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The endoplasmic reticulum stress-inducible protein Niban regulates eIF2α and S6K1/4E-BP1 phosphorylation

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Abstract

The *Niban*/*NIBAN* gene is specifically expressed in hereditary renal carcinomas of model animals and in human malignancies, including renal cancers. Although the expression profiles of *Niban*/*NIBAN* suggest that it plays an important role in carcinogenesis, no functional information has yet been reported. In this study, we found that the levels of *Niban*/*NIBAN* mRNA and protein were induced by treatment with tunicamycin, an inducer of endoplasmic reticulum (ER) stress. To elucidate Niban's *in vivo* function, we generated a *Niban* knockout mouse. *Niban*$^{-/-}$ mouse showed no obvious phenotype. Unexpectedly, we found that eukaryotic translational initiation factor (eIF) 2α phosphorylation, which is up-regulated during ER stress, was increased in *Niban*$^{-/-}$ cells relative to wild-type control cells. In addition, decreased phosphorylation of p70 ribosomal S6 subunit kinase (S6K) 1 and eukaryotic initiation factor 4E-binding protein (4E-BP) 1 was also detected in *Niban*$^{-/-}$ cells. Similar effects were observed following transfection of *NIBAN*-specific interfering RNAs in HeLa cells. Thus, Niban positively affects protein translation machineries. Additionally, suppression of *NIBAN* expression in HeLa cells promoted apoptosis. Together these results suggest that Niban is involved in the ER stress response, and that Niban can modulate cell death signaling by regulating translation.

Keywords: Niban; eIF2α; ER stress; S6K1; 4E-BP1; Apoptosis; Tunicamycin; Knockout mouse

We have studied the mechanism of multi-step carcinogenesis using the Eker rat model of hereditary renal carcinomas [1]. The Eker rat has a germline mutation in the rat homologue of human *TSC2*, a causative gene of the tumor-prone disease, tuberous sclerosis [1]. The *Tsc2* gene product forms a complex with the product of *Tsc1*, another tuberous sclerosis gene, and inhibits small GTP-binding protein Rheb∼mTOR (the mammalian target of rapamaycin kinase) pathway [2]. *Tsc1*/*TSC1*- or *Tsc2*/*TSC2*-deficient hamartomas/tumors show mTOR activation and increased phosphorylation of two downstream substrates, p70 ribosomal S6 subunit kinase (S6K) 1 and eukaryotic initiation factor 4E-binding protein (4E-BP) 1 [3,4]. Activation of this pathway is directly linked to the deficiency of causative genes and is one of the early events implicated in carcinogenesis.

To identify genes associated with the ensuing "steps" during carcinogenesis, we cloned genes showing tumor-specific expression in the Eker rat [5,6]. *Niban* is one of such genes and is expressed even in early pre-neoplastic lesions [6,7]. *Niban* is also expressed specifically in renal tumors from other animal models of hereditary renal carcinogenesis [7]. The human homologue of *Niban* (*NIBAN*) also shows tumor-specific expression, suggesting that the

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Niban/*NIBAN* gene product plays an important role in carcinogenesis [7,8].

The rat *Niban* encodes a 937-amino-acid polypeptide with no significant homology with known functional domains. The highest level of amino-acid conservation among rat, mouse and human Niban homologues is at the amino-terminal region, which suggests that this region may encompass functionally important sequences. Human Niban shows 42% amino-acid identity across its entire length with human C9orf88, a putative polypeptide predicted from a cloned full-length cDNA (GenBank Accession No. AB210016). Niban also has limited homology (~28%) with human BCNP1, identified by proteomic analysis as a membrane component in chronic lymphocytic leukemia cells [9]. Although the functions of C9orf88, BCNP1 and Niban proteins cannot be predicted from their primary amino-acid sequences, these proteins may constitute a family of functionally related polypeptides.

To better understand the function of Niban, we measured the expression levels of *Niban* mRNA and protein in cell lines. We also used gene targeting to characterize the *Niban*'s *in vivo* function.

