NM23-H1 Tumor Suppressor Physically Interacts with Serine-Threonine Kinase Receptor-associated Protein, a Transforming Growth Factor-β (TGF-β) Receptor-interacting Protein, and Negatively Regulates TGF-β Signaling*

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NM23-H1 is a member of the NM23/NDP kinase gene family and a putative metastasis suppressor. Previously, a screen for NM23-H1-interacting proteins that could potentially modulate its activity identified serine-threonine kinase receptor-associated protein (STRAP), a transforming growth factor (TGF)-β receptor-interacting protein. Through the use of cysteine to serine amino acid substitution mutants of NM23-H1 (C4S, C109S, and C145S) and STRAP (C152S, C270S, and C152S/C270S), we demonstrated that the association between these two proteins is dependent on Cys145 of NM23-H1 and Cys152 and Cys270 of STRAP but did not appear to involve Cys4 and Cys109 of NM23-H1, suggesting that a disulfide linkage involving Cys145 of NM23-H1 and Cys152 or Cys270 of STRAP mediates complex formation. The interaction was dependent on the presence of dithiothreitol or β-mercaptoethanol but not H2O2. Ectopic expression of wild-type NM23-H1, but not NM23-H1(C145S), negatively regulated TGF-β signaling in a dose-dependent manner, enhanced stable association between the TGF-β receptor and Smad7, and prevented nuclear translocation of Smad3. Similarly, wild-type NM23-H1 inhibited TGF-β-induced apoptosis and growth inhibition, whereas NM23-H1(C145S) had no effect. Knockdown of NM23-H1 by small interfering RNA stimulated TGF-β signaling. Coexpression of wild-type STRAP, but not STRAP(C152S/C270S), significantly stimulated NM23-H1-induced growth of HaCaT cells. These results suggest that the direct interaction of NM23-H1 and STRAP is important for the regulation of TGF-β-dependent biological activity as well as NM23-H1 activity.

The NM23 family of genes is characterized by reduced expression in certain highly metastatic cell lines and tumors (1).

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In humans, the eight NM23 genes that have been identified to date, NM23-H1, NM23-H2, NM23-H3, NM23-H4, NM23-H5, NM23-H6, NM23-H7, and NM23-H8, encode NDP kinases or homologous isoforms (2). However, although NM23-H1 was initially identified as a putative metastasis suppressor, its enzymatic activity does not appear to be responsible for its function as a metastasis suppressor during tumor progression (3). Studies of NM23 family proteins in other species have provided evidence for their role in proliferation, differentiation, apoptosis, development, and endocytosis (4). In Drosophila, for example, abnormal wing discs (awd) is an NDP kinase, and the killer-of-prune mutation of awd (awd<sup>km</sup>) causes abnormalities in cell morphology and differentiation (5). Recently, both NM23-H1 and NM23-H2 have been reported to play a role in endocytosis (6). In addition, NM23 has been associated with the differentiation of human MDA-MB-435 breast carcinoma cells (7). The ability of NM23 family proteins to regulate such a diverse set of cellular processes has recently been linked to their ability to modulate signal transduction by a diverse set of growth factors, such as transforming growth factor-β1 (TGF-β1), nerve growth factor, platelet-derived growth factor, and insulin-like growth factor-1 (8). However, the mechanism of regulation of these signaling pathways by NM23 family proteins is unknown. To date, NM23 family proteins have been shown to associate with several cellular proteins, including glyceraldehyde-3-phosphate dehydrogenase (9), Hsc70 (70-kDa heat shock cognate protein) (10), telomere (11), RORα (retinoid acid receptor-related orphan receptor α)/RZRβ (retinoid Z receptor β) (12), Rad, a Ras-related small GTPase (13), creatine kinase and antioxidant protein (14), and thromboxane A2 receptor, a G protein-coupled receptor (6). These results suggest that the identification of additional binding partners of NM23 proteins will provide greater insight into the regulation and biological function of NM23 family proteins.

STRAP interacts with both PDK1 (3-phosphoinositide-dependent protein kinase-1) and TGF-β receptor. Through this interaction, STRAP positively regulates PDK1 and negatively regulates TGF-β signaling by stabilizing the association...
between TGF-β receptor and Smad7 (15, 16). Here, we report that the physical association of NM23-H1 with STRAP in vivo is important for the negative regulation of TGF-β-mediated signaling as well as NM23-H1 tumor suppressor activity.

MATERIALS AND METHODS

Cell Culture, Cell Line Construction, and Reagents—293T, HeLa, HepG2, Hep3B, HaCaT, and HCT116 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) as described previously (17). Porcine TGF-β1 and anti-Smad7 antibody were purchased from R&D Systems (Minneapolis, MN). Anti-GST, anti-β-actin, anti-FLAG (M2), and anti-STRAP antibodies were described previously (15). Anti-NM23-H1, anti-CDK4, anti-cyclin D1, anti-PAI-1, and anti-histone (H2B) antibodies used in immunoprecipitation and immunoblot analyses were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor-594 anti-mouse secondary antibody and Alexa Fluor-488 anti-rabbit secondary antibody were purchased from Molecular Probes, Inc. (Eugene, OR). Propidium iodide, RNase A, isopropyl-β-D-thiogalactopyranoside, dithiothreitol (DTT), aprotinin, phenylmethylsulfonyl fluoride, hydroxyurea, and anti-His antibody were purchased from Sigma. Polyvinylidene difluoride membranes were obtained from Millipore Corp. (Bedford, MA). [γ-32P]ATP was purchased from PerkinElmer Life Sciences.

DNA Construction and Plasmids—The NM23-H1 mutants were generated by the PCR method. In brief, pBluescript containing a full-length NM23-H1 (pBS-NM23-H1; GenBank™ accession number X73066) was used as the template for amplification with either the T3 (5′-AAATACCTTCACTAAAG-3′) or T7 (5′-ATATCGACTAGTATAG-3′) primer, in conjunction with one of the following mutant primers containing alterations in the nucleotide sequence of NM23-H1: for NM23-H1 Cys4 → Ser (NM23-H1(C4S)), sense 5′-ATGGCCCAACTCGAGCCGTACC-3′, antisense 5′-GGTAGACGCTGATGTTTCG-3′; for NM23-H1 Cys109 → Ser (NM23-H1(C109S)), sense 5′-GGGAGACTTCTCGATACAAGTT-3′, antisense 5′-GTTCACTGCTGATGTTCG-3′; for NM23-H1 Cys145 → Ser (NM23-H1(C145S)), sense 5′-ATGACCGACTGCTGATGTTCG-3′, antisense 5′-GGGAGACTTCTCGATACAAGTT-3′; for NM23-H1 Cys152 → Ser (NM23-H1(C152S)), sense 5′-GGGAGACTTCTCGATACAAGTT-3′, antisense 5′-GTTCACTGCTGATGTTCG-3′; for NM23-H1 Cys270 → Ser (NM23-H1(C270S)), sense 5′-GGGAGAUCGGCUUGUGGUUTT-3′; for NM23-H1 Cys321 → Ser (NM23-H1(C321S)), sense 5′-GGGAGACGCGUGUGUGUUTT-3′ and 5′-GCUUGCGAAGUUCUCATT-3′ containing alterations in the nucleotide sequence of STRAP (GenBank™ accession number BC000162); for STRAP Cys152 → Ser (STRAP[C152S]), sense 5′-AGCTTGTGGTCAGTGAGGATA-3′, antisense 5′-TAT CTCACCTGACCACAAGACT-3′; for STRAP Cys770 → Ser (STRAP[C770S]), sense 5′-TCCTATTCTACTAGTGATTTA-3′, antisense 5′-TAAATCTCACTTAGTAATAGGA-3′. To generate the double substitution mutant of STRAP (STRAP[C152S/C770S]), pBS-STRAP(C270S) was used as the template, and the STRAP(C152S) sense and antisense primers were used for PCR amplification. To generate the STRAP deletion constructs (STRAP-WD4–6, STRAP-WD4–8, and STRAP(C152S/C770S)), a third round of PCR amplification was then performed using full-length STRAP, STRAP-WD4–6, and STRAP(C152S/C770S) as templates. The forward primers for STRAP-WD4–6 (5′-GGCATTTCTGCTGGCCACAAGC-3′), -WD4 (5′-GGGAGATCTCCATAGAAATAATTGGT-3′), -WD5 (5′-GGGAGATCTCCATAGAAATAATTGGT-3′), and -CT (5′-GAAGGCTGTGGTAGAAGGAACCCAC-3′) each contained an EcoRI site (underlined). The reverse primers for STRAP-WD1–3 (5′-GCCTCGAGCAATCTATAATGCTGAATTA-3′), -WD4 (5′-GCCTCGAGCATACTGATGATCACAATGAGCC-3′), -WD5 (5′-GCCTCGAGCTCCACTTACTACAGTATGATCACAATGAGCC-3′), and -CT (5′-GCCTCGAGGAGGCCTTAAACGTAGAACC-3′) each contained an XhoI site (underlined). All PCR products were confirmed by DNA sequencing of both strands (Bioneer Corp., Cheongwon, Korea). The p3TP-Lux reporter plasmid was a kind gift from Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York). The p21-Luc and Smad7-Luc reporter plasmids were kindly provided by H-S. Choi (Chonnam National University, Kwangju, Korea). GST-tagged and FLAG-tagged STRAP plasmids were described previously (15).

