Molecular Cell
Article

SUMOylation-Dependent Localization of IKKε in PML Nuclear Bodies Is Essential for Protection against DNA-Damage-Triggered Cell Death

Florian Renner,[1] Rita Moreno,[1] and M. Lienhard Schmitz[1,*]
[1]Medical Faculty, Institute of Biochemistry, Friedrichstrasse 24, Justus-Liebig-University, 35392 Giessen, Germany
*Correspondence: lienhard.schmitz@biochemie.med.uni-giessen.de
DOI 10.1016/j.molcel.2010.01.018

SUMMARY

The IKK-related kinase IKKε contributes to the antiviral response and can function as an oncogene that is frequently amplified in breast cancer. Here we report on an additional role of IKKε as a mediator protecting from DNA-damage-induced cell death. Genotoxic stress allows for kinase-dependent entry of IKKε into the nucleus, where IKKε-dependent PML phosphorylation is a prerequisite for retention of this kinase in PML nuclear bodies. Within these subnuclear structures IKKε inducibly colocalizes with TOPORS, which functions as a SUMO E3 ligase mediating SUMOylation of IKKε at lysine 231. SUMO modification of IKKε is required to trigger phosphorylation of nuclear substrates including NF-κB p65, thereby contributing to the antiapoptotic function of NF-κB in response to DNA damage.

INTRODUCTION

DNA-strand breaks are generated during several physiological processes such as replication or lymphocyte development, but also by genotoxic agents (Bredemeyer et al., 2008; Harper and Elledge, 2007). Several mechanisms have evolved to cope with dangerous DNA insults. A signaling pathway called the DNA-damage response (DDR) senses DNA lesions and initiates either cell-cycle arrest and DNA repair or alternatively apoptosis when the damage is severe (Vousden, 2006). DNA double-strand breaks (DSBs) are recognized by multiprotein complexes that recruit protein kinases such as ataxia telangiectasia-mutated (ATM), which phosphorylates numerous substrates in the cell and thereby triggers the NF-κB and the p53 signaling pathways (Lavin, 2008; Wu and Miyamoto, 2007; Wu et al., 2006). One of the p53 target genes is promyelocytic leukemia protein (PML), a tumor suppressor protein that occurs in differentially spliced isoforms (de Stanchina et al., 2004; Shen et al., 2006) and nucleates the assembly of PML nuclear bodies (PML-NBs). These subnuclear structures harbor several constitutive and transient residents and have been implicated in a variety of biological functions including the regulation of apoptosis and senescence (Bernardi and Pandolfi, 2007). PML-NBs have been proposed to function as DNA-damage sensors, as DSBs cause an increase in PML-NB size and number (Dellaire et al., 2006). PML can be conjugated to small ubiquitin-related modifier (SUMO), a small protein that is attached to the ε-amino group of lysines by an ATP-consuming enzymatic reaction that employs E1, E2, and E3 enzymes (Boddy et al., 1996; Meulmeester and Melchior, 2008; Yeh, 2009). SUMO E3 ligases are structurally unrelated proteins that interact with the E2 enzyme ubiquitin-conjugating enzyme 9 (Ubc9) and SUMO to increase the rate of substrate modification (Johnson and Gupta, 2001). Several isopeptidases cleave SUMO from its target proteins, thus rendering SUMOylation a highly dynamic process. PML can bind all three SUMO isoforms in a covalent and noncovalent manner, the latter being important for the physical integrity of PML-NBs. Association of SUMO with specialized SUMO interaction motifs (SIMs) allows the generation of protein/protein interaction networks that ensure the integrity of PML-NBs and constitute a nucleation event for subsequent recruitment of further SUMOylated proteins (Hecker et al., 2006; Shen et al., 2006). Besides SUMOylation, the function of PML is also steered by phosphorylation, which can be exerted by several kinases including ATM and Rad3 related (ATR) and homeodomain-interacting protein kinase 2 (HIPK2) (Dellaire et al., 2006; Gresko et al., 2009).

One of the DDR-induced transcription factors is NF-κB, which functions as a central cellular stress sensor that is activated by adverse conditions (Hayden and Ghosh, 2008; Janssens et al., 2005; Mabb et al., 2006). As DNA-damage-activated NF-κB serves to allow for cell survival, it is an important factor accounting for chemotherapy resistance during cancer treatment. Despite this high clinical relevance, the signaling pathways leading to NF-κB activation during the DDR remain incompletely defined. All NF-κB-activating signaling pathways share a critical step that involves the proteasome-dependent generation of DNA-binding subunits (Hacker and Karin, 2006; Hayden and Ghosh, 2008). Induction of DSBs leads in parallel to the activation of ATM and to the nuclear accumulation of the adaptor protein NF-κB essential modulator (NEMO). SUMOylation of nuclear NEMO is enhanced by the SUMO E3 ligase protein inhibitor of activated STAT y (PIASy) and by p53-inducible death domain-containing protein (PIDD) (Janssens et al., 2005; Mabb et al., 2006). ATM phosphorylates NEMO at serine 85, resulting in NEMO monoubiquitination at the lysines that were conjugated to SUMO before (Huang et al., 2003; Wu et al., 2006). The ATM/NEMO complex subsequently translocates to the cytoplasm and allows for activation of the IκB kinase (IKK) complex. The IKK

complex consists of the adaptor NEMO and the catalytically active kinases IKKα and IKKβ. The activated IKK complex mediates phosphorylation of the inhibitory IκB proteins, which in turn allows for ubiquitin/proteasome-dependent elimination of IκB and inducible DNA binding of NF-κB (Habraken and Piette, 2006; Janssens and Tschopp, 2006). Five different NF-κB DNA-binding subunits assemble in various dimer combinations, but the p50/p65 heterodimer is the most frequently detected form. NF-κB activity is regulated not only by controlled liberation from the IκB proteins, but also by posttranslational modification of the DNA-binding subunits by phosphorylation, acetylation, and ubiquitination (Neumann and Naumann, 2007; Perkins, 2006). A number of p65 phosphorylation sites have been revealed such as serine 468, which is contained in a C-terminal transactivation domain. Inducible phosphorylation of this site is exerted by IKKβ and the noncanonical IKK family member IKKε, also called IKKi (Mattioli et al., 2006; Schwabe and Sakurai, 2005). Besides its function as a NF-κB kinase, IKKε has been identified as an important mediator of the antiviral interferon response (Chau et al., 2008; Fitzgerald et al., 2003; Matsui et al., 2006; Sharma et al., 2003) and as a breast cancer oncogene (Adli and Baldwin, 2006; Boehm et al., 2007).

Here we reveal the IKK-related kinase IKKε as a further member of the NF-κB activation cascade in response to DNA damage. DNA-strand breaks allow for inducible SUMOylation of IKKε, which is essential for its localization in PML-NBs. Nuclear IKKε specifies antiapoptotic gene expression upon phosphorylation of nuclear substrates such as NF-κB p65.

RESULTS

SUMOylation of IKKε at Lysine 231 Depends on Its Kinase Activity

In a screen for SUMO substrates in the NF-κB pathway, we identified IKKε to be modified by SUMOylation. HEK293T cells were transfected to express Flag-tagged IKKε either alone or along with plasmids encoding SUMO-1 tagged with His_6 or green fluorescent protein (GFP). Equal amounts of protein in cell lysates were separated by SDS-PAGE and subsequently analyzed by western blotting. These experiments showed that coexpression of His_6- or GFP-tagged SUMO-1 caused upshifted IKKε bands at the expected molecular weights, while a conjugation-defect SUMO-1 point mutant was not attached to IKKε (Figure 1A). Accordingly, SUMO modification also occurred for immunoprecipitated IKKε (see Figure S1A available online). Further experiments showed that the expression of SUMO-2 and SUMO-3 can lead to the SUMOylation of IKKε as well (Figure S1B), thus revealing that all three SUMO isoforms can be attached to this kinase. In order to identify additional components of the cellular machinery regulating IKKε SUMOylation, further experiments were conducted. Expression of the SUMO E2 protein Ubc9 resulted in an increased IKKε SUMOylation, while the isopeptidase sentrin-specific protease 1 (SENP1) caused the deconjugation of SUMO from IKKε (Figure 1B). To investigate a possible relevance of the IKKε kinase activity for its SUMOylation, the wild-type form was compared to point mutants that are either totally inactive (IKKε-K38A and IKKε-S172A) or partially active (IKKε-F40C and IKKε-F40I), as confirmed in control experiments determining their differential abilities to phosphorylate their substrate protein NF-κB p65 (Figure S1C). Analysis of IKKε SUMOylation after coexpression of the respective IKKε variants with His_6-SUMO revealed a strict correlation between kinase activity and SUMOylation (Figure 1C). To map the SUMOylation site, charge-conserving lysine to arginine IKKε point mutants were tested for SUMOylation. These experiments revealed largely absent IKKε SUMOylation for the K231R point mutant (Figure 1D) even after enrichment of SUMOylated proteins on Ni-NTA columns (Figure S1D). In order to ensure that absent SUMOylation of IKKε-K231R is not due to a defect kinase function, we compared its activity to that of the wild-type kinase in reporter gene assays. As IKKε induces interferon β expression (Fitzgerald et al., 2003; Sharma et al., 2003), the mutants were coexpressed with an interferon β promoter-controlled luciferase reporter gene. Luciferase assays showed that the SUMOylation-defective IKKε-K231R was as active as the wild-type kinase, while the kinase-inactive IKKε-K38A mutant failed to trigger gene expression (Figure 1E), thus identifying lysine 231 as a bona fide SUMOylation site.