Materials and methods

Construction of a Niban targeting vector and generation of knockout mice. A *Niban* gene fragment was cloned from a mouse genomic library (129SvJ strain) using a rat *Niban* cDNA probe and used to construct a targeting vector with a replacement of exons 7 and 8 with neomycin resistance gene-expression cassette (see Results). Introduction of targeting vector and selection of cells were carried out using standard protocols and Southern blot analysis (see below) was used to screen homologously recombinant cells. Male germline chimaeras were established, and F1 mice were obtained from crosses between those male chimaeras and female C57BL/6 mice.

Southern and Northern blot analyses and polymerase chain reaction (PCR). Southern and Northern blot analyses were performed as described previously [10]. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and OneStep RT-PCR kit (QIAGEN, Hilden, Germany) was used to perform RT-PCR. Primers used for genotyping and RT-PCR were listed in Supplementary Materials and methods.

Primary antibodies. Rabbit polyclonal antibodies for phospho-eIF2α Ser51, eIF2α, phospho-S6K1 Thr389, phospho-4E-BP1 Thr37/46, 4E-BP1, cleaved caspase-3 Asp175, and cleaved-PARP Asp214 were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibody for p70 S6K1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-HA and mouse monoclonal anti-α- and β-actin antibodies were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibodies against human and rat Niban were described previously [7]. For Western blot analysis, see Supplementary Materials and methods.

Cell culture, drug treatment, and metabolic labeling. Cell lines and media for cell culture are listed in Supplementary Materials and methods. Tunicamycin (TM) (Sigma) was dissolved to 2 mg/ml in dimethyl sulfoxide (DMSO) and was used at a final concentration of 2 μg/ml in culture medium. For the other culture conditions, see Supplementary Materials and methods. For treatment of mice, TM was injected subcutaneously at a dose of 1 mg/kg body weight, and mice were then sacrificed for analysis 24 h later. For [^{35}S]metabolic labeling, 24 h or 48 h after transfection of siRNA, HeLa cells were placed for 30 min in methionine and cysteine-free DMEM (Cell Science & Technology Institute, Japan), and labeled by addition of [^{35}S]methionine and cysteine (20 μCi/ml; GE Biosciences) to the culture medium for 2 h. The protein concentration was measured after harvest with lysis buffer (see Supplementary Materials and methods). The [^{35}S] incorporation was analyzed by FUJI BAS2500 image analyzer (Fuji Film).

Cell proliferation assay and detection of apoptotic cells. For cell proliferation assay, see Supplementary Materials and methods. To count individual living cells, cells were subjected to staining with Trypan blue. Significant differences in survival data were determined using *t*-test analysis. Apototic cells were detected using the AnnexinV-FITC fluorescent microscopy kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Positive signals were viewed with an Olympus IX70 microscope (Olympus, Tokyo, Japan).

RNA interference. The target sequences for siRNA against the human *NIBAN* gene was 5′-GCATTTCCCAGACCCTCTTTT-3′ (sense strand). The random control sequence was 5′-GCTGCAATCGATTGATAGC-3′ (sense strand). HeLa cells were transfected with individual siRNAs (25 nM final) and Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Results

Induction of Niban gene expression by tunicamycin

We examined *Niban*/*NIBAN* mRNA and protein expression patterns under various conditions using HeLa cells and renal tumor (RT) cell lines from model animals (see Supplementary Materials and methods). We found that TM, a potent endoplasmic reticulum (ER) stress inducer, increased the levels of *Niban*/*NIBAN* mRNA and protein, although basal levers and relative increases differed between cell lines (Fig. 1A and B).

The effect of TM on *Niban* expression was analyzed by subcutaneous administration in mice. In the absence of TM, levels of *Niban* mRNA were different in each organ (Fig. 1C). In the liver, kidney and cerebrum, TM-treated mice showed increased amounts of *Niban* mRNA relative to control mice, with no obvious difference in other organs tested (Fig. 1C). These results suggest that ER stress induces an increase in the levels of *Niban* mRNA in some cell types in animal body.