Yeast Two-hybrid Screen—The yeast genetic screen for the isolation of NM23-H1-interacting proteins was carried out as described previously (17).

Transfection, in Vivo Binding Assay, and Native Polyacrylamide Gel Electrophoresis—293T, HeLa, HaCaT, Hep3B, or HepG2 cells were transfected with the indicated expression plasmids, using WelFect-Ex™ Plus (WelGENE, Daegu, Korea). GST-tagged and FLAG-tagged STRAP plasmids were described previously (15).

siRNA Experiment—The NM23-H1-specific siRNA oligonucleotides (a, 5′-GGGACGCGCGUUGUUGUUUTT-3′; b, 5′-GCUUGCGAAGUUCUCATT-3′) corresponding to two coding regions (a, amino acids 129–134; b, amino acids 43–48) of human NM23-H1, and a nonspecific control siRNA (5′-GCCTCGGAGGCAGUUGUUGUUTT-3′) were synthesized at SamChully Pharmaceutical Ltd. (Seoul, Korea). The sense and antisense oligonucleotides for each siRNA were annealed as described previously (15). HeLa, HepG2, or HaCaT cells
10^5 cells/well) were plated in 6-well flat-bottomed microplates (Nunc, Rochester, NY) the day before transfection. Cells were transfected with siRNA oligonucleotides using the WelFect-Ex™ Plus method. After 48 h of incubation with the siRNAs, cells were analyzed by immunoblot to confirm down-regulation of target proteins.

Preparation of Recombinant Proteins—Recombinant GST- or His<sub>6</sub>-tagged human STRAP (wild type and the C152S, C270S, and C152S/C270S mutants) and NM23-H1 (wild type and the C4S, C109S, and C145S mutants) were generated by subcloning the corresponding cDNA fragments of NM23-H1 and STRAP into pGEX4T-1 (Amersham Biosciences) and pQE30 (Qiagen, Valencia, CA), respectively. Proteins were purified by affinity chromatography using glutathione-Sepharose 4B columns (Amersham Biosciences) or a His-Bind Resin column (Novagen, Madison, WI), as described previously (17).

Spectroscopic Measurement—The far-UV CD spectra were recorded over the range of 190–250 nm on a Jasco J-715 spectropolarimeter at the following settings: 10 millidegrees sensitivity, 0.2 nm resolution, 3 units accumulation, 1 s response, and scanning speed 100 nm/min. The protein was assayed at a concentration of 0.058 mg/ml in a 1-mm path length cylindrical quartz cell.

Luciferase Reporter Assay—HepG2 cells were transiently cotransfected using the WelFect-Ex™ Plus method with p3TP-Lux, p21-Luc, or Smad7-Luc, along with the indicated expression vectors, and luciferase activity was monitored with a luciferase assay kit from Promega (Madison, WI), according to the manufacturer’s instructions and as described previously (15).

Apoptosis Assay—Assays for apoptosis were performed as previously described (15). Briefly, cells grown on sterile coverslips were transfected with an expression vector encoding green fluorescent protein (GFP), together with the indicated expression vectors. After 24 h, the cells were treated with TGFB-β1 (10 ng/ml for HeLa, 2 ng/ml for HaCaT) for 20 h and then fixed with ice-cold 100% methanol and washed three times with PBS. The cells were then stained with a bisbenzimide (Hoechst 33258) and visualized under a fluorescence microscope as described previously (15). The percentage of apoptotic cells was determined by counting the number of GFP-positive cells with apoptotic nuclei and dividing this by the total number of GFP-positive cells.

Preparation of Nuclear Fractions—Hep3B cells (~4 × 10^5 cells/60-mm dish) transfected with the indicated combinations of expression vectors (for Smad3, STRAP, wild-type NM23-H1, and NM23-H1(C145S)) were harvested and washed twice with PBS and then resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride). Cells were solubilized by pipetting, and the lysate was incubated on ice for 10 min and then centrifuged at 15,000 rpm for 6 min at 4 °C. The resulting supernatant (nuclear fraction) was collected in a chilled microcentrifuge tube.

Autophosphorylation and Phosphotransferase Assay of NM23-H1—To assay autophosphorylation of NM23-H1, ~3 μg of purified recombinant wild-type or mutant NM23-H1 were incubated with 1 μM [γ-<sup>32</sup>P]ATP (0.2 μCi/ml) at room temperature for 10 min in a 20-μl reaction volume containing TMD buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 1 mM DTT). The reaction was stopped by the addition of 1 volume of SDS-PAGE sample buffer (pH 8.8) and incubated at room temperature for an additional 10 min. Proteins were resolved by 15% SDS-PAGE, and the wet gel was subjected to autoradiography at ~70 °C. To assay the phosphotransferase activity of NM23-H1, ~3 μg of purified recombinant His-NM23-H1 were incubated with 5 μCi of [γ-<sup>32</sup>P]ATP for 15 min at room temperature in a final volume of 100 μl of TMD buffer. The reaction products were concentrated with a Centricon-30 (Millipore, Billerica, MA) to a final volume of less than 50 μl. Five μl of the concentrated sample were incubated with unlabeled wild-type or mutant NM23-H1 (12 μg) in a final volume of 30 μl of TMD buffer for 10 min at room temperature. The reaction was stopped by the addition of 0.5 volumes of SDS-PAGE sample buffer (pH 8.8), incubated at room temperature for an additional 10 min, and then resolved by 10% SDS-PAGE. The wet gel was subjected to autoradiography at ~70 °C.

Assay of NM23-H1 NDP Kinase Activity—The NDP kinase assay was performed in TMD buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 1 mM DTT) as described previously (20). In brief, 3 μg of purified recombinant wild-type or mutant NM23-H1 were incubated with 1 μM [γ-<sup>32</sup>P]ATP (0.2 μCi/ml) and 100 μM GDP in TMD buffer in a final volume of 20 μl for 10 min at room temperature. The reaction was stopped by the addition of 20 μl of 50 mM EDTA (pH 8.0). The reaction mixture (2 μl) was spotted onto 20 × 20-cm polyethyleneimine-cellulose TLC plates (Merck), and reaction products were resolved by capillary action in 0.75 mM KH<sub>2</sub>P<sub>2</sub>O <sub>7</sub> pH 3.65. The TLC plates were dried and exposed to film, and the formation of [γ-<sup>32</sup>P]GTP was visualized by autoradiography.

Fluorescence-activated Cell Sorting Analysis—HaCaT cells (2 × 10<sup>5</sup> cells/60-mm dish) transiently transfected with the indicated expression vectors or siRNA duplexes (NM23-H1 or control siRNA) were washed with ice-cold PBS and then treated with hydroxyurea (2 mM) for 20 h to synchronize the cells in G<sub>i</sub>/G<sub>1</sub>. Cell cycle distribution was analyzed after treatment with 10% serum for 24 h in the presence or absence of TGFB-β1 (2 ng/ml) as follows. Cells were trypsinized, washed twice with ice-cold PBS, and then incubated at 37 °C for 30 min with a solution (1 mM Tris-HCl, pH 7.5) containing 50 μg/ml propidium iodide and 1 mg/ml RNase A. Flow cytometry was carried out using a FACS Calibur-S system (BD Biosciences), and the data were analyzed using the ModFit LT version 3.0 (PMacro) program.

Immunofluorescence Cytochemistry—Immunohistochemistry was performed as described previously (15). In brief, Hep3B cells growing on sterile coverslips were transfected with the indicated expression vectors. The coverslips were placed on ice, and cells were washed three times with ice-cold PBS and then incubated with 4% paraformaldehyde for 10 min at room tem-
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RESULTS

NM23-H1 Physically Interacts with STRAP in Vivo—To identify proteins that interacted with NM23-H1, we carried out a yeast two-hybrid screen using the full-length human NM23-H1 as a bait, as described previously (18). Screening of a human HeLa cDNA library led to the identification of eight distinct positive clones (data not shown). Among them, one clone was found to encode STRAP, a TGF-β receptor-interacting protein (23).

Based on this previous result, we investigated whether NM23-H1 interacted with STRAP in mammalian cells by performing a set of cotransfection experiments using GST- and FLAG-tagged eukaryotic expression vectors. FLAG-STRAP was detected in association with NM23-H1 only when coexpressed with GST-NM23-H1 (Fig. 1A, top). These data corroborated the results of the yeast two-hybrid screen and demonstrated that NM23-H1 physically interacts with STRAP in cells.

To confirm the physical association between NM23-H1 and STRAP, we performed a coimmunoprecipitation analysis of endogenous proteins using an anti-STRAP antibody or preimmune IgG as a control. NM23-H1 was present in anti-STRAP immune complexes from all cell lines examined, including 293T, Hep3B, and SK-N-BE(2)C (18) cells (Fig. 1B), demonstrating that NM23-H1 physically interacts with STRAP in vivo.

