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TITLE: Characterization of a SUMO Ligase that is Essential for DNA Damage-Induced NF-Kappa B Activation

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### Title: Characterization of a SUMO Ligase that is Essential for DNA Damage-Induced NF-Kappa B Activation

**Abstract:**

It has been recently proposed that inhibition of NF-κB may be a therapeutic target for the treatment of ER-breast cancers. As of now, the majority of NF-κB inhibitors focus on the key signal integrating complex known as the IκB kinase (IKK) complex. Since NF-κB plays a major role in many essential physiological processes in the cell, global inhibition of NF-κB at the central IKK complex could allow for increased risk of side effects as well as the desirable effects on cancer cell death. Hence, identification of specific, novel molecular targets in the NF-κB signaling pathway may lead to the identification of more specific NF-κB inhibitors. Our hypothesis is that PIASy, a SUMO ligase, is essential for DNA damage induced NF-κB activation, however is not critical for classical activation of NF-κB, leaving the more physiological pathway intact. We reveal that PIASy is signaling at the level of NEMO SUMOylation, a posttranslational modification that we recently identified being critical for DNA damage induced activation of NF-κB. Reduction of PIASy through siRNA caused inhibition of NF-κB in response to multiple DNA damaging agents commonly used in anti-cancer therapy. We provide strong evidence that PIASy is working at the level of NEMO SUMOylation and propose that PIASy is the SUMO ligase for NEMO. Furthermore, we show that the catalytic activity of PIASy is essential for NF-κB activation and hence suggest that inhibition of PIASy may be used as a more specific inhibitor in anti-cancer therapy to treat ER-breast cancer.

**Subject Terms:**

PIAS•/PIASy, NEMO, NF-κB, SUMO
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Annual Summary Report

I. Introduction

Background:
Nuclear Factor-κB (NF-κB) is a transcription factor that regulates a diverse subset of genes involved in immune function, growth control, development, and regulation of apoptosis. NF-κB exists in the cell in inactive cytoplasmic complexes, with the predominant complex being p65/p50 dimers. NF-κB can be activated by a wide variety of stimuli at the cell surface such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and lipopolysaccharide (LPS). NF-κB can also be activated in response to various DNA damaging agents, such as ionizing radiation and Topoisomerase II inhibitors (e.g. etoposide and doxorubicin/adriamycin). Activation of NF-κB in response to various DNA damaging agents is less rapid than extracellular stimuli and generally results in the activation of anti-apoptotic genes. The early key molecular events involved in NF-κB activation with various stimuli are diverse; however a key component in this signaling pathway with most stimuli is the IKK complex that is composed of three major proteins in the cytoplasm of the cell. One protein, NEMO serves as an adaptor/scaffolding component which is essential for IKK complex activity to ultimately activate NF-κB. NF-κB activation has also been suggested to play a role in resistance to therapy in advanced breast cancers. Importantly, estrogen receptor negative (ER-) breast cancers tend to have high constitutive levels of active NF-κB and activate NF-κB in response to DNA damaging anticancer agents. It has been recently proposed that inhibition of NF-κB may be a potential therapeutic target for ER- breast cancers. As of now, the majority of NF-κB inhibitors focus inhibiting the IKK complex. One potential issue with inhibiting the IKK complex is that NF-κB mediates a variety of physiologically vital processes besides those involved in the regulation of apoptosis. As a result, nonspecific inhibition of NF-κB’s various target genes could allow for an increased risk of side effects and unpredictable effects on apoptosis of breast cancer cells. One solution to this dilemma is inhibition of specific NF-κB pathways upstream of IKK activation in response to DNA damaging agents. If one could selectively inhibit the NF-κB signaling pathways induced by DNA damaging anticancer agents and leave other important NF-κB signaling pathways (such as TNFα and IL-1 signaling) intact, then enhanced cancer cell death may be achieved with fewer side effects.

Objective/Hypothesis: PIASγ/PIASy is the SUMO ligase for NEMO and inhibition of this specific ligase may provide a novel target for chemotherapeutic treatment regimens against ER-breast cancer.

II. Body

Aim1: To understand the mechanisms governing NEMO SUMOylation
In this grant, we proposed if PIASy was directly affecting NEMO SUMOylation, then it should be able to enhance NEMO SUMOylation in vitro. We initially showed that in vitro translated NEMO in rabbit reticulocyte extracts could be SUMO modified by immunopurified PIASy. However, we were unable to show that GST-NEMO could be SUMOylated in vitro and were unsuccessful in purifying His-PIASy with high purity and quantity. We were able to obtain
highly purified recombinant His-PIASy in collaboration with Dr. Yoshiaka Azuma. We utilized this His-PIASy in an *in vitro* SUMOylation assay with purified recombinant His-NEMO that we generated in the lab (Figure 1). We found that PIASy can promote NEMO SUMOylation in a complete recombinant system and this could be reversed by the catalytic domain of a SUMO protease, Senp1.

**Figure 1:**

![In vitro SUMOylation assay](image)

Figure 1: *In vitro* SUMOylation assay was performed using 0.75 µg of recombinant His-NEMO and 1 µg of recombinant His-PIASy at 30°C for 75 min. His-SENP1 was added after SUMOylation in lane 4. Samples were terminated in 2xSDS sample buffer, run on an SDS-PAGE gel and blotted with anti-NEMO (top), anti-SUMO-1 (middle) and anti-His antibodies (bottom three blots).

We have previously shown that PIASy can inducibly interact with endogenous NEMO. However, we would like to examine and isolate an endogenous NEMO:PIASy complex to identify novel binding partners and understand how this complex is functioning in the cell. Since a suitable commercial antibody is not available, we decided to generate our own for our future studies with PIASy. In collaboration with Neoclone, (a Madison based company), we provided them with purified recombinant His-PIASy. Ascites with cross reactivity for His-PIASy was given to us to test for reactivity against PIASy. We currently have ascites that tests positive against human PIASy that will be suitable for our future studies. Furthermore, we have identified RSK1, a protein recently shown to be required for DNA damage induced NF-κB activation as a binding partner for PIASy (Figure 2).