Generation and characterization of Niban knockout mice

To elucidate the *in vivo* function of *Niban*, we generated *Niban* knockout mice. *Niban* exons 7 and 8, part of the conserved region among vertebrate homologues that may be important for function (Supplementary Fig. 1), were deleted (Fig. 2A). The *Niban*-deficiency did not appear result in embryonic lethality (n; $Niban^{+/+} = 32$, $Niban^{+/-} = 70$, $Niban^{-/-} = 34$, Fig. 2B and C and Supplementary Fig. 2).

During a 1-year observation period, no obvious, specific abnormalities in appearance and microscopic phenotype were observed in $Niban^{-/-}$ mice (Supplementary Fig. 3A and data not shown). We have not detected significant differences in proliferation between $Niban^{-/-}$ mouse embryonic fibroblasts (MEFs) and control MEFs in response to various conditions employed, including TM treatment (Supplementary Fig. 3B), heat shock and serum starvation (data not shown). These results strongly

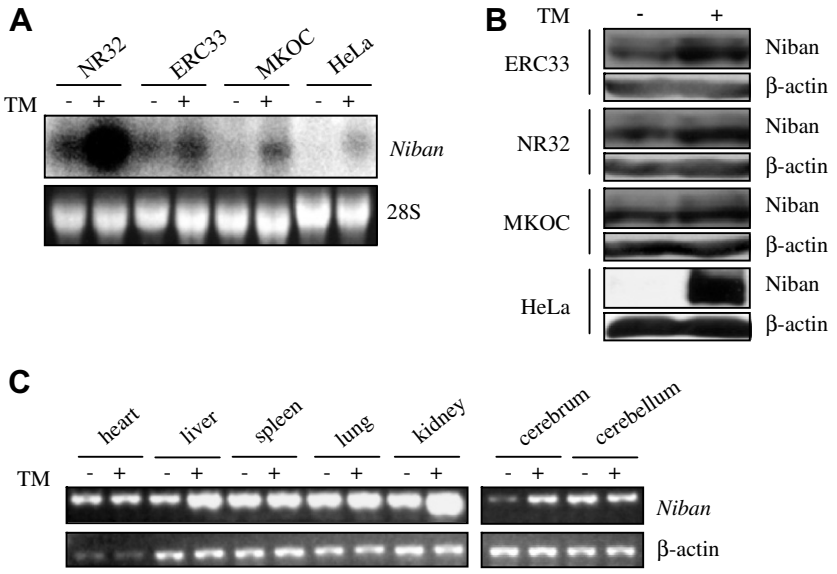

Fig. 1. Increased levels of *Niban*/*NIBAN* following tunicamycin treatment. (A) Northern blot analysis. NR32, ERC33, and MKOC1-277 (MKOC) RT cells and HeLa cells were cultured either with 2 μg/ml tunicamycin (TM +) or vehicle only (−) for 20 h. RNAs were analyzed for the 6.5 kb *Niban*/*NIBAN* mRNA (*Niban*). Control 28S ribosome RNAs are shown below. (B) Western blot analysis. Cells were cultured as in (A) and analyzed with Niban antibodies (upper panel). The lower panel shows β-actin. (C) RT-PCR analysis of mouse organs. After 24 h TM treatment, *Niban* cDNA was amplified by RT-PCR (upper panel). Lanes, (+); TM-treated mouse, (−); vehicle-treated mouse. β-Actin cDNA was used as a control (lower panel). Results were confirmed with two-independent experiments.