DNA-Damage-Inducible SUMOylation of IKKε Depends on TOPORS

In the search for a physiologically relevant stimulus regulating the IKKε SUMOylation status, no changes were detected in response to most identified regulators of IKKε activity including lipopolysaccharide, double-stranded RNA, or phorbol ester (data not shown). By contrast, induction of single-strand DNA breaks by the DNA topoisomerase I inhibitor camptothecin or of DSBs by the DNA topoisomerase II inhibitors doxorubicin or etoposide (ETO) allowed a robust induction of IKKε SUMOylation (Figure 2A). Immunofluorescence experiments revealed that ETO treatment triggered an accumulation of endogenous IKKε in nuclear bodies (Figure 2B). To reveal a possible contribution of IKKε SUMOylation to this assembly in subnuclear structures, the localization of wild-type IKKε was compared to that of kinase-inactive K38A and SUMOylation-defective K231R point mutants. IKKε-deficient mouse embryonic fibroblasts (MEFs) were reconstituted to express the various IKKε mutants at physiological levels and remained either untreated or were stimulated with ETO. Immunofluorescence analysis showed that the wild-type kinase faithfully recapitulated the ETO-induced recruitment of endogenous IKKε to nuclear bodies, while both point mutants entirely remained in the cytosol (Figure 2C). Is SUMOylation of IKKε sufficient to trigger accumulation in nuclear bodies? To test this possibility, *IKKε*$^{-/-}$ MEFs were reconstituted with expression vectors encoding Flag-SUMO-1-IKKε or two variants thereof that were point mutated either in lysine 38 or the SUMO acceptor lysine 231. Analysis of the intracellular localization showed that SUMO fusion was sufficient to cause nuclear body localization of SUMO-IKKε and SUMO-IKKε-K231R even in untreated cells. In contrast, the SUMO-IKKε-K38A mutant stayed exclusively cytosolic even after ETO treatment (Figure 2D). These experiments suggest that the kinase function of IKKε is a prerequisite for its nuclear import, while the SUMOylation of IKKε serves to allow localization in nuclear bodies. In support of this notion, forced SUMOylation of IKKε upon expression of GFP-SUMO-1 and the conjugating E2 protein Ubc9 was

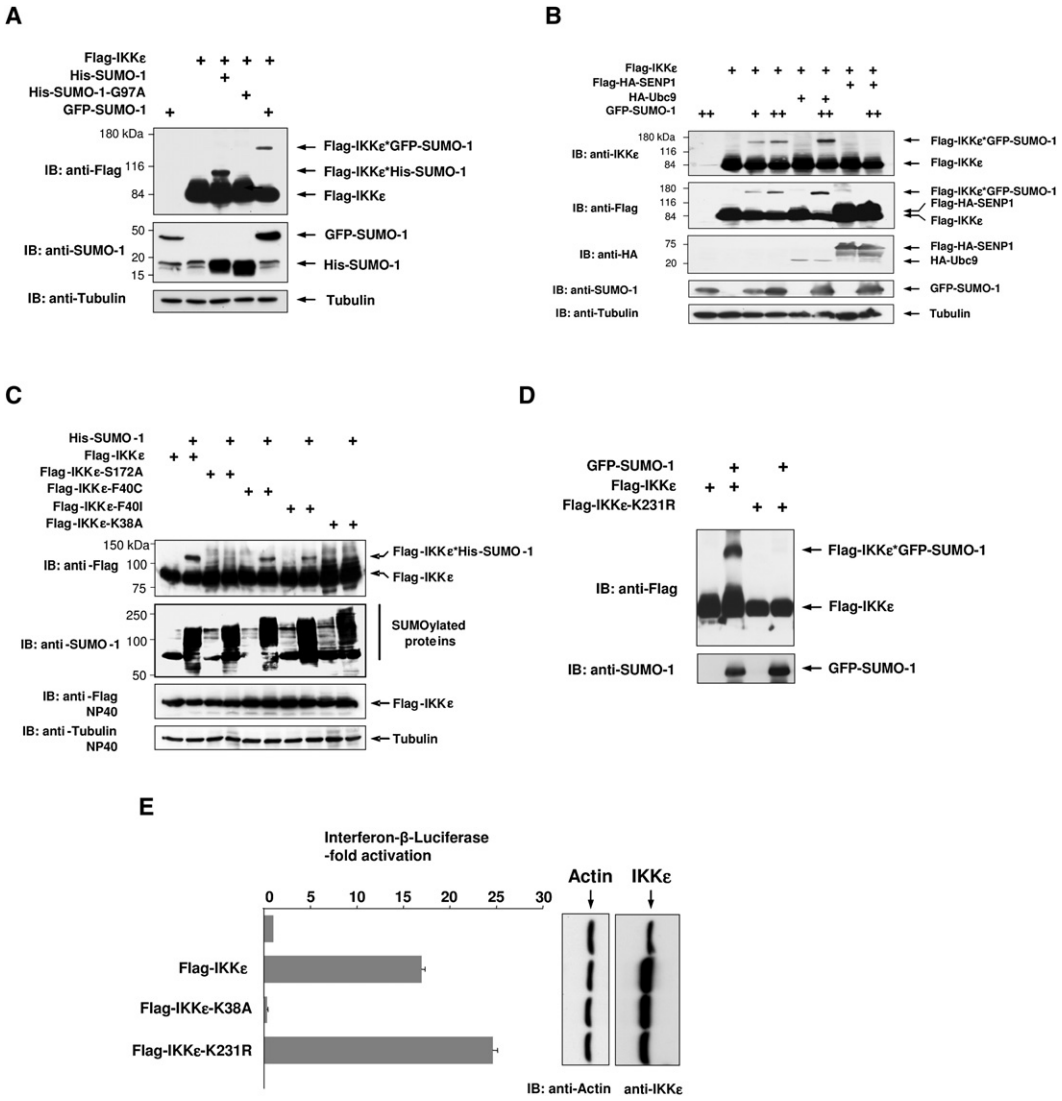

Figure 1. SUMOylation of IKKε

(A) HEK293T cells were transfected with expression vectors as shown and lysed in 1× SDS buffer to maintain SUMOylation. Equal amounts of proteins were analyzed for the occurrence and SUMOylation of IKKε by immunoblotting (IB). Molecular weight markers are displayed on the left of the blots.

(B) Epitope-tagged versions of IKKε, Ubc9, SENP1, and SUMO-1 were coexpressed in various combinations as displayed and analyzed by western blotting for IKKε SUMO modification.

(C) IKKε and the indicated point-mutated versions thereof were expressed in HEK293T cells together with His_6-tagged SUMO-1 and analyzed by immunoblotting for IKKε SUMOylation.

(D) IKKε and IKKε-K231R were coexpressed with GFP-SUMO-1 as shown and tested for SUMO modification of IKKε by immunoblotting.

(E) HEK293T cells were transfected with expression vectors encoding IKKε or variants thereof along with an interferon-β (IFN-β)-dependent firefly luciferase reporter gene. A fraction of the extracts was analyzed for adequate expression of IKKε variants by western blotting as shown; another fraction was tested for luciferase activity. Values are expressed as fold induction relative to cells transfected only with the reporter gene. Error bars depict standard deviations from three different experiments.

sufficient to cause IKKε relocalization to nuclear bodies (Figure S2). We then conducted a screen in order to identify the cognate SUMO E3 ligase for IKKε. SUMOylation remained unchanged in the presence of the SUMO E3 ligases polycomb 2 (Pc2), protein inhibitor of activated STAT 1 (PIAS1), PIASxα, PIASxβ, PIASy, TNF receptor-associated factor 7 (TRAF7), and the SUMO E3 domain of Ran-binding protein 2 (RanBP2). In contrast, the SUMO E3 ligase topoisomerase I-binding arginine serine-rich protein (TOPORS) enhanced IKKε SUMOylation in a dose-dependent manner (data not shown). Mapping experiments showed that the TOPORS domain containing E3 ligase function localizes to the region between amino acids 437 and 555, while a more C-terminal fragment encompassing amino acids 491–574 acts as an inactive mutant (Figure 3A), which is in line with the previously reported inactivity of this region for p53 SUMOylation (Weger et al., 2005). A comparison of the