**Figure 2:**

![Western blot](image)

Figure 2: HEK293 cells were transfected with FLAG-RSK1, Myc-NEMO, and HA-PIASy. Cells were lysed and immunoprecipitated using anti-FLAG antibody. Western blotting was performed using anti-Myc, anti-HA, and anti-FLAG antibodies.
We are currently trying to understand the role of RSK1 kinase in the PIASy:NEMO complex and how it signals in the DNA damage induced NF-κB activation pathway.

**Aim2: Determine if PIASy/y dictates the ability of breast cancer cells to activate NF-κB basally and in response to DNA damaging agents.**

We initially proposed that PIASy expression levels may dictate the ability of breast cancer cells to activate NF-κB and have high constitutive NF-κB activity. For instance, we have found that the MDA-MB-231(ER-) breast cancer cell line efficiently activates NF-κB in response to DNA damaging agents. Interestingly, MDA-MB-231 breast cancer cell lines have high constitutive NF-κB activity and this activity is increased upon stimulation with DNA damaging agents. In contrast, MCF-7 (ER+) breast epithelial cell lines are extremely inefficient to activate NF-κB in response to DNA damaging agents, even though these cells are capable of activating NF-κB when stimulated with the non-DNA damaging agent TNFα. We proposed that PIASy protein levels may also dictate whether or not cell lines can activate NF-κB in response to DNA damaging agents. We have recently been able to obtain a minimal amount of human PIASy antibody from Dr. Mary Dasso at the NIH. As a result, we used this PIASy antibody for protein profiling of various breast cancer cell lines along with other cell lines that are known to variably activate NF-κB in response to genotoxic stress agents. We utilized cellular extracts from the ER positive cell line MDA-MB-231 and the negative breast cancer cell line MCF-7 to determine if PIASy levels may dictate the ability for these cells to activate NF-κB in response to DNA damage (Figure 3). However, surprisingly we found that PIASy protein levels were much lower in the ER- line compared to the ER+ cell line. Levels were even lower than in HEK293 cells which also efficiently activate NF-κB in response to DNA damaging agents. We also observed no major correlation between cells that can efficiently activate NF-κB with DNA damaging agents and the expression of PIASy levels in the cell. This suggests that PIASy expression levels alone may not be a good predictor of whether or not a cell can activate NF-κB with DNA damaging agents. However, recent work has examined the role of a protein known as PIDD in DNA damage induced NF-κB activation. Expression of a PIDD fragment (PIDD-C) resulted in enhanced NF-κB activation with the DNA damaging agents, etoposide and doxorubicin. Interestingly, expression of the PIDD-C fragment is absent in the majority of cell lines that cannot activate NF-κB by DNA damaging agents with the exception of the HeLa cell line. Although we are still confident that PIASy is still critical for DNA damage induced NF-κB activation since we have observed that knockdown of PIASy can inhibit DNA damage induced NF-κB activation in HEK293, HeLa, and CEM cell lines.
However, we still believe that a reduction in PIASy protein levels may sensitize ER- breast cancer cell lines to DNA damaging agents but not through mere enhanced expression as proposed previously. Interestingly, we have found that even minimal protein expression levels of PIASy protein are sufficient to activate NF-κB in response to DNA damaging agents in HEK293 cells suggesting that residual levels of PIASy in ER- breast cancer cell lines are sufficient to activate NF-κB in response to DNA damaging agents (Figure 4).

We are still working out the conditions to examine the effects of loss of PIASy protein levels on NF-κB activation in the the ER- breast cancer cell line MDA-MB-231. Since the most potent siRNA oligonucleotide (used in figure 4) gave us a modest decrease in PIASy protein levels in MDA-MB-231 cells, we are now utilizing a vector based shRNA construct that can efficiently reduce PIASy protein levels in MDA-MB-231 breast cancer cell lines that can be transfected with 80% efficiency. Studies examining its effects on basal and DNA damage induced NF-κB activation and its consequence on cell survival are currently under way. Moreover, we initially proposed that PIASy levels in MCF-7 cells exposed to antiestrogens (such as tamoxifen) will be examined to establish a link between ERα and PIASy expression. These experiments are currently being employed to see if tamoxifen may regulate PIASy expression levels.

III. Key Research Accomplishments and Conclusions:
1. Complete recombinant in vitro SUMOylation of NEMO
2. Mouse monoclonal antibodies against human PIASy
3. Identification of a new PIASy interacting partner, RSK1
4. Protein expression profiling of PIASy in ER+ and negative cell lines

IV. Reportable Outcomes

2. Abstract: A SUMO E3 PROMOTES NEMO SUMOYLATION AND NF-κB ACTIVATION IN RESPONSE TO GENOTOXIC STRESS. Keystone Symposia: NF-κB: 20 Years on the Road from Biochemistry to Pathology, 2006

V. Conclusions

Aim1: We have shown that PIASy can promote NEMO SUMOylation directly in a complete recombinant system. We have further generated our own PIASy antibody since there are no reliable commercially available antibodies. Our PIASy antibody will be used in future studies to examine novel proteins in complex with PIASy. We have further identified a new protein, RSK1 as an interacting partner for PIASy and are currently assessing its role in PIASy regulation and DNA damage induced NF-κB signaling.

Aim2: We have analyzed protein extracts from multiple cell lines including the ER+ cell line, MCF7 and the ER- cell line MDA-MB-231. We have found initially that there is no correlation between expression levels of PIASy and whether or not these cell lines can activate NF-κB in response to DNA damaging agents. We have however shown that the formation of a PIDD fragment, PIDD-CC recently implicated in DNA damage induced NF-κB activation is absent in the MCF7 cell line but not the MDA-MB-231 cell line suggesting that PIDD expression levels may play a role in whether these cell lines activate NF-κB with DNA damaging agents. We have also shown that just a residual amount of PIASy is sufficient to activate NF-κB with DNA damaging agents in HEK293 cells.