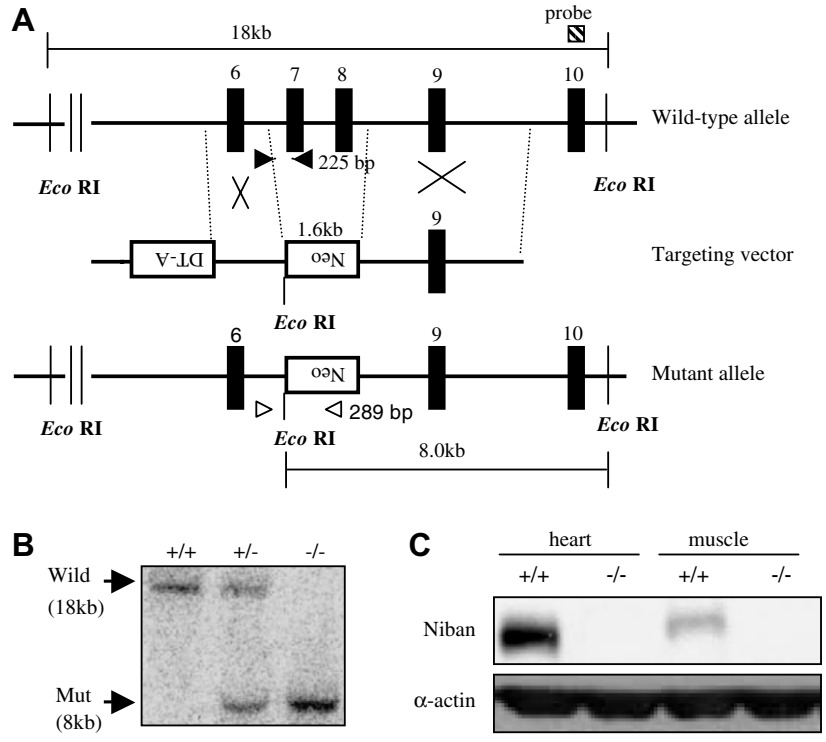

Fig. 2. Generation of a *Niban* knockout mouse. (A) The structure of the targeting vector and wild-type and mutant *Niban* alleles. Exons are denoted by filled and numbered boxes. Neomycin (Neo) and diphtheria toxin A-chain gene (DT-A) expression cassettes are depicted as open boxes. Arrowheads indicate the coordinates of two primer pairs used for genotyping. (B) Southern blot analysis of mouse genomic DNA. *Eco*RI-digested DNAs from *Niban*$^{+/+}$, *Niban*$^{+/-}$, and *Niban*$^{-/-}$ mice were analyzed. Restriction fragments from wild-type (Wild) and mutant *Niban* alleles (Mut) are indicated on the left. (C) Western blot analysis of mouse organs. Representative results of an anti-Niban immunoblot of heart and skeletal muscle proteins from *Niban*$^{+/+}$ and *Niban*$^{-/-}$ mice are shown. Lower panel; control α-actin.

suggest that *Niban* is not required for normal mouse growth and development.

Suppression of eIF2α phosphorylation by Niban

We next hypothesized that Niban may affect the phosphorylation of eukaryotic initiation factor (eIF) 2α that occurs in response to certain stressors to inhibit translation [11,12]. Surprisingly, eIF2α phosphorylation in normal culture conditions increased in *Niban*$^{-/-}$ MEFs relative to control MEFs, suggesting that Niban suppresses eIF2α phosphorylation (Fig. 3A). Under this condition, MEFs expressed substantial amounts of Niban protein, and eIF2α phosphorylation and Niban protein levels both increased after TM treatment. However, levels of phosphorylated eIF2α (p-eIF2α) were similar in untreated *Niban*$^{-/-}$ MEFs and TM-treated *Niban*$^{+/+}$ MEFs (Fig. 3B). The level of eIF2α phosphorylation increased further in TM-treated *Niban*$^{-/-}$ MEFs, suggesting that *Niban*-deficiency and TM treatment have an additive effect on eIF2α phosphorylation in MEFs.