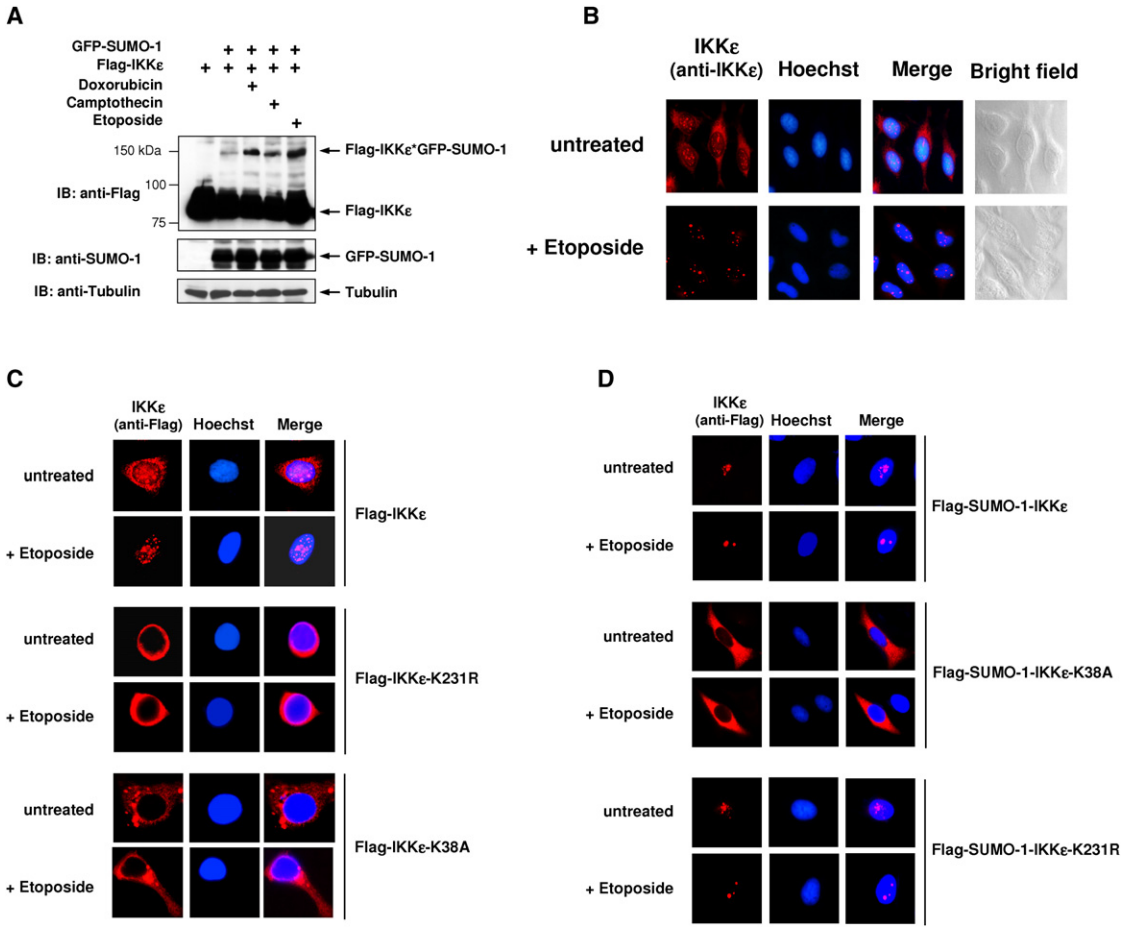

Figure 2. DNA-Damage-Inducible Translocation of IKKε to Nuclear Bodies

(A) Cells expressing IKKε and GFP-SUMO-1 were treated for 8 hr with camptothecin (2 μM), ETO (2 μM), or doxorubicin (0.25 μg/ml) and analyzed for SUMO modification of IKKε by western blotting.

(B) HeLa cells were stimulated with ETO as shown, followed by the analysis of IKKε localization with the indicated antibodies using indirect immunofluorescence. Nuclear DNA was visualized by Hoechst staining, and whole cells are displayed in bright field pictures. The merge shows an overlay between IKKε and Hoechst staining.

(C) IKKε-deficient MEFs were reconstituted to express moderate amounts of the indicated IKKε variants and treated with ETO as shown. Localization of proteins was revealed by immunofluorescence.

(D) *IKKε*$^{-/-}$ MEFs were reconstituted with expression vectors encoding Flag-SUMO-1-IKKε or two variants thereof that were point mutated either in lysine 38 or the SUMO acceptor lysine 231. Cells were left untreated or exposed to genotoxic stress as shown. The subcellular localization of the fusion proteins was visualized by indirect immunofluorescence. Nuclear DNA was revealed by Hoechst staining, and an overlay of both pictures is shown in the right part. All cells analyzed by immunofluorescence were treated with ETO (2 μM) for 8 hr.

intracellular localization of IKKε and TOPORS revealed only a partial overlap in untreated cells, while the induction of DSBs resulted in a largely overlapping localization of both proteins in nuclear speckles (Figure 3B). As SUMO E3 ligases bind their substrate proteins (Meulmeester and Melchior, 2008), a possible interaction between the endogenous IKKε and TOPORS proteins was tested by coimmunoprecipitation experiments. ETO-treated or control cells were lysed, followed by immunoprecipitation of IKKε from nuclear extracts. Western blotting allowed us to detect strongly increased amounts of TOPORS in IKKε immunoprecipitates from ETO-treated cells (Figure 3C), thus revealing an inducible interaction between both proteins. In a next step, the role of TOPORS for SUMOylation of the endogenous IKKε protein was investigated. Most of the SUMO-modified endogenous IKKε protein was found in the insoluble nuclear fraction of cell lysates (data not shown), raising the need to employ a purification procedure under denaturing conditions. Cells were transfected with a vector directing the synthesis of a TOPORS-specific shRNA or with the empty vector as a control along with a vector encoding His_6-tagged SUMO-1. Cells were treated with ETO and exposed to a protein crosslinker in order to stabilize the SUMOylated form, followed by lysis of the insoluble pellet containing SUMOylated IKKε under denaturing conditions. SUMOylated IKKε was enriched on Ni-NTA agarose columns and the eluted proteins were further analyzed by immunoblotting. These experiments showed that ETO-induced SUMO

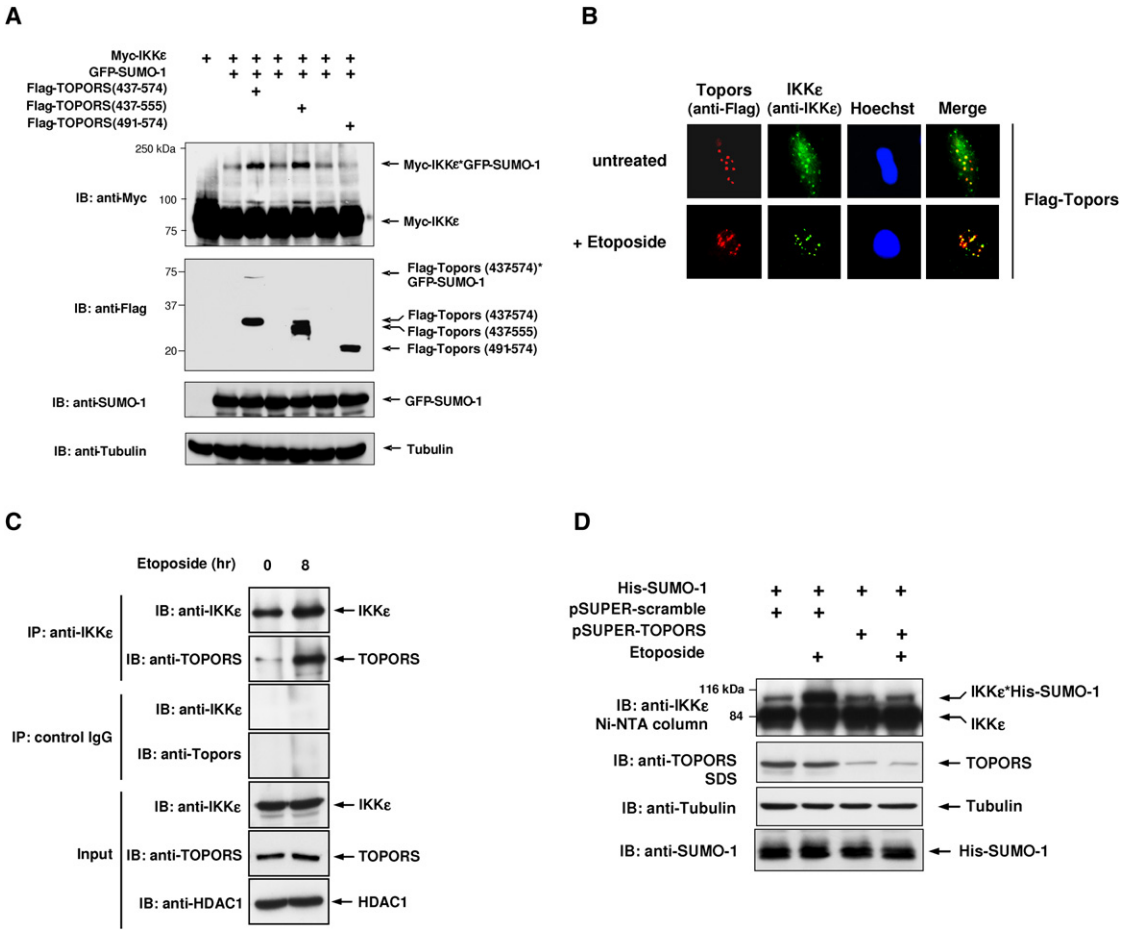

Figure 3. DNA-Damage-Inducible SUMOylation of IKKε Depends on TOPORS

(A) Active or inactive TOPORS fragments were coexpressed with IKKε and GFP-SUMO-1 as shown, followed by the analysis of IKKε SUMOylation via immunoblotting.

(B) HeLa cells transiently expressing Flag-tagged TOPORS were treated with ETO and analyzed for the subcellular localization of TOPORS or IKKε as displayed.

(C) HeLa cells were treated with 2 μM ETO as shown, followed by cell lysis and preparation of nuclear extracts. Equal amounts of IKKε in nuclear extracts were either tested for adequate protein expression, or used for immunoprecipitation (IP) with anti-IKKε or control antibodies. After elution of bound proteins in 1× SDS sample buffer, coprecipitated TOPORS was visualized by immunoblotting as shown.

(D) Cells were transfected with expression vectors for a TOPORS-specific shRNA and His_6-tagged SUMO-1 as shown. After 2 days, cells were treated for 8 hr with ETO (2 μM) and 60 min with a cell-permeable crosslinker. The insoluble pellets were lysed in SDS sample buffer or in guanidine-HCl containing buffer allowing the enrichment of the SUMOylated kinase on Ni-NTA columns. The proteins from the indicated fractions were further analyzed by immunoblotting.

modification of endogenous IKKε depends on the TOPORS protein (Figure 3D).

Molecular Mechanisms of DNA-Damage-Inducible Accumulation of IKKε in PML-NBs

TOPORS mainly localizes to PML-NBs (Rasheed et al., 2002), raising the possibility that IKKε also is recruited to this class of subnuclear structures. To address this question, the localization of IKKε was investigated in cells expressing the PML splice variant PML-IV, which has the ability to recruit other proteins to PML-NBs (Bischof et al., 2002). Immunofluorescence analysis showed that expression of PML-IV caused translocation of IKKε to PML-NBs even in untreated cells (Figure 4A), thus revealing this kinase as an additional transient component of these subnuclear substructures. The importance of PML was also evident by a loss-of-function approach. Expression of a PML-specific shRNA precluded ETO-induced recruitment of IKKε to PML-NBs, emphasizing the relevance of PML in an independent experimental setting (Figure 4B). To study the colocalization of the endogenous proteins, we used a preextraction procedure in order to allow visualization of the TOPORS protein in nuclear bodies. In untreated cells, PML and TOPORS showed only a marginal colocalization with IKKε. In contrast, ETO treatment resulted in the induction of PML phosphorylation and the complete accumulation of IKKε and TOPORS in PML-NBs (Figure 4C).