Appendices: First author published Nature Cell Biology paper, see attached.
PIASy mediates NEMO sumoylation and NF-κB activation in response to genotoxic stress

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Protein modification by SUMO (small ubiquitin-like modifier) is an important regulatory mechanism for multiple cellular processes¹,². SUMO-1 modification of NEMO (NF-κB essential modulator), the IκB kinase (IKK) regulatory subunit, is critical for activation of NF-κB by genotoxic agents¹. However, the SUMO ligase, and the mechanisms involved in NEMO sumoylation, remain unknown. Here, we demonstrate that small interfering RNAs (siRNAs) against PIASy (protein inhibitor of activated STATy) inhibit NEMO sumoylation and NF-κB activation in response to genotoxic agents, overexpression of PIASy enhances these events. PIASy preferentially stimulates site-selective modification of NEMO by SUMO-1, but not SUMO-2 and SUMO–3, PIASy–NEMO interaction is increased by genotoxic stress and occurs in the nucleus in a manner mutually exclusive with IKK interaction. In addition, hydrogen peroxide (H₂O₂) also increases PIASy–NEMO interaction and NEMO sumoylation, whereas antioxidants prevent these events induced by DNA-damaging agents. Our findings demonstrate that PIASy is the first SUMO ligase for NEMO whose substrate specificity seems to be controlled by IKK interaction, subcellular targeting and oxidative stress conditions.

We previously showed that SUMO-1 modification of NEMO–IKKγ is required for the activation of NF-κB in response to etoposide (VP16) and camptothecin (CPT), but not by lipopolysaccharide or tumor necrosis factor α (TNFα)³. This sumoylation is associated with nuclear accumulation of IKK-free NEMO and is followed by phosphorylation on Ser 85 increased by VP16 (Fig. 1a). To examine whether PIASy can function as a direct SUMO E3 for NEMO, amino-terminally HA-tagged NEMO substrate was translated in rabbit reticulocyte extracts and incubated with sumoylation reaction mixtures containing recombinant sumoylation components. Of the four major bands translated (see Supplementary Information, Fig. 5a), only the slowest migrating band could be immunoprecipitated with an anti-HA antibody. Thus, this band represents the full-length HA–NEMO protein.
LETTERS

Figure 1 PIASy is necessary for NF-κB activation by genotoxic agents. (a) HEK293 cells were transfected with control or siRNAs against genes encoding different SUMO ligases. RNA was isolated and the expression level of mRNAs was determined using RT–PCR. GAPDH served as a loading control. (b) HEK293 cells were transfected as stated above with control or PIASy siRNAs. Cells were treated with 10 ng ml–1 TNFα for 15 min, 10 μM VP16 for 90 min and 10 μM CPT for 120 min. Total cell extracts were made and NF-κB activity was measured by EMSA. EMSA with an Oct-1 probe was used as a control. Western blots of total protein extracts were probed with anti-PIASy antibody. (c) HEK293 cells were transfected with control or two different siRNA oligonucleotides against PIASy (oligo #2 and oligo #4). Cells were treated with 10 μM VP16 for 90 min. Total cell extracts were analysed as in b. (d) HEK293 cells were transfected as stated in a. Cells were treated with 20 Gy of ionizing radiation (IR) for 90 minutes, 25 μM doxorubicin (D) for 105 minutes and 60 J m–2 UV (254 nm) for 135 minutes. Total cell extracts were analysed as in b. (e) HEK293 cells were transfected twice with control siRNA, PIASy siRNAs, Ubc9 siRNAs, or the super-repressor 1xBcr32023A along with 3x-xB–Luc. NF-κB reporter gene and 24 h after the second transfection cells were treated with VP16 for 8 h. The cell extracts were used for the luciferase assay and relative luciferase activities were plotted by comparing fold induction with untreated control. Western blots of the same cell extracts with anti-PIASy, anti-Ubc9, and anti-HA antibodies are shown to demonstrate knockdown efficiency. Means ± s.e.m. are shown (n = 3). Mean values were compared to the VP16-treated control siRNA using an unpaired t-test and determined to be statistically significant. *, P < 0.01; **, P < 0.03; *** P < 0.01. (f) HEK293 cells were transfected with control or PIASy siRNAs and treated as stated above with increasing doses of VP16. RNA was analysed for IL-8 expression using quantitative real time RT–PCR. Means ± s. d. are shown (n = 4). Median values were compared to the VP16-treated control siRNA using the Mann-Whitney Rank Sum test and determined to be statistically significant. *, P < 0.03. An uncropped scan of the top gel in b is shown in the Supplementary Information, Fig. S5a.

whereas the smaller bands may represent translation products derived from internal methionines or degradation products. After the sumoylation reaction, three major slower-migrating bands were observed (see Supplementary Information, Fig. S2b), of which the two highest bands could be immunoprecipitated with an anti-HA antibody. These bands also reacted with anti-NEMO and anti-SUMO-1 antibodies when analysed by western blotting (data not shown), indicating that they are sumoylated full-length NEMO species. The nature of the third band could not be determined by these analyses.

When Flag–PIASy expressed in and immunopurified from HEK293 cells was added to the in vitro sumoylation reaction under the conditions in which the sumoylation of NEMO was minimal with only E1 and E2 (Fig. 3a), an enhancement of NEMO sumoylation was observed that was dependent on all components of the reaction mixture. Similarly prepared PIASyCA failed to enhance NEMO sumoylation. Quantification of enhancement in repeated experiments demonstrated a 2.5-fold enhancement by PIASy, but not by PIASyCA.

In accordance with the immunopurified PIASy described above, recombinant human His–PIASy purified from Escherichia coli showed enhancement of NEMO sumoylation in vitro (not shown). However, we were unable to purify it to near homogeneity. Thus, a recombinant Xenopus PIASy protein that shares 77% sequence identity and 84% homology with the human protein (His–PIASy, Fig. 3d) and that was previously described for in vitro sumoylation of topoisomerase II, was used4. His–xPIASy, purified to near homogeneity, enhanced sumoylation of in vitro translated NEMO, which showed major bands similar to those observed with FLAG–PIASy (Fig. 3c), as well as high molecular weight smears or ladders. All modified NEMO species could be cleaved by the catalytic domain of recombinant SENP1, a SUMO protease (Fig. 3c). Importantly, His–xPIASy promoted SUMO-1 isomorph-specific modification of NEMO, similar to genotoxic stress conditions in vivo, as it failed to efficiently sumoylate NEMO in the presence of SUMO-2 or SUMO-3 (see Supplementary Information, Fig. S2e).

