Inhibition of SMG-8, a subunit of SMG-1 kinase, ameliorates nonsense-mediated mRNA decay-exacerbated mutant phenotypes without cytotoxicity

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Nonsense-mediated mRNA decay (NMD) is an mRNA quality surveillance mechanism that eliminates aberrant mRNAs carrying premature termination codons (PTCs). NMD inhibits the production of aberrant proteins that still retain, at least in part, wild-type function as well as dominant-negative peptides. Therefore, the selective inhibition of NMD has the potential to ameliorate NMD-exacerbated mutant phenotypes. However, we do not have sufficient knowledge of how to effectively suppress NMD with minimum cytotoxic effects. In this study, we aimed to identify NMD-related factors that can be targeted to efficiently inhibit NMD without causing significant cytotoxicity to restore the levels of truncated but partially functional proteins. We evaluated the knockdown of 15 NMD components in Ullrich congenital muscular dystrophy fibroblasts, which have a homozygous frameshift mutation causing a PTC in the collagen type VI α2 gene. Of the 15 NMD factors tested, knockdown of SMG-8 produced the best effect for restoring defective mRNA and protein levels without affecting cell growth, cell-cycle progression, or endoplasmic reticulum stress. The efficacy of SMG-8 knockdown to improve the mutant phenotype was confirmed using another cell line, from a cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy patient who carries a PTC-containing mutation in Htra serine peptidase 1. Our results suggest that SMG-8 is an appropriate target for inhibiting NMD to improve NMD-exacerbated mutant phenotypes. NMD inhibition by knockdown of SMG-8 may also be useful to induce synergy in combining the use of read-through drugs for patients with nonsense mutation-associated diseases.

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complex can interact with the downstream EJC, it remodels itself to form the decay-inducing complex (DECID) and causes the degradation of the mRNA (22–25). Upf1 is an ATP-dependent mRNA helicase, and SMG-1 is the crucial kinase required for the progression of the recognition of the PTC (26). SMG-1 kinase activity is inhibited by two subunits of the SMG-1 kinase complex, SMG-8 and SMG-9 (24, 27). SMG-8 is also involved in the recruitment of inactive SMG-1 to the SURF complex. After DECID formation, SMG-1 phosphorylates specific serine residues in Upf1, resulting in the remodeling of the mRNA surveillance complex, enabling it to discriminate between PTC-containing mRNA and normal mRNA (24). Phosphorylated Upf1 then recruits SMG-5, SMG-6, and SMG-7 and is subsequently dephosphorylated (28, 29). Subsequent phosphorylation and dephosphorylation of Upf1 are thought to be crucial steps in the initiation of NMD.

The above progress on NMD-related factors and the molecular mechanism of NMD prompted us to identify proteins that can be targeted to effectively inhibit NMD while inducing minimum cytotoxicity to rescue the phenotype of PTC-related genetic diseases. In this study, we used Ullrich disease fibroblasts (11, 14) as a model cell line of PTC-related genetic diseases exacerbated by NMD. Among 15 NMD-related factors, we identified that SMG-8 can suppress NMD efficiently with minimum cytotoxic effects. The ability of SMG-8 knockdown to efficiently suppress NMD was confirmed using another PTC-containing cell line from a patient with cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) (12, 30).

**Results**

**Effect of siRNA-Mediated Knockdown of 15 NMD-Related Factors on Expression of the Intracellular Collagen VI Triple Helix.** To identify a target for the efficient and selective inhibition of NMD, we focused on 15 proteins that have been reported to be involved in NMD. The first group of proteins contains the core NMD components Upf1 and Upf2, which are conserved from human to yeast. The second group contains components of the SMG-1 complex, SMG-1 and its regulator SMG-8. The third group contains SMG-6 and SMG-7, which are required for dephosphorylation of Upf1. The fourth group contains the release factors eRF1, eRF3a, and eRF3b, which are involved in the recognition of the termination codons. The last group contains the core EJC components eIF4A3, Y14, MAGOH/MAGOHB (MAGOH/b), MLN51, and RNPS1 (1–4). To evaluate the potency of RNAi for down-regulating NMD-related proteins, we used Ullrich disease fibroblasts, which contain a PTC-generating mutation in COL6A2 (11, 14). The nontreated cell line shows very faint staining using an antibody that selectively recognizes the collagen VI triple helix, whereas knockdown of SMG-1 or Upf1 resulted in strong staining, as reported previously (Fig. 1) (14). Silencing of the other NMD components also rescued the mutant phenotype, although the degree of the rescue varied; RNAi of eRF1, eRF3b, or RNPS1 was less effective (Fig. 1).