To investigate a contribution of IKKε to PML phosphorylation, cells were transfected to express PML-IV together with IKKε and IKKε-K38A, respectively. Subsequent immunoblot experiments using an antibody that we raised to specifically recognize PML phosphorylated at serine 38 (Gresko et al., 2009) showed that

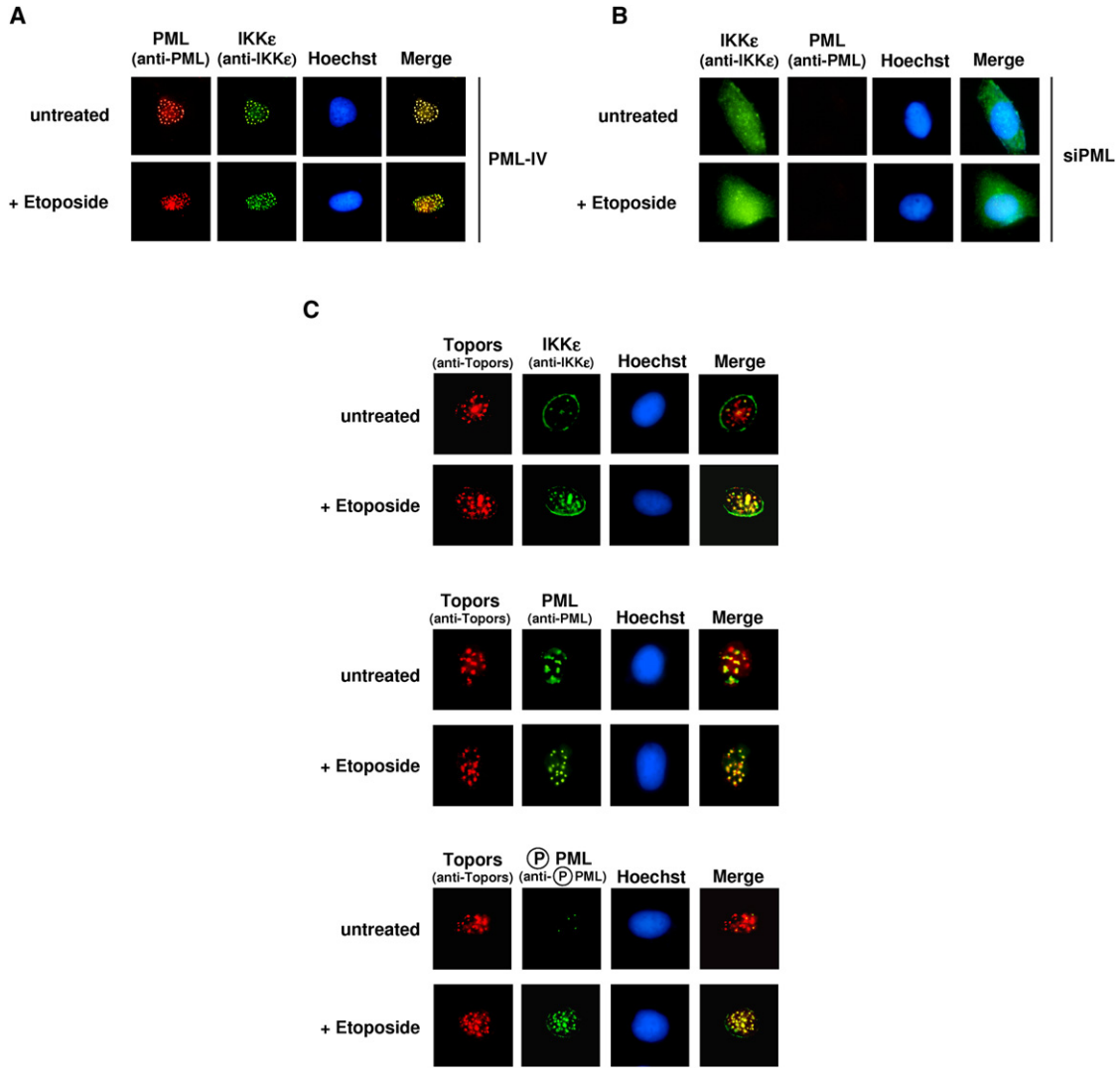

Figure 4. DNA Damage Triggers Recruitment of IKKε to PML-NBs

(A) HeLa cells were transfected to express PML-IV and treated with ETO as shown, followed by the detection of endogenous IKKε (green) and PML-IV (red) using immunofluorescence microscopy.

(B) HeLa cells transfected with a plasmid to express a PML-specific shRNA were treated with ETO and analyzed for subcellular localization of IKKε.

(C) HeLa cells were left untreated or stimulated with of ETO as shown. Cytoplasmic and nucleoplasmic background signals obtained with the TOPORS antibody were reduced upon pretreatment with a cytoskeletal stripping buffer before fixation. Localization of the indicated proteins was revealed by indirect immunofluorescence. All stimulations were performed with 2 μM ETO for 8 hr.

IKKε expression results in PML phosphorylation (Figure 5A). However, in vitro kinase assays with purified proteins revealed that IKKε does not phosphorylate PML directly (Figure S3A), thus suggesting that IKKε employs a downstream kinase to cause PML phosphorylation. The functional relevance of PML phosphorylation for its IKKε-recruiting ability was investigated upon expression of a PML-IV variant that is mutated in serine 38 and two neighboring residues that can be targeted by compensatory phosphorylations after mutation of serine 38 (Gresko et al., 2009). Immunofluorescence analysis showed adequate localization of the PML-IV phosphomutant in PML-NBs but a strongly impaired retention of IKKε in PML-NBs (Figure 5B). To test the potential role of SUMO/SIM interactions for the recruitment step, it was then interesting to investigate the ability of the SUMOylation-deficient PML-IV mutant to mediate PML-NB localization of endogenous IKKε. These experiments revealed the strict dependence of PML SUMOylation for the retention of IKKε in PML-NBs (Figure 5C). Collectively, these experiments show the necessity of PML phosphorylation and SUMOylation for the accumulation of IKKε in PML-NBs. In further experiments, we observed elevated PML levels upon overexpression of IKKε (data not shown), but the occurrence of this effect also for the phosphorylation-deficient PML variant (Figure S3B) excludes any relevance of this phosphorylation

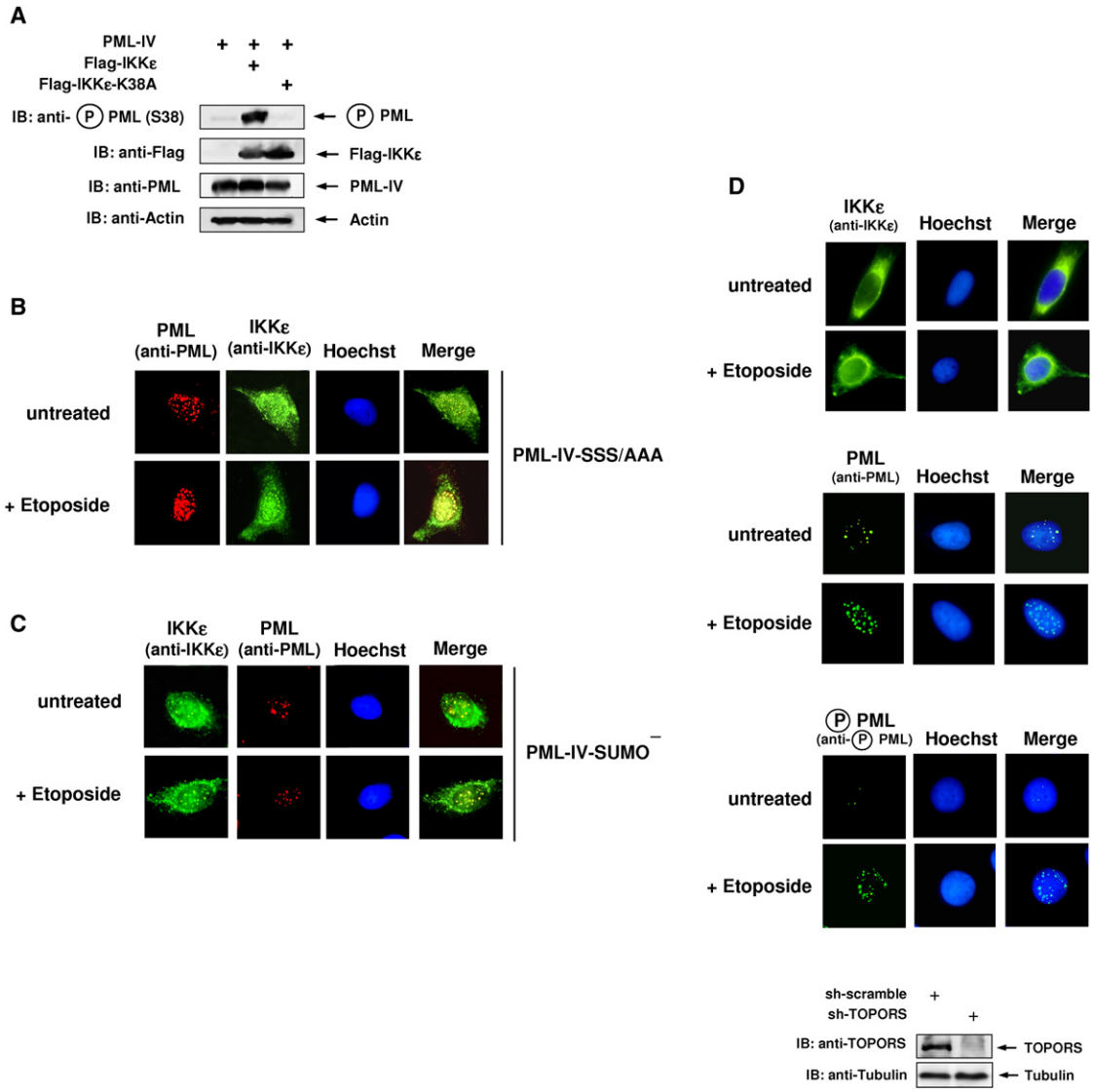

Figure 5. Phosphorylation and SUMOylation of PML Are Required for IKKε Accumulation in PML-NBs

(A) PML-IV was expressed alone or along with wild-type or kinase-inactive IKKε in HEK293 cells as shown. Equal amounts of PML-IV were analyzed for PML-IV serine 38 phosphorylation using a phospho-specific antibody.