To determine the subcellular localization of NM23-H1 and STRAP, immunofluorescence confocal microscopy was performed using Hep3B cells transfected with expression vectors encoding FLAG-STRAP and FLAG-NM23-H1. Both STRAP and NM23-H1 were distributed mainly in the cytoplasm and colocalized with each other, as seen in the merged image in Fig. 1C. These results indicated that NM23-H1 interacts with and colocalizes with STRAP in vivo.

FIGURE 1. NM23-H1 binding to STRAP in vivo. A, 293T cells were transfected with pEBG (GST) and pEBG-NM23-H1 (GST-NM23-H1), along with an expression vector encoding FLAG-tagged STRAP (FLAG-STRAP). GST fusion proteins were purified on glutathione-Sepharose beads (GST purification), and complex formation (top) and the starting amount of FLAG-STRAP in the in vivo binding assay (bottom; Lysate) were determined by immunoblot (WB) using an anti-FLAG antibody. B, cell lysates from untransfected parental 293T, Hep3B, and SK-N-BE(2)C cells were subjected to immunoprecipitation (IP) using either rabbit preimmune serum (preimm.) or rabbit anti-STRAP antibody (α-STRAP), followed by immunoblot analysis using an anti-NM23-H1 antibody. As a control, the expression levels of NM23-H1 and STRAP in total cell lysate were analyzed by immunoblot using anti-NM23-H1 and anti-STRAP antibodies, respectively. C, colocalization of STRAP and NM23-H1. Hep3B cells were transiently transfected with expression vectors encoding FLAG-Smad3 and FLAG-NM23-H1. Cells were immunostained using anti-FLAG(M2) (to detect STRAP) or anti-NM23-H1 antibodies, followed by Alexa Fluor-594 anti-mouse secondary antibody (for STRAP, in red) or Alexa Fluor-488 anti-rabbit secondary antibody (for NM23-H1, in green) and then examined using confocal microscopy. Nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI). The yellow color in the merged image represents colocalization of STRAP and NM23-H1. Data are representative of at least four independent experiments.
Cysteine Residues of NM23-H1 and STRAP Participate in NM23-H1-STRAP Complex Formation—Protein-protein interactions are mediated by disulfide linkages as well as prototypical protein binding domains in the interacting proteins (17). Although STRAP contains six WD40 repeat regions (23), NM23-H1 lacks any of the typical protein binding domains that would potentially mediate its interaction with the WD40 domains of STRAP. We speculated that the interaction of NM23-H1 and STRAP is mediated by cysteine residues present in the two proteins. 293T cells were transiently transfected with expression vectors encoding FLAG-NM23-H1 or the FLAG-tagged NM23-H1 substitution mutants NM23-H1(C4S), NM23-H1(C109S), and NM23-H1(C145S), together with an expression vector for GST-tagged wild-type STRAP (GST-STRAP). GST-STRAP was precipitated using glutathione-Sepharose beads, and complex formation between NM23-H1 and STRAP was examined by immunoblot using an anti-FLAG antibody (Fig. 2A).

There was a dramatic decrease in complex formation in cells expressing NM23-H1(C145S), compared with wild-type NM23-H1, whereas complex formation was not influenced in cells expressing NM23-H1(C4S) and NM23-H1(C109S) (Fig. 2A, top, lanes 5–8). These results suggested that Cys145 of NM23-H1 plays a critical role in the association of NM23-H1 with STRAP. The amount of STRAP and NM23-H1 in each sample was similar (Fig. 2A, middle and bottom), indicating that the observed difference in complex formation was not due to differences in STRAP and NM23-H1 expression levels. The WD40 repeats of STRAP are functional motifs that are generally involved in protein-protein interactions and the assembly of multiprotein complexes (24). To determine whether cysteine residues within the WD40 repeat regions of STRAP (Cys152 and Cys270) affected complex formation between NM23-H1 and STRAP, 293T cells were cotransfected with expression plasmids encoding wild-type and mutant forms of GST-NM23-H1 and FLAG-STRAP. Coexpression of NM23-H1(C145S) with either STRAP(C152S) or STRAP(C270S) resulted in a significant decrease in complex formation between NM23-H1 and STRAP (Fig. 2B, top, lanes 4 and 6), whereas coexpression of wild-type NM23-H1 with either STRAP(C152S) or STRAP(C270S) resulted in only a slight decrease in complex formation, as compared with the expression of wild-type NM23-H1 and wild-type STRAP (Fig. 2B, top, lane 1 versus lanes 3 and 5). These results indicated that both Cys152 and Cys270 of STRAP play an important role in its association with NM23-H1. In addition, mutation of Cys270 of β-ME on ice for 0.5–1 h and subjected to immunoprecipitation using an anti-NM23-H1 antibody (IP), and immune complexes were analyzed for the presence of STRAP by immunoblot using an anti-FLAG antibody (top). The amount of immunoprecipitated NM23-H1 and the expression level of STRAP in total cell lysates were determined by immunoblot analysis using an anti-NM23-H1 antibody (middle) and an anti-FLAG antibody (bottom), respectively. D, native polyacrylamide gel electrophoresis of NM23-H1-STRAP complexes. Autophosphorylation of purified recombinant NM23-H1 was performed in TMD buffer as described under “Materials and Methods.” Autophosphorylated NM23-H1 was incubated with unlabeled STRAP (wild-type or STRAP(C152S/C270S)) in the presence of 5 mM H2O2 at room temperature for 1 h. For native polyacrylamide (12%) gel electrophoresis, the procedure was the same as SDS-PAGE, with the exception that SDS and β-mercaptoethanol were not included in any solutions, and samples were not boiled before loading. The data are representative of at least four independent experiments.

**FIGURE 2.** Effect of mutations in cysteine residues of NM23-H1 and STRAP on NM23-H1-STRAP complex formation. A and B, 293T cells were transiently transfected with expression plasmids encoding the indicated proteins. GST fusion proteins were purified on glutathione-Sepharose beads (GST purification), and complex formation between NM23-H1 and STRAP (top) was analyzed by immunoblot (WB) using an anti-FLAG antibody, as described for Fig. 1. C, effect of reductants (DTT and β-mercaptoethanol (β-ME)) and H2O2 on the interaction of STRAP with endogenous NM23-H1. 293T cells were transiently transfected with an expression vector encoding FLAG-STRAP, and the cell lysates were treated with the indicated concentrations of H2O2, DTT, and β-ME on ice for 0.5–1 h and subjected to immunoprecipitation using an anti-NM23-H1 antibody (IP), and immune complexes were analyzed for the presence of STRAP by immunoblot using an anti-FLAG antibody (top). The amount of immunoprecipitated NM23-H1 and the expression level of STRAP in total cell lysates were determined by immunoblot analysis using an anti-NM23-H1 antibody (middle) and an anti-FLAG antibody (bottom), respectively. D, native polyacrylamide gel electrophoresis of NM23-H1-STRAP complexes. Autophosphorylation of purified recombinant NM23-H1 was performed in TMD buffer as described under “Materials and Methods.” Autophosphorylated NM23-H1 was incubated with unlabeled STRAP (wild-type or STRAP(C152S/C270S)) in the presence of 5 mM H2O2 at room temperature for 1 h. For native polyacrylamide (12%) gel electrophoresis, the procedure was the same as SDS-PAGE, with the exception that SDS and β-mercaptoethanol were not included in any solutions, and samples were not boiled before loading. The data are representative of at least four independent experiments.
STRAP, which is within the sixth WD40 repeat, had a somewhat stronger effect on complex formation compared with mutation of Cys\(^{152}\), which is within the fourth WD40 repeat (Fig. 2B, lane 1 versus lanes 3 and 5, lane 2 versus lanes 4 and 6). To further examine the roles of Cys\(^{152}\) and Cys\(^{270}\) of STRAP in its association with NM23-H1, we generated a double substitution mutant of STRAP, STRAP(C152S/C270S), and examined its binding properties in the in vivo binding assay. Expression of STRAP (C152S/C270S) dramatically inhibited complex formation between NM23-H1 and STRAP (Fig. 2B, lanes 7 and 8). Collectively, these results strongly suggested that cysteine residues, especially Cys\(^{152}\) and Cys\(^{270}\) of STRAP and Cys\(^{145}\) of NM23-H1, play a critical role in the association of NM23-H1 and STRAP in vivo.