A similar result was obtained using His–NEMO protein expressed in E. coli and purified to near homogeneity (Fig. 3d, e). Thus, these results demonstrated that PIASy enhanced SUMO-1 modification of NEMO in a highly purified in vitro system. In addition, overexpression of Xenopus PIASy could enhance NF-κB activation in HEK293 cells and could also
Figure 2 PIASy modulates NEMO sumoylation and NF-κB activation in response to genotoxic stress. (a) HEK293 Myc–NEMO stable cells were transfected with control or PIASy siRNAs. Cells were left untreated or treated with 10 μM VP16 for indicated times in minutes. Cell lysates were boiled in 1% SDS and 10% input samples were taken. The remaining samples were then diluted to 0.1% SDS and immunoprecipitated with anti–Myc antibody. The precipitates were blotted with anti-SUMO-1 and anti-Myc antibodies. Inputs were blotted with anti-PIASy antibody. (b) HEK293 cells were transfected with control or PIASy siRNAs and treated with 10 ng ml–1 TNFα for 15 min, 10 μM CPT for 75 min or 10 μM VP16 for 60 min. Total cell extracts were examined by immunoblotting with an anti-ATM and anti-phospho-Ser 1981-ATM antibodies. PIASy protein was examined by immunoblotting with an anti-PIASy antibody. The precipitates were blotted with anti-SUMO-1 and anti-Myc antibodies. Inputs were blotted with anti-PIASy antibody. (c) HEK293 cells were transfected as stated in a and treated with 10 μM VP16 for the indicated times. Total cell extracts were examined by immunoblotting with an anti-PIASy and anti-PIASyCA expression vector. Cells were processed as in (a). (d) HEK293 Myc–NEMO stable cells were transfected with 0.5 and 1.5 μg of a PIASy expression vector. Cells were left untreated or treated with 10 μM VP16 for 60 min. Cell lysates were prepared and immunoprecipitated as in a. The precipitates were blotted with anti-SUMO-1 and anti-Myc antibodies. 10% inputs were blotted with anti-HA antibody. (e) HEK293 cells were transfected with 0.5, 1.5 and 4.0 μg of a PIASy expression vector. Cells were treated with 10 μM VP16 for 60 min and 10 ng ml–1 TNFα for 15 min. Total cell extracts were measured for NF-κB activity by EMSA. Cell lysates were probed with anti–IκBα, anti-NEMO and anti-Myc antibodies. n.s., nonspecific band. (f) HEK293 Myc–NEMO stable cells were transfected with a PIASy expression vector. Cells were processed as in a. Precipitates were blotted as in d. (g) HEK293 cells were transfected with 0.5, 1.5 and 4.0 μg of a PIASy expression vector. Cells were analysed as in e. An uncropped scan of the top gel in a is shown in the Supplementary Information, Fig. S5b.

NEMOκKO lost the slower migrating of the two sumoylated bands (closed circle). These results suggested that the upper of the two bands represented sumoylated NEMO on Lys 309 and the lower band on Lys 277. When both lysines were mutated (NEMOκKO), the two bands migrated differently from those seen in the wild-type NEMO protein (single and double stars). Thus, it is likely that mutation of Lys 277 and Lys 309 results in sumoylation of NEMO on alternative sites.
Figure 3 PIASy promotes sumoylation of NEMO in vitro. (a) HEK293 cells were transfected with Flag-PIASy WT and CA constructs or vector control (V). Cell lysates were immunoprecipitated with an anti-Flag antibody and washed extensively using high salt buffer. Immunopurified Flag–PIASy WT and CA mutant, and vector control used for in vitro translated 35S HA–NEMO, and the reaction mixtures were incubated at 30 °C for 90 min. Reaction products were separated by SDS–PAGE and the position 311 (Fig. 3f). To further examine site-selective sumoylation in vitro, Ala 276 was mutated to isoleucine to create a more hydrophobic residue at the −1 position. This resulted in increased sumoylation of the putative Lys 277 band compared with wild-type NEMO (Fig. 3g). In contrast, when Asp 311 was mutated to glutamate, sumoylation of the Lys 309 band was not greatly affected, suggesting that PIASy promotes sumoylation of NEMO at Lys 309 equivalently with either aspartate or glutamate at the +2 position. Mutations within the carboxy-terminal zinc finger domain of NEMO severely hinder NEMO sumoylation position 311 (Fig. 3f). To further examine site-selective sumoylation in vitro, Ala 276 was mutated to isoleucine to create a more hydrophobic residue at the −1 position. This resulted in increased sumoylation of the putative Lys 277 band compared with wild-type NEMO (Fig. 3g). In contrast, when Asp 311 was mutated to glutamate, sumoylation of the Lys 309 band was not greatly affected, suggesting that PIASy promotes sumoylation of NEMO at Lys 309 equivalently with either aspartate or glutamate at the +2 position. Mutations within the carboxy-terminal zinc finger domain of NEMO severely hinder NEMO sumoylation.
was present in NEMO precipitates without any stimulation (Fig. 4a and see Supplementary Information, Fig. S3b) and this interaction was further augmented on treatment with VP16. Myc–NEMO mutants (Fig. 4b) and Flag–PIASy were co-expressed and their interactions were examined to determine the region(s) of NEMO that are critical for PIASy interaction. Successive C-terminal deletions demonstrated that NEMO1–126 was sufficient to interact with PIASy (Fig. 4c and see Supplementary Information, Fig. S3c). A deletion of the first 120 amino-acid residues of NEMO abrogated its interaction with PIASy. These results showed that the amino terminus of NEMO is both necessary and sufficient for PIASy association.

The N-terminal region of NEMO is also essential for IKKβ interaction. Coimmunoprecipitation experiments with NEMO mutants and Flag–IKKβ demonstrated that association of the NEMO mutants with IKKβ was nearly identical to interaction with PIASy (Fig. 4d). Cotransfection and coimmunoprecipitation experiments further demonstrated that NEMO–PIASy and NEMO–IKK interactions are mutually exclusive (Fig. 4e and see Supplementary Information, Fig. S3d). Moreover, subcellular fractionation studies revealed a NEMO–PIASy complex in the nuclear fraction, but the majority of IKKβ was located in the cytoplasm (Fig. 4f). Even though a small amount of IKKβ was observed in the nuclear fraction under these conditions, an IKKβ–PIASy interaction was not detected. These results suggested that PIASy–NEMO interaction occurs predominantly in the nucleus in a manner mutually exclusive with NEMO–IKKβ interaction.

