NM23-H1 Tumor Suppressor and Its Interacting Partner STRAP Activate p53 Function*

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p53 plays a critical role in a variety of growth inhibitory responses, including cell cycle arrest, differentiation, and apoptosis, and contributes to tumor suppression. Here we show that NM23-H1 and its binding partner STRAP (serine-threonine kinase receptor-associated protein) interact with p53 and potentiate p53 activity. Both NM23-H1 and STRAP directly interact with the central DNA binding domain within residues 113–290. The use of NM23-H1 and STRAP mutants revealed that Cys145 of NM23-H1 and Cys152 (or Cys270) of STRAP were responsible for p53 binding. Furthermore, Cys176 and Cys135 of p53 were required to bind NM23-H1 and STRAP, respectively. Ectopic expression of wild-type NM23-H1 and STRAP, but not NM23-H1(C145S) and STRAP(C152S/C270S), positively regulated p53-mediated transcription in a dose-dependent manner. Knockdown of endogenous NM23-H1 or STRAP produced an opposite trend and inhibited the p53-mediated transcription. Similarly, NM23-H1 and STRAP stimulated p53-induced apoptosis and growth inhibition, whereas the NM23-H1(C145S) and STRAP(C152S/C270S) mutants had no effect. We also demonstrated that p53 activation by NM23-H1 and STRAP was mediated by removing Mdm2, a negative regulator of p53, from the p53-Mdm2 complex. These results suggest that NM23-H1 and its interacting partner STRAP physically interact with p53 and positively regulate its functions, including p53-induced apoptosis and cell cycle arrest.

The p53 tumor suppressor plays an important role in cellular processes such as growth arrest, senescence, and apoptosis, in response to a broad array of cellular damage (1–4). It is usually maintained at low levels in cells to allow normal growth. The transcriptional activity and stability of p53 are highly regulated by post-translational mechanisms involving protein–protein interactions, phosphorylation, acetylation, and ubiquitination (5–8). An important regulator of p53 activity is Mdm2 (9, 10), which binds to the transactivation domain of p53 and inhibits its function. Several proteins thwart the p53-Mdm2 interaction by binding directly to p53 (11) or Mdm2 (12–14). For example, p14ARF (p19ARF in mice) is predominantly located within nucleoli, where it promotes p53 accumulation by binding to and sequestering Mdm2 and acts as a direct inhibitor of the E3 ubiquitin ligase activity of Mdm2 (15). However, in response to stress, p53 stabilization may also occur through Mdm2-independent mechanisms. For example, calpain (16), β-catenin (17), Sin3a (18), JNK (c-Jun N-terminal kinase) (19), NQO1 (20, 21), and MdmX (14) have been shown to stabilize p53 independently of Mdm2. These results suggest that the identification of additional interacting partners of p53 or Mdm2 will provide greater insight into the regulation of p53 activity.

NM23-H1 was initially identified as a putative metastasis suppressor on the basis of its reduced expression in certain highly metastatic cell lines and tumors, even though its enzymatic activity provided no evidence for a role as a metastasis suppressor in tumor progression (22). In humans, eight NM23 genes have been identified as follows: NM23-H1, NM23-H2, NM23-H3, NM23-H4, NM23-H5, NM23-H6, NM23-H7, and NM23-H8. These genes encode for nucleoside diphosphate kinases or for homologous isoforms (23). Extensive studies using NM23 proteins have shown that they participate in the regulation of a broad spectrum of cellular responses, including development, differentiation, proliferation, endocytosis, and apoptosis (24). The molecular mechanisms underlying the role of NM23-H1 as a metastasis suppressor, however, have so far remained unclear.

Serine-threonine kinase receptor-associated protein (STRAP), known as a transforming growth factor-β receptor-interacting protein, is a positive regulator of 3-phosphoinositide-dependent protein kinase-1 (PKD1) and negatively regulates TGF-β signaling by stabilizing the association between TGF-β receptor and Smad7 (25). Recent studies also suggest that STRAP might be involved in tumorigenesis and development (26, 27). We have recently reported that STRAP-induced inhibition of TGF-β signaling was enhanced by direct interaction with the NM23-H1 tumor suppressor, suggesting a possible

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2The abbreviations used are: Mdm2, mouse double minute 2; TGF-β, transforming growth factor-β; STRAP, serine-threonine kinase receptor-associated protein; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; GFP, green fluorescent protein; DTT, dithiothreitol; PBS, phosphate-buffered saline; siRNA, small interfering RNA; HA, hemagglutinin; 5FU, 5-fluorouracil; DBD, DNA binding domain.
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link between TGF-β and NM23-H1-mediated signaling pathways (28). TGF-β signaling has also been connected with p53-mediated signaling in which p53 cooperated with Smads for TGF-β gene responses (29). In this study, we found that the NM23-H1 tumor suppressor and its interacting partner STRAP may play an important role in the regulation of a p53-induced signaling pathway in which they act as positive regulators of p53 activity by dissociating Mdm2, a known negative regulator of p53.

MATERIALS AND METHODS

Reagents—Anti-GST, anti-NM23-H1, anti-β-actin, anti-FLAG (M2), anti-histone (H2B), and anti-STRAP antibodies were described previously (28, 30). Anti-p53, anti-p21, and anti-Bax antibodies used in immunoprecipitation and immunoblot analyses were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor-594 anti-mouse secondary antibody and Alexa Fluor-488 anti-rabbit secondary antibody were purchased from Molecular Probes (Eugene, OR). Propidium iodide, RNase A, isopropyl β-D-thiogalactopyranoside, dithiothreitol (DTT), aprotinin, and phenylmethylsulfonyl fluoride were purchased from Sigma. Polyvinylidene difluoride membranes were obtained from Millipore Corp. (Bedford, MA). [γ-32P]ATP was purchased from PerkinElmer Life Sciences.