To test the hypothesis that a decrease in the levels of Niban could result in greater phosphorylation of eIF2α, *NIBAN* gene expression was knocked down using small interference RNA (siRNA) in HeLa cells. In cells transfected with control RNA (control cells), there was an increase in the levels of Niban protein relative to non-transfected cells, which we attribute to transfection-mediated cell stress (Fig. 3C and data not shown). The levels of Niban protein decreased significantly in cells transfected with *NIBAN*-specific siRNA, and eIF2α phosphorylation levels increased relative to control cells, even under normal culture conditions (Fig. 3C). Furthermore, we found the level of eIF2α phosphorylation was decreased significantly in COS7 cells that co-expressed Niban (Supplementary Fig. 4). These results suggest that Niban regulates eIF2α phosphorylation.

Decrease of S6K1 and 4E-BP1 phosphorylation by Niban suppression

S6K1 and 4E-BP1, which have been implicated in certain stress signals, also regulate translation [13]. Surprisingly, S6K1 and 4E-BP1 phosphorylation were suppressed in *Niban*$^{-/-}$ MEFs relative to control MEFs (Fig. 3B). In

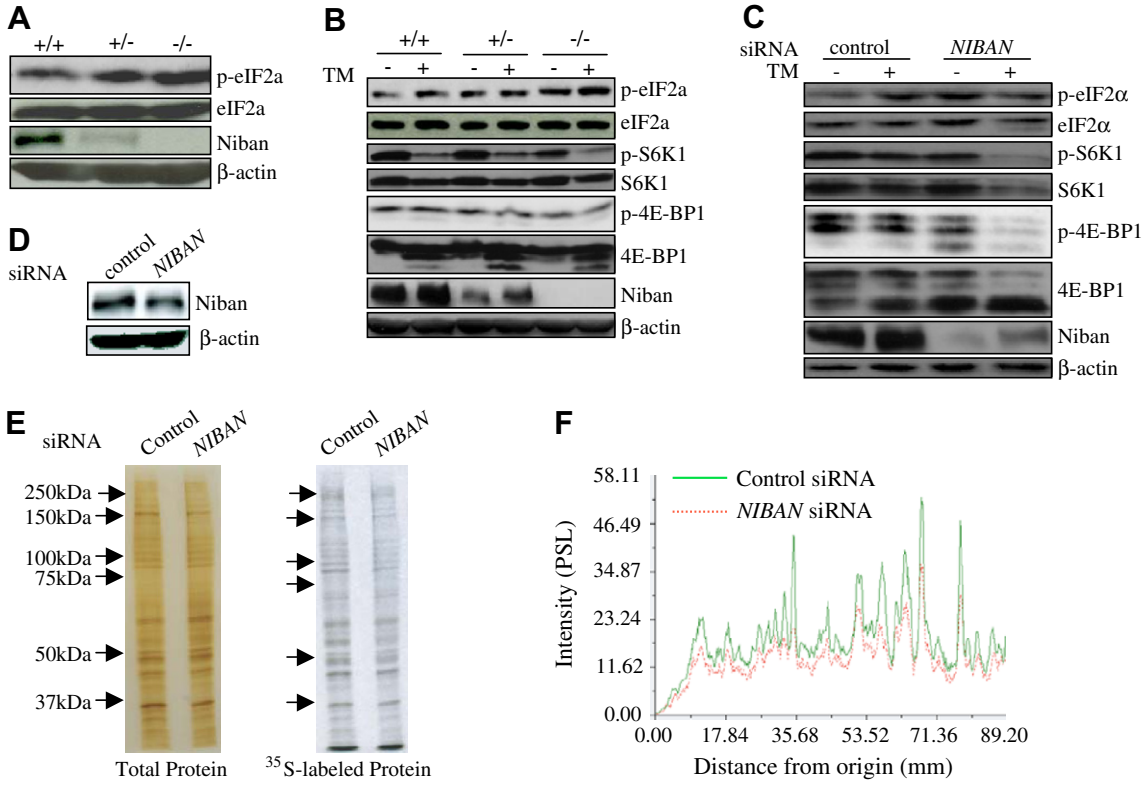

Fig. 3. Regulation of eIF2α, S6K1, and 4E-BP1 phosphorylation and protein synthesis by Niban. (A) eIF2α phosphorylation in MEFs. MEFs were analyzed by Western blot for the phosphorylated form of eIF2α (p-eIF2α), total eIF2α or Niban. (B) Western analysis of MEFs. MEFs were cultured either with tunicamycin (TM +) or vehicle only (−) for 20 h, and then analyzed for p-eIF2α, eIF2α, Niban, the phosphorylated form of S6K1 (p-S6K1) and 4E-BP1 (p-4E-BP1), total S6K1 and 4E-BP1. (C) Modulation of phosphorylation by *NIBAN* knockdown. HeLa cells were transfected with a *NIABN*-specific siRNA or control siRNA. Twenty-eight hours after transfection, cells were added either TM (+) or vehicle only (−) for an additional 20 h incubation and analyzed as in (B). (D) Expression of Niban protein in HeLa cells 24 h after transfection of siRNA. (E) Analysis of ^{35}S-labeled proteins by SDS–PAGE. 24 h after transfection of siRNA, HeLa cells were subjected to [^{35}S] metabolic labeling (2 h). Equal