To determine whether the interaction of NM23-H1 and STRAP was redox-dependent, we examined complex formation in 293T cells transiently expressing FLAG-STRAP using in vivo binding assays. The presence of the reductants DTT and \(\beta\)-mercaptoethanol markedly decreased the amount of STRAP that communoprecipitated with endogenous NM23-H1, whereas the oxidant \(\text{H}_2\text{O}_2\) had no effect (Fig. 2C, top). These results indicated that the in vivo association of NM23-H1 with STRAP is dependent on the redox state of the two proteins. We also analyzed the association of purified, recombinant NM23-H1 and STRAP using non-denaturing polyacrylamide gel electrophoresis. Purified NM23-H1 was subjected to an autophosphorylation using radiolabeled ATP and then incubated with unlabeled, recombinant STRAP. As shown in Fig. 2D, a shift in the mobility of autophosphorylated NM23-H1 was clearly evident upon incubation in the presence of wild-type STRAP but was undetectable when autophosphorylated NM23-H1 was incubated in the absence of STRAP (Fig. 2D).
lane 1 versus lane 2), providing additional evidence of a physical association between NM23-H1 and STRAP. The band shift was not observed when NM23-H1 was incubated with STRAP(C152S/C270S) (Fig. 2D, lane 1 versus lane 3). Together, these results suggested that complex formation between NM23-H1 and STRAP requires cysteine residues present in each of the two proteins.

**Determination of the NM23-H1 Interaction Domain of STRAP—**To map the domain(s) within STRAP required for its association with NM23-H1, we generated a set of eight STRAP deletion mutants (Fig. 3A) and examined their ability to interact with NM23-H1 in an in vivo binding assay. NM23-H1 interacted with STRAP-WD4-6, containing the three C-terminal WD40 repeats (amino acids 129–350), but not with STRAP-WD1–3, which contained the N-terminal three WD40 repeats (amino acids 1–129) (Fig. 3B, top). These results indicated that the interaction of STRAP with NM23-H1 is mediated via the C-terminal WD40 repeats of STRAP. To more precisely define the NM23-H1-binding motif of STRAP, we examined the binding properties of six additional STRAP deletion constructs: STRAP-WD4, -WD4(C152S), -WD5, -WD6, -WD6(C270S), and -CT (Fig. 3A). The binding of NM23-H1 to STRAP-WD4, STRAP-WD5, and STRAP-WD6 was readily apparent (Fig. 3C, top), whereas NM23-H1 binding to STRAP-WD4(C152S) and STRAP-WD6(C270S) was undetectable (Fig. 3C, top). This result was in good agreement with the previous result (Fig. 2) that Cys(Cys152 and Cys270 of STRAP play a critical role in its association with NM23-H1. Other cysteine residues within STRAP, including Cys10, Cys36, Cys305, and Cys340, appeared to have no role in the association between NM23-H1 and STRAP (Fig. 3B and C). Together, these results clearly demonstrated that STRAP binds to NM23-H1 through Cys(Cys152, within the fourth WD40 repeat, and Cys270, within the sixth WD40 repeat.

**Conformation of NM23-H1 and STRAP Is Not Altered by Cys → Ser Mutations—**Previous studies have shown that NM23-H1 and NM23-H2 can form homohexamers in vitro and
Functional Association between NM23-H1 and STRAP

**A**

| GST            | FLAG-Nm23-H1(WT) | FLAG-Nm23-H1(C4S) | FLAG-Nm23-H1(C109S) | FLAG-Nm23-H1(C145S) |
|----------------|------------------|-------------------|---------------------|---------------------|
| GST Purification | WB : anti-FLAG   | WB : anti-FLAG    | WB : anti-FLAG      | WB : anti-FLAG      |
| Lyse           | WB : anti-GST    | WB : anti-GST     | WB : anti-GST       | WB : anti-GST       |

**B**

| GST            | FLAG-Nm23-H1(WT) | FLAG-Nm23-H1(C4S) | FLAG-Nm23-H1(C109S) | FLAG-Nm23-H1(C145S) |
|----------------|------------------|-------------------|---------------------|---------------------|
| GST Purification | WB : anti-FLAG   | WB : anti-FLAG    | WB : anti-FLAG      | WB : anti-FLAG      |
| Lyse           | WB : anti-GST    | WB : anti-GST     | WB : anti-GST       | WB : anti-GST       |

**C**

| GST            | GST-Nm23-H1 | GST-Nm23-H2 | FLAG-Nm23-H1 | FLAG-STRAP |
|----------------|-------------|-------------|--------------|------------|
| GST Purification | WB : anti-FLAG | WB : anti-FLAG | WB : anti-FLAG | WB : anti-FLAG |
| Lyse           | WB : anti-GST | WB : anti-GST | WB : anti-GST | WB : anti-GST |

**FIGURE 4. Effect of the Cys → Ser mutations on the conformation of NM23-H1 and STRAP.**

A and B, effect of Cys → Ser mutations of NM23-H1 on the association between NM23-H1 and NM23-H1 (A) or between NM23-H1 and NM23-H2 (B). 293T cells were transiently transfected with expression vectors encoding the indicated proteins. GST fusion proteins were purified on glutathione-Sepharose beads (GST purification), and proteins were resolved by SDS-PAGE. Complex formation was analyzed by immunoblot (WB) using an anti-FLAG antibody (top panels). The membrane was then stripped and reprobed with an anti-GST antibody to confirm the precipitation of equivalent amounts of GST fusion protein (middle); the expression levels of FLAG-tagged proteins were analyzed by immunoblot using an anti-FLAG antibody (bottom). C, effect of STRAP on the association between NM23-H1 and NM23-H1 or between NM23-H1 and NM23-H2. 293T cells were transiently transfected with the indicated combinations of expression vectors for GST-NM23-H1, GST-NM23-H2, FLAG-NM23-H1, and FLAG-STRAP, and cell lysates were subjected to precipitation with glutathione-Sepharose beads (GST purification). Complex formation between NM23-H1 and NM23-H1 or between NM23-H1 and NM23-H2 was analyzed by immunoblot using an anti-FLAG antibody (top). The amounts of GST-tagged NM23-H1 and NM23-H2 (middle) and FLAG-tagged STRAP and NM23-H1 (bottom) in the in vivo binding assay were analyzed by immunoblot using the indicated antibodies. D and E, CD spectra of wild type (WT) and the indicated mutant forms of NM23-H1, as well as wild type and the indicated mutant forms of STRAP. Each protein was present at a concentration of 0.058 mg/ml in 10 mM potassium phosphate buffer (pH 7.0). F, effect of Cys mutations of STRAP on its homo-oligomerization. 293T cells were transiently transfected with expression vectors encoding the indicated proteins, and then GST fusion proteins were purified on glutathione-Sepharose beads (GST purification) and resolved by SDS-PAGE. The self-association of STRAP was analyzed by immunoblot using an anti-FLAG antibody (top). G, effect of Cys → Ser mutations of STRAP on the association between activated type 1 TGF-β receptor and STRAP. 293T cells were transiently transfected with expression vectors encoding the indicated proteins, and in vivo binding assays were performed as described for Fig. 1. The membrane was then stripped and reprobed with an anti-FLAG antibody to confirm the precipitation of equivalent amounts of GST-TβR1 (middle); the expression levels of FLAG-STRAP were analyzed by immunoblot using an anti-FLAG antibody (bottom). The data are representative of at least four independent experiments.

**in vivo**, as well as heterohexamers possessing different ratios of H1 and H2 subunits (9, 25–30). In light of these observations, we were interested in the role of the cysteine residues of NM23-H1 in complex formation between NM23-H1 and NM23-H2 or between NM23-H1 and NM23-H2. The expression of the NM23-H1 mutants NM23-H1(C4S), NM23-H1(C109S), and NM23-H1(C145S) had no effect on complex formation between NM23-H1 and NM23-H1 (Fig. 4A) or NM23-H1 and NM23-H2 (Fig. 4B), suggesting that the Cys → Ser mutations in these proteins do not alter the conformational state of NM23-H1 that is required for oligomerization. To confirm that the Cys → Ser mutations of NM23-H1 do not alter its normal conformation, we examined whether coexpression of STRAP influenced complex formation between NM23-H1 and NM23-H1 or between NM23-H1 and NM23-H2. We did not detect any significant changes in complex formation between NM23-H1 and NM23-H1 (Fig. 4C, top, lane 3 versus lane 4) or NM23-H1 and NM23-H2 (Fig. 4C, top, lane 5 versus lane 6) in the presence of STRAP, indicating that, in contrast to the association of NM23-H1 and STRAP, homo-oligomerization of NM23-H1 and hetero-oligomerization of NM23-H1 with NM23-H2 does not require Cys145. We also carried out CD spectroscopy to examine the secondary structures of the wild-type and mutant forms of NM23-H1 and STRAP. The results clearly showed that the CD spectra of NM23-H1(C4S), NM23-H1(C109S), and NM23-H1(C145S) and of STRAP(C152S), STRAP(C270S), and STRAP(C152S/C270S) almost completely overlapped that of wild-type NM23-H1 and wild-type STRAP, respectively (Fig. 4D, E, and F), indicating that the secondary structures of each of the NM23-H1 and STRAP mutants, as determined by CD spectroscopy, were not affected by the mutations. We also analyzed whether the Cys → Ser mutations of STRAP influenced the homo-oligomerization of STRAP (16). As shown in Fig. 4F, no changes in the self-association of STRAP were detected in the presence of the STRAP mutants. These results indicated that the Cys → Ser mutations of STRAP do not affect its ability to homo-oligomerize and have no effect on the normal conformation of STRAP. Previous studies have
shown that STRAP associates with the TGF-β type 1 receptor (16, 23). To determine whether the STRAP Cys → Ser substitution mutants could form complexes with the TGF-β type 1 receptor, 293T cells were transfected with expression vectors for wild-type and mutant forms of STRAP, together with a GST fusion protein of a constitutively active form of the TGF-β type 1 receptor, GST-TβR1(TD). Cell lysates were incubated with glutathione-Sepharose beads, and protein complexes were analyzed by immunoblot using an anti-FLAG antibody. Similar amounts of STRAP were detected in every precipitate (Fig. 4, top), indicating that, similar to wild-type STRAP, the STRAP mutants also associate with the TGF-β type 1 receptor. These results provided additional evidence that the normal conformation of STRAP is not affected by the Cys → Ser mutations. Taken together, these data suggested that NM23-H1 physically interacts with STRAP through intermolecular disulfide linkages involving cysteine residues and that the Cys → Ser mutations of both NM23-H1 and STRAP do not affect the normal conformation of these proteins.