As many double-strand break (DSB)-inducing agents also induce oxidative stress, we examined whether such a stress could be critical for NEMO sumoylation and NF-κB activation. The antioxidants N-acetylcysteine (NAC) and pyrrolidinedithiocarbamate (PDTC) prevented sumoylation of endogenous NEMO and NF-κB activation. The antioxidants with 2% inputs are also shown. (f) HEK293 cells were transfected with Flag-PIASy, Myc–NEMO or empty vector. Cytoplasmic and nuclear extracts were isolated and PIASy was immunoprecipitated with anti-Flag antibody. 2% input was also probed with anti-Myc antibody. (d) HEK293 cells were transfected with Flag-IKKβ and Myc–NEMO mutants. Extracts were immunoprecipitated with Flag-IKKβ, Myc–NEMO and increasing amounts of HA–PIASy construct. Cell extracts were immunoprecipitated with an anti-Flag antibody and probed with anti-Flag and HRP-conjugated anti-HA and anti-Myc antibodies. Immunoblots with 2% inputs are also shown. (f) HEK293 cells were transfected with Flag-PIASy, Myc–NEMO or empty vector. Cytoplasmic and nuclear extracts were isolated and PIASy was immunoprecipitated with anti-Flag antibody. Samples were immunoblotted with anti-Flag, anti-Myc, anti-IKKβ and anti-tubulin antibodies.

**Figure 4** NEMO interacts with PIASy in a manner that is mutually exclusive with IKKβ binding. (a) HEK293 cells were transfected with HA–PIASy or empty vector. Lysates were immunoprecipitated with anti-NEMO antibody or a mouse IgG control. Samples were immunoblotted with anti-HA and anti-NEMO antibodies. (b) Schematic representation of NEMO mutants used for coimmunoprecipitation analysis with human Flag-PIASy and human Flag-IKKβ. Amino acids 63–193 represent coiled-coil 1 (CC1), 258–298 represent coiled-coil 2 (CC2), 319–347 represent a leucine zipper and 397–418 represent a zinc finger. (c) HEK293 cells were transfected with Flag-PIASy and Myc–NEMO mutants. Cells extracts were immunoprecipitated with an anti-Flag antibody and probed with an HRP-conjugated anti-Myc antibody. 2% input was also probed with anti-Myc antibody. (d) HEK293 cells were transfected with Flag-IKKβ and Myc–NEMO mutants. Extracts were immunoprecipitated as in c. (e) HEK293 cells were transfected with Flag-IKKβ, Myc–NEMO and increasing amounts of HA–PIASy construct. Cell extracts were immunoprecipitated with an anti-Flag antibody and probed with anti-Flag and HRP-conjugated anti-HA and anti-Myc antibodies. Immunoblots with 2% inputs are also shown. (f) HEK293 cells were transfected with Flag-PIASy, Myc–NEMO or empty vector. Cytoplasmic and nuclear extracts were isolated and PIASy was immunoprecipitated with anti-Flag antibody. Samples were immunoblotted with anti-Flag, anti-Myc, anti-IKKβ and anti-tubulin antibodies.
In summary, we have shown that PIASy meets the minimal requirements of a SUMO ligase for NEMO in the genotoxic stress-induced NF-κB signalling pathway. First, a reduction of the expression of endogenous PIASy by siRNAs reduces the cellular capacity to cause SUMO-1 modification of NEMO and activate NF-κB in response to several DNA-damaging agents. In contrast, some of the other E3s tested showed reproducible increases in NF-κB activation (for example, PIAS1 and PIAS3). It is unclear how these siRNAs cause this increase, but PIAS1 and PIAS3 have been shown to cause inhibition of p65 DNA binding activity\(^{18,19}\). Although we cannot exclude the possibility that other SUMO E3s also contribute to NEMO sumoylation in specific cellular contexts, our study demonstrates that PIASy is critical for NF-κB activation by genotoxic agents in multiple cell types.

In addition, we found that the N-terminal domain of NEMO is both necessary and sufficient for PIASy interaction. Interestingly, this overlaps with the region of NEMO where IKKβ associates\(^{12}\). IKKβ and PIASy fail to coimmunoprecipitate in the presence of NEMO, suggesting that NEMO–PIASy and NEMO–IKKβ complexes are mutually exclusive. The interaction between NEMO and PIASy is largely nuclear, as opposed to the IKKβ–NEMO interaction that is mostly cytoplasmic\(^{12}\). Thus, cell compartmentalization is an additional means to segregate these complexes. Our previous study indicated that IKK-free NEMO present in the human genome identified only CYLD (cylindromatosis tumor suppressor protein) as functioning in cytokine signalling\(^{17}\). Similarly, our RNAi screen against SUMO E3s found that only siRNAs against PIASy had inhibitory effects on NF-κB activation by genotoxic stress inducers. In contrast, some of the other E3s tested showed reproducible increases in NF-κB activation (for example, PIAS1 and PIAS3). It is unclear how these siRNAs cause this increase, but PIAS1 and PIAS3 have been shown to cause inhibition of p65 DNA binding activity\(^{18,19}\).