(B) HeLa cells were transfected to express an expression vector encoding phosphorylation-deficient PML-IV mutated in serines 8, 36, and 38 (PML-IV SSS/AAA). Localization of the mutant PML-IV protein and its ability to recruit endogenous IKKε in unstimulated or ETO-treated cells were analyzed by immunofluorescence.

(C) HeLa cells were transfected to express a PML-IV variant that was mutated in all three SUMO acceptor lysines (PML-IV-SUMO^{-}) and treated as in (B).

(D) After lentiviral delivery of a TOPORS-specific shRNA, HeLa cells were left untreated or DSBs were induced by exposure to ETO. Intracellular distribution of the endogenous IKKε, PML, and phospho-PML proteins was revealed by immunofluorescence, and a fraction of the cells were lysed and tested by immunoblotting to ensure efficient TOPORS knockdown (lower). All ETO treatments in this figure were performed with 2 μM for 8 hr.

site for PML stabilization. As recent findings revealed that SUMOylation can modulate substrate protein stability (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008), the potential involvement of such a mechanism was tested. Expression of IKKε stabilized the wild-type PML protein and also a non-SUMOylatable PML variant (Figure S3C), thus suggesting alternative stabilization mechanisms.

To investigate the contribution of TOPORS for PML-NB recruitment of IKKε, ETO-induced IKKε relocalization was investigated in cells where endogenous TOPORS was knocked down by shRNA. While the loss of TOPORS remained without consequences for PML location and its phosphorylation, the ETO-triggered relocalization of IKKε to PML-NBs was lost (Figure 5D). These data indicate that TOPORS is not only important for SUMOylation of IKKε, but also for retention of this kinase in PML-NBs. Conversely, the knockdown of IKKε remained without any impact on TOPORS localization in PML-NBs (Figure S3D). Also, colocalization between TOPORS and PML was unchanged

in *IKKε*$^{-/-}$ cells (Figure S3E), thus showing the hierarchical organization of the recruitment procedure.

SUMOylation of IKKε Is Important for Its Prosurvival Function during the DDR

Is the SUMOylation-dependent subcellular localization of IKKε also affecting target protein phosphorylation? To address this question, we compared IKKε and the IKKε-K231R mutant for their ability to trigger phosphorylation of the NF-κB p65 protein. Both IKKε variants were re-expressed in *IKKε*$^{-/-}$ cells which then were either left untreated or stimulated with ETO. Analysis of p65 phosphorylation by immunofluorescence showed that the exclusively cytosolic IKKε-K231R protein had lost the ability to trigger p65 serine 468 phosphorylation, which predominantly occurred in the nucleus (Figure 6A), a finding which is in line with biochemical fractionation experiments (Mattioli et al., 2006). The importance of nuclear IKKε functions identified here is underscored by experiments showing the induction of NF-κB-dependent gene expression by the nuclear SUMO-IKKε-K231R protein, but not by the cytosolic IKKε-K231R variant (Figure 6B). The relevance of intracellular distribution for nuclear IKKε function was revealed by the observation that genetic fusion of SUMO did not only cause constitutive recruitment of the fusion protein to nuclear structures (Figure 2D) but also restored the lost NF-κB activation of the IKKε-K231R mutant (Figure 6B). To investigate the relevance of IKKε SUMOylation for DNA-damage-triggered expression of endogenous target genes, *IKKε*$^{-/-}$ cells were retransfected to express IKKε or the SUMOylation-deficient point mutant. After induction of DSBs, gene expression was determined by real-time PCR experiments. As shown for the ETO-inducible *Cdkn1a* and *Mdm2* genes, the IKKε-K231R mutant has lost the ability to restore ETO-induced gene expression (Figure 6C). The relevance of IKKε for DNA-damage-induced cell death was investigated by a loss-of-function approach. HEK293 cells were transfected with a vector directing the synthesis of small hairpin RNAs (shRNAs) specific for IKKε or adequate control vectors. After selection of stably transfected cells, limited doses of ETO were added to induce moderate cell death. Quantitative analysis of cell survival by MTT (thiazolyl blue tetrazolium bromide) assays showed a strongly decreased viability in the absence of IKKε in a dose- and time-dependent fashion (Figure 7A, Figure S4A), thus revealing an important contribution of IKKε for prosurvival signaling during DNA-damage-induced cell death. What is the role of IKKε SUMOylation for its function during the DDR? To address this question, IKKε-deficient MEFs were retransfected to express IKKε wild-type or the IKKε-K38A and IKKε-K231R mutants or each of these proteins in a fusion with SUMO-1. Quantification of ETO-induced cell death showed that expression of wild-type IKKε allowed restoration of the apoptosis-resistant status. By contrast, cells expressing SUMOylation-defective IKKε-K231R or kinase-inactive IKKε-K38A failed to regain ETO resistance. Genetic fusion of SUMO-1 to IKKε-K231R allowed full protection of cells from ETO-induced cell death (Figure 7B), thus revealing the importance of IKKε SUMOylation for its prosurvival function. Similar results were obtained by an alternative experimental approach upon shRNA-mediated knockdown of endogenous IKKε, followed by retransfection with different codon-exchanged, shRNA-resistant IKKε forms (Figure S4B), emphasizing the importance of SUMOylation for the antiapoptotic effect of IKKε. To reveal a functional role of TOPORS for DSB-triggered cell death, cells were depleted for endogenous TOPORS by siRNA and compared for ETO-induced cell death with adequately treated controls. These experiments revealed that knockdown of TOPORS caused slightly reduced cell viability (Figure 7C). It is important to note that downregulation of TOPORS will not only blunt the antiapoptotic IKKε function but will also enhance the proapoptotic p53 response as it functions as a negative regulator of p53 (Lin et al., 2005; Rajendra et al., 2004). Therefore, this experiment clearly shows that the antiapoptotic function of TOPORS overrides its proapoptotic activity. To identify relevant IKKε substrates in the nucleus, we compared cells selected to express an IKKε downregulating shRNAs to adequate controls for DNA-damage-induced NF-κB signaling. While ETO-triggered phosphorylation of IκBα remained unchanged in the absence of IKKε, ETO-induced p65 serine 536 phosphorylation was slightly altered and serine 468 phosphorylation was strongly diminished (Figure 7D). To investigate a possible role of the inducible phosphorylation site for the antiapoptotic p65 function, p65-deficient MEFs stably reconstituted with wild-type p65 or a nonphosphorylatable p65 serine 468 to alanine mutant were treated with limited doses of ETO. Cells expressing the mutant p65 protein were more sensitive toward ETO-triggered cell death and not phosphorylated at serine 468 (Figure 7E). These results show the contribution of this phosphorylation site for the prosurvival effect of NF-κB.

DISCUSSION

Molecular Mechanisms Allowing IKKε Uptake in PML-NBs

Our results collectively demonstrate an important role of IKKε for the protection from DNA-damage-induced cell death, as schematically shown in Figure 7F. In unstressed cells, IKKε localizes in the cytosol and, to a minor extent, to the nucleus (Figures 2B and 2C). In a first step, DNA-damage-induced IKKε activation results in the nuclear translocation of the kinase. The activation of IKKε is still an unresolved issue, as recent evidence shows that serine 172 phosphorylation in the activation loop does not employ autophosphorylation but rather transphosphorylation by a not-yet-identified upstream kinase (Clark et al., 2009). As IKKε does not contain a classical nuclear localization signal, the subsequent nuclear import may occur indirectly via association with other proteins. It will be important to determine the molecular mechanism for nuclear translocation of IKKε in future experiments. In the second step of this activation cascade, an IKKε-dependent kinase phosphorylates PML at serine 38, a process that does not require TOPORS or retention in PML-NBs. Subsequent SUMOylation of IKKε is the third step in this ordered process that allows trapping of IKKε in PML-NBs. This model is also supported by experiments showing that PML-IV fails to recruit kinase-inactive IKKε and also SUMOylation-deficient IKKε to PML-NBs (Figure S4C). Along this line, fusion of SUMO-1 to the exclusively cytoplasmic IKKε-K231R mutant allows trapping of this fusion protein in nuclear bodies (Figure 2D) and also PML-IV-mediated recruitment of these