**Effect of siRNA Knockdown of NMD-Related Factors on Upf1 Phosphorylation and Accumulation of PTC-Containing COL6A2 mRNA.** To confirm that the above effects reflect the inhibition of NMD, we next examined the phosphorylation of Upf1 upon depletion of NMD components. As mentioned above, the process of NMD involves phosphorylation and dephosphorylation of Upf1. Although the phosphorylation status of Upf1 does not exactly parallel NMD inhibition, it provides a useful biochemical measure of NMD status. As expected, knockdown of proteins that are involved before Upf1 phosphorylation—Upf1, Upf2, SMG-1, SMG-8, eRF3a, eIF4A3, Y14, MAGOH/b, and MLN51—suppressed the phosphorylation of Upf1. On the other hand, knockdown of proteins required for Upf1 dephosphorylation, SMG-6 or SMG-7, caused an increase in the phosphorylation of Upf1 (Fig. 2 A and B). These results support the notion that all of the siRNAs tested inhibit NMD in Ullrich disease fibroblasts.

To quantitatively evaluate the efficiency of NMD inhibition, we next assessed the accumulation of PTC-containing COL6A2 mRNA by real-time PCR analysis. Knockdown of Upf1, SMG-1, SMG-8, SMG-6, SMG-7, MAGOH/b, and MLN51 caused an increase in PTC-containing COL6A2 mRNA (Fig. 2C). The functional effect on NMD was evidenced by the up-regulation of an endogenous NMD target, non–protein-coding growth arrest-specific 5 (GASS) mRNA, harboring PTCs, except SMG-7 (Fig. S1B). The result with SMG-7 might be because SMG-6 acts redundantly with the SMG-5–SMG-7 complex for GASS, as reported (31). However, knockdown of eRF1a, eRF4A3, Upf2, or Y14 did not show a clear increase in COL6A2 mRNA levels (Fig. 2D), although NMD suppression was evidenced by up-regulation of GASS mRNA harboring PTCs in eRF3a, Upf2, or Y14 knockdown cells (Fig. S1C). The apparent difference between this assay and that shown in Fig. 1 might be caused by differences in the knockdown methods: Synthetic siRNA was used in this experiment, and vector-produced shRNA was used in the experiments presented in Fig. 1 (Fig. S1D).

**Cytotoxic Effect of siRNA Knockdown of NMD-Related Factors.** Some NMD-related factors are essential for cell growth during embryogenesis (18–20); therefore, we evaluated whether knockdown of NMD-related factors causes cytotoxicity due to suppression of normal cellular functions. We first analyzed cell growth following knockdown of eight NMD-related factors: Upf1, SMG-1, SMG-8, SMG-6, SMG-7, MAGOH/b, and MLN51. The effect of the knockdown of each NMD-related factor on the growth rate of Ullrich disease fibroblasts was compared with that of epithelioid carcinoma cells (HeLa cells). The growth rates of SMG-8–knocked down cells were not significantly different from those of the nonsilencing siRNA transfectants in either Ullrich disease fibroblasts or HeLa cells (Fig. 3). In contrast, cell-growth rates of the other seven NMD component-knocked down cells were significantly suppressed at 6 d after siRNA transfection in both cell lines (Fig. 3 C and D). Interestingly, growth-rate suppression was much more severe in HeLa cells than in Ullrich disease fibroblasts for all seven proteins. Western blot analyses confirmed knockdown of the respective proteins in HeLa cells (Fig. S2).