amounts of proteins were resolved by SDS–PAGE and was stained by silver staining (left panel) and then subjected to autoradiography (right panel). (F) Comparison of ^{35}S-radioactivity. Radioactivities of lanes shown in (E) were measured by image analyzer and plotted according to the molecular weight. Total area representing radioactivity was calculated and used for comparison. In all Western panels, an anti-β-actin immunoblot is shown as a control.

TM-treated MEFs, S6K1/4E-BP1 phosphorylation levels were markedly reduced, irrespective of *Niban* genotype (Fig. 3B). Thus, there was an additive effect of TM treatment and *Niban*-deficiency in MEFs with regard to the phosphorylation state of S6K1 and 4E-BP1.

The relationship between Niban function and S6K1/4E-BP1 phosphorylation was also investigated using *NIBAN* knockdown in HeLa cells. *NIBAN*-suppressed HeLa cells contained relatively less phosphorylated S6K1 (p-S6K1) and 4E-BP1 (p-4E-BP1) than control cells, again revealing a positive correlation between increased Niban expression and increased S6K1/4E-BP1 phosphorylation (Fig. 3C). There was an additive effect of TM similar to MEFs in HeLa cells (Fig. 3C). These results suggest that Niban has a positive effect on S6K1/4E-BP1 phosphorylation.

Inhibition of protein synthesis by NIBAN suppression

To test the postulation that the silencing of *NIBAN* may inhibit overall protein synthesis, [^{35}S]-labelling was used to quantify the rate of protein synthesis after *NIBAN* knockdown (Fig. 3C and D). Because we observed promotion of apoptosis by *NIBAN* suppression (see below), we examined the status of protein synthesis 24 h (no difference in apoptosis) and 48 h after transfection. Although suppression of *NIBAN* was not complete 24 h after transfection (Fig. 3D), ^{35}S-labeled protein were reduced ~25% relative to control cells (Fig. 3E and F), whereas 48 h after transfection, ^{35}S-labeled protein were reduced ~53% (data not shown). These results suggest that *NIBAN* knockdown inhibits overall protein synthesis. However, as apoptosis was increased 48 h after transfection (see below), the inhibition may include influences from apoptosis at that time.