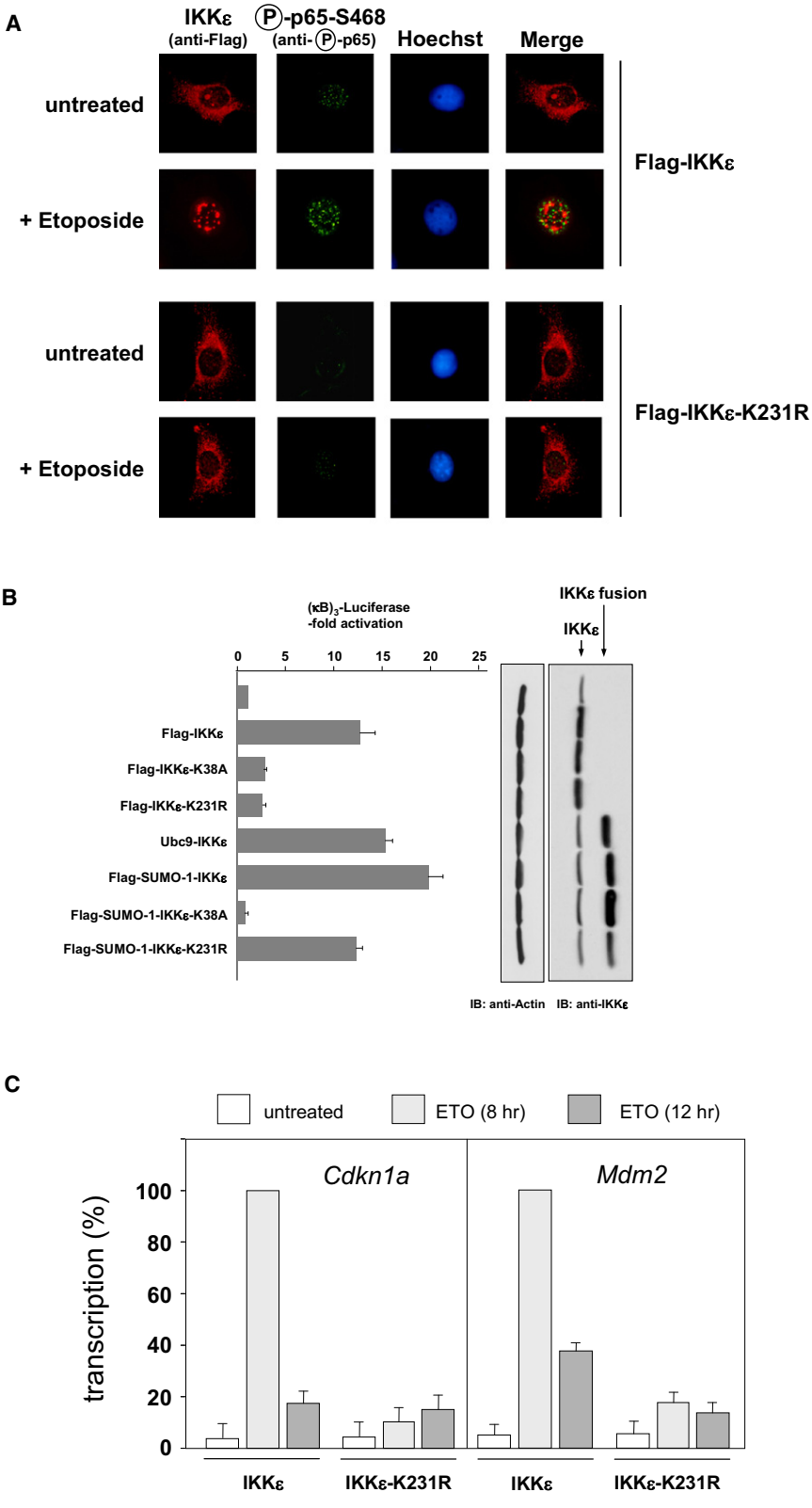

Figure 6. NF-κB-Triggered Gene Expression Depends on SUMOylation of IKKε

(A) *IKKε*$^{-/-}$ cells transfected to express IKKε or IKKε-K231R were left untreated or exposed for 8 hr to ETO (2 μM). Localization of IKKε and serine 468-phosphorylated NF-κB p65 was revealed by indirect immunofluorescence as shown.

(B) The indicated IKKε variants were compared for their ability to stimulate NF-κB target gene transcription. The experiment was performed as in Figure 1E with the difference that a luciferase reporter gene under the control of three NF-κB-binding sites ($[κB]_3$ luciferase) was used instead of the IFN-β reporter. Error bars show standard deviations from three independent experiments.

(C) *IKKε*$^{-/-}$ MEFs were retransfected to express equal amounts of IKKε or IKKε-K231R. After 36 hr, cells were stimulated for 8 or 12 hr with ETO (3 μM), followed by quantitative analysis of *Cdkn1a* and *Mdm2* gene expression by real-time PCR. In order to facilitate comparison, full gene activation was arbitrarily set as 100%. Experiments were performed in triplicates, and error bars display standard deviations.

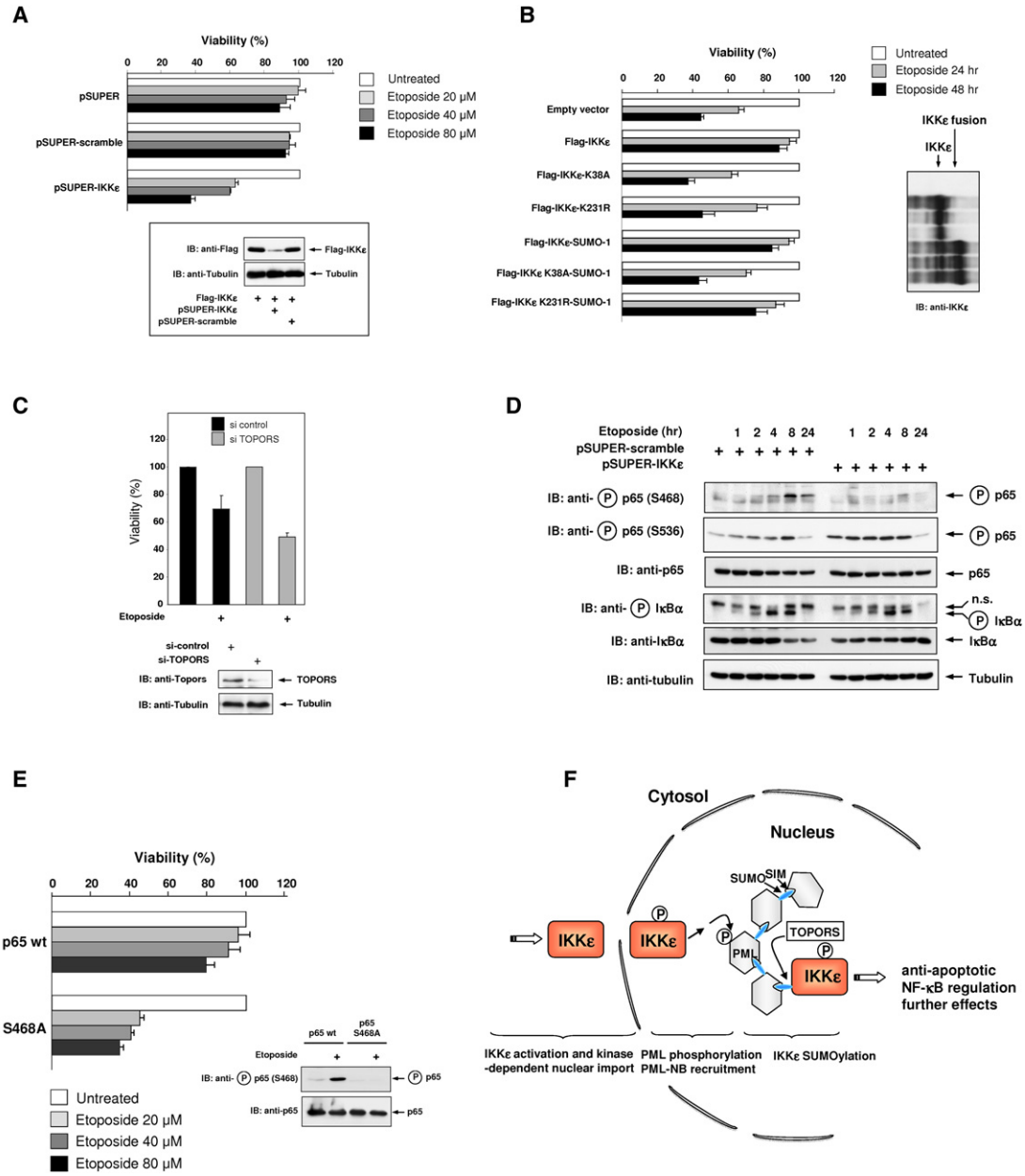

Figure 7. SUMOylation-Dependent Antiapoptotic Function of IKKε

(A) HEK293 cells were transfected with the indicated vectors producing an IKKε-specific shRNA (pSUPER-IKKε), a scrambled sequence (pSUPER-scramble), or the empty vector (pSUPER). After 3 days of puromycin selection, cells were treated with the indicated ETO concentrations for 24 hr and cell viability was measured by MTT assays performed in triplicates. The inset shows the functionality of the used IKKε shRNA.

(B) *IKKε*$^{-/-}$ cells were retransfected to express the indicated proteins. A fraction of the cells was analyzed for adequate protein expression by western blotting, while the remaining cells were treated with 80 μM ETO and cell viability measured as in (A).

(C) Cells were treated either with TOPORS-specific siRNA or a scramble control, followed by a control western blot ensuring TOPORS knockdown (lower part) or treatment of cells for 36 hr with 10 μM of ETO and determination of cell viability by MTT assays.

(D) IKKε knockdown cells or control HEK293 cells were treated for different periods with ETO (2 μM), followed by immunoblotting to test phosphorylation of IKKε substrate proteins.

(E) *p65*$^{-/-}$ MEFs stably reconstituted with wild-type (WT) and point-mutated p65 were treated for 24 hr with the indicated ETO concentrations and analyzed as in (A). Error bars for all experiments measuring cell viability in this figure show standard deviations from two experiments performed in duplicates. A control western blot showing p65 serine 468 phosphorylation in ETO treated cells (2 μM, 8 hr) is also displayed.