Retention of Normal Function of NM23-H1 and STRAP after Cys → Ser Mutations—The stimulation of PDK1 activity upon expression of STRAP is a well-established assay for the biological activity of STRAP (15). To determine whether the STRAP Cys → Ser mutants influenced PDK1 activity when coexpressed with PDK1, 293T cells were transiently cotransfected with expression vectors for FLAG-tagged wild-type and mutant forms of STRAP, along with GST-PDK1. PDK1 was immunoprecipitated from the transfected cells, and its activity was measured in an in vitro kinase assay using serum- and glucocorticoid-inducible kinase as a substrate (31). Similar to wild-type STRAP, coexpression of STRAP mutants resulted in a significant increase in PDK1 activity (Fig. 5A), indicating that the Cys → Ser mutations of STRAP do not affect the ability of STRAP to stimulate PDK1 activity.

Biochemical studies have shown that NM23-H1 has several types of enzymatic activity, including autophosphorylation, phosphotransferase activity, and NDP kinase activity (32). To determine whether the Cys → Ser mutations of NM23-H1 affected its biochemical properties, we examined the autophosphorylation (Fig. 5B), phosphotransferase (Fig. 5C), and NDP kinase (Fig. 5D) activities of wild-type and mutant forms of NM23-H1. The activities of the substitution mutants were similar to those of wild-type NM23-H1 in every assay, indicating that the Cys → Ser mutations of NM23-H1 do not modulate its biochemical properties. Collectively, these results suggested that the Cys → Ser mutations of both NM23-H1 and STRAP do not alter the normal function of these proteins.

NM23-H1 Negatively Regulates TGF-β-induced Transcription—It has previously been shown that STRAP exists as a homo- or hetero-oligomer and can negatively regulate TGF-β-induced transcription in a dose-dependent manner (16). To investigate the physiological significance of the interaction of NM23-H1 and STRAP, we examined the effect of NM23-H1 expression on TGF-β-induced transcription using three TGF-β-responsive reporter plasmids: p3TP-Lux (Fig. 6A), p21-Luc...
Functional Association between NM23-H1 and STRAP

(Fig. 6B), and Smad7-Luc (Fig. 6C). Similar to STRAP (23), overexpression of NM23-H1 in HepG2 cells suppressed TGF-β-induced luciferase activity in a dose-dependent manner (Fig. 6, A–C, left). We next examined whether the interaction of NM23-H1 with STRAP influenced TGF-β-induced transcription. Expression of NM23-H1 with STRAP enhanced the inhibition of TGF-β-mediated transcription in a dose-dependent manner, as compared with the effect of expression of STRAP alone (Fig. 6, A–C, right), suggesting that NM23-H1 and STRAP cooperate in the inhibition of TGF-β signaling. To determine if STRAP is required for NM23-H1-induced inhibition of TGF-β signaling, we performed knockdown experiments using siRNAs that targeted STRAP. As shown in Fig. 6D, siRNA-mediated knockdown of STRAP resulted in a significant increase in TGF-β-induced transcription, and the effect was proportional to the amount of STRAP-specific siRNA used in the transfection. There was very little inhibitory effect on TGF-β signaling upon expression of NM23-H1 in STRAP knockdown cells (Fig. 6D, right), indicating that the direct interaction between NM23-H1 and STRAP plays an important role in NM23-H1-induced inhibition of TGF-β signaling. To examine further whether the inhibition of TGF-β-induced transcription by NM23-H1 is directly or indirectly dependent on its interaction with STRAP, we examined the effect of expression of the NM23-H1 substitution mutants on TGF-β-induced transcription. As shown in Fig. 6E, expression of NM23-H1(C145S) did not affect TGF-β-induced transcription, whereas expression of wild-type NM23-H1, NM23-H1(C4S), and NM23-H1(C109S) significantly decreased TGF-β-induced transcription to a similar extent in a dose-dependent manner. Since Cys145 of NM23-H1 is also involved in the association with STRAP (Fig. 2), this result suggested that the direct interaction between NM23-H1 and STRAP plays an important role in the modulation of TGF-β signaling. We also performed knockdown experiments of NM23-H1 using two NM23-H1-specific siRNAs (a and b). As shown in Fig. 6F (top), transfection of HepG2 cells with siRNA duplexes targeting NM23-H1, but not a nonspecific control siRNA, resulted in a significant increase of TGF-β-induced transcription, and the effect was proportional to the amount of siRNA used in the transfection. Immunoblot analysis confirmed that the level of endogenous NM23-H1 was significantly reduced in cells transfected with NM23-H1 siRNA duplexes, compared with untransfected cells (−), or cells transfected with the nonspecific control siRNA (Fig. 6F, bottom). These results indicated that down-regulation of endogenous NM23-H1 stimulates TGF-β-induced transcription. We extended this analysis to an investigation of the effect of stable expression of NM23-H1 on TGF-β-mediated signaling (Fig. 6F). We generated HCT116 cells stably expressing NM23-H1 (+NM23-H1) and examined gene expression in these cells in response to TGF-β signaling.
induced by a constitutively active form of type I TGF-β receptor, TβR1(TD). Overexpression of NM23-H1 attenuated TGF-β-mediated up-regulation of PAI-1, p21, and Smad7 and the TGF-β-mediated down-regulation of CDK4 and cyclin D1, proteins whose genes are normally up-regulated and down-regulated, respectively, by TGF-β (Fig. 6G, left versus right). To confirm the effect of stable expression of NM23-H1 on TGF-β signaling, we generated HCT116 cells stably expressing an siRNA targeting NM23-H1 (NM23-H1) and analyzed them for TGF-β-induced gene expression. Compared with control HCT116 cells treated with TβR1(TD), knockdown of NM23-H1 significantly increased the expression of PAI-1, p21, and Smad7 and decreased the expression CDK4 and cyclin D1 (Fig. 6H). Similar results were also observed in HeLa cells transiently transfected with an NM23-H1-specific siRNA (Fig. 6I), confirming the results we obtained with the stable transfectants (Fig. 6, G and H). Collectively, these data suggested that NM23-H1 physically associates with STRAP and potentiates STRAP-induced inhibition of TGF-β signaling.

NM23-H1 Is Involved in the Negative Regulation of TGF-β-induced Apoptosis and Growth Inhibition—STRAP has been shown to positively regulate a PDK1-mediated signaling pathway that is crucial for cell survival, cell growth, and protein synthesis and to negatively regulate TGF-β-induced transcription (15, 16). Since NM23-H1 also appeared to inhibit TGF-β-induced transcription (Fig. 6), we examined whether NM23-H1 was involved in regulating TGF-β-induced apoptosis. HeLa and HaCaT cells were transiently transfected with expression vectors encoding GFP, NM23-H1, and STRAP, and apoptosis was assessed in the presence of purified wild-type and mutant NM23-H1 proteins (−3 μg) in TMD buffer (D). The data are representative of at least four independent experiments.