Cell Culture and Cell Line Construction—p53/Mdm2 double null MEF cells were kindly provided by Dr. G. Lozano (University of Texas, M. D. Anderson Cancer Center). 293T, HeLa, HepG2, MCF7, H1299, HCT116, and p53/Mdm2 double null MEF cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) as described previously (28, 30). Both HCT116 cells stably expressing pcDNA3-His empty vector, NM23-H1, or STRAP and HCT116 cells stably expressing NM23-H1- or STRAP-specific siRNA were screened in the presence of 850 μg/ml G418 until control parental HT116 cells completely died. For confirmation of the bulk transfectant cell lines, culture plates containing G418-resistant colonies were trypsinized, and the presence of endogenous and exogenous NM23-H1 and STRAP proteins was examined by immunoblot analysis.

Plasmids and DNA Construction—The p53-Luc reporter plasmid was a kind gift from Dr. Y.-I. Yeom (Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea). The GST-tagged and FLAG-tagged STRAP plasmids were used for the preparation of recombinant proteins. The GST-DBD was generated after subcloning of the EcoRI/BamHI site of KS-DBD. The GST-DBD was generated after subcloning of the C176S, C238S, C242S, and C277S) were generated by the PCR method as described previously (28). The following mutant primers containing alterations in the nucleotide sequence of wild-type p53(DBD) were used for PCR as follows: C135S, sense 5′-AAGAGTTTTTCTACACTTGGCC-3′, antisense 5′-GGCCAGTTTGGAAAAACATCTTT-3′; C176S, sense 5′-ATGAGGTGCGCTCAACCCCACC-3′, antisense 5′-ATGAGGTGCGCTCAACCCCACC-3′; C238S, sense 5′-AACGTTCCTCAATGGGGCCGC-3′, antisense 5′-GGCGCCATTGGAGAAGCTT-3′; C242S, sense 5′-AACGTTCCTCAATGGGGCCGC-3′, antisense 5′-GGCGCCATTGGAGAAGCTT-3′; C277S, sense 5′-ATGAGGTGCGCTCAACCCCACC-3′, antisense 5′-TCTCCAGGTAGGGCACAAAAC-3′. The identity of all the PCR products was confirmed by nucleotide sequencing analysis on both strands (Bioneer Corp., Cheongwon, Korea).

In Vivo Binding Assay and Native PAGE—Each plasmid DNA indicated under “Results” was transiently transfected into 293T, HCT116, H1299, MCF7, or HeLa cells with WelFect-Ex™ Plus (WelGENE, Daegu, Korea), according to the manufacturer’s instructions (28, 30). In vivo binding assays were performed as described previously (25). p53 was translated in vitro using the TN reticulocyte lysate system from Promega. In vitro translated 35S-labeled p53 was incubated with unlabeled recombinant wild-type and mutant forms of NM23-H1 or STRAP in the presence of 5 mM H2O2 at room temperature for 1 h. The native PAGE (8%) was carried out as described previously (28).

siRNA Experiments—The NM23-H1 and STRAP siRNA oligonucleotides were described previously (25, 28). The sense and antisense oligonucleotides for each siRNA were annealed as described previously (25). In brief, HeLa or HCT116 cells were plated in 6-well flat-bottomed microplates (Nunc) at a concentration of 2 × 104 cells per well the day before transfection. siRNA oligonucleotides were transfected into cells using the WelFect-Ex™ Plus method. 48 h after transfection, immunoblotting was carried out to confirm the down-regulation of target proteins.

Preparation of Recombinant Proteins—Recombinant GST- or hexahistidine His6-tagged human NM23-H1 (wild-type and the C4S, C109S, and C145S mutants) and STRAP (wild-type, and the C152S, C270S, and C152S/C270S mutants) were generated by subcloning the corresponding cDNA fragments of NM23-H1 and STRAP into pGEX4T-1 (Amersham Biosciences) and pQE30 (Qiagen, Valencia, CA) as described previously (31).

Preparation of Nuclear and Cytoplasmic Fractions—HeLa cells (~4 × 105 per 60-mm dish) transfected with the indicated combinations of expression vectors (wild-type and mutant forms of NM23-H1 and STRAP) were used for the preparation of nuclear and cytoplasmic fractions that were used in immunoblot analyses, as described previously (28).

Luciferase Reporter Assay—HeLa (32, 33), HCT116, and MCF7 cells were transiently transfected according to the WelFect-Ex™ Plus method with the p53-Luc reporter plasmid,
along with the appropriate plasmids as indicated. Luciferase activity was monitored with a luciferase assay kit (Promega) following the manufacturer’s instructions as described previously (25).

**In Vivo Ubiquitination Assay**—p53-null HCT116 cells were transiently transfected with expression plasmids encoding p53, NM23-H1, STRAP, and HA-tagged ubiquitin either alone or in combination using WelFect-Ex™ Plus. 44 h after transfection, the cells were treated with 10 μg/ml MG132 (Calbiochem) for 4 h, harvested, and then washed twice with phosphate-buffered saline (PBS, pH 7.4), lysed in 200 μl of Tris-buffered saline, pH 7.4, containing 2% SDS, and incubated at 95 °C for 10 min. Tris-buffered saline containing 1% Triton X-100 (800 μl) was added to the lysate, and the mixture was incubated on ice after sonication and centrifugation at 4 °C for 30 min to remove cellular debris. Lysates (500 μg) were incubated with anti-p53 antibody. The immunoprecipitates were collected with protein A-Sepharose 4B, washed three times with Triton-buffered saline, separated by 8% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with the indicated antibodies and then developed using an ECL detection system (Amersham Biosciences).

**Apoptosis Assay**—The apoptosis assay was performed as described previously (25). HeLa cells (32) undergoing apoptosis were quantified using the GFP system. Briefly, cells grown on sterile coverslips were transfected with pEGFP, an expression vector encoding GFP, together with expression vectors as indicated. 24 h after transfection, the cells were treated with 5-fluorouracil (3.8 mM), fixed with ice-cold 100% methanol, and then washed three times with PBS. The cells were then stained with bisbenzimide (Hoechst 33258) and visualized under a fluorescence microscope. The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells.