Figure 5 Oxidative stress seems to be required for NEMO sumoylation in response to VP16. (a) CEM cells were pretreated for 3 h with 50 mM NAC and for 30 min with 100 μM PDTC. Cells were then treated for 1 h with 10 μM VP16. Cell lysates were boiled in 1% SDS and then diluted to 0.1% SDS and immunoprecipitated with anti-NEMO antibody. Precipitates were immunoblotted with anti-SUMO-1 and anti-NEMO antibodies. (b) CEM cells were treated as above and cell lysates were analysed by EMSA and western blotted with an anti-ATM and anti-phospho-Ser 1981-ATM antibodies. (c) 1.3E2 NEMO-deficient murine pre-B cells stably expressing Myc–NEMO were pretreated with NAC and PDTC as above. Cells were treated with 2 mM H₂O₂ for 45 min. Cell lysates were immunoprecipitated with anti-Myc antibody and precipitates were immunoblotted with anti-SUMO-1 and anti-Myc antibodies. (d) HEK293 cells were transfected with Myc–PIASy and 24 h later they were treated with 2 mM H₂O₂ for 45 min. Cell lysates were immunoprecipitated with an anti-NEMO antibody and the precipitates were immunoblotted with HRP-conjugated anti-Myc and anti-NEMO antibodies. (e) Schematic representation of a model for genotoxic stress-induced NF-κB activation. Genotoxic agents cause both DSBs and oxidative stress. DSBs lead to ATM activation, whereas oxidative stress may promote NEMO–PIASy interaction and NEMO sumoylation. These parallel events together promote NEMO ubiquitination and activation of IKK and NF-κB. An uncropped scan of the top gel in a is shown in the Supplementary Information, Fig. S5c.
becomes sumoylated and SUMO-modified NEMO accumulates in the nucleus. The mutually exclusive interactions between NEMO–IKKβ and NEMO–PIASy partly explain why only IKK–free NEMO is targeted for sumoylation in vivo.

Nuclear interaction of PIASy–NEMO and increased basal sumoylation and NEMO nuclear accumulation (data not shown) on PIASy overexpression also suggest that NEMO sumoylation may occur in the nuclear compartment. As SUMO–NEMO fusion proteins displayed tendencies to accumulate in the nucleus, SUMO modification was suggested to promote NEMO nuclear targeting. The zinc finger domain of NEMO was required for both sumoylation and nuclear targeting, further suggesting that this domain is required for sumoylation to take place. However, our current data demonstrate that the zinc finger is not required for NEMO interaction with PIASy in vivo or sumoylation in vitro. Instead, it may be critical for events involved in oxidative-stress signalling to promote NEMO nuclear localization (Fig. 5e) or PIASy interaction of NEMO that is already located in the nucleus. Alternatively, the zinc finger domain may be necessary for stabilization of sumoylated NEMO, possibly by interfering with its interaction with a SUMO protease. PIDD (p53-inducible death domain) and RIP1 (receptor interacting protein 1) may be involved in the regulation of nuclear accumulation and sumoylation of NEMO in a manner dependent on the zinc-finger domain. As activation of NF-κB by a variety of anti-cancer DNA-damaging agents is linked to modulation of malignant behaviours (including resistance to chemos- and radiation therapy), further understanding of the enzymatic components and mechanisms involved may facilitate the development of novel anti-cancer agents targeting this signalling pathway.

METHODS
Reagents. Etoposide (VP16), camptothecin (CPT), doxorubicin, 2, 5-diphenyloxazole (PPO), phenylmethylsulphonylfluoride (PMSF), aprotinin, N-acetylcyesteine, N-ethylmaleim ide and iodoacetamide were obtained from Sigma (St Louis, MO). Pyrrolidinedithio-carbamate was obtained from Alexis Corporation (Lausanne, Switzerland). Human recombinant TNFα and anti-tubulin antibody were obtained from Calbiochem (La Jolla, CA). Antibodies for horseradish peroxidase (HRP)-conjugated anti-goat, IκBα, c-Myc and NEMO were obtained from Santa Cruz (Santa Cruz, CA). Anti-c-Myc and anti-HA peroxidase antibodies were obtained from Roche (Indianapolis, IN). Anti-NEMO antibody was obtained from BD Pharmingen (San Jose, CA). Anti-Flag antibody was obtained from Sigma. Anti-GMP-1 (SUMO-1), SUMO-2 and SUMO-3 antibodies were obtained from Zymed Laboratories (San Francisco, CA). ATM antibody was purchased from Genetex (San Antonio, TX) and anti-phospho-ATM (Ser1881) was obtained from R & D Systems (Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse antibodies, Gamma bind G-Sepharose beads and protein G–Sepharose beads were obtained from Amersham (Piscataway, NJ). SUMO-1 antibody, human recombinant SUMO-1, SUMO-2, SUMO-3, Saccharomyces cerevisiae SUMO E1 (Aps1p/Uba1p), human recombinant SUMO E2 (Ubc9) and human recombinant His–SENP1 catalytic domain (CD) proteins were purchased from Boston Biochem (Cambridge, MA).

Cell culture. HEK293 and CEM cells were maintained as previously described. HEK293 6× Myc–NEMO and 1.3E2 6× Mym–NEMO stable cell lines were generated as previously described.

siRNAs, RNA isolation and RT–PCR. siRNA-mediated knockdown of polycomb 2 (Pc2), PIAS1, PIAS3, PIASxα/β, PIASy and MMS21-like was performed by calcium phosphate transfection of 200 pmol of siGENOME SMARTpool double-stranded RNA oligonucleotides (Dharmacon, Lafayette, CO) or control siRNA (Dharmacon). A second transfection was also performed after 24 h to increase RNA interference efficiency. RNA from these transfected cells was isolated using the QiAshredder and RNeasy mini kits (Qiagen, Valencia, CA) 24 h after the second transfection. Multiplex RT–PCR was performed using Qiagen's OneStep RT-PCR kit according to the manufacturers’ protocol. RT–PCR primers and siRNA oligonucleotide sequences are provided in the Supplementary Information, Fig. S4.

Cloning of PIASy and NEMO wild type and mutants. Generation of pcDNA3.1(+) 6×Myc–NEMO and pcDNA3.1(+) 2×HA–NEMO was described previously. His–NEMO was cloned into pET28a(+) from Novagen (Madison, WI) using restriction sites HindIII and Xhol. pcDNA3.1(+) 2×HA–NEMO A276I and D311E mutants were generated using site directed mutagenesis from pcDNA3.1(+)(ref. 3) into pcDNA3 2×HA–NEMO template. pcDNA3 2×HA–NEMO K277A and K309A mutants were generated through subcloning of pcDNA3 6×Myc–NEMO K277A and K309A (ref. 3) into pcDNA3 2×HA (Invitrogen) vector using restriction sites BamHI and Xhol. Flag–Myc and HA-tagged full-length PIASy was cloned through PCR amplification of PIASy from ATCC (Mammalian Genome Collection, Manassa, VA; IMAGE ID: 5176540) into pFlag–CMV-2 (Sigma) using NotI–Xhol restriction sites. PIASy was cloned into pcDNA3.1(+) 6×Myc (a gift from Z. Chen, UT-Southwestern, Dallas, TX) and pcDNA3.1(+)(ref. 3) into pcDNA3 2×HA constructs using BamHI–EcoRI restriction sites. PIASy C342–347A (CA) mutant was generated using two-step PCR mutagenesis from the original PIASy template. Primers used for cloning are available on request. All constructs were verified by DNA sequencing. Xenopus pET28a–PIASy was subcloned into the pcDNA3–Myc vector using EcoRI and Xhol restriction sites. Generation of a Flag–IKKβ construct was previously described.