Promotion of apoptosis by NIBAN knockdown in HeLa cells

During RNA interference analysis, we noticed that *NIBAN*-suppressed HeLa cells underwent cell death. There were 45% fewer *NIBAN*-suppressed cells relative to control cells 42 h after transfection (Fig. 4A). After TM treatment, cell numbers decreased further (~50%) in both control and *NIBAN*-suppressed cells (Fig. 4A). Under normal conditions, levels of cleaved caspase-3 and PARP were increased in *NIBAN*-suppressed cells relative to control cells (Fig. 4B). Following TM treatment, levels of these apoptosis markers increased further in both control and *NIBAN*-suppressed cells (Fig. 4B). *NIBAN*-suppressed cells exhibited approximately 2.8-fold more annexinV-positive apoptotic signals relative to control cells 48 h, but not 24 h after transfection (Fig. 4C and D and data not shown). These results suggest that *NIBAN* knockdown promotes apoptosis in HeLa cells.

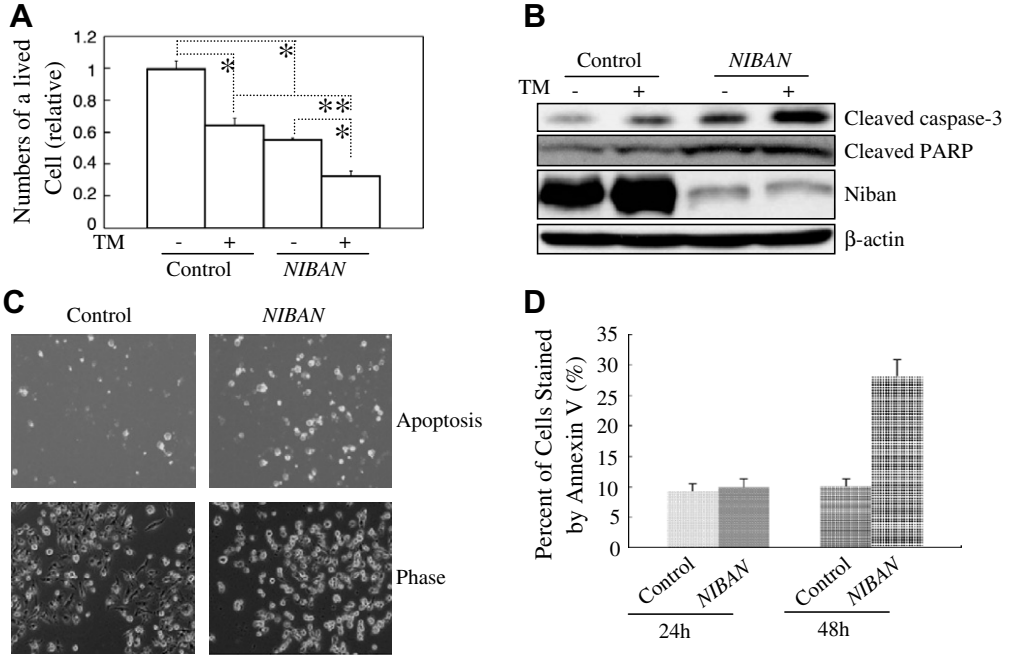

Fig. 4. Increased apoptosis with *NIBAN* knockdown in HeLa cells. (A) Increase cell death with a combination of NIBAN knockdown and TM. HeLa cells were transfected with either a *NIBAN*-specific siRNA (*NIBAN*) or a control RNA (Control). Twenty-eight hours after transfection of siRNA, TM (+) or vehicle only (−) was added, and cells cultured for an additional 20 h were counted to calculate percent viability. Data represent the mean ± SE calculated from 6 wells for each case in two-independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$). (B) Analysis of marker proteins for apoptosis. Cells were transfected and treated with TM as in (A) and were analyzed to assess levels of cleaved caspase-3, cleaved PARP and Niban. β-Actin is shown as a control. (C) Detection of apoptotic cells. 42 h after transfection of siRNA, apoptotic cells were detected by staining with annexinV-FITC. Panels show representative results of fluorescent images that depict apoptotic cells (upper panels), and phase contrast images (lower panels) of the same fields. (D) The percentage of annexinV-FITC-positive cells. At least six different fields for each case were counted.