**FACS Analysis**—HeLa cells (32) transiently expressing wild-type and mutant forms of NM23-H1 and STRAP either alone or in combination, together with transfectants expressing p53 or empty vector alone as controls, were washed with ice-cold PBS and then treated with 5-fluorouracil (3.8 mM) for 30 h or with doxorubicin (6 ng/ml) for 24 h. The trypsinized cells were washed twice with ice-cold PBS and incubated at 37 °C for 30...
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RESULTS

NM23-H1 and STRAP Physically Interact with p53 in Vivo—We have found previously that the NM23-H1 tumor suppressor physically interacts with STRAP, a TGF-β receptor interacting protein and inhibits TGF-β signaling in cells (28). p53 activation is associated with its tumor suppressor function (1), as well as with possible cross-talk between TGF-β- and p53-induced signaling (29). Based on these data, we reasoned that NM23-H1 and its interacting partner STRAP might interact with p53 in intact cells. To examine whether NM23-H1 and STRAP directly bind to p53, we performed in vivo binding assays and coimmunoprecipitation experiments using exogenous or endogenous proteins in 293T, HeLa, and HepG2 cells. The interaction of FLAG-tagged NM23-H1 and STRAP proteins with endogenous p53 was analyzed by immunoprecipitation with an anti-p53 antibody, followed by immunoblotting with an anti-FLAG antibody. Both NM23-H1 and STRAP were detected in the p53 immunoprecipitate (Fig. 1A), indicating that NM23-H1 and STRAP physically interact with p53 in cells. To confirm the interaction of NM23-H1 and STRAP with p53 in vivo, we next performed coimmunoprecipitation experiments with endogenous proteins using an anti-p53 antibody, or preimmune IgG as a control. Both NM23-H1 and STRAP were only present in anti-p53 immune complexes from all cell lines tested, including 293T, HeLa, and HepG2 cells (Fig. 1B), demonstrating that NM23-H1 and STRAP physically interact with p53 in vivo. Furthermore, to verify a specific interaction between NM23 proteins and p53, we performed in vivo binding assays using five members of NM23 gene family, NM23-H1, NM23-H2, NM23-H3, NM23-H5, and NM23-H6. Coprecipitations between NM23-H1 and p53 and between NM23-H2 and p53 were only detected when NM23 proteins were expressed, whereas NM23-H3-p53, NM23-H5-p53, and NM23-H6-p53 interactions were not detectable (Fig. 1C). This result suggests that p53 selectively binds to NM23-H1 and -H2 among NM23 proteins.

Physical Association between NM23-H1 and p53 or STRAP and p53 Is Affected by Cysteine Residues—Protein-protein interactions can be mediated by disulfide linkages as well as prototypical protein binding domains in interacting proteins (28, 31). Although STRAP contains six WD40 repeat regions that may be important for p53 binding, NM23-H1 lacks any of the typical protein binding domains that would potentially mediate its interaction with p53. In addition, it has been demonstrated recently that NM23-H1 and STRAP could be bridged through interchain disulfides (28). Based on this, we speculated that the interaction of NM23-H1 and STRAP with p53 might be mediated by cysteine residues present in each of these two proteins. 293T cells were transiently transfected with expression vectors encoding GST-tagged wild-type NM23-H1 (GST-NM23-H1(WT)) or the GST-tagged NM23-H1 substitution mutants NM23-H1(C4S), NM23-H1(C109S), or NM23-H1(C145S), together with an expression vector for FLAG-tagged wild-type p53 (FLAG-p53). GST-NM23-H1 proteins were precipitated using glutathione-Sepharose beads, and complex formation between NM23-H1 and p53 proteins was examined by immunoblot analysis using an anti-FLAG antibody (Fig. 2A, left panel). There was a dramatic decrease in