**FIGURE 5.** Effect of Cys → Ser mutations on the activity of NM23-H1 and STRAP. A, effect of expression of STRAP mutants on PDK1 kinase activity. 293T cells were cotransfected with expression vectors for GST-PDK1 and either wild-type or mutant STRAP proteins. Cell lysates were subjected to precipitation with glutathione-Sepharose beads, and protein complexes were analyzed for PDK1 activity by an in vitro kinase assay using serum- and glucocorticoid-inducible kinase as a substrate. A circled P followed by SGK indicates the position of phosphorylated serum- and glucocorticoid-inducible kinase. The membrane was then stripped and reprobed with the indicated antibodies to confirm equivalent amounts of substrate (third panel) and GST-PDK1 (second panel) in each assay; the expression levels of FLAG-STRAP proteins were analyzed by immunoblot (WB) using an anti-FLAG antibody (bottom panel). B–D, effect of expression of NM23-H1 mutants on NM23-H1 enzymatic activities. Autophosphorylation was performed in TMD buffer using purified recombinant NM23-H1 proteins (B). Purified GST-tagged wild-type and NM23-H1 mutants (−3 μg) were incubated with 1 μM [γ-32P]ATP at room temperature for 10 min in a final volume of 20 μl of TMD buffer. Phosphotransferase assays were performed with the indicated wild type (WT) and NM23-H1 mutants (C). A circled P followed by GST-NM23-H1 or His-NM23-H1 indicates the position of phosphorylated GST-NM23-H1 or His-NM23-H1, respectively. NDP kinase activity was assayed in the presence of purified wild-type and mutant NM23-H1 proteins (−3 μg) in TMD buffer (D). The data are representative of at least four independent experiments.
Functional Association between NM23-H1 and STRAP

STRAP alone (Fig. 7A, bars 3 and 4 versus bar 5). In these experiments, expression of STRAP consistently had a somewhat stronger effect on the suppression of TGF-β-induced apoptosis when compared with NM23-H1 (Fig. 7A, bar 3 versus bar 4). The suppressive effect of NM23-H1 on TGF-β-induced apoptosis was also examined using siRNAs targeting NM23-H1.

A

![Image](https://example.com/image1.png)

B

![Image](https://example.com/image2.png)

**FIGURE 6. Enhancement of STRAP-induced inhibition of TGF-β signaling by NM23-H1.** A–C, HepG2 cells were transiently transfected with 0.3 μg of p3TP-Lux (A), 0.3 μg of p21-Luc (B), 0.3 μg of Smad7-Luc (C), 0.1 μg of an expression plasmid for β-galactosidase as an internal control, and increasing amounts of expression vectors for the indicated NM23-H1 proteins in the presence or absence of STRAP and subsequently stimulated by TGF-β1 (100 pM). D, regulation of TGF-β-induced transcription by STRAP-specific siRNA. HepG2 cells were transfected with 0.3 μg of p3TP-Lux and increasing amounts (50, 100, and 200 nM) of STRAP-specific siRNA (left), or 200 nM of STRAP-specific siRNA (right) as indicated in the presence (right) or absence (left) of coexpressed NM23-H1. The data are representative of at least three independent experiments. As a control, the expression level of endogenous STRAP was analyzed by immunoblot using an anti-STRAP antibody. E, HepG2 cells were transfected with 0.3 μg of p3TP-Lux, 0.6 μg of STRAP expression plasmid, and increasing amounts of expression vectors encoding wild-type and mutant NM23-H1 proteins and then stimulated by TGF-β1. Luciferase activity was measured 48 h after transfection and normalized to β-galactosidase activity. The fold increase relative to control cells transfected with STRAP alone is presented. The S.D. values are less than 5%. The data are representative of at least four independent experiments. As a control, the expression level of endogenous STRAP was analyzed by immunoblot using an anti-STRAP antibody. F, HepG2 cells were transfected with 0.3 μg of p3TP-Lux, 0.6 μg of STRAP expression plasmid, and increasing amounts of expression vectors encoding wild-type and mutant NM23-H1 proteins and then stimulated by TGF-β1. Luciferase activity was measured 48 h after transfection and normalized to β-galactosidase activity. The fold increase relative to control cells transfected with STRAP alone is presented. The S.D. values are less than 5%. The data are representative of at least four independent experiments. G and H, effect of NM23-H1 on the expression of TGF-β target genes. Parental HCT116 cells (HCT116), HCT116 cells stably expressing NM23-H1 (+NM23-H1), and HCT116 cells stably expressing an NM23-H1-specific siRNA (−NM23-H1) were lysed and subjected to immunoblot analysis using anti-PAI-1, anti-p21, anti-Smad7, anti-CDK4, anti-cyclin D1, and anti-NM23-H1 antibodies. A constitutively active form of the type I TGF-β receptor, TβRI(TD), was used to alternatively initiate TGF-β signaling (+TGF-β1). I. modulation of TGF-β target genes by NM23-H1-specific siRNA. HepG2 cells were transiently transfected with 200 nM control siRNA (Con.) or NM23-H1-specific siRNA(b) (NM23-H1) as indicated and then treated with 10 ng/ml TGF-β1 for 20 h. Cell lysates were subjected to immunoblot analysis using anti-PAI-1, anti-p21, anti-Smad7, anti-CDK4, anti-cyclin D1, and anti-NM23-H1 antibodies. Equal amounts of protein in each lane were confirmed by immunoblotting with an anti-β-actin antibody (G–I, bottom panels). The data are representative of at least four independent experiments.

Apoptotic cell death was significantly increased by transfection with NM23-H1-specific siRNAs in a dose-dependent manner (Fig. 7B), implying that NM23-H1, like STRAP, positively regulates cell survival through the suppression of TGF-β-induced apoptotic signaling. As additional evidence that NM23-H1 plays an important role in the negative regulation of TGF-β-
Functional Association between NM23-H1 and STRAP

C

![Graph showing the functional association between NM23-H1 and STRAP](image)

D

![Graph showing the effect of STRAP siRNA on NM23-H1 expression](image)

E

![Graph showing the fold increase in expression](image)

FIGURE 6 — continued
induced apoptosis, we transfected cells with increasing amounts of wild-type NM23-H1(WT) and NM23-H1(C145S) and examined the effect on TGF-β-induced apoptosis (Fig. 7C). Similar to STRAP (16), the ability of wild-type NM23-H1 to suppress TGF-β-induced apoptosis was dose-dependent (Fig. 7C, bar 3 versus bars 4 and 5). However, expression of NM23-H1(C145S) had no effect on TGF-β-induced apoptosis (Fig. 7C, bar 3 versus bars 6 and 7), consistent with the results of the luciferase reporter assays (Fig. 6E).

These results indicated that the Cys145 of NM23-H1 plays a critical role in the regulation of TGF-β-induced apoptosis, probably by mediating a direct interaction with STRAP.

To explore further the functional significance of the association between NM23-H1 and STRAP, we investigated the effect of wild-type NM23-H1 and NM23-H1(C145S) on TGF-β-induced growth inhibition using flow cytometry. As shown in Fig. 7D, ~32% of HaCaT cells expressing STRAP accumulated in S phase after 24 h of serum stimulation in the presence of TGF-β (Fig. 7D, upper bottom panels, STRAP), compared with ~55% in the absence of TGF-β (Fig. 7D, upper middle panels, STRAP). Furthermore, in the presence of TGF-β, a higher percentage of cells (~41%) were in S phase when STRAP was coexpressed with wild-type NM23-H1 (Fig. 7D, upper bottom panels, STRAP/NM23-H1), compared with the expression of STRAP alone (~32%; Fig. 7D, upper bottom panels, STRAP). Coexpression of NM23-H1(C145S) had no such stimulatory effect, since there was no difference in the percentage of cells in S phase between cells expressing STRAP alone and STRAP plus NM23-H1(C145S) in the presence of TGF-β (Fig. 7D, upper bottom panels; ~32% versus ~34%). Statistical analysis of S phase cell fractions in the presence of TGF-β is shown in the bar graph in Fig. 7D (bottom). These results indicated that Cys145 of NM23-H1 plays an important role in the positive regulation of STRAP-induced growth stimulation in response to TGF-β.

We also analyzed HaCaT cells transiently transfected with an NM23-H1-specific siRNA by flow cytometry. As shown in Fig. 7E, reducing the level of endogenous NM23-H1 by NM23-H1 knockdown resulted in a significant decrease in the percentage of cells present in S phase (Fig. 7E, upper bottom panels, ~43% versus ~34%), providing additional support for a role of
NM23-H1 in the regulation of STRAP-induced growth stimulation in response to TGF-β. Statistical analysis of S phase cell fractions under these conditions is shown in the bar graph in Fig. 7E (bottom). Collectively, these results suggested that NM23-H1 is involved in the negative regulation of TGF-β-induced apoptosis and growth inhibition through an inhibition of TGF-β signaling.