Knockdown of either SMG-1 or Upf1 affects the cell cycle in U2O2 osteosarcoma cells (21, 22); therefore, we determined whether knockdown of NMD-related factors affected cell-cycle progression in Ullrich disease fibroblasts and HeLa cells. For both cell lines, cell-cycle distribution analysis by flow cytometry 48 h after transfection of siRNAs showed that knockdown of each of the eight NMD-related factors generated cells with a
profile similar to that of nonsilencing siRNA-treated cells. Quantitative analysis revealed that the ratio of G2/M to G0/G1 cells for each NMD component knockdown was not significantly different from that observed in cells treated with nonsilencing siRNA (Fig. S3).

We then examined whether NMD suppression causes endoplasmic reticulum (ER) stress, because it is possible that the accumulation of aberrant mRNAs by NMD suppression increases the production of unfolded proteins, which leads to ER stress (33). To test this possibility, we analyzed the expression of ER

**Fig. 2.** Effect of siRNA-mediated knockdown of 12 NMD-related factors on the suppression of NMD and accumulation of PTC-containing COL6A2 mRNA. (A) Transfection of siRNAs caused knockdown of each protein in Ullrich disease fibroblasts. Knockdown of Upf1, SMG-1, SMG-8, MAGOH/b, and MLN51 caused suppression of phosphorylation of Upf1, whereas knockdown of SMG-6 or SMG-7 resulted in hyper-phosphorylation of Upf1. The results indicate that NMD was suppressed in all of these siRNA-mediated knocked-down cells. p-Upf1, anti-phospho-Upf1 antibody. (B) Transfection of siRNAs targeting eRF3a, eIF4A3, Upf2, and Y14 caused the knockdown of each protein and suppression of phosphorylation of Upf1, indicating that NMD was suppressed in all of these knocked-down cells. (C) Effect of siRNA-mediated knockdown of eight NMD-related factors on the expression of COL6A2 mRNA (∼1.4-fold increase). The histogram depicts the levels of COL6A2 mRNA normalized to the G6PDH mRNA band represented as approximate fold-increase over nonsilencing siRNA treatment. Values shown are means ± SEM of four separate experiments. (D) Effect of siRNA-mediated knockdown of four NMD-related factors on the expression of COL6A2 mRNA. Knockdown of eRF3a, eIF4A3, Upf2, and Y14 did not show an ∼1.4-fold increase in COL6A2 mRNA. Values shown are means ± SEM of four separate experiments.

**Fig. 3.** Effect of knockdown of eight NMD-related factors on cell growth in Ullrich disease fibroblasts (A and C) and HeLa cells (B and D). Fb, fibroblasts. Values shown are means ± SD of six wells. (A and B) Cell-growth rate curve. The growth rate of SMG-8–knocked down cells was the same as nonsilencing siRNA transfectants in both Ullrich fibroblasts and HeLa cells. (C and D) Histogram of the growth rate on the sixth day after transfection with siRNAs. Cell-growth rates of Upf1–, SMG-1–, SMG-7–, MAGOH/b–, and MLN51–knocked down cells were more severely suppressed 6 d after knockdown in HeLa cells than in Ullrich disease fibroblasts.
stress-related proteins, including phosphorylated eIF2α, activating transcription factor 4 (ATF4), and ER chaperone glucose-regulated protein of 78 kDa (GRP78) using NMD-suppressed Ullrich disease fibroblasts and HeLa cells. However, neither of these cell lines showed signs of ER stress responses; ER stress-related proteins were not up-regulated under NMD suppression, demonstrating that NMD suppression via transfection of siRNAs targeting NMD-related factors does not by itself induce ER stress in these cell lines (Fig. S4).