FIGURE 2. Effect of cysteine residues of NM23-H1 and STRAP on the association of NM23-H1 and STRAP with p53. A, 293T cells were transiently transfected with the appropriate expression plasmids, and GST fusion proteins were purified on glutathione-Sepharose beads (GST purification), and a complex formation between NM23-H1 and p53 (left, top panel) or STRAP and p53 (right, top panel) was determined by Western blot (WB) analysis using an anti-FLAG antibody as described in Fig. 1. B, native PAGE of NM23-H1-p53 and STRAP-p53 complexes. In vitro translated 35S-labeled p53 was prepared with the Tnt reticulocyte lysate system as described under “Materials and Methods.” 35S-Labeled p53 was incubated with unlabeled recombinant wild-type and mutant forms of NM23-H1 (left panel) or STRAP (right panel) in the presence of 5 mM H2O2, at room temperature for 1 h. For native (B) PAGE, 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. C, effect of reductants (DTT and β-mercaptoethanol (β-Me)) and H2O2 on the interaction between NM23-H1 and p53 or STRAP and p53. Cell lysates from HCT116 and MCF7 cells were treated with the indicated concentrations of H2O2, DTT, and β-mercaptoethanol on ice for 0.5–1 h and subjected to immunoprecipitation (IP) using an anti-p53 antibody, and immune complexes were analyzed for the presence of NM23-H1 or STRAP by immunoblotting using an anti-NM23-H1 antibody or an anti-STRAP antibody (top panels). The amounts of immunoprecipitated p53 and the expression level of NM23-H1 or STRAP in total cell lysates were determined by immunoblot analysis using an anti-p53 antibody (middle panels) and an anti-NM23-H1 antibody or an anti-STRAP antibody (bottom panels), respectively. WB, Western blot. D, effect of DTT on the interactions of p53 with wild-type and mutant forms of NM23-H1 or STRAP. For reduction, the binding mixture of in vitro translated 35S-labeled p53 with the recombinant wild-type and mutant forms of NM23-H1 or STRAP (each 3 μg) was reduced with 5 mM DTT (→ DTT) for 1 h at room temperature. For a supershift assay, the binding mixture was further incubated with anti-NM23-H1 antibody or anti-STRAP antibody (each 1 μg) for 1 h at room temperature. Rabbit preimmune serum was used as a negative control. E, identification of specific cysteine residues within the DBD domain of p53 for NM23-H1 and STRAP binding. 293T cells were transiently transfected with expression vectors encoding FLAG-tagged wild-type p53(3RDB) (FLAG-p53(3RDB)) or one of its substitution mutants (FLAG-C135S, FLAG-C176S, FLAG-C2385, FLAG-C2425, or FLAG-C2775), together with wild-type GST-NM23-H1 or GST-STRAP. GST-NM23-H1 and GST-STRAP were precipitated using glutathione-Sepharose beads (GST purification), and NM23-H1-p53 and STRAP-p53 complex formations were analyzed by immunoblotting using an anti-FLAG antibody (top panels).
complex formation in cells expressing NM23-H1(C145S) as compared with wild-type NM23-H1 (Fig. 2A, left top panel, 2nd versus 5th lane), whereas complex formation was not significantly influenced in cells expressing NM23-H1(C4S) and NM23-H1(C109S) (Fig. 2A, left top panel, 2nd versus 3rd and 4th lanes). These results suggest that Cys145 of NM23-H1 plays a critical role in the association of NM23-H1 with p53. We next determined whether STRAP interacts with p53 via a disulfide linkage because the cysteine residues within the WD40 repeat regions of STRAP (Cys152 and Cys270) affected complex formation between NM23-H1 and STRAP (28). 293T cells were cotransfected with expression plasmids encoding wild-type or mutant forms of GST-tagged STRAP, together with wild-type FLAG-p53. Coexpression of p53 with STRAP(C152S/270S) resulted in a remarkable decrease in complex formation between STRAP and p53 (Fig. 2A, right top panel, 2nd versus 5th lane), whereas coexpression of p53 with either STRAP(C152S) or STRAP(C270S) resulted in only a slight decrease in complex formation as compared with the expression of wild-type STRAP and p53 (Fig. 2A, right top panel, 2nd versus 3rd and 4th lanes). These results indicate that both Cys152 and Cys270 of STRAP play an important role in its association with p53. We also analyzed the association of purified, recombinant NM23-H1 or STRAP with p53 using nondenaturing PAGE. In vitro translated 35S-labeled p53 was incubated with unlabeled, recombinant NM23-H1 or STRAP. A shift in the mobility of 35S-labeled p53 was clearly evident upon incubation in the presence of wild-type NM23-H1 and STRAP, NM23-H1(C4S), NM23-H1(C109S), STRAP(C152S), and STRAP(C270S), but it was undetectable when 35S-labeled p53 was incubated in the absence of NM23-H1 or STRAP (Fig. 2B, 1st versus 2nd to 4th lanes). Additionally, the bandshift was not observed when 35S-labeled p53 was incubated with NM23-H1(C145S) or STRAP(C152S/C270S), providing additional evidence of a physical association between NM23-H1 and p53, and STRAP and p53 (Fig. 2B, 1st versus 5th lane). To determine whether the interaction between NM23-H1 and p53 or STRAP and p53 was redox-dependent, we examined complex formation in HCT116 and MCF7 cells using in vivo binding assays. The presence of the reductants DTT and β-mercaptoethanol markedly decreased the amount of endogenous NM23-H1 or STRAP that coimmunoprecipitated with endogenous p53, whereas the oxidant H2O2 had no effect (Fig. 2C, top panels). These results indicate that the in vivo association of NM23-H1 or STRAP with p53 is dependent on the redox state of the two proteins. To further confirm the redox dependence of the interaction, we also investigated the association of purified, recombinant NM23-H1 or STRAP with in vitro translated 35S-labeled p53 in the absence or presence of DTT using nondenaturing PAGE. A band shift of p53 that was observed in the absence of DTT when incubated with wild-type NM23-H1 and STRAP, NM23-H1(C4S), NM23-H1(C109S), STRAP(C152S), and STRAP(C270S) was completely abolished in the presence of DTT (Fig. 2D, left panels, 2nd to 4th versus 7th to 9th lanes). Furthermore, the binding of p53 with NM23-H1 or STRAP could be supershifted by an anti-NM23-H1 antibody or by an
anti-STRAP antibody, indicating again that NM23-H1 and STRAP are able to directly associate with p53 (Fig. 2D, right panels). To examine the specific cysteines within p53 that are required for its association with NM23-H1 or STRAP, we generated a set of five p53(DBD) substitution mutants (35–37), and we examined their ability to interact with NM23-H1 or STRAP using in vivo binding assays in 293T cells. NM23-H1 interacted with wild-type p53(DBD), C238S, and C242S but not with C176S (Fig. 2E, left top panel), indicating that the interaction of NM23-H1 with p53 is mediated via the Cys$^{176}$ of the DBD of p53 within residues 113–290. On the other hand, STRAP, unlike NM23-H1, did not interact with wild-type and all mutant forms of p53(DBD) tested, with the exception of C135S (Fig. 2E, middle and right top panels), indicating that the Cys$^{135}$ of p53 DBD is responsible for STRAP binding. Collectively, these results suggest that complex formation between NM23-H1 and p53 and between STRAP and p53 requires cysteine residues present in each of the two proteins.
Regulation of the Association between NM23-H1 and p53 or STRAP and p53 by 5FU—Previous studies have shown that the p53 expression is induced in response to various genotoxic stresses, including UV and a host of anticancer agents (38, 39). Based on this, we assessed whether genotoxic stresses, such as 5FU and doxorubicin, can influence the NM23-H1-p53 or STRAP-p53 complex formation using in vivo binding assays in HeLa cells following 5FU treatment. Upon 5FU treatment, the association between NM23-H1 and p53 or STRAP and p53 was considerably increased in cells treated with 5FU as compared with control cells untreated with 5FU (Fig. 3, A and B, left top panels, 3rd versus 4th lane). Similar results were also observed in the absence of exogenous NM23-H1 (or STRAP) and p53 (Fig. 3, A and B, right panels). These data demonstrate that the interaction between NM23-H1 and p53 or STRAP and p53 appears to be dependent on stimulation by 5FU. Given the effect of 5FU on the interaction between NM23-H1 and p53 or STRAP and p53, we conducted further assays to examine the effect of doxorubicin on the modulation of the physical association between NM23-H1 and p53 or STRAP and p53. Similarly, the interaction between NM23-H1 and p53 or STRAP and p53 appears to be increased by doxorubicin treatment (data not shown). These data suggest that NM23-H1 and its interacting partner STRAP are involved in the p53 signaling pathway.