NM23-H1 Modulates the Nuclear Translocation of Smad3—It has previously been shown that STRAP blocks TGF-β signaling by stabilizing the complex of activated TGF-β type 1 receptor and Smad7 (16). To investigate the mechanism of NM23-H1-mediated inhibition of TGF-β signaling, we examined whether NM23-H1, like STRAP, stabilized the interaction between TGF-β type 1 receptor and Smad7 in an in vivo binding assay. 293T cells were transiently transfected with expression plasmids encoding GST-TBR1(TD), Smad7, STRAP, and NM23-H1, and the effect on complex formation was assessed by precipitation with glutathione-Sepharose 4B. As shown in Fig. 8A, the association between TBR1(TD) and Smad7 was significantly increased upon expression of increasing amounts of wild-type NM23-H1, as compared with control cells that did not express NM23-H1 (Fig. 8A, left top panel, lane 5 versus lanes 6–8). When we examined the effect of NM23-H1(C145S), which is unable to physically associate with STRAP, we found that expression of NM23-H1(C145S) had no affect on the association between TBR1(TD) and Smad7 (Fig. 8A, right top panel, lane 2 versus lanes 3–5), indicating that the physical association between NM23-H1 and STRAP is important for stabilizing the TBR1-

![Image](60x247 to 552x559)

**FIGURE 7. Effect of NM23-H1 on TGF-β-induced apoptosis and growth inhibition.** A, effect of NM23-H1 on TGF-β-induced apoptosis. HeLa or HaCaT cells were transiently transfected with expression vectors encoding NM23-H1 (3 μg) and/or STRAP (3 μg), along with an expression vector encoding GFP (2 μg), as indicated. Transfected cells and untransfected parental HeLa or HaCaT cells were incubated for 24 h and then treated with TGF-β1 (HeLa, 10 ng/ml; HaCaT, 2 ng/ml) for 20 h to induce apoptosis. GFP-positive cells were examined for the presence of apoptotic nuclei using fluorescence microscopy. B, effect of NM23-H1-specific siRNA on TGF-β-induced apoptosis. HeLa or HaCaT cells were transiently transfected with NM23-H1-specific siRNA(b) (white bars) or a nonspecific control siRNA (black bars), along with an expression vector encoding GFP (2 μg). Transfected cells and untransfected parental HeLa or HaCaT cells (−) were incubated for 24 h and then treated with TGF-β1 as described for A to induce apoptosis. Data represent the means ± S.D. of triplicate assays and are representative of at least three independent experiments. C, effect of wild-type NM23-H1 or NM23-H1(C145S) on TGF-β-mediated inhibition of TGF-β-induced apoptosis. HeLa or HaCaT cells were transiently transfected with increasing amounts (2 and 4 μg) of expression vectors for wild-type NM23-H1 (WT) or NM23-H1(C145S), as indicated, in the presence (+) or absence (−) of 3 μg of STRAP expression vector, together with an expression vector encoding GFP (2 μg). Transfected cells were treated with TGF-β1 (HeLa, 10 ng/ml; HaCaT, 2 ng/ml) for 20 h to induce apoptosis, and apoptosis was assessed as described for A (top). Expression levels of STRAP and NM23-H1 were analyzed by immunoblot using an anti-FLAG antibody (middle and bottom). D, effect of NM23-H1 (WT or the C145S mutant) on STRAP-mediated inhibition of TGF-β-induced growth arrest. HaCaT cells (2 × 10⁵ cells/60-mm dish) transfected with a control vector or expression vectors for STRAP, STRAP/NM23-H1, or STRAP/NM23-H1(C145S) were synchronized in G1/G0 by hydroxyurea treatment (2 mm) for 20 h. Cells were collected before (0-h starvation) or after 10% serum treatment for 24 h in the absence (24-h serum stimulation) or presence of 2 ng/ml TGF-β1 (24-h serum stimulation + TGF-β1). The percentage of cells in G1, S, and G2/M was analyzed by flow cytometry. The fraction of cells in S phase (24-h serum stimulation + TGF-β1) was quantitated and is presented in the bar graphs at the bottom. E, effect of NM23-H1-specific siRNA on TGF-β-mediated inhibition of TGF-β-induced growth arrest. HaCaT cells (2 × 10⁵ cells/60-mm dish) transfected with the indicated siRNA (NM23-H1-specific siRNA(b) or control siRNA), together with an expression vector for STRAP, were synchronized in G1/G0 by hydroxyurea treatment as described for D. Cells were collected before (0-h starvation) or after 10% serum treatment for 24 h in the absence (24-h serum stimulation) or presence of 2 ng/ml TGF-β1 (24-h serum stimulation + TGF-β1), and the percentage of cells in G1, S, and G2/M was analyzed by flow cytometry. The fraction of cells in S phase (24-h serum stimulation + TGF-β1) was quantitated and is presented in the bar graph at the bottom. Data represent the means ± S.D. of duplicate assays and are representative of at least three independent experiments.
Functional Association between NM23-H1 and STRAP

C

![Graph showing the functional association between NM23-H1 and STRAP in HeLa and HaCaT cell lines.](image)

D

![Graph showing cell cycle analysis in different conditions.](image)

FIGURE 7—continued
Smad7 complex and blocking TGF-β signaling. We also examined whether expression of NM23-H1 affected the association between STRAP and Smad7, since STRAP, in conjunction with Smad7, has been shown to be involved in preventing Smad2 and Smad3 access to the TGF-β receptor (16). As shown in Fig. 8B, the interaction of STRAP with Smad7 was considerably increased upon coexpression of wild-type NM23-H1, compared with control cells that did not express NM23-H1, and this effect was dependent on the amount of NM23-H1 expression plasmid used in the transfection (Fig. 8B, left top panel, lane 2 versus lanes 3 and 4). This result implied that NM23-H1 negatively regulates TGF-β signaling by assisting in the recruitment of Smad7 to the TGF-β receptor by STRAP. There was no effect on the association between STRAP and Smad7 in 293T cells expressing NM23-H1(C145S) (Fig. 8B, right top panel, lane 1 versus lanes 2 and 3), in agreement with the above result (Fig. 8A). Smad3 also plays a key role in TGF-β signaling. We next investigated whether NM23-H1 modified Smad3 translocation...
FIGURE 8. NM23-H1 inhibits nuclear translocation of Smad3. A and B, effect of NM23-H1 on the association of activated type I TGF-β receptor and Smad7 (A) and STRAP and Smad7 (B). 293T cells were transfected with the indicated combinations of expression vectors for TβRI(TD), Smad7, and STRAP plus increasing amounts of wild-type NM23-H1 or NM23-H1(C145S) (0.2, 0.4, and 0.6 μg) (A) or with the indicated combinations of expression vectors for STRAP and Smad7 plus increasing amounts of wild-type NM23-H1 or NM23-H1(C145S) (0.1 and 0.3 μg) (B). Complex formation was determined by immunoblot (WB) using an anti-FLAG antibody, as described for Fig. 1 (top). C and D, modulation of Smad3 localization by NM23-H1. Hep3B cells were transiently transfected with the indicated combinations of expression vectors for Smad3 and NM23-H1 in the presence or absence of 100 pM TGF-β1. Cells were immunostained with anti-FLAG(M2) (to detect Smad3) or anti-NM23-H1 (to detect NM23-H1) antibodies, followed by Alexa Fluor-594 anti-mouse secondary antibody (for Smad3, red) or Alexa Fluor-488 anti-rabbit secondary antibody (for NM23-H1, green) and analyzed by confocal microscopy. Nuclei were visualized with 4',6'-diamidino-2-phenylindole (DAPI). E, effect of NM23-H1(C145S) on Smad3 localization. Hep3B cells were transiently transfected with the indicated combinations of expression vectors for Smad3, STRAP, wild-type NM23-H1, and NM23-H1(C145S) in the presence or absence of 100 μg TGF-β1. Cells were immunostained with anti-FLAG(M2) antibody (to detect Smad3), followed by Alexa Fluor-594 anti-mouse secondary antibody (for Smad3, red), and examined by confocal microscopy (top). Smad3 localization was also analyzed by immunoblot (bottom). Cytoplasmic and nuclear fractions of Hep3B cells transfected with the indicated expression vectors in the presence of TGF-β1 were analyzed by immunoblot using the indicated antibodies. Cytoplasm and Nucleus, the cytoplasmic and nuclear fraction, respectively. Quantification of the blots was done by using densitometry analysis software (Labworks 4.0 software, UVP Inc., Upland, CA), and the fold increase relative to control samples that did not express NM23-H1 is presented (lower bottom panel). The data are representative of at least three independent experiments.
changes in NM23-H1 localization upon expression of Smad3, in the presence or absence of TGF-β (Fig. 8D, bottom, lane 1 versus lane 3, lane 2 versus lane 4). To further examine NM23-H1-mediated inhibition of Smad3 nuclear translocation, we assessed the movement of Smad3 in cells expressing NM23-H1(C145S), Smad3, and STRAP using confocal microscopy. As shown in Fig. 8E, in the presence of TGF-β, expression of NM23-H1(C145S) had the opposite effect of wild-type Nm32-H1 (Fig. 8E, top, lane 2 versus lane 3), further supporting the significance of the physical interaction between NM23-H1 and STRAP in the regulation of TGF-β signaling. We also performed immunoblot analysis of cytoplasmic and nuclear fractions of cell extracts to determine the subcellular localization of Smad3 in the presence or absence of wild-type NM23-H1 or NM23-H1(C145S). In the presence of TGF-β, Smad3 accumulated in the nuclear fraction of untransfected, control Hep3B cells, and this effect was significantly decreased upon expression of wild-type NM23-H1 (Fig. 8E, lower top panel, lane 4 versus lane 5). As expected, there was a corresponding increase in the accumulation of Smad3 in the cytoplasmic fraction (Fig. 8E, lower top panel, lane 1 versus lane 2), consistent with the confocal microscopy data (Fig. 8, C and E). No difference was observed in the subcellular localization of Smad3 between untransfected control cells and cells expressing NM23-H1(C145S) (Fig. 8E, lower top panel, lane 1 versus lane 3, lane 4 versus lane 6). Together, these results suggested that expression of NM23-H1 prevents the translocation of Smad3 from the cytoplasm to the nucleus in response to TGF-β.

