AKT-dependent phosphorylation of Niban regulates nucleophosmin- and MDM2-mediated p53 stability and cell apoptosis

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Although Niban is highly expressed in human cancer cells, the cellular functions of Niban remain largely unknown. We demonstrate here that ultraviolet irradiation induces phosphorylation of Niban at S602 by AKT, which increases the association of Niban with nucleophosmin and disassociation of nucleophosmin from the MDM2 complex. This leads to the promotion of MDM2–p53 interaction and subsequent p53 degradation, thereby providing an antiapoptotic effect. Conversely, depletion of or deficiency in Niban expression promotes stabilization of p53 with increased cell apoptosis. Our findings illustrate a pivotal role for AKT-mediated phosphorylation of Niban in protecting cells from genotoxic stress-induced cell apoptosis.

Keywords: Niban; AKT; nucleophosmin; p53; MDM2; apoptosis

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INTRODUCTION

The *Niban* gene, also known as family with sequence similarity 129, member A (FAM129A), consists of 14 exons located on

human chromosome 1. *Niban* was originally identified from a rat with renal carcinoma having the *TSC2* gene mutation [1]. Overexpression of Niban has been detected in patients with many types of cancer, including thyroid, head and neck, renal, and liver cancer. Niban is highly expressed in the early stages of cancer development and remains overexpressed throughout the cancer progression [2–5]. Niban is also upregulated in its mRNA and protein expression in response to endoplasmic reticulum stress and regulates protein translation machinery by modulating phosphorylation of eukaryotic translational initiation factor 2 α , p70 ribosomal S6 subunit kinase 1, and eukaryotic initiation factor 4E-binding protein 1 [6]. However, how Niban executes its cellular functions and its exact role in cell survival and apoptosis remain largely unknown.

P53 has a key role in the regulation of cell-cycle progression and promotes apoptosis in response to multiple stress signals, including DNA damage, oxidative stress, metabolic stress, and deregulated oncogene activation [7–9]. In unstressed normal cells, p53 activity is maintained at low levels through a combination of p53 degradation and transcriptional restraint, which are primarily mediated by murine double minute 2 (MDM2) [10]. MDM2 regulates p53 activity through both ubiquitination-dependent p53 degradation and repression of p53 transcriptional activity [11,12]. Interestingly, the *MDM2* gene in turn is transcriptionally activated by p53, constituting a feedback regulatory loop [13].

P53 stability and transcriptional activity can be regulated through control of its interaction with MDM2 by distinct mechanisms. ADP-ribosylation factor interferes with MDM2–p53 interaction, leading to stabilization and activation of p53 [12]. The other prominent regulator of MDM2 besides ADP-ribosylation factor is MDMX (also known as Mdm4). MDM2 and MDMX interact with each other through their C-terminal really interesting new gene (RING) domains, and MDMX promotes the E3-ligase activity of MDM2 for p53 degradation [12]. In addition, nucleophosmin (NPM)/B23, a

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Fig 1 | AKT interacts with and phosphorylates Niban at S602. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. (A) The indicated cell lines were treated with ultraviolet irradiation (100 J/m²) and then incubated for the indicated period of time. (B) U87 cells were pretreated with or without AKT inhibitor IV (10 μ M) or U0126 (20 μ M) for 30 min before irradiation with or without ultraviolet. The cells were collected 3 h after ultraviolet irradiation. (C) U87 cells transiently expressing HA-AKT1 were pretreated with or without AKT inhibitor IV (10 μ M) for 30 min before irradiation with or without ultraviolet. The cells were collected 1 h after ultraviolet irradiation. (D) *In vitro* kinase assays were performed by mixing purified bacterially expressed WT His-Niban or the His-Niban S602A with or without purified active AKT1. (E) Vectors expressing HA-AKT1 DD or HA-AKT1 AAA were transfected into U87 cells. (F) U87 cells were pretreated with or without AKT inhibitor IV (10 μ M) for 30 min before irradiation with or without ultraviolet. The cells were collected 3 h after ultraviolet irradiation. (G) Vectors expressing FLAG-tagged WT Niban or Niban S602A mutant were transfected into 293 T cells. HA, haemagglutinin A; His, histidine; IP, immunoprecipitation; WB, western blot; WT, wild type.