NM23-H1 and STRAP Enhance p53-mediated Transcription—Because NM23-H1 and STRAP interacted with p53 and the association was modulated by genotoxic stresses, such as 5FU (see Figs. 1–3), we explored the possibility of a functional link between alterations in p53 activity and NM23-H1 (or STRAP). Interestingly, p53-dependent transcriptional activity was elevated by transfection with NM23-H1 or STRAP in a dose-dependent manner in the absence of p53 (Fig. 4A). Moreover, STRAP consistently showed a somewhat stronger effect in enhancing p53 activity than did NM23-H1. The stimulatory effect of NM23-H1 or STRAP on p53-mediated transcription was also observed in the presence of p53 (Fig. 4B). We performed siRNA experiments to confirm the role of NM23-H1 and STRAP in controlling p53 transcriptional activity. When
HeLa cells were transfected with siRNAs of NM23-H1 (a and b) or STRAP (334 and 515), the two different forms of NM23-H1- or STRAP-siRNA resulted in a significant reduction of p53 transcriptional activity in a dose-dependent manner (Fig. 4C, upper panels), further supporting the positive role of NM23-H1 and STRAP in the control of p53 activity. As a control, the amount of endogenous NM23-H1 and STRAP was reduced dose-dependently in cells transfected with NM23-H1- and STRAP-siRNAs as compared with the cells transfected with a control siRNA or untransfected control cells (Fig. 4C, lower panels). To analyze the role of NM23-H1 and STRAP in the regulation of p53-mediated signaling, we examined the effect of NM23-H1 or STRAP overexpression on p53-mediated gene responses using HeLa, p53-null H1299, and p53-null HCT116 cells transiently transfected with wild-type NM23-H1 and STRAP, NM23-H1(C145S), or STRAP(C152S/C270S). Overexpression of wild-type NM23-H1 or STRAP resulted in the upregulation of p53 as well as its targets, including p21 and Bax, in HeLa cells, although this did not occur with NM23-H1(C145S) or STRAP(C152S/C270S) (Fig. 4D, left panels). However, this effect was not observed in H1299 and HCT116 p53 null cells (Fig. 4D, middle and right panels). These results suggest that direct interaction with p53 plays an important role in the stimulation of p53 activity by NM23-H1 or STRAP. A similar result was also obtained with HCT116 cells stably expressing NM23-H1 (NM23-H1(OE)) or STRAP (STRAP(OE)) (Fig. 4E, left panel). Consistently, knockdown of NM23-H1 or STRAP by transfection with NM23-H1- or STRAP-specific siRNA in HCT116 cells decreased the expression of p53, p21, and Bax (Fig. 4E, middle panel). A similar result was also observed in HCT116 cells stably expressing an siRNA targeting NM23-H1 (NM23-H1(KD)) or STRAP (STRAP(KD)) (Fig. 4E, right panel). As a control, expression levels of NM23-H1 or STRAP were determined by Western blot analysis. HCT116 cells stably expressing an siRNA targeting NM23-H1 (NM23-H1(KD)) or STRAP (STRAP(KD)) displayed a significant decrease in the
amount of endogenous NM23-H1 or STRAP as compared with the control parent HCT116 cells (Fig. 4F). Together, these data suggest that NM23-H1 and its interacting partner STRAP physically associate with p53 and enhance p53 activity.

**NM23-H1 and STRAP Stimulate the Nuclear Translocation of p53**—Given that expression of NM23-H1 and STRAP enhance p53-mediated transcription in a dose-dependent manner (Fig. 4), we investigated whether NM23-H1 and STRAP modify the intracellular localization of p53. Immunofluorescence microscopy analysis was performed using HeLa cells (34) transfected with wild-type or mutant forms of NM23-H1 and STRAP in the presence of p53. Although p53 exhibited both a cytoplasmic and nuclear distribution in the absence of NM23-H1, it was detected most abundantly in the nucleus (data not shown). Expression of wild-type NM23-H1, NM23-H1(C4S), and NM23-H1(C109S) significantly increased the nuclear localization of p53 (Fig. 5A, 2nd panel, 1st 3 columns), whereas NM23-H1(C145S) mutant had no effect on the nuclear translocation of p53, similar to the distribution of p53 in the absence of NM23-H1 (Fig. 5A, 2nd panels, 4th and 5th column). To further verify whether NM23-H1 could alter the subcellular localization of p53, HeLa cells transfected with wild-type or mutant forms of NM23-H1 were separated into nuclear and cytoplasmic fractions. Each fraction was analyzed by Western...
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results using Western blot analysis with nuclear and cytoplasmic fractions of STRAP HeLa transfectants (Fig. 5E). To further examine the role of the physical interaction of NM23-H1 and STRAP with p53 in the modulation of p53 translocation, we performed confocal microscopy analysis (Fig. 5F), as well as Western blot analysis (Fig. 5G), using HeLa cells expressing wild-type NM23-H1 or STRAP along with the wild-type and mutant form of NM23-H1 or STRAP. Coexpression of wild-type NM23-H1 and STRAP showed a stronger effect on the nuclear translocation of p53 as compared with control cells expressing NM23-H1 or STRAP alone (Fig. 5G, left top panels, 2nd versus 3rd lane). In contrast, transfection of cells with STRAP(C152S/C270S) or NM23-H1(C145S) had no effect on the increase in the nuclear translocation of p53 (Fig. 5G, left, top panels, 2nd versus 4th lane), again supporting the importance of the direct interaction between NM23-H1 and p53 or STRAP and p53 in the regulation of p53 activity. A similar result was also observed for the translocation of endogenous p53 in the absence of exogenous p53 (Fig. 5G, right panels, endogenous p53). These results suggest that NM23-H1 and STRAP activate p53 function through the stimulation of the nuclear translocation of p53.