Discussion

The levels of *Niban*/*NIBAN* m\RNA increase in TM-treated cell cultures as well as in mice. We also noted an increase in *Niban*/*NIABN* mRNA levels in cells treated with another ER-stress inducer, thapsigargin (Sun et al., unpublished observation) [12]. It is presently not known whether known ER stress-regulated transcription factors are involved in *Niban*/*NIBAN* induction [14]. In the absence of TM, each cell line and mouse tissue display a unique expression profile for *Niban*/*NIBAN* mRNA and protein, suggesting that *Niban*/*NIBAN* expression is regulated in a cell type-specific manner *in vivo*. Induction of *Niban*/*NIBAN* expression during renal carcinogenesis may be related to the regulation of *Niban*/*NIBAN* expression during ER stress. The positive and/or negative implications of stress conditions during tumor development have been discussed [15,16], and the expression of *Niban*/*NIBAN* in tumors may reflect a cell stress during carcinogenesis.

Although the physiological activity of Niban protein has yet to be identified, our results demonstrate a relationship between Niban function and the phosphorylation of proteins involved in translational regulation. Phosphorylation of eIF2α, which is essential for ER stress-induced suppression of translation, is mediated by pancreatic eIF2α kinase (PERK) [17]. Paradoxically, TM treatment of cultured cells increased levels of both eIF2α phosphorylation and Niban protein, and the effects of TM treatment and Niban suppression showed an additive effect on eIF2α phosphorylation. These data lead us to hypothesize that translational suppression during ER stress may be counteracted by Niban via some type of negative feedback mechanism. The characteristics of Niban, its increase by TM treatment and inhibition of eIF2α phosphorylation, are similar to those of $P58^{IPK}$, which is induced by ATF6-dependent manner and acts as an inhibitor of PERK [18,19]. Presently, it is not known whether Niban inhibits eIF2α phosphorylation by modulation of PERK activity directly or through the activity of another factor.

Our data demonstrate a positive effect of Niban on S6K1/4E-BP1 phosphorylation. Currently, it is not known whether Niban functions upstream or downstream of mTOR. Interestingly, the fact that TM treatment reduced S6K1/4E-BP1 phosphorylation suggests that ER stress, via an unknown mechanism, can inhibit the mTOR∼S6K1/4E-BP1 pathway. The negative effect of hypoxia on the mTOR pathway has been found to be mediated by the REDD1/TSC1/TSC2 and AMPK/TSC1/TSC2 pathways [20], and hypoxia induces increased eIF2⟨ phosphorylation [15,16]. Although the relationship between the mTOR/S6K1/4E-BP1 pathway and ER stress is not fully understood, it is possible that Niban may function to link and coordinate mTOR/S6K1/4E-BP1 and ER stress.

NIBAN knockdown increased the frequency of apoptosis in HeLa cells, perhaps as a result of changes in translation. It is possible that in *NIBAN*-suppressed HeLa cells, the signal from some other pro-apoptotic pathway is enhanced. As noted above, the up-regulation of *NIBAN* expression that was observed during HeLa cell transfection may be a response to stressful conditions that function synergistically with *NIBAN* knockdown to promote apoptosis. Nonetheless, the targeted knockouts indicate that *Niban* is not essential for normal mouse growth and development, and that *Niban*-deficient MEF cells can be maintained in culture. Based on the observed differences in Niban expression in various cell lines and tissues, the requirement for Niban function may vary between cell types, as well as in various environmental conditions, and the induction of apoptosis in response to a *Niban*/*NIBAN*-deficiency is strongly context-dependent. Niban's function in tumor cells may be to protect cells from death-inducing stress [15,16] by modulating protein translation. Genetic crosses between *Niban* knockout mice and *Tsc2* knockout mice will be a feasible approach to elucidating Niban's role in tumorigenesis [10]. In addition, phenotypical analyses of *Niban* knockout mice in various stress conditions may also provide new insights into Niban function *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.021.

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