nucleolar phosphoprotein that constantly shuttles between the nucleolus and cytoplasm, binds to human MDM2 and inhibits MDM2's function through regulation of MDM2-p53 interaction, thereby stabilizing p53 and increasing its transcriptional activity [14,15]. However, the mechanism underlying the regulation of NPM is unknown.

In the study reported herein, we demonstrated that ultraviolet irradiation results in phosphorylation of Niban at S602 by AKT, which promotes the association of Niban with NPM and inhibits the regulatory effect of NPM on p53-MDM2 complexes, thereby destabilizing p53 and inhibiting p53-dependent apoptosis.

RESULTS

AKT interacts with and phosphorylates Niban at S602

Protein phosphorylation acts as a key posttranslational modification to modulate protein-protein interactions and, subsequently, their functions [16,17]. To determine whether Niban is phosphorylated in response to genotoxic stress, we subjected U87 and D54 human glioblastoma cells and H460 human lung cancer cells, all of which contain wild-type (WT) *TP53*, to ultraviolet irradiation [18,19]. Immunoprecipitation of Niban with an antiNiban antibody followed by immunoblotting with antiphosphorylated serine, threonine, and tyrosine antibodies

revealed that ultraviolet irradiation induced phosphorylation of Niban at serine but not tyrosine or threonine (Fig 1A).

To identify the protein kinase that phosphorylates Niban, we analysed the amino-acid sequence of Niban using the Scansite online search programme (<http://scansite.mit.edu/>) [20] and found that potential AKT, extracellular signal-regulated protein kinase (ERK), and protein kinase A (PKA) phosphorylation sites are present in Niban. Treatment of U87 cells with AKT inhibitor IV, the MEK/ERK inhibitor U0126, or the adenosine 3',5'-cyclic monophosphorothioate (a PKA inhibitor) before ultraviolet irradiation inhibited ultraviolet-induced phosphorylation of AKT, ERK1/2 (Fig 1B), and PKA (data not shown), respectively. However, inhibition of AKT but not ERK or PKA (data not shown) blocked irradiation-induced serine phosphorylation of Niban. In addition, immunoprecipitation of endogenous Niban followed by immunoblot analysis with an antihaemagglutinin A (HA) antibody for transiently expressed HA-AKT1 showed that ultraviolet irradiation enhanced the binding of AKT1 to Niban, which was blocked by pretreatment with AKT inhibitor IV (Fig 1C). These results indicate that AKT binds to and phosphorylates Niban in response to ultraviolet irradiation.

To determine which Niban residues are phosphorylated by AKT, we analysed the Niban amino-acid sequence and found that the ⁵⁴⁷PARRAS⁶⁰² sequence of human Niban is closely related to the

Fig 2 | AKT phosphorylation of Niban S602 increases its binding to NPM. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. (A) *In vitro* kinase assays were performed by mixing purified bacterially expressed WT His–Niban or His–Niban S602A mutant with or without AKT1, which was followed by GST pull-down analyses with purified GST or GST–NPM. (B) Purified bacterially expressed WT His–Niban, His–Niban S602A mutant, or His–Niban S602D mutant was mixed with immobilized GST–NPM. A GST pull-down assay was performed. (C) Niban or NPM was immunoprecipitated from D54 cells treated with or without ultraviolet radiation (100 J/m²) and incubated for 1 or 3 h. (D) U87 cells were pretreated with or without AKT inhibitor IV (10 μM) for 30 min and with or without ultraviolet irradiation. The cells were collected 3 h after ultraviolet irradiation. (E) Vectors expressing FLAG–tagged WT Niban, Niban S602A, or Niban S602D were transfected into 293 T cells. GST, glutathione *S*-transferase; His, histidine; NPM, nucleophosmin; WB, western blot; WT, wild type.