STRAP Potentiates NM23-H1-induced Cell Growth—The involvement of NM23-H1 in cell growth and differentiation has been demonstrated in several cell systems (4, 32). To determine whether NM23-H1 was involved in the regulation of cell growth in HaCaT cells, we performed a preliminarily analysis of cell growth using flow cytometry. We found that the expression of NM23-H1 in HaCaT cells led to an enhancement of cell growth.
growth in a dose-dependent manner, as demonstrated by the percentage of cells in S phase (data not shown), indicating that HaCaT cells can be used as a model system for examining the regulation of cell growth by NM23-H1. Parental HaCaT cells and cells transfected with a control vector or expression vectors encoding NM23-H1, NM23-H1/STRAP, or NM23-H1/STRAP(C152S/C270S) were stained with propidium iodide and examined by flow cytometry. Compared with HaCaT cells expressing NM23-H1 alone, HaCaT cells coexpressing NM23-H1 and STRAP had a significantly increased percentage of cells in S phase after 24 h of serum stimulation (Fig. 9, middle, NM23-H1 and NM23-H1/STRAP, ~37% versus ~50%), indicating that STRAP cooperates with NM23-H1 in the enhancement of NM23-H1-induced cell growth. Coexpression of STRAP(C152S/C270S) had little effect on NM23-H1-induced cell growth (Fig. 9, middle, NM23-H1 and NM23-H1/C152S/C270S, ~37% versus ~38%). This result was confirmed by statistical analysis of the fraction of cells in S phase in the presence of serum (Fig. 9, bottom). Together, these results suggested that Cys152 and Cys270 of STRAP probably play an important role in the regulation of NM23-H1-induced cell growth, probably by mediating the physical interaction between STRAP and NM23-H1.

DISCUSSION

Several reports have implicated NM23 family proteins in a variety of biological processes, including proliferation, differentiation, metastasis, and development (4). In an attempt to determine the mechanism of action of NM23 proteins, several biochemical activities and NM23-interacting proteins have also been reported (33). The eight NM23 genes identified to date encode NDP kinases (34). In addition, NM23 proteins also possess phosphotransferase activity for the phosphorylation of heterologous proteins (35). A recent study has shown that NM23-H1 forms a complex with glyceraldehyde-3-phosphate dehydrogenase in vivo and that formation of this complex is important for the protein phosphotransferase activity of NM23-H1 (9). Postel et al. (36, 37) discovered that NM23-H2 possesses transcriptional regulatory activity, and DNA-cleaving activity has also been identified in NM23 family proteins, including NM23-H1, NM23-H2, and E. coli NM23 (38–40).

Cellular NM23-H1 forms homo- or heterohexameric complexes composed of NM23-H1 and NM23-H2 (9, 25–30). The formation of hexameric NM23-H1 appears to be mediated primarily by noncovalent interactions, since it is completely dissociated into monomers during SDS-PAGE under reducing and nonreducing conditions. It was recently reported that disulfide bonding and homodimerization of NM23-H1 is mediated by Cys109 of NM23-H1 and that the NDP kinase activity of a C109A substitution mutant of NM23-H1 is similar to wild-type NM23-H1 (41), strongly suggesting the existence of intermolecular disulfide bridges between NM23-H1 and its interacting partners. All eukaryotic NM23 family proteins have been shown to interact with several cellular proteins (6, 9–14). Given these observations, we searched for potential binding partners of NM23-H1 in order to understand the mechanism of regulation of the diverse activities of NM23 family proteins. In this study, we identified and characterized STRAP as a binding partner of NM23-H1.

STRAP was originally isolated as a TGF-β receptor-interacting protein. The association of STRAP with Smad7 has a synergistic effect on the inhibition of TGF-β signaling by these proteins (16, 23). We recently reported that STRAP can act as an intermediate molecule linking PDK1 and TGF-β signaling pathways (15). We found that expression of STRAP significantly potentiated PDK1 kinase activity, suggesting that STRAP may function to regulate the enzymatic activities of its binding partners. Based on these previous results, the association of STRAP with NM23-H1 represented an intriguing potential mechanism of regulation of both NM23-H1 activity and STRAP-mediated TGF-β signaling. In the current study, we demonstrated that formation of NM23-H1-STRAP complexes significantly inhibited several NM23-H1-associated biochemical activities, including autophosphorylation, phosphotransferase activity, and NDP kinase activity (data not shown), as well as NM23-H1-induced cell proliferation (Fig. 9). These results demonstrate the functional significance of the interaction between STRAP and NM23-H1. As shown in Fig. 9 and in
data not shown, two cysteine residues (Cys\textsuperscript{152} and Cys\textsuperscript{270}) within the WD\textsuperscript{40} repeat regions of STRAP that are involved in the direct interaction between STRAP and NM23-H1 are equally critical in the regulation of the biochemical and biological activities of NM23-H1, suggesting that the direct interaction between these two proteins plays an important role in the modulation of NM23-H1 activity.

Several reports suggest that NM23 family proteins are involved in diverse signal transduction processes (42, 43). It is believed that the regulation of such biological processes as proliferation, differentiation, and development by NM23 proteins derives from their ability to modulate the signaling pathways that govern these processes (8). However, the mechanisms by which signaling pathways are modulated by NM23-H1 is unknown. As one approach to investigate putative mechanisms of regulation of NM23 family proteins, we performed a yeast two-hybrid screen using full-length NM23-H1 as a bait and identified STRAP as an NM23-H1-interacting protein. In the current study, using an \textit{in vivo} binding assay, we demonstrated that NM23-H1-STRAP complexes were clearly detectable in intact cells (Fig. 1). Moreover, we showed that the interaction of NM23-H1 with STRAP led to an enhancement of the inhibitory effect of STRAP on TGF-\(\beta\)-mediated signaling (Figs. 6–8), suggesting that NM23-H1 acts as a potential negative regulator of TGF-\(\beta\) signaling through direct binding to STRAP. However, we cannot exclude the possibility that other cellular proteins that interact with STRAP could be the critical regulator of TGF-\(\beta\) signaling, since we have recently demonstrated that PDK1, another STRAP binding partner, also potentiates STRAP-induced inhibition of TGF-\(\beta\) signaling (15). We showed that all of the amino acid substitution mutants of NM23-H1, with the exception of NM23-H1(C145S), significantly enhanced STRAP-induced inhibition of TGF-\(\beta\) signaling (Fig. 6\(E\)), strongly indicating that the direct physical interaction between NM23-H1 and STRAP, most likely mediated through disulfide linkages of cysteine residues, plays a pivotal role in the regulation of STRAP-mediated TGF-\(\beta\) signaling. However, we cannot exclude the possibility that other cellular proteins that interact with STRAP could be the critical regulator of TGF-\(\beta\) signaling, since we have recently demonstrated that PDK1, another STRAP binding partner, also potentiates STRAP-induced inhibition of TGF-\(\beta\) signaling (15). We showed that all of the amino acid substitution mutants of NM23-H1, with the exception of NM23-H1(C145S), significantly enhanced STRAP-induced inhibition of TGF-\(\beta\) signaling (Fig. 6\(E\)), strongly indicating that the direct physical interaction between NM23-H1 and STRAP, most likely mediated through disulfide linkages of cysteine residues, plays a pivotal role in the regulation of STRAP-mediated TGF-\(\beta\) signaling. This is consistent with the observed effect of NM23-H1(C145S) expression on TGF-\(\beta\)-mediated apoptosis and growth inhibition (Fig. 7). We identified a putative mechanism of inhibition of TGF-\(\beta\) signaling by NM23-H1, demonstrating that coexpression of wild-type NM23-H1, but not NM23-H1(C145S), significantly increased the interaction between activated type I TGF-\(\beta\) receptor and Smad7 in a dose-dependent manner (Fig. 8\(A\)). These results suggested that wild-type NM23-H1, like PDK1 (15), contributes to the stabilization of activated type I TGF-\(\beta\) receptor-Smad7 complexes in inhibiting TGF-\(\beta\) signaling.

In summary, the results of the current study demonstrate that NM23-H1 physically interacts with STRAP \textit{in vivo} and that NM23-H1-STRAP complex formation requires the participation of cysteine residues of both NM23-H1 and STRAP, making this complex redox status-dependent. We presented evidence of the functional significance of the association between NM23-H1 and STRAP, demonstrating a role for the STRAP-NM23-H1 complex in STRAP-induced inhibition of TGF-\(\beta\)-mediated apoptosis and growth inhibition, modulation of the biochemical activities of NM23-H1, including autophosphorylation, phosphotransferase, and NDP kinase activities (data not shown), as well as NM23-H1-induced cell proliferation. More-
Functional Association between NM23-H1 and STRAP

over, our results also point to a mechanism of NM23-H1-mediated regulation of TGF-β signaling and biological functions.

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