**Effect of SMG-8 Knockdown on the Accumulation of ECM Collagen VI Protein.** The above experiments suggested that SMG-8 is the most appropriate target for suppressing NMD without causing cytotoxicity. To further examine rescue from the mutant phenotype, we evaluated the function of accumulated aberrant collagen VI protein by SMG-8 knockdown. Subconfluent cultures were cultured for 6 d in the presence of 0.25 mM ascorbic acid to induce the secretion of triple-helical collagen VI, as performed previously (14). Immunocytochemistry using an anti-collagen VI antibody revealed much dot- or thread-like staining of collagen VI in the ECM of SMG-8-knockdown cells compared with nonsilencing siRNA transfectants (Fig. 4). The results suggest that the accumulated aberrant collagen VI protein is secreted and integrated into the ECM in Ullrich disease fibroblasts (Fig. S1A). Taken together, these results indicate that depletion of SMG-8 in Ullrich disease fibroblasts rescues the NMD-exacerbated mutant phenotype without causing cytotoxicity.

**Effect of NMD Suppression on CARASIL Fibroblasts.** To test the general applicability of SMG-8 as a target for selective NMD inhibition, we next examined whether the depletion of SMG-8 rescues the expression of aberrant protein in other mutant cell types. For this purpose, we analyzed another PTC-containing cell line from a patient with CARASIL, which is characterized by cerebral small-vessel arteriopathy with subcortical infarcts, alopecia, and spondylosis (12, 30). Gene analysis of the case revealed a nonsense mutation with a PTC in the human HtrA serine peptidase 1 (HTRA1) gene (12, 30). HTRA1 possesses dual activities as chaperones and serine proteases, and represses TGF-β family signaling by binding and cleaving the prodomain of pro-TGF-β1 in the ER, resulting in a reduction of mature TGF-β1 (12). In this case, mutant PTC-mRNA is expected to be degraded by NMD, resulting in an enhancement of TGF-β1 secretion and abnormal TGF-β1 autocrine effects. Thus, NMD inhibition might restore expression of HTRA1 mRNA and protein and cleave the prodomain of pro-TGF-β1, resulting in the suppression of TGF-β1 signaling (Fig. S5A).

To test this possibility, we first assessed the effect of NMD suppression by the knockdown of Upf1, SMG-1, SMG-8, or SMG-7 on the accumulation of PTC-containing HTRA1 mRNA. Western blot analysis revealed that transfection of siRNA targeting Upf1, SMG-1, SMG-8, or SMG-7 into CARASIL fibroblasts knocked down these proteins. NMD inhibition was estimated by the decrease (in the case of Upf1, SMG-1, and SMG-8 knockdown) or increase (in the case of SMG-7 knockdown) in Upf1 (Fig. S4). Real-time PCR analysis showed that knockdown of Upf1, SMG-1, SMG-8, or SMG-7 up-regulated HTRA1 mRNA levels (1.6- to 2.3-fold increase) (Fig. S5B). Up-regulation of HTRA1 protein was visualized by immunostaining with an anti-HTRA1 antibody (Fig. S5C).

To evaluate the function of accumulated aberrant HTRA1 protein in CARASIL fibroblasts, we examined the effect of HTRA1 protein on the TGF-β1 pathway (Fig. S5). Knockdown of SMG-1, SMG-8, or SMG-7, but not Upf1, resulted in accumulation of cleaved inactivated products from pro–TGF-β1, suggesting a decrease in the secretion of mature TGF-β1 (Fig. 6A). This was supported by a decrease in TGF-β1 signaling, as measured by the phosphorylation status of Smad2 or Smad3 (Fig. 6B). The functional effect on NMD was evidenced by the up-regulation of an endogenous NMD target GAS5 mRNA harboring PTCs except SMG-7 (Fig. S5B). In addition, the decreasing of TGF-β1 signaling caused by SMG-8 knockdown was canceled by the HTRA1 co-knockdown. The results provide evidence to prove that NMD inhibition by SMG-8 knockdown does restore HTRA1 (Fig. S5C and D). Taken together, these results suggest that accumulation of aberrant HTRA1 protein by knockdown of SMG-1, SMG-8, or SMG-7 rescues the defect in CARASIL fibroblasts. On the other hand, Upf1 knockdown did not alter pro–TGF-β1 cleavage or TGF-β1 signaling. This is probably due to an uncharacterized Upf1-specific function.