Western blotting, immunoprecipitation and EMSA. Cell lysates production and western blotting was performed as previously described. EMSA analysis and image quantification was also performed as previously described. For NEMO–PIASy immunoprecipitations, cell lysates were preclariated overnight with Gamma Bind G-Sepharose. Preclarayed lysates were then immunoprecipitated as previously described.

Luciferase reporter assay. HEK293 cells were transfected with 25 ng of 3×κB–Luc, 25 ng of CMV–β-Gal, and 200 pmol of PIASy siRNA or control siRNA as stated above. Cells were treated with 10 µM VP16 for 8 h and harvested according to Promega’s luciferase assay system kit. β-galactosidase activity was measured using the Galecton-Plus kit purchased from Tropix (Bedford, MA). The transfection efficiency was normalized within each cell line with β-galactosidase activity. Results were displayed as fold induction from untreated control.

Quantitative RT–PCR analysis. HEK293 cells were transiently transfected with control and PIASy siRNA as stated above and treated with increasing doses of VP16 for 3 h. Generation of total RNA from treated cells, cDNA synthesis from total RNA and quantitative real-time RT–PCR was previously described. PCR primers used in this study are provided in the Supplementary Information, Fig. S4. Product accumulation was monitored by SYBR green fluorescence, with the relative expression levels determined from a standard curve of serial dilutions of cDNA. All samples were normalized to GAPDH levels and results were displayed as fold induction from untreated control.

Purification of recombinant His–NEMO. His–NEMO protein was generated in BL21 Rosetta 2 strain cultured in LB broth containing 25 µg ml–1 kanamycin and 34 µg ml–1 chloramphenicol. Cultures were induced at 30°C with 1 mM IPTG for 3 h at 37°C. Cell pellets were resuspended in equilibration/wash buffer (50 mM sodium phosphate, 300 mM NaCl at pH 7.0) and lyzed by sonication (4 rounds of 10 s bursts on ice). Cell debris and inclusion bodies were removed by centrifugation. The resulting supernatant was added to BD Talon Co2+ metal affinity resin (BD Biosciences). Bound protein was washed three times in equilibration/wash buffer and eluted in equilibration/wash buffer containing 150 mM imidazole. His–NEMO protein was then dialyzed against 50 mM Tris at pH 7.6 and 5 mM MgCl2.

In vitro and in vivo sumoylation assays. In vitro sumoylation of NEMO was performed by in vitro translation of HA–NEMO protein using the TNT T7 Coupled Reticulocyte Extract System (Promega) in the presence of Redivue 35S-methionine (Amersham). In vitro sumoylation assays were performed using 7.5 µg ml–1 SUMO1 E1 (Aps1/Uba1), 50 µg ml–1 SUMO E2 (Ubc9) and 50 µg ml–1 of SUMO-1 added to 4 µl of in vitro translated NEMO in the presence of an ATP regenerating system (10 mM creatine phosphate, 10 units creatine kinase, 1 unit inorganic pyrophosphatase and 2 mM ATP) in 50 mM Tris at pH 7.6 and 5 mM MgCl2.
in a 40 µl reaction with 1 µg ml⁻¹ aprotinin and 1 mM PMSE. The reaction was incubated at 30 °C for 180 min. End products were terminated with SDS sample buffer and separated by SDS–PAGE gels. Sumoylated products were detected by fixing SDS–PAGE gels in acetic acid followed by addition of the scintillant PPO. Gels were dried and exposed to autoradiograph film (Kodak, Rochester, NY). For in vitro sumoylation with recombinant His–NEMO and His–xPIASy reaction conditions were performed as stated above with the exception of using 0.75 µg His–NEMO substrate and 1 µg His–xPIASy in a 40 µl reaction. End products were treated as above except SDS–PAGE gels were transferred and immunoblotted with goat anti-NEMO, rabbit anti-SUMO and His anti-mouse antibodies. For proteolysis of SUMO modified NEMO, SUMO reaction end products (40 µl were treated as above except SDS–PAGE gels were transferred and immunoblotted as stated above except SDS sample buffer and reaction products separated by SDS–PAGE gels.

To determine the effects of PIASy on NEMO sumoylation in vitro, pFlag–CMV-2 PIASy wild-type and pFlag–CMV-2 PIASyC242A expression constructs were transiently transfected into HEK293 cells. Cell extracts were prepared from these cells 24 h following transfection in immunoprecipitation buffer (20 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA, 250 mM NaCl at pH 7.4). PIASy protein was immunoprecipitated by addition of anti-Flag antibody and GammaBind G–Sepharose. The immunoprecipitates were washed in high salt immunoprecipitation buffer (20 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA and 500 mM NaCl at pH 7.4) with 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM DTT, 1 µg aprotinin per ml and 0.5 mM PMSE four times, followed by washing two times with in vitro sumoylation buffer (50 mM Tris and 5 mM MgCl₂ at pH 7.6) with 1 µg aprotinin per ml and 0.5 mM PMSE. Immune purified PIASy beads (20 µl) were added to the sumoylation reaction as stated above but incubated at 30 °C for 110 min.

To detect in vivo sumoylation of NEMO, cells were rinsed twice with PBS, harvested and pelleted by centrifugation. The cell pellets were then lysed in an immunoprecipitation buffer (20 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA and 250 mM NaCl at pH 7.4) in the presence of 1% SDS, protease and phosphatase inhibitors as well as 10 mM N-ethylmaleimide and 3 mM iodoacetamide.