(F) Schematic model summarizing the mechanisms and functions of IKKε during genotoxic stress.

fusion proteins to PML-NBs (Figure S4D) even in absence of DNA damage. Collectively, these data reveal clear evidence that SUMOylation is essential for the localization of IKKε in PML-NBs. These subnuclear structures were previously described as subcellular domains containing many SUMOylated proteins that are kept in association by a complex network of SUMO/SIM interactions. PML-NBs are multiprotein assemblies that are characterized by extremely densely packed protein aggregates, a feature that is termed macromolecular crowding and has an important impact on the biochemical behavior of macromolecules (Richter et al., 2008). As most of these insoluble proteins escape classical biochemical purification procedures under mild lysis conditions, approaches such as crosslinking and subsequent purification under denaturing conditions will allow identification of more PML-NB-resident proteins and elucidation of their role. Like IKKε, various DNA repair and checkpoint proteins dynamically colocalize with PML-NBs which also recruit many regulators of cell death including p53 (Bernardi et al., 2008). PML-NBs were shown to function as catalytic surfaces and allow for posttranslational modifications including p53 phosphorylation and acetylation (Bernardi and Pandolfi, 2007). Accordingly, PML-knockout cells have profound defects in DNA-damage-induced cell death (Guo et al., 2000; Pearson et al., 2000).

The Role of SUMOylation for DNA-Damage-Triggered NF-κB Induction

Previous work showed that SUMOylation is also employed for further steps in the DSB-triggered NF-κB activation pathway. DNA damage induces PIDD- and receptor-interacting protein 1 (RIP1)-dependent SUMOylation of NEMO, which is important for its subsequent phosphorylation by ATM in the nucleus (Janssens et al., 2005; Mabb et al., 2006). This raises the possibility that SUMOylation is a more generally applied principle to control the nuclear localization of NF-κB signaling proteins during the DDR. This hypothesis is in line with the pervasive role of SUMO modification for the proteins mediating DNA repair or damage avoidance pathways (Bergink and Jentsch, 2009). The relevance of SUMOylation during the DDR is also seen by the intriguing finding that Rad60 proteins contain integral SUMO-like domains that act as SUMO mimetics and are required to mediate DNA repair (Prudden et al., 2009).

Emerging Functions for IKKε

The importance of nuclear IKKε functions identified here is underscored by experiments showing the induction of NF-κB-dependent gene expression by the nuclear SUMO-IKKε-K231R protein, but not by the cytosolic IKKε-K231R variant (Figures 6B and 6C). In support of this notion, a functional role of IKKε in the nucleus was revealed in a recent study that showed inducible recruitment of IKKε to distinct NF-κB target genes in order to allow clearance of the corepressor NCoR from target promoters (Huang et al., 2009). Our data reveal a critical role of IKKε-mediated p65 serine 468 phosphorylation for the induction of cell death, but it is reasonable to assume that further phosphorylation targets in the nucleus will contribute to the prosurvival function of this kinase. A list of candidate proteins was recently defined by an unbiased screen that allowed the identification of an IKKε consensus phosphorylation motif. This motif is also contained in the tumor suppressor protein cylindromatosis (CYLD), which is directly phosphorylated by IKKε at serine 418. Phosphorylation of this site decreases its deubiquitinase activity and is necessary for IKKε-driven tumorigenic transformation (Hutti et al., 2009). The repertoire of functions identified for the IKKε protein is steadily growing. While initial experiments revealed the importance of IKKε for the production of type I interferons and also secondary interferon-mediated effects (Chau et al., 2008), recent results suggest that this kinase also sustains chronic low-grade inflammation that occurs in obesity (Chiang et al., 2009). Genetic screens identified the IKKε-encoding gene as an oncogene that is frequently amplified and overexpressed in breast cancers and ensures survival of the cancer cells (Boehm et al., 2007). Given the relevance of regulated IKKε translocation to the nucleus revealed in this study, it will be interesting to investigate whether its function in the nucleus is also important for oncogenic transformation of breast cancer cells. In summary, the data presented here raise the possibility that IKKε inhibition or interference with its inducible SUMOylation would not only eliminate the proproliferative effects of IKKε but also sensitize cancer cells to chemotherapeutic drugs.

EXPERIMENTAL PROCEDURES

Plasmids

The plasmids pcDNA3-His_6-SUMO-2&3 (Ron T. Hay, University of Dundee), pSG5-His_6-SUMO-1-G97A (Luca Cardone, University of Strasbourg), pcDNA3-Flag-HA-SENP1 (Peter O'Hare, MCRI, London), pCATCH-Flag-TOPORS and its derivatives, pSUPER-TOPORS (Stefan Weger, Charite, Berlin), pcDNA3-Flag-IKKε-S172A (Tom Maniatis, Harvard University, Boston), pSIREN-retroQ-shPML (Thomas Stamminger, University of Erlangen), and pGL3-IFN-beta-Luc (Stefan Ludwig, University of Münster) were kind gifts from the indicated persons. The other plasmids are given in the Supplemental Experimental Procedures.

Immunofluorescence Analysis

Cells were grown on coverslips and fixed in a 1:1 solution methanol:acetone, rehydrated in phosphate-buffered saline (PBS), and subsequently blocked with 10% goat serum in PBS. The cells were incubated with primary and fluorescence-coupled secondary antibodies essentially as described (Roscic et al., 2006). To allow visualization of endogenous TOPORS in nuclear bodies and to reduce the background signals in the cytoplasm and nucleoplasm, cells were pretreated with cytoskeletal stripping buffer before fixation as described (Mirzoeva and Petrini, 2001). Further details are given in the Supplemental Experimental Procedures.

Reagents and Antibodies

Details about all reagents and the suppliers are listed in the Supplemental Experimental Procedures.

siRNA- and shRNA-Meditated Knockdown

Small interfering RNAs previously published to efficiently target human TOPORS (Guan et al., 2008) and the AllStars Negative Control siRNA were obtained from QIAGEN. HEK293 cells were transfected with siRNA overnight directly after seeding in 6 cm dishes using HiPerfect following the instructions given by the manufacturer (QIAGEN). The next day, cells were transfected overnight for a second time. Lentiviruses for the knockdown of IKKε (TRCN0000010027) and TOPORS (TRCN0000007519), respectively, were produced in HEK293T by cotransfecting packaging and integration vectors as recommended by The RNAi Consortium (TRCN).

Cell Culture, Transfections, and Luciferase Assays

All cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 1% (v/v) penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Transient transfections were done with Rotifect according to the instructions of the manufacturer (Roth). Luciferase assays were done using the Promega Dual Luciferase System as described in further detail in the Supplemental Experimental Procedures.

Cell Extracts, Immunoprecipitation, and Enrichment of His-Tagged Proteins

Nuclear extracts were prepared essentially as described (Mattioli et al., 2006), and equal amounts of IKKε contained in the nuclear fraction were further analyzed by coimmunoprecipitation. Crosslinking experiments were done upon addition of 0.25 mM of the membrane-permeable crosslinker dimethyl-3-3′-dithiobispropionimidate 2-HCl (Pierce) for 60 min as described (Roscic et al., 2006), followed by preparation of NP-40 buffer extracts containing 10 mM N-ethylmaleimide. Following centrifugation, cell pellets strongly enriched in SUMOylated IKKε were dissolved in Gu-HCl lysis buffer, and His-tagged proteins were further purified on Ni-NTA columns. His_6-SUMO-modified proteins expressed in eukaryotic cells were enriched after lysis under denaturing conditions using Ni-NTA agarose beads as described in the Supplemental Experimental Procedures.

Real-Time PCR

IKK$\varepsilon^{-/-}$ cells were electroporated to express equal amounts of IKKε or IKKε-K231R using the MEF nucleofector II kit (Amaxa). After 36 hr, electroporated cells were lysed, and total RNA was extracted using the RNeasy mini kit (QIAGEN). After cDNA synthesis, gene expression was quantified as described in further detail in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2010.01.018.

ACKNOWLEDGMENTS

We thank Daniela Stock and Stephanie Steitz for excellent technical assistance and Dr. M. Krause (Marburg, Germany) for help with gene expression analysis. We are indebted to Dr. S. Akira (Osaka, Japan) for *IKK*$\varepsilon^{-/-}$ MEFs, and Drs. S. Weger (Berlin, Germany), R.T. Hay (Dundee, UK), L. Cardone (Strasbourg, France), P. O'Hare (London, UK), T. Maniatis (Boston, USA), R. Niedenthal (Hannover, Germany), T. Stamminger (Erlangen, Germany), and S. Ludwig (Münster, Germany) for plasmids. Work from the lab is supported by grants from the Deutsche Forschungsgemeinschaft projects SCHM 1417/4-2, SCHM 1417/5-1, SCHM 1417/7-1, GRK 1566/1, and the Excellence Cluster Cardio-Pulmonary System (ECCPS).

Received: July 2, 2009
Revised: September 30, 2009
Accepted: November 25, 2009
Published: February 25, 2010

REFERENCES

Adli, M., and Baldwin, A.S. (2006). IKK-i/IKKepsilon controls constitutive, cancer cell-associated NF-kappaB activity via regulation of Ser-536 p65/RelA phosphorylation. J. Biol. Chem. *281*, 26976–26984.

Bergink, S., and Jentsch, S. (2009). Principles of ubiquitin and SUMO modifications in DNA repair. Nature *458*, 461–467.

Bernardi, R., and Pandolfi, P.P. (2007). Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nat. Rev. Mol. Cell Biol. *8*, 1006–1016.

Bernardi, R., Papa, A., and Pandolfi, P.P. (2008). Regulation of apoptosis by PML and the PML-NBs. Oncogene *27*, 6299–6312.

Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P.G., and Dejean, A. (2002). Deconstructing PML-induced premature senescence. EMBO J. *21*, 3358–3369.