Because knockdown of NMD components, except for SMG-8, had a weak cytotoxic effect in Ullrich disease fibroblasts (Fig. 3), we investigated the cell-growth rate of CARASIL fibroblasts under NMD suppression. As shown in Fig. 6 C and D, cell-growth rates of Upf1-, SMG-1–, and SMG-7–knocked down cells were significantly suppressed at 6 d after the knockdown. In contrast, the growth rate of SMG-8–knocked down cells was not significantly different from that of nonsilencing siRNA transfectants. This result further supports the notion that SMG-8 is a promising target for NMD inhibition to improve NMD-exacerbated mutant phenotypes without affecting cell growth in PTC-containing CARASIL fibroblasts (Fig. S5A).

**Discussion**

In this study using two different PTC-containing mutant cell lines, Ullrich disease fibroblasts and CARASIL fibroblasts, we identified SMG-8 as the best target among 15 NMD-related factors examined for NMD inhibition to improve NMD-exacerbated mutant phenotypes without causing cytotoxicity. The two cell lines carried different PTC-generating mutations: a homozygous frameshift mutation in COL6A2 in Ullrich disease fibroblasts, and a nonsense mutation in HTRA1 in CARASIL fibroblasts. The basal level of mRNA abundance in both fibroblasts was around 20% of the wild-type allele (Fig. S6). We demonstrated that the defects in cellular function due to these different PTC-generating mutations were rescued in part by up-regulation of mutated mRNAs and proteins caused by appropriate NMD suppression following knockdown of SMG-8.

Importantly, SMG-8 knockdown did not cause a defect in cell growth in the fibroblasts of either patient or in HeLa cells, in contrast to the other seven NMD factors tested (Figs. 3 and 6C and D). Our previous study demonstrated a milder effect of SMG-8 knockdown on NMD inhibition in HeLa TetOff cells (24). The reason why SMG-8 knockdown does not induce suppression of cell growth may be due to a more limited inhibition of NMD compared with the other seven NMD-related factors, although SMG-8 knockdown-induced NMD suppression might vary among cells and tissues. In addition, we observed no effects of SMG-8 knockdown on cell-cycle progression or induction of ER stress in Ullrich disease fibroblasts or HeLa cells (Figs. S3 and S4). Furthermore, we have generated a stable SMG-8–knocked down human skeletal muscle cell line (HSKMC) that shows cell growth with a remarkable decrease in phospho-Upf1, which suggested NMD inactivation (Fig. S7A). Up-regulation of ER stress-related proteins was not observed in this cell line even after 2 mo of culture (Fig. S7B). From these results, we concluded that SMG-8 is a promising target for efficiently inhibiting
NMD to improve NMD-exacerbated mutant phenotypes without affecting essential physiological cell functions.

NMD inhibition without causing cytotoxicity may rescue many other patients with PTC-related diseases where the mutant proteins retain some residual activity and partially normal protein functions. Such a strategy might be applicable to some Duchenne muscular dystrophy patients with mutations near the 3′ end of the dystrophin gene (8) and to type 2 long-QT syndrome patients (34). Furthermore, increases in mRNA levels by SMG-8 knockdown-induced NMD inhibition may bring a synergistic effect on read-through efficiency driven by drugs causing translational read-through (17).