Cell fractionation. Briefly, nuclear and cytoplasmic extracts were generated through hypotonic shock followed by nuclear extraction with salt as previously described.

Statistical analysis. Statistical analysis was performed with SigmaStat 3.0 software (SYSTAT Software, Inc., Richmond, CA). Mean values with equal variance were compared using and unpaired t-test. Mean values with unequal variance were compared using the Many-Whitney Rank Sum test.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.

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Figure S1: a. HEK293 cells were transfected with control or siRNAs against different SUMO ligases. Cells were treated with 10 μM VP16 for 60 minutes or 10ng/mL TNFα for 15 minutes. Total cell extracts were made and NF-κB activity was measured using EMSA. b. HEK293 and HEK293 IκBα-S32/36A stable cells were treated with 10 μM VP16 for 3 hours. RNA from cells was isolated and RT-PCR was performed using IL-8 and GAPDH primers. c. HEK293 cells were transfected with control or PIASy siRNAs and treated with increasing doses of VP16 for 3 hours. RNA was isolated and analyzed for IκBα using quantitative real time RT-PCR. d. CEM cells were transfected with control or PIASy siRNAs and treated with 10 μM VP16 for 6 hours. Samples were analyzed for p21 using quantitative real time RT-PCR.
**Figure S2**: a. Light exposure of *in vitro* translated NEMO assay performed in b. 

*b.* *In vitro* translated 35S-methionine-labeled HA-NEMO was added to *in vitro* SUMOylation reaction. SUMO E1 and E2 were added as stated in materials and methods. Samples were run on an SDS-PAGE gel, fixed, and exposed to film. Alternatively, reaction products were boiled in 1% SDS and immunoprecipitated with an anti-HA antibody and analyzed as stated above. 

*c.* HEK293 cells were transfected with 0.15, 0.5, 1.5, and 4.0 µg of Xenopus PIASy (Myc-PIASy) DNA. Cells were treated with 10 µM VP16 for 75 minutes. Total cell extracts were measured for NF-kB activity by EMSA. 

*d.* HEK293 cells were transfected with PIASy siRNA (oligo#2) and 0.2 µg Myc-PIASy DNA. Cells were treated with 10 µM VP16 for 120 minutes. NF-kB activity was measured as in c. 

*e.* *In vitro* translated 35S-methionine-labeled HA-NEMO was added to SUMO reaction as in a. Reactions were performed in the presence of different SUMO protein isoforms: SUMO-1 (lanes 1-3), SUMO-2 (lanes 4-6), or SUMO-3 (lanes 7-9). 0.5 µg of His-PIASy was added to the SUMO reaction. The mixture was incubated at 30 °C for 75 minutes and terminated in 2xSDS sample buffer. Samples were run on an SDS-PAGE gel, fixed, and exposed to film. Alternatively, terminated reaction end products were western blotted and probed with mouse anti-SUMO-1 and rabbit anti-SUMO-2 (which also recognizes SUMO-3), showing that His-PIASy can promote modification of reticulocyte proteins by SUMO-1, -2 and -3 even though it preferentially promotes SUMO-1 modification of NEMO.
**Figure S3:** a. A similar experiment as shown in Figure 3g was performed using immunopurified human Flag-PIASy instead of His-xPIASy. The SUMOylation banding patterns of different NEMO mutants are nearly indistinguishable from those shown in Figure 3g. b. HEK293 cells were transfected with Flag-PIASy, Myc-NEMO, and Flag-PIASy-CA. Cells were harvested and lysed, and cell extracts were immunoprecipitated using anti-Flag antibody. Western blotting was performed using anti-Myc and anti-FLAG antibodies. c. HEK293 cells were transfected with Flag-PIASy and Myc-NEMO mutants. Cells were immunoprecipitated with an anti-Flag antibody and probed with an HRP-conjugated anti-Myc antibody. d. HEK293 cells were transiently transfected with HA-PIASy, Flag-I KKβ, and Myc-NEMO constructs. Flag-I KKβ was titrated in increasing DNA ratios with PIASy. Cell extracts were immunoprecipitated with an anti-HA antibody and probed with anti-Flag and HRP-conjugated anti-HA and anti-Myc antibodies.
### RT-PCR Primers

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<th>- strand</th>
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<td>5' - ACCCCTTCATAGCCAACCTCAACTAC-3'</td>
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<td>5' - GCACTCCAGGGAACACCTTGATA-3'</td>
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#### siRNA Oligonucleotides

| Control: 5' - UACCGUCUCACACUUGAUUGdTdT-3' | 5' - CGAUCAAGGGGAACGGAUCdTT-3' |
| PIASy oligo #2: 5' - CAAGACAGGGAGGGUGGAUUU-3' | 5' - PAUCAACUCACCCCGCUUGUUU-3' |
| PIASy oligo #4: 5' - AAGCGUUGCGUUCUUAAUUU-3' | 5' - PAUUAAGAAACGGCAGCUUUU-3' |

#### Real Time RT-PCR Primers

| GAPDH: 5' - GAAAGTCGG-AGTCAACCGGATT-3' | 5' - GAATTTGGCCATGGGATGAAAT-3' |
| IL-8: 5' - GCCAGCTCTGTGGTGGGCTAC-3' | 5' - CGCAGTGTGGTCCACTCTCA-3' |
| IkBα: 5' - GCTACCACTCAATGGCCCACA-3' | 5' - TAGCCATGGATAGGGCTAAGTGAGA-3' |
| p21: 5' - GCAGACCCAGCATGACAGATTTC-3' | 5' - GGGAATAGGGCTTCTCCTT-3' |

*Figure S4:* Sequences of RT-PCR primers and some of the siRNA oligos used in this study.
**Figure S5**: Full scans of original EMSA gel and Western blots. 

**a.** Full scan of the top panel of Figure 1b EMSA gel. n.s. refers to a nonspecific band. 

**b.** Full scan of the top panel of Figure 2a SUMO-1 Western blot. h.c. refers to IgG heavy chain and l.c. refers to light chain. 

**c.** Full scan of the top panel of Figure 5a SUMO-1 Western blot.