NM23-H1 and STRAP Stimulate p53 Activity by the Removal of Mdm2 from the p53-Mdm2 Complex—To address how NM23-H1 and STRAP stimulate p53 activity, we next examined the effect of NM23-H1 and STRAP on the association between p53 and Mdm2, a negative regulator of p53 (9, 10). p53 was cotransfected with Mdm2 into HeLa cells in the presence or absence of the wild-type and mutant form of NM23-H1 (Fig. 6A) or STRAP (Fig. 6B). Compared with the control cells coexpressing p53 and Mdm2 in the absence of NM23-H1 or STRAP, the expression of wild-type NM23-H1 or STRAP significantly decreased the association between p53 and Mdm2 in a dose-dependent manner (Fig. 6A and B, upper left panels, 1st versus 2nd to 4th lanes). In contrast, NM23-H1(C145S) or STRAP(C152S/C270S) that are unable to bind with p53 had no effect on the association of the proteins (Fig. 6A and B, lower left panels, 1st versus 2nd to 4th lanes). These results suggest that NM23-H1 and STRAP stimulate p53 activity by dissociating Mdm2 from the p53-Mdm2 complex. Furthermore, expression of wild-type NM23-H1 or STRAP decreased the physical interaction between the two endogenous p53 and Mdm2 proteins in intact cells (Fig. 6A and B, upper right panels, 1st versus 2nd to 4th lanes), but the expression of NM23-H1(C145S) or STRAP(C152S/C270S) did not affect the interaction between endogenous p53 and Mdm2 proteins (Fig. 6A and B, lower right panels, 1st versus 2nd to 4th lanes). To verify whether the knockdown of endogenous NM23-H1 or STRAP could contribute to the alteration of the p53-Mdm2 complex formation, HeLa cells transfected with p53 and Mdm2, together with an NM23-H1-specific siRNA or a STRAP-specific siRNA, were subjected to immunoprecipitation using an anti-Mdm2 antibody, followed by immunoblot analysis using an anti-p53 antibody. The association between p53 and Mdm2 was significantly increased in a dose-dependent manner in NM23-H1- or STRAP-knockdown cells as compared with the control cells expressing a nonspecific control siRNA (Fig. 6C, top panels, 1st versus 2nd and 3rd lanes). A similar result was also observed in

blot analysis. The accumulation of p53 in the nuclear fraction was significantly increased in cells expressing wild-type NM23-H1, NM23-H1(C45S), and NM23-H1(C109S) compared with the control cells untransfected with NM23-H1 (Fig. 5B, left top panel, 1st versus 2nd to 4th lanes), whereas the cytoplasmic accumulation of p53 was markedly decreased (Fig. 5B, left, 4th panel, 1st versus 2nd to 4th lanes). However, such a change in p53 localization was not observed in cells expressing NM23-H1(C145S) (Fig. 5B, left, top panel, 1st versus 5th lane), consistent with the above confocal microscopy data (Fig. 5A). To provide further evidence that NM23-H1 is physiologically responsible for the modulation of the intracellular localization of endogenous p53, we also performed Western blot analysis in the absence of exogenous p53 under the same conditions. A similar result was also observed for the translocation of p53 (Fig. 5B, endogenous p53). To be certain that these data are correct, we also determined whether anticancer agents (38, 39), including 5FU and doxorubicin, can influence the nuclear accumulation of p53 using Western blot analysis in HeLa cells transfected with p53 and Mdm2, together with an NM23-H1 or STRAP double mutant, like NM23-H1(C145S), had no effect on the intracellular localization of p53 (Fig. 5D, 2nd panels, 4th versus 5th lane). We also confirmed these

FIGURE 6—continued

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HCT116 cells stably expressing an siRNA targeting \textit{NM23-H1} (NM23-H1(KD)) or STRAP (STRAP(KD)) (Fig. 6D, \textit{top panels}). This was further confirmed by measuring the p53 stability using Western blot analysis in HeLa and p53/Mdm2 double null MEF cells. The addition of NM23-H1 or STRAP considerably increased the p53 stability in HeLa cells as compared with the control (vector) (Fig. 6E, \textit{upper top panel, Vector versus NM23-H1 and STRAP}). In addition, the coexpression of...
NM23-H1 with STRAP potentiated the p53 stability as compared with the effect of the expression of NM23-H1 or STRAP alone (Fig. 6E, upper top panel, NM23-H1 and STRAP versus NM23-H1/STRAP). Similar trend was also observed in p53/Mdm2 double null MEF cells transfected with the indicated expression vectors for p53, Mdm2, NM23-H1, and STRAP (Fig. 6E, lower top panel). We then investigated the role of NM23-H1 and STRAP in Mdm2-mediated p53 ubiquitination. As expected, coexpression of NM23-H1 with STRAP significantly decreased p53 ubiquitination (Fig. 6F, top panel, 2nd versus 5th lane), whereas the p53 ubiquitination was affected to a lesser extent in the presence of NM23-H1 or STRAP alone (Fig. 6F, top panel, 2nd versus 3rd and 4th lanes). Together, these data suggest that NM23-H1 and its interacting partner STRAP physically interact with p53 and stimulate the dissociation of Mdm2 from the p53-Mdm2 complex, resulting in the enhancement of p53 stability.