AKT phosphorylation motifs RXX(S/T) [21] and RXX(S/T) [22,23]. Incubation of purified active AKT1 and purified WT Niban or Niban S602A, in which S602 was mutated to an alanine, showed that AKT1 phosphorylated WT Niban but not the Niban S602A mutant (Fig 1D). In addition, phosphorylation of Niban at S602 was recognized by an antibody that specifically recognized phosphorylated Niban at this residue. These results indicate that AKT phosphorylates Niban at S602.

To determine whether AKT can phosphorylate Niban *in vivo*, we transfected a constitutively active HA–AKT1 DD (T308D/S473D) or HA–AKT1 AAA (K179A/T308A/S473A) kinase-dead mutant into U87 cells. Immunoblotting of immunoprecipitated Niban with an antiphosphorylated Niban S602 antibody showed that expression of the HA–AKT1 DD, but not the HA–AKT1 AAA mutant, phosphorylated Niban at S602 (Fig 1E). In addition, ultraviolet irradiation-induced phosphorylation of WT Niban was blocked by pretreatment with AKT inhibitor IV (Fig 1F) and MK-2206, an allosteric AKT inhibitor [24] (supplementary Fig S1A online), or introduction of a S602A mutation (Fig 1G). Pretreatment with LY294002, a phosphoinositide-3-kinase inhibitor, largely reduced ultraviolet-induced Niban phosphorylation at S602, indicating that phosphoinositide-3-kinase upstream from AKT regulates Niban S602 phosphorylation (supplementary

Fig S1A online). Similar to ultraviolet irradiation, epidermal growth factor stimulation induced Niban S602 phosphorylation, which was significantly blocked by pretreatment with LY294002 and MK-2206 (supplementary Fig S1B online). These results indicate that AKT, which is activated by ultraviolet irradiation [25] or epidermal growth factor, phosphorylates Niban at S602.

Phosphorylated Niban increases its binding to NPM

To determine the effect of phosphorylation of Niban by AKT on the function of Niban in response to ultraviolet irradiation, we immunoprecipitated Niban from ultraviolet-irradiated U87 cells. Mass spectrometry analysis of a tryptic digest of the Niban-coprecipitated proteins detected two peptides—MTDQEAIQDLWQWR and MSVQPTVSLGGFEITPPVVLRL—that match the amino-acid sequence of NPM. To determine whether the interaction of Niban with NPM can be promoted by AKT-dependent phosphorylation of Niban, we performed glutathione *S*-transferase (GST) pull-down analyses by mixing purified GST–NPM with purified WT histidine (His)–Niban or the His–Niban S602A mutant in the presence or absence of purified active AKT1. Immunoblot analysis with an antiNiban antibody showed that binding of both WT Niban and Niban S602A to NPM in the absence of AKT was limited. However, inclusion of AKT1 significantly enhanced the binding of

Fig 3 | Niban inhibits binding of NPM to MDM2 and promotes MDM2–p53 interaction. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. (A) A control or Niban shRNA was stably expressed in U87 cells. (B) U87 cells with or without expression of Niban shRNA were irradiated with or without ultraviolet (100 J/m^2) and then incubated for 3 h. (C) pCMV5–MDM2 was co-transfected with a control vector or a vector expressing FLAG-tagged WT Niban, Niban S602A, or Niban S602D into HCT116 cells. The cells were collected 3 h after ultraviolet (100 J/m^2) irradiation. (D) Purified GST–NPM on glutathione agarose beads and purified His–MDM2 were mixed with or without purified His–Niban and/or active AKT1 in the presence or absence of AKT inhibitor IV ($10\text{ }\mu\text{M}$). GST, glutathione *S*-transferase; His, histidine; IP, immunoprecipitation; MDM2, murine double minute 2; NPM, nucleophosmin; shRNA, short hairpin RNA; WB, western blot; WT, wild type.