The severity of cell-growth suppression by NMD inhibition appears to differ between noncancer and cancer cells (Figs. 3 and 6). It is possible that the effects of NMD suppression on cellular metabolism are different between noncancer and cancer cells. Although we did not detect any effect of NMD suppression on the cell cycle or ER stress in HeLa cells (Figs. S2 and S3), it is predicted that survival of some cancer cells is aided by NMD (35, 36). These mutations are expected to affect not only nonessential genes but also essential genes required for cancer cell survival. NMD can eliminate these aberrant mRNAs with PTCs and can maintain cancer cell homeostasis (36). Remarkable suppression of cell growth in NMD-inhibited HeLa cells (Fig. 3D) may be due to the disturbance of cell homeostasis. In addition, unnatural polypeptides synthesized from mutated mRNAs with PTCs may act as antigens on the surface of cancer cells, which could potentially activate the antitumor immune system if they are not eliminated by NMD (35, 37). Based on these observations, NMD repression may be a potential therapeutic target for certain cancers.
Occurrence of NMD usually depends on the location of PTCs. Normal termination codons or PTCs in the last exon do not efficiently elicit NMD, because at least one intron downstream of a PTC is required to strongly promote NMD in mammalian cells (1–4). In general it is thought that an aberrant mRNA carrying a PTC that is located more than ~55 nt upstream of the last exon–exon junction is targeted by NMD (1–4). However, there have been many clinical cases where the location of PTC mutations is not consistent with the above finding (1–4). Kerr et al. reported muscular dystrophy cases carrying the same PTC mutation in the dystrophin gene, but with different phenotypes: severe Duchenne muscular dystrophy or milder Becker muscular dystrophy (8). Furthermore, it has been reported that NMD activity differs among tissues (38, 39) as well as cell lines (40, 41), whereas the stabilities of PTC-mRNAs encoding aberrant proteins are allele-dependent in human cultured cells (11, 26, 42). These findings strongly indicate that there are additional factors that affect the phenotypes of PTC-generating mutations. From our findings that knockdown of 15 NMD factors showed different effects on NMD activity, rescue of mutant mRNAs and proteins, and cellular function defects, we suggest that the additional factors may include polymorphisms in genes encoding NMD-related factors or their regulators, or reflect their different expression levels in different cellular contexts.

In conclusion, we identified SMG-8 as a promising target for efficient and selective NMD inhibition to rescue partially functional truncated peptides in PTC-related, NMD-exacerbated diseases. There may also be synergy in combining NMD inhibition by knockdown of SMG-8 and the use of read-through drugs for patients with nonsense mutation-associated diseases.

**Materials and Methods**

Patients and cell culture were described in SI Materials and Methods. SI Materials and Methods also provides details for siRNA preparation and transfection, real-time quantitative PCR, Western blot analysis, and antibodies. See SI Materials and Methods for analyses of immunocytochemistry, flow cytometry for cell cycle, and cell growth.

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Supporting Information

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SI Materials and Methods

**Patients and Cell Culture.** Ullrich’s disease fibroblasts were described previously (1, 2), and CARASIL fibroblasts were obtained from a patient with CARASIL carrying the R370X mutation in HTRA1 (3). Informed consent was obtained by each patient. HeLa cells were obtained from American Type Culture Collection (ATCC). Human skeletal muscle cells (HSKMC) were purchased from Cell Applications. Cells were cultured as described previously (1). For HTRA1, TGF-β1, phospho-smad 2, and smad2 analyses, cells were cultured in serum-free Cosmedium 001 (Cosmo Bio) supplemented with 0.5 mM ascorbic acid for an additional 30 h prior to analysis. The experiment was approved by National Institute for Minamata Disease.

**siRNA Preparation and Transfection.** The following siRNA target sequences were used: Upf1, GATGCACTTGCCTCATAT; SMG-1, GTGTATHTGCGAAGAAGATA; SMG-8, siGENOME duplex D-016480-02 (Dharmacon); SMG-6, GGGTCACATGTGCTGCTGAACT; SMG-7, CAGCACAGCTCAACAGCCA; eRF3a, GAGGGAAATCTTGGTGTT; eIF4A3, AGCAATACGAGCTAGAT; Y14, HP GenomeWide siRNA 1027440 (Qiagen); MAGOHb, GATGTCGCAACGGCAGACCA; and MLN51, CATTGCTTCAGCTCATAAT. MAGOH is encoded by 2 genes, MAGOH and MAGOHb, and knockdown of only MAGOH could not induce NMD suppression; therefore, we designed an siRNA targeting both MAGOH and MAGOHb genes (Fig. S8). Annealing for duplex siRNA formation was performed using a licensed method (Qiagen). HTRA1 siRNA was obtained from Thermo Scientific. NS siRNA, an all Star Negative Control siRNA, which is guaranteed to show no knockdown activity, was obtained from Qiagen.