**NM23-H1 and STRAP Induce p53-mediated Apoptosis and Cell Cycle Arrest**—We next asked whether the expression of NM23-H1 and STRAP affects p53-mediated apoptosis and cell cycle arrest. First, we examined whether NM23-H1 has an effect on p53-mediated apoptosis using a GFP assay system (25), because NM23-H1 interacted with p53 and enhanced p53-mediated transcriptional activation (see Figs. 1–4). Apoptotic cells were scored by changes in their nuclear morphology among GFP-positive cells after inducing apoptosis by treatment with 5FU. The addition of NM23-H1 resulted in an increase in p53-mediated apoptosis that was directly proportional to the amount of NM23-H1 added (Fig. 7A, left panel, 2nd versus 3rd and 4th lanes), regardless of the fact that 5FU is an inducer of p53-mediated apoptosis (38), together with the finding that functional cooperation exists between NM23-H1 and STRAP in p53-mediated apoptosis (Fig. 7A, left panel, 3rd versus 5th and 6th lanes). However, coexpression of the STRAP (C152S/C270S) mutant, which is unable to physically associate with p53, had no effect on p53-mediated apoptosis induced by NM23-H1 (Fig. 7A, left panel, 3rd versus 7th and 8th lanes). Similarly, STRAP also led to a significant increase in p53-mediated apoptosis, and the stimulatory effect of STRAP was elevated in a dose-dependent manner in the presence of p53 (Fig. 7A, right panel, 2nd versus 3rd and 4th lanes). The coexpression of wild-type NM23-H1 with STRAP also resulted in a marked increase in p53-mediated apoptosis (Fig. 7A, right panel, 3rd versus 5th and 6th lanes), whereas coexpression of NM23-H1 (C145S) did not affect p53-mediated apoptosis induced by STRAP (Fig. 7A, right panel, 3rd versus 7th and 8th lanes). In both cases, we observed that the level of p53-mediated apoptosis was substantially higher after 5FU treatment than before 5FU treatment because of the induction of p53 activation by 5FU. To further confirm the positive roles of NM23-H1 and STRAP in p53-mediated biological functions, we next performed flow cytometry analysis in HeLa cells using 5FU to induce apoptosis (Fig. 7B, left panel) or doxorubicin (39) to induce cell cycle arrest (Fig. 7B, right panel). As a result, HeLa transfectants expressing NM23-H1 and/or STRAP considerably increased p53-mediated apoptosis as compared with the control transfectants expressing an empty vector (Vector) or p53 alone (p53) (Fig. 7B, left panel, 2nd versus 3rd to 5th lanes). These results were similar to those found for p53-mediated apoptosis as determined by the GFP system (Fig. 7A). The expression of NM23-H1 and/or STRAP also enhanced the G1 arrest as compared with the control empty vector (Vector) or p53 alone (p53) (Fig. 7B, right panel, 2nd versus 3rd to 5th lanes). To investigate further whether the modulation of p53-mediated biological functions by NM23-H1 and STRAP is dependent on its direct interaction with p53, we examined the effect of NM23-H1 or STRAP substitution mutants on p53-mediated apoptosis and cell cycle arrest. Expression of NM23-H1(C145S) or STRAP(C152S/C270S) had no effect on p53-mediated apoptosis (Fig. 7C, left panel) and cell cycle arrest (Fig. 7C, right panel). This result indicates that the direct interaction of NM23-H1 and STRAP with p53 is important for the regulation of p53 signaling. To further demonstrate the positive role of NM23-H1 and STRAP in p53 signaling, we also performed flow cytometry using HCT116 cells stably expressing NM23-H1 (NM23-H1(OE)) or STRAP (STRAP(OE)), as well as HCT116 cells stably expressing an siRNA targeting NM23-H1 (NM23-H1(KD)) or STRAP (STRAP(KD)). As expected, overexpression of NM23-H1 or STRAP increased p53-mediated apoptosis and cell cycle arrest, compared with cells transfected with an empty vector or p53 alone (Fig. 7D, left and right panels, 2nd versus 3rd and 5th lanes), indicating that the up-regulation of NM23-H1 or STRAP stimulates p53 signaling. Consistently, knockdown of NM23-H1 or STRAP decreased p53-mediated apoptosis and cell cycle arrest (Fig. 7D, left and right panels, 2nd versus 4th and 6th lanes). Taken together, these data clearly indicate that both NM23-H1 and STRAP play a critical role in the positive regulation of p53 activity.

**FIGURE 7. Effect of NM23-H1 and STRAP on p53-mediated apoptosis and cell cycle arrest**. A, effect of NM23-H1 and STRAP on p53-mediated apoptosis. HeLa cells were transiently transfected with increasing amounts of NM23-H1 (wild-type and C145S mutant) or STRAP (wild-type and C152S/C270S mutant) as indicated, together with 2 μg of p53 and 3 μg of GFP, in the presence (black bars) or absence (white bars) of 5FU. Apoptotic cell death was determined using the GFP expression system, as described previously (28, 30). GFP-positive cells were examined for the presence of apoptotic nuclei with a fluorescence microscope. The data shown are the mean ± S.D. of duplicate assays and are representative of at least four independent experiments. B, effect of NM23-H1 and STRAP on cell cycle distribution. HeLa cells (32) expressing NM23-H1 alone (NM23-H1), STRAP alone (STRAP), and NM23-H1 and STRAP (NM23-H1/STRAP), together with p23, were treated with 3.6 mm 5FU for 30 h or 6 ng/ml doxorubicin for 24 h, and sub-G1 DNA content was analyzed by FACScan. Apoptotic cells in each sample that had been untreated, or treated with 5FU, are shown as the sub-G1 population (left panel). The indicated percentages represent the G0/G1 (white bars) and G2/M (black bars) arrest in response to doxorubicin (right panel). C, effect of NM23-H1 (C145S) and STRAP (C152S/C270S) mutants on cell cycle distribution. HeLa cells expressing the indicated plasmids were treated with 5FU and doxorubicin as described above, and sub-G1, (left panel) and G0/G1 (right panel) populations were analyzed by FACScan. D, effect of overexpression and knockdown of NM23-H1 and STRAP on cell cycle distribution. HCT116 cells stably expressing pcDNA-His empty vector (p53), NM23-H1 (NM23-H1(OE)), or STRAP (STRAP(OE)), as well as HCT116 cells stably expressing NM23-H1-specific siRNA (NM23-H1(KD)) or STRAP-specific siRNA (STRAP(KD)), were transiently transfected with p53. As a negative control, pcDNA-His empty vector stable transfectants that were not transfected with p53 (Vector) are indicated. The cells were treated with 0.38 mm 5FU (left panel) or 6 ng/ml doxorubicin (right panel), and sub-G1 and G0/G1 populations were analyzed by FACScan. These experiments were independently performed at least three times with similar results.
DISCUSSION