NPM to WT Niban, but not to Niban S602A (Fig 2A). In addition, a phosphorylation-mimic Niban S602D mutant, in which the S602 was mutated into an aspartic acid, increased its binding to GST–NPM compared with WT His–Niban and His–Niban S602A mutant (Fig 2B). The Niban S602A mutant showed reduced binding to NPM, suggesting that S602 of Niban is structurally involved in interacting with NPM and this interaction is enhanced by phosphorylation of Niban at S602.

The interaction between Niban and NPM was also analysed *in vivo*. Immunoblotting of immunoprecipitated endogenous Niban from D54 (Fig 2C, upper panel) with an antiNPM antibody showed that ultraviolet irradiation induced binding of Niban to endogenous NPM. These findings were further supported by reciprocal NPM immunoprecipitation followed by immunoblotting with an antiNiban antibody (Fig 2C, lower panel). In addition, ultraviolet irradiation–increased binding of Niban to NPM was blocked by pretreatment of the cells with AKT inhibitor IV (Fig 2D). In line with the increase amount of Niban S602D mutant pull-downed by GST–NPM *in vitro* (Fig 2B), a coimmunoprecipitation assay showed that Niban S602D exhibited greatly enhanced binding to NPM compared with WT Niban and the Niban S602A mutant (Fig 2E). These results indicate that phosphorylation of Niban at S602 by AKT in response to ultraviolet irradiation increased the binding of Niban to NPM.

Niban promotes MDM2–p53 interaction

NPM interrupts MDM2–p53 complex formation [15]. To determine whether AKT-mediated association of Niban with NPM affects the MDM2–p53 interaction regulated by NPM, we depleted Niban in

U87 cells by expressing its short hairpin RNA (Fig 3A). Immunoblotting of immunoprecipitated MDM2 with antibodies against NPM or p53 showed that Niban depletion increased basal and ultraviolet irradiation–induced interaction between MDM2 and NPM, but inhibited the interaction between MDM2 and p53 (Fig 3B). In addition, in contrast to overexpression of WT Niban and Niban S602D, Niban S602A overexpression, similar to Niban depletion, increased the binding of MDM2 to NPM, but reduced the association between MDM2 and p53 (Fig 3C). These results indicate that phosphorylation of Niban by AKT disrupts the binding of NPM to MDM2 and promotes MDM2–p53 interaction.

To determine whether Niban competes with MDM2 for binding to NPM, we incubated purified GST–NPM and MDM2 in the presence or absence of purified Niban and/or active AKT1. As shown in Fig 3D, the presence of Niban reduced the interaction between NPM and MDM2, and this effect was significantly enhanced by inclusion of AKT1, but impaired by pretreatment with AKT inhibitor IV. These results strongly indicate that AKT promotes Niban binding to NPM and this in turn sequesters NPM from interaction with MDM2. Thus, more free MDM2 would be available to interact with p53 protein and to regulate p53 expression. These results indicate that Niban inhibits the binding of NPM to MDM2 and increases MDM2–p53 interaction.

Niban promotes p53 downregulation and cell survival

P53 activation in response to stress signals has a central role in cell apoptosis [10]. Niban regulates the interaction between MDM2 and p53, suggesting that Niban mediates p53 stability, thereby