For immunostaining intercellular collagen VI protein, we used a vector-produced siRNA system because it gives much stronger staining of intercellular collagen VI than synthetic siRNA (1). Plasmid-expressed shRNAs targeting SMG-1 [siRNA sequence: GTGATATGTGCCAAGAATA], Upf2 [siRNA sequence: CACAGGCTCTTACAGATC], SMG-8 [siRNA sequence: siGENOME duplex D-016480-02 (Dharmacon)], SMG-6 [siRNA sequence: GGGTCACATGTGCTGCTGAACT], SMG-7 [siRNA sequence: CACAGCTCTACAGCCA], eRF3a [siRNA sequence: GAGGGAAATCTTGGTGTT], eIF4A3 [siRNA sequence: GTGTCGCAACGGGCAACCA], Y14 [siRNA sequence: HP GenomeWide siRNA 1027440 (Qiagen)], MAGOH [siRNA sequence: CATTGCTTCAGCTCATAAT], or RNPS1 [siRNA sequence: GGAAGACCGTGAAAGAA] were constructed in the vector psiLentGene-Puro (Promega). Plasmid-expressed shRNA targeting Upf1 was described previously (4). Plasmid-expressed shRNA targeting hRenilla luciferase (psiLentGene-puro-hRenilla; GGCCTCTCAGTCCTCCTAC) was used as control. HiPerFect Transfection Reagent (QIAGEN) or Lipofectamin RNAiMAX (Invitrogen) was used for the transfection of synthetic siRNAs, and Lipofectamine 2000 (Life Technologies) for plasmid-constructed siRNAs. Cells were analyzed 48 h after transfection, and results were obtained from more than three independent experiments.

For the production of stable cell lines, the HSKMC cell line was transfected with Nucleofector (Amaza) using the Human Dermal Fibroblast Nucleofector Kit (Amaza) according to the optimized protocol. Stable cell lines were selected with puromycin (Sigma).

**Immunocytochemistry.** Fibroblasts were cultured on gelatin-coated coverslips and prepared as described previously (1). Intracellular and ECM collagen VI were stained using a mouse monoclonal anti-collagen VI antibody recognizing the stereoscopic triple-helical stalk structure (Daichii Fine Chemical) as described previously (1). HTRA1 protein was stained using a mouse monoclonal anti-HTRA1 antibody (R & D). The samples were examined with a Nikon TE800 fluorescence microscope. The immunofluorescence experiments shown in the separate panels in each figure were performed simultaneously under the same conditions.

**Real-Time Quantitative PCR.** Total RNA was extracted 52 h after siRNA transfection and first-strand cDNA was prepared as described previously (5). Quantitative real-time PCR was performed using a LightCycler DX 400 System (Roche). COL6A2 and HTRA1 mRNAs were amplified as described previously (1, 3). Primer sets for GASS (GenBank accession number: NR_002578) was as follows: 5′-GGATGCACTTGTGCTGCTGATA-3′ (nucleotides 45–66) and 5′-TGTGTGCAATGCGTGTAGTAG-3′ (nucleotides 134–156). Values are shown as ± SE of more than four separate experiments.

**Western Blot analysis and Antibodies.** Cell lysates were prepared as described previously (1) and separated by 5.5% (wt/vol) SDS/PAGE or by 10% (wt/vol) SDS/PAGE in the presence of DTT and transferred to nitrocellulose membranes (Bio-Rad). The membranes were then incubated with the indicated antibodies: monoclonal mouse anti-Upf1 (5C3) (6), monoclonal mouse anti- phospho-Upf1 (7H1) (7), polyclonal rabbit anti-SMG-1 (Bethyl), polyclonal rabbit anti-SMG-8, polyclonal rabbit anti-Upf1 (8) (Bethyl), polyclonal rabbit anti-SMG-6 (7), polyclonal rabbit anti-Magoh (Abcam), polyclonal rabbit anti-eRF3a (Abcam), monoclonal mouse anti-eRF4A3 (Abcam), monoclonal mouse anti-Y14 (Abcam), polyclonal rabbit anti-CASC3 (MLN51) (Sigma), mouse monoclonal anti-a-tubulin (Santa Cruz), polyclonal rabbit anti-Elf2α (Cell Signaling), polyclonal rabbit anti-phospho-Elf2α (Cell Signaling), polyclonal rabbit anti-ATF4 (Santa Cruz), polyclonal rabbit anti-GRF78 (Cell Signaling), polyclonal goat anti-LAP-TGF-β1 (R & D), polyclonal rabbit anti-GAPDH (Cell Signaling), polyclonal rabbit anti-phospho-Smad2 (Cell Signaling), polyclonal rabbit anti-smad2 (Cell Signaling), or polyclonal rabbit anti-smad3 (Cell Signaling). Proteins were detected as described previously (1) or with EzWestLumi (ATTO).

