puro | MDAPanc28/IkBαM |
|---|---|---|
| Plk3 mRNA level | | |
| 0  | 12 | 24 | 48 | 0 | 12 | 24 | 48 | 48(h) |
| 100 | 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 | 1000 |

Duration of Doxycycline Stimulation (h)
Figure 2. Li et al

**a**

|       | HEK293/Puro | HEK293/IκBαM |
|-------|-------------|--------------|
| Dox 0 |             |              |
| 12    |             |              |
| 24    |             |              |
| 48    |             |              |
| Plk3  |             |              |
| rRNA |             |              |

**b**

|       | MDAPanc28/Puro | MDAPanc28/IκBαM |
|-------|----------------|-----------------|
| TNFα 0 |                |                |
| 1     |                |                |
| 6     |                |                |
| 12    |                |                |
| 24    |                |                |
| 48    |                |                |
| Plk3  |                |                |
| rRNA |                |                |

**c**

|       | NIH/3T3 |
|-------|---------|
| FGF-1 |         |
| PMA   |         |
| Plk3  |         |
| β-actin |        |
Figure 3. Li et al

a) NF-κB

|        | Sp1-269 | Sp1-265 | +1 |
|--------|---------|---------|----|
| TGGAGTTCC | GGGGCAGGCC | GAGGCGGGGT |
| -1927 -1130 -1121 | -278 -256 |
| GC-rich region | ATG |

b) p65 Peptide

|        |
|--------|
| Anti-p65 Ab |
| Cold WT-oligo |
| Cold MT-oligo |
| MT probe |
| WT probe |
| Dox |
| p65 S.S. |
| p65/p50 |
| p50/p50 |
| N.S. |
| F.P. |

---

c) MDAPanc28/Puro

|        | MDAPanc28/IκBαM |
|--------|----------------|
| Anti-p65 |
| Dox. |
| CHIP |
| Input |

---

d) WT-MEFs

WT-MEFs | Plik3P-Luc WT | Plik3P-Luc MT

---

e) Plik3P-Luc WT

MDAPanc28/Puro | MDAPanc28/IκBαM

---

f) Plik3P-Luc WT

Time (h) | WT-MEFs | RelA−/− MEFs

|        |
|--------|
Figure 4. Li et al

|                  | MDAPanc28/Puro | MDAPanc28/\(\kappa\)B\(\alpha\)M |
|------------------|----------------|----------------------------------|
| Plk3 siRNA/Dox  | -              | +                                |
| Plk3             | +              | +                                |
| Ser-20p-p53      | +              | +                                |
| \(\beta\)-actin  | +              | -                                |

1 2 3 4 5

|                  | MDAPanc28/Puro | MDAPanc28/\(\kappa\)B\(\alpha\)M |
|------------------|----------------|----------------------------------|
| Plk3 siRNA/Dox  | -              | +                                |
| p21\(\text{waf1}\) | +              | -                                |
| \(\beta\)-actin  | +              | +                                |

1 2 3 4 5

|                  |                  |                  |
|------------------|------------------|------------------|
| Flag-Plk3/Dox    | Flag-Plk3\(K91R\) | Flag-Plk3\(K91R\) |
| Flag             | +                | +                |
| p53              | +                | +                |
| IB               | Flag             | Flag             |

1 2 3

|                  |                  |                  |
|------------------|------------------|------------------|
| Input p53        | Plk3            | Plk3\(K91R\)    |
| +                | +                | +                |
| Dox              | Plk3            | Plk3\(K91R\)    |
| +                | +                | +                |
| Phos-p53         | Flag            | Flag             |

1 2
Figure 5. Li et al

(a) HCT116p53+/+ and HCT116p53-/- cells treated with Dox at 0, 24, 48, and 72 hours.

(b) HCT116-p53+/+ and HCT116-p53-/- cells transfected with Vec or Plk3-WT at 24, 48, and 72 hours.

(c) HCT116-p53+/+ cells transfected with Vec, Plk3K91R, and Plk3ΔN at 24, 48, and 72 hours.
Figure 6. Li et al

(a) HEK293 cells
Vec-CTL  Pik3\(^{\Delta N}\)

(b) Pik3\(^{K91R}\)  Pik3 WT

(c) HCT116 cells
Vec-CTL  Pik3 WT

(d) HCT116p53\(^{-/-}\) cells
Vec-CTL  Pik3 WT

(e) Colony Numbers
\[
\begin{align*}
\text{Vec-CTL} & \quad \Delta N & \quad K91R & \quad WT \\
\hline
\text{Pik3} & \quad 400 & \quad 300 & \quad 200 & \quad 100 & \quad 0
\end{align*}
\]

(f) Colony Numbers
\[
\begin{align*}
\text{Vec-CTL} & \quad \text{Pik3} & \quad \text{Vec-CTL} & \quad \text{Pik3} \\
\hline
\text{HCT116} & \quad 500 & \quad 400 & \quad 300 & \quad 200 & \quad 100 & \quad 0 \\
\text{HCT116p53\(^{-/-}\)} & \quad 400 & \quad 300 & \quad 200 & \quad 100 & \quad 0
\end{align*}
\]

(h) Relative Cell Numbers
\[
\begin{align*}
\text{Vec-CTL} & \quad \Delta N & \quad K91R & \quad WT \\
\hline
\text{HEK293} & \quad 120 & \quad 90 & \quad 60 & \quad 30 & \quad 0
\end{align*}
\]

(i) Pik3  WT  K91R  \(\Delta N\)

1  2  3
Figure 7. Li et al
Figure 8. Li et al

**a**

Pik3 SIRNA
CTL SIRNA
Dox.

**b**

Pik3 SIRNA
CTL SIRNA
Dox.

**c**

Pik3 SIRNA
CTL SIRNA
TNF-α

**d**

EGFP
γ-Tublin
DAPI
Overlay

**e**

Doxycycline

Superoxide

NF-κB/IκBα

Survival

p53

Pik3

Apoptosis
Function of polo-like kinase 3 in NF-κB-mediated proapoptotic response
Zhongkui Li, Jiangong Niu, Tadashi Uwagawa, Bailu Peng and Paul J. Chiao

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