Fig 4 | Niban promotes downregulation of p53 expression and cell survival. (A,B) HCT116 cells transfected with a vector expressing Niban were irradiated with or without ultraviolet (100 J/m²) and then incubated for 6 h (A) or 24 h (B). (A) Immunoblotting analyses were performed with the indicated antibodies. (B) The cells were stained with annexin V-FITC-PI and analysed using a FACSCalibur flow cytometer. The data represent the means $\pm$ standard deviations from three independent experiments, * P < 0.05. (C,D) U87 cells with or without Niban shRNA expression were irradiated with or without ultraviolet (100 J/m²) and then incubated for 16 h. (C) Immunoblotting analyses were performed with the indicated antibodies. (D) The cells were stained with annexin V-FITC-PI and analysed using a FACSCalibur flow cytometer. The data represent the means $\pm$ standard deviations from three independent experiments, * P < 0.05. (E,F) *Niban*^{-/-} and *Niban*^{+/+} MEFs were irradiated with or without ultraviolet (100 J/m²) and then incubated for 8 h. (E) Immunoblotting analyses were performed with the indicated antibodies. (F) The cells were stained with annexin V-FITC-PI and analysed using a FACSCalibur flow cytometer. The data represent the means $\pm$ standard deviations from three independent experiments, ** P < 0.01. (G,H) *Niban*^{-/-} MEFs with or without reconstituted expression of WT Niban, Niban S602A, or Niban S602D were irradiated with or without ultraviolet (100 J/m²) and then incubated for 8 h. (G) Immunoblotting analyses were performed with the indicated antibodies. (H) The cells were stained with annexin V-FITC-PI and analysed using a FACSCalibur flow cytometer. The data represent the means $\pm$ standard deviations from three independent experiments. Significance of the results between ultraviolet-irradiated *Niban*^{-/-} MEFs and *Niban*^{-/-} MEFs with reconstituted expression of WT Niban, Niban S602A, and Niban S602D was calculated using the two-tailed Student's *t*-test, * P < 0.05. FITC, fluorescein isothiocyanate; MEF, mouse embryonic fibroblast; NS, no significant difference; PI, propidium iodide; shRNA, short hairpin RNA; WB, western blot; WT, wild type.

affecting cell survival. To test this hypothesis, we overexpressed Niban in HCT116 cells (containing WT *TP53*) [26], which have low expression of Niban (Fig 4A, middle panel). Overexpression of Niban reduced ultraviolet irradiation-induced p53 expression (Fig 4A, upper panel). In addition, Niban overexpression partially inhibited ultraviolet irradiation-induced apoptosis (Fig 4B). Conversely, depletion of Niban by expression of its short hairpin RNA increased ultraviolet irradiation-induced p53 expression in U87 cells (Fig 4C). In addition, Niban depletion enhanced ultraviolet irradiation-induced apoptosis (Fig 4D). In line with these findings, ultraviolet irradiation induced a much greater increase in p53 expression in *Niban*^{-/-} mouse embryonic

fibroblasts (MEFs) than in *Niban*^{+/+} cells (Fig 4E). Furthermore, a much larger fraction of *Niban*^{-/-} cells than *Niban*^{+/+} cells underwent apoptosis in response to ultraviolet irradiation (Fig 4F). These results indicate that Niban downregulates p53 expression and provides antiapoptotic effect against ultraviolet-induced cell apoptosis.

To investigate whether AKT-regulated phosphorylation of Niban has a role in regulation of p53 expression and cell survival, we reconstituted expression of WT Niban, Niban S602A, and Niban S602D in *Niban*^{-/-} cells (Fig 4G). Consistent with the results showing that phosphorylation of Niban increases MDM2-p53 interaction, expression of WT Niban and Niban S602D, but not

Fig 5 | A mechanism underlying the antiapoptotic effect regulated by AKT-mediated Niban phosphorylation. Ultraviolet irradiation results in phosphorylation of Niban at S602 by AKT. Phosphorylated Niban binds to NPM, thereby preventing NPM from binding to MDM2. The resulting free MDM2 interacts with p53, leading to p53 degradation, which in turn inhibits UV-irradiation induced cell apoptosis. MDM2, murine double minute 2; NPM, nucleophosmin.

Niban S602A, suppressed both basal and ultraviolet irradiation-induced p53 expression and ultraviolet irradiation-induced cell apoptosis (Fig 4H). These results indicate that Niban phosphorylation by AKT promotes p53 degradation and provided a survival effect during ultraviolet irradiation-induced apoptosis.