**Flow Cytometry for Cell Cycle Analysis.** siRNA-transfected fibroblasts (10⁶ cells/35-mm dish) cultured for 51 h in growth medium were harvested with 0.2% Triton X-100/ Ca²⁺- and Mg²⁺- free PBS that was pre-warmed at 37 °C. Cells were then prepared for cell cycle distribution analysis. Briefly, the samples were incubated with RNase (final concentration: 0.5%) (Life Technologies) and propidium iodide (final concentration: 50 µg/mL) (BD Biosciences, USA), and then analyzed on a FACS analyzer (BD Biosciences, USA). Three plates were examined for each sample.

**Cell Growth Analysis.** siRNA-transfected fibroblasts were re-plated at a density of 10³ cells/well on a 96-well plate 24 h after transfection with synthetic siRNAs. Viability was determined by using a Cell Counting Kit (DOJINDO, Kumamoto, Japan).

**Fig. S1.** (A) Effect of NMD suppression on a homozygous frameshift mutation causing a premature termination codon (PTC) in COL6A2 in Ullrich disease fibroblasts. (B and C) Effect of siRNA targeting NMD-related factors on an endogenous NMD target, growth arrest-specific 5 (GASS) mRNA, harboring PTCs in Ullrich disease fibroblasts. (D) Immunocytochemistry for COLVI using synthetic siRNA (eRF3a, eIF4A3, Upf2, Y14) transfectants. Weak staining of triple-helical COLVI was observed compared with that of SMG-1-, Upf1-, or SMG-8-knocked down cells.
Fig. S2. Western blot analyses of NMD-related factors and NMD activity in HeLa cells transfected with siRNA targeting six NMD-related factors. Cells were harvested 48 h after transfection with the respective siRNA. Cells transfected with siRNA targeting MAGOH/b could not be analyzed because of cell disruption.
Fig. S3. Effect of NMD-related factor knockdown on cell-cycle progression in Ullrich disease fibroblasts (A) and HeLa cells (B). Cell-cycle distribution was analyzed by flow cytometry. Cell-cycle profiles of nonsilencing siRNA transfectants, Upf1-, SMG-1–, and SMG-8–knocked down cells are presented. The table shows the G2/M:G0/G1 ratio of cells for each NMD-related factor knockdown. Cell-cycle distribution was not affected by knockdown of any of the NMD-related factors.
Fig. S4. Western blot analyses of endoplasmic reticulum (ER) stress-related proteins in NMD-related factor-knocked down Ullrich disease fibroblasts (A) and HeLa cells (B). Neither cell line showed up-regulation of ER stress-related proteins. p-eIF2α, anti-phospho-eIF2α antibody.
Fig. S5. (A) Effect of NMD suppression on a nonsense mutation causing a PTC in HTRA1 in cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) fibroblasts. (B) Effect of siRNA targeting NMD-related factors on an endogenous NMD target, GAS5 mRNA, harboring PTCs in CARASIL fibroblasts. Values shown are means ± SEM of four separate experiments. (C and D) Rescue experiment of the expression of siRNA targeted to HTRA1 in SMG-8–knocked down cells. (C) Effect of siRNA targeted to HTRA1 in CARASIL fibroblasts. Values