Boddy, M.N., Howe, K., Etkin, L.D., Solomon, E., and Freemont, P.S. (1996). PIC 1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukaemia. Oncogene *13*, 971–982.

Boehm, J.S., Zhao, J.J., Yao, J., Kim, S.Y., Firestein, R., Dunn, I.F., Sjostrom, S.K., Garraway, L.A., Weremowicz, S., Richardson, A.L., et al. (2007). Integrative genomic approaches identify IKBKE as a breast cancer oncogene. Cell *129*, 1065–1079.

Bredemeyer, A.L., Helmink, B.A., Innes, C.L., Calderon, B., McGinnis, L.M., Mahowald, G.K., Gapud, E.J., Walker, L.M., Collins, J.B., Weaver, B.K., et al. (2008). DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes. Nature *456*, 819–823.

Chau, T.L., Gioia, R., Gatot, J.S., Patrascu, F., Carpentier, I., Chapelle, J.P., O'Neill, L., Beyaert, R., Piette, J., and Chariot, A. (2008). Are the IKKs and IKK-related kinases TBK1 and IKK-epsilon similarly activated? Trends Biochem. Sci. *33*, 171–180.

Chiang, S.H., Bazuine, M., Lumeng, C.N., Geletka, L.M., Mowers, J., White, N.M., Ma, J.T., Zhou, J., Qi, N., Westcott, D., et al. (2009). The protein kinase IKKepsilon regulates energy balance in obese mice. Cell *138*, 961–975.

Clark, K., Plater, L., Peggie, M., and Cohen, P. (2009). Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. J. Biol. Chem. *284*, 14136–14146.

de Stanchina, E., Querido, E., Narita, M., Davuluri, R.V., Pandolfi, P.P., Ferbeyre, G., and Lowe, S.W. (2004). PML is a direct p53 target that modulates p53 effector functions. Mol. Cell *13*, 523–535.

Dellaire, G., Ching, R.W., Ahmed, K., Jalali, F., Tse, K.C., Bristow, R.G., and Bazett-Jones, D.P. (2006). Promyelocytic leukemia nuclear bodies behave as DNA damage sensors whose response to DNA double-strand breaks is regulated by NBS1 and the kinases ATM, Chk2, and ATR. J. Cell Biol. *175*, 55–66.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M., and Maniatis, T. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat. Immunol. *4*, 491–496.

Gresko, E., Ritterhoff, S., Sevilla-Perez, J., Roscic, A., Frobius, K., Kotevic, I., Vichalkovski, A., Hess, D., Hemmings, B.A., and Schmitz, M.L. (2009). PML tumor suppressor is regulated by HIPK2-mediated phosphorylation in response to DNA damage. Oncogene *28*, 698–708.

Guan, B., Pungaliya, P., Li, X., Uquillas, C., Mutton, L.N., Rubin, E.H., and Bieberich, C.J. (2008). Ubiquitination by TOPORS regulates the prostate tumor suppressor NKX3.1. J. Biol. Chem. *283*, 4834–4840.

Guo, A., Salomoni, P., Luo, J., Shih, A., Zhong, S., Gu, W., and Pandolfi, P.P. (2000). The function of PML in p53-dependent apoptosis. Nat. Cell Biol. *2*, 730–736.

Habraken, Y., and Piette, J. (2006). NF-kappaB activation by double-strand breaks. Biochem. Pharmacol. *72*, 1132–1141.

Hacker, H., and Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. Sci. STKE *2006*, re13.

Harper, J.W., and Elledge, S.J. (2007). The DNA damage response: ten years after. Mol. Cell *28*, 739–745.

Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344–362.

Hecker, C.M., Rabiller, M., Haglund, K., Bayer, P., and Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. J. Biol. Chem. *281*, 16117–16127.

Huang, T.T., Wuerzberger-Davis, S.M., Wu, Z.H., and Miyamoto, S. (2003). Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. Cell *115*, 565–576.

Huang, W., Ghisletti, S., Perissi, V., Rosenfeld, M.G., and Glass, C.K. (2009). Transcriptional integration of TLR2 and TLR4 signaling at the NCoR derepression checkpoint. Mol. Cell *35*, 48–57.

Hutti, J.E., Shen, R.R., Abbott, D.W., Zhou, A.Y., Sprott, K.M., Asara, J.M., Hahn, W.C., and Cantley, L.C. (2009). Phosphorylation of the tumor suppressor CYLD by the breast cancer oncogene IKKepsilon promotes cell transformation. Mol. Cell *34*, 461–472.

Janssens, S., and Tschopp, J. (2006). Signals from within: the DNA-damage-induced NF-kappaB response. Cell Death Differ. *13*, 773–784.

Janssens, S., Tinel, A., Lippens, S., and Tschopp, J. (2005). PIDD mediates NF-kappaB activation in response to DNA damage. Cell *123*, 1079–1092.

Johnson, E.S., and Gupta, A.A. (2001). An E3-like factor that promotes SUMO conjugation to the yeast septins. Cell *106*, 735–744.

Lallemand-Breitenbach, V., Jeanne, M., Benhenda, S., Nasr, R., Lei, M., Peres, L., Zhou, J., Zhu, J., Raught, B., and de The, H. (2008). Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. Nat. Cell Biol. *10*, 547–555.

Lavin, M.F. (2008). Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nat. Rev. Mol. Cell Biol. *9*, 759–769.

Lin, L., Ozaki, T., Takada, Y., Kageyama, H., Nakamura, Y., Hata, A., Zhang, J.H., Simonds, W.F., Nakagawara, A., and Koseki, H. (2005). topors, a p53 and topoisomerase I-binding RING finger protein, is a coactivator of p53 in growth suppression induced by DNA damage. Oncogene *24*, 3385–3396.

Mabb, A.M., Wuerzberger-Davis, S.M., and Miyamoto, S. (2006). PIASy mediates NEMO sumoylation and NF-kappaB activation in response to genotoxic stress. Nat. Cell Biol. *8*, 986–993.

Matsui, K., Kumagai, Y., Kato, H., Sato, S., Kawagoe, T., Uematsu, S., Takeuchi, O., and Akira, S. (2006). Cutting edge: role of TANK-binding kinase 1 and inducible IkappaB kinase in IFN responses against viruses in innate immune cells. J. Immunol. *177*, 5785–5789.

Mattioli, I., Geng, H., Sebald, A., Hodel, M., Bucher, C., Kracht, M., and Schmitz, M.L. (2006). Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKK epsilon. J. Biol. Chem. *281*, 6175–6183.

Meulmeester, E., and Melchior, F. (2008). Cell biology: SUMO. Nature *452*, 709–711.

Mirzoeva, O.K., and Petrini, J.H. (2001). DNA damage-dependent nuclear dynamics of the Mre11 complex. Mol. Cell. Biol. *21*, 281–288.

Neumann, M., and Naumann, M. (2007). Beyond IkappaBs: alternative regulation of NF-kappaB activity. FASEB J. *21*, 2642–2654.

Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P.P., et al. (2000). PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature *406*, 207–210.

Perkins, N.D. (2006). Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene *25*, 6717–6730.

Prudden, J., Perry, J.J., Arvai, A.S., Tainer, J.A., and Boddy, M.N. (2009). Molecular mimicry of SUMO promotes DNA repair. Nat. Struct. Mol. Biol. *16*, 509–516.

Rajendra, R., Malegaonkar, D., Pungaliya, P., Marshall, H., Rasheed, Z., Brownell, J., Liu, L.F., Lutzker, S., Saleem, A., and Rubin, E.H. (2004). Topors functions as an E3 ubiquitin ligase with specific E2 enzymes and ubiquitinates p53. J. Biol. Chem. *279*, 36440–36444.

Rasheed, Z.A., Saleem, A., Ravee, Y., Pandolfi, P.P., and Rubin, E.H. (2002). The topoisomerase I-binding RING protein, topors, is associated with promyelocytic leukemia nuclear bodies. Exp. Cell Res. *277*, 152–160.

Richter, K., Nessling, M., and Lichter, P. (2008). Macromolecular crowding and its potential impact on nuclear function. Biochim. Biophys. Acta *1783*, 2100–2107.

Roscic, A., Moller, A., Calzado, M.A., Renner, F., Wimmer, V.C., Gresko, E., Ludi, K.S., and Schmitz, M.L. (2006). Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. Mol. Cell *24*, 77–89.

Schwabe, R.F., and Sakurai, H. (2005). IKKbeta phosphorylates p65 at S468 in transactivaton domain 2. FASEB J. *19*, 1758–1760.

Sharma, S., tenOever, B.R., Grandvaux, N., Zhou, G.P., Lin, R., and Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. Science *300*, 1148–1151.

Shen, T.H., Lin, H.K., Scaglioni, P.P., Yung, T.M., and Pandolfi, P.P. (2006). The mechanisms of PML-nuclear body formation. Mol. Cell *24*, 331–339.

Tatham, M.H., Geoffroy, M.C., Shen, L., Plechanovova, A., Hattersley, N., Jaffray, E.G., Palvimo, J.J., and Hay, R.T. (2008). RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. Nat. Cell Biol. *10*, 538–546.

Vousden, K.H. (2006). Outcomes of p53 activation—spoilt for choice. J. Cell Sci. *119*, 5015–5020.

Weger, S., Hammer, E., and Heilbronn, R. (2005). Topors acts as a SUMO-1 E3 ligase for p53 in vitro and in vivo. FEBS Lett. *579*, 5007–5012.

Wu, Z.H., and Miyamoto, S. (2007). Many faces of NF-kappaB signaling induced by genotoxic stress. J. Mol. Med. *85*, 1187–1202.

Wu, Z.H., Shi, Y., Tibbetts, R.S., and Miyamoto, S. (2006). Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. Science *311*, 1141–1146.

Yeh, E.T. (2009). SUMOylation and De-SUMOylation: wrestling with life's processes. J. Biol. Chem. *284*, 8223–8227.