NM23 proteins are implicated in signal transduction processes (40, 41), and their biological functions, including metastasis, proliferation, development, and differentiation, can be regulated by their ability to modulate the diverse signaling pathways that are involved in these biological activities (42). However, the mechanism by which these signals are modulated by NM23-H1 is still unknown. We have recently shown that NM23-H1 interacts with STRAP, a TGF-β receptor-interacting protein, and inhibits TGF-β signaling (28). In addition, previous reports indicate that p53 and TGF-β signaling can be linked with each other (29). Based on these results, it seems that a possible cross-talk between NM23-H1 and p53 tumor suppressors may occur in cells. This notion is further supported by the finding that NM23-H1 can associate with p53 (Figs. 1–3) and modulate the binding between p53 and its known negative regulator Mdm2 (Fig. 6). To address how NM23-H1 and its interacting partner STRAP dissociate Mdm2 from the p53-Mdm2 complex, we examined whether the binding domain(s) of p53 for NM23-H1 and STRAP and p53, resulting in the removal of Mdm2 from p53-Mdm2 complex and a stimulation of p53-mediated apoptosis and cell cycle arrest.

FIGURE 8. A model for the positive role of NM23-H1 and STRAP in p53 signaling pathway. p53 signals induce the dissociation of the NM23-H1-STRAP complex that is maintained in the basal state, which in turn promotes the association between NM23-H1 and p53 or STRAP and p53, resulting in the removal of Mdm2 from p53-Mdm2 complex and a stimulation of p53-mediated apoptosis and cell cycle arrest.

It has been shown that the central DNA binding domain of p53 functions as a protein binding domain for several proteins, including simian virus 40 (SV40) large T antigen (43), 53BP1, 53BP2, and members of the Bcl2 protein family (44–46). Here we showed that NM23-H1 and its interacting partner STRAP also interact with the DNA binding domain of p53, which contains all the cysteine residues of p53, through the Cys145 of NM23-H1 or Cys152 (or Cys270) of STRAP. In addition, 80–90% of tumor mutations occur within the central DNA binding domain of p53 and produce an alteration in its wild-type conformation (47). Based on these data, we imagine that the conformational effect that is induced by intermolecular disulfide linkages between cysteine residues of NM23-H1 (or STRAP) and p53 is likely to play an important role in the modulation of p53-Mdm2 complex formation. Consistent with this, the results of our present study show that NM23-H1(C145S) or STRAP(C152S/C270S) mutants, which are unable to bind with p53, had no effect on the association of p53 with Mdm2, whereas a considerable decrease in the association was observed in the presence of wild-type and other substitution mutants of NM23-H1 or STRAP (Fig. 6 and data not shown). Nevertheless, we cannot rule out the possibility that NM23-H1, but not STRAP, directly interferes with Mdm2 binding to p53 because NM23-H1, unlike STRAP, physically interacts with Mdm2 through its Cys145 residue (data not shown). These observations further support the notion that p53 is differentially regulated by NM23-H1 and STRAP.

We have previously shown that NM23-H1 physically interacts with STRAP through a disulfide linkage involving Cys145 of NM23-H1 (or STRAP) and p53 is likely to play an important role in the modulation of p53-Mdm2 complex formation. Consistent with this, the results of our present study show that NM23-H1(C145S) or STRAP(C152S/C270S) mutants, which are unable to bind with p53, had no effect on the association of p53 with Mdm2, whereas a considerable decrease in the association was observed in the presence of wild-type and other substitution mutants of NM23-H1 or STRAP (Fig. 6 and data not shown). Nevertheless, we cannot rule out the possibility that NM23-H1, but not STRAP, directly interferes with Mdm2 binding to p53 because NM23-H1, unlike STRAP, physically interacts with Mdm2 through its Cys145 residue (data not shown). These observations further support the notion that p53 is differentially regulated by NM23-H1 and STRAP.
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5FU treatment (data not shown), indicating that the physical association between NM23-H1 and STRAP that is maintained in the resting state contributes to the sequestration of NM23-H1 and STRAP from the pool available for activating p53 function until cells are stimulated by DNA damage and other stresses (Fig. 8). These data suggest that NM23-H1 and its interacting partner STRAP are directly involved in p53 activation through physical interaction with p53 but that binding of NM23-H1 to STRAP may prevent NM23-H1- and STRAP-induced p53 activation, probably because this blocks the direct binding of these two proteins to p53.

In summary, our results show that NM23-H1 and its interacting partner STRAP directly bind and activate p53 to induce p53-mediated signaling through the stimulation of the nuclear translocation of p53. However, at this moment we cannot rule out the possibility that NM23-H1 and STRAP prevent nuclear export of p53 by inhibiting Mdm2-mediated monoubiquitination of p53 (48). They further suggest that both NM23-H1 and STRAP may influence the nuclear export of p53 by inhibiting Mdm2-mediated monoubiquitination through physical interaction with p53 but that binding of these two proteins to p53.

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