DISCUSSION

NPM is directly implicated in tumorigenesis and embryonic development [15,27,28]. NPM interacts with the MDM2-p53 complex, leading to stabilization of p53 [14,15]. Our findings revealed that Niban, which is overexpressed in many types of human cancer cells, is phosphorylated by AKT in response to ultraviolet irradiation. Phosphorylated Niban binds to NPM, thereby preventing NPM from binding to MDM2. The resulting increase in the amount of free MDM2 interacts with p53, leading to p53 polyubiquitination and degradation (Fig 5). Thus, Niban functions as an antiapoptotic protein, having an instrumental role in cellular responses to extracellular stress through regulation of the interactions between NPM and MDM2, and association of MDM2 with p53, as well as promotion of p53 degradation.

Phosphatidylinositol 3-kinase and AKT are critical regulators of cell proliferation and survival [29,30]. AKT activation, which can be induced by activation of growth factor receptors or mutation of the tumour suppressor phosphatase and tensin homologue, occurs in many types of cancer cells, where it transmits a potent proliferation and survival signal [29]. In response to extracellular stress signalling, such as that induced by chemotherapy and irradiation (including ultraviolet irradiation), AKT signalling can provide an antiapoptotic effect, thereby contributing to resistance of cancer cells to chemotherapy and radiotherapy [25,31]. AKT promotes cell survival by affecting several downstream targets, including regulation of components of the apoptotic machinery,

such as Bad and caspase 9; phosphorylation of substrates that indirectly inhibit apoptosis, such as forkhead transcription family members; and progression of the cell cycle through cytoplasmic sequestration of p21 and stabilization of cyclin D [31,32]. In addition, AKT directly phosphorylates MDM2 and enhances MDM2-mediated ubiquitination and degradation of p53 [33,34]. We did not detect significantly changes in AKT-dependent phosphorylation of MDM2 in response to ultraviolet irradiation. Instead, our results revealed that activated AKT phosphorylates Niban, leading to abrogation of the inhibitory effect of NPM on MDM2-dependent p53 degradation. Regulation of Niban expression or its phosphorylation status is sufficient to alter p53 expression and cell apoptosis strongly suggesting that AKT-phosphorylated Niban has a pivotal role in ultraviolet irradiation-induced cell apoptosis.

Mutations and transcriptional abnormalities of p53 occur in a significant percentage of human cancers. For instance, loss of p53 transcriptional activity was found in 88% of head and neck squamous cell carcinomas [35]. In contrast, ubiquitous expression of Niban was observed in head and neck squamous cell carcinomas compared with in normal tissue [3]. These findings suggest that Niban has p53-independent cellular activities in tumour development and that some types of cancer favour one survival pathway over another in a cell- and stress type-dependent manner.

AKT activation in response to both radiotherapy and chemotherapy has an important role in the resistance of cancer cells to these treatments [29,36]. Our results revealed a new mechanism, in which AKT contributes to resistance of cancer cells through Niban phosphorylation-promoted p53 degradation. Thus, a therapeutic approach targeting AKT-regulated and Niban-dependent p53 degradation might overcome therapy resistance of cancer cells with WT p53 expression.

METHODS

Materials. Rabbit antibodies recognizing p53 (Ab-6), HA tag, Niban, and phosphorylated Niban S602 were obtained from Signalway Biotechnology (Pearland, TX, USA). Antibodies against NPM (FC82291), MDM2 (SMP14), p-Thr (H-2), p-ERK1/2, and ERK1/2 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Purification of recombinant proteins. WT and mutant His-Niban, His-MDM2, and GST-NPM proteins were expressed in bacteria and purified as described previously [37].

In vitro kinase assays. Kinase reactions were performed as described previously [38].

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: This study was conceived by Z.L.; Z.L. wrote the manuscript, designed the experiments, and analysed the data; H.J. wrote the manuscript, designed the experiments, performed the experiments, and analysed the data; Z.D. and D.H. performed the experiments; and D.X., B.-H.J. and D.M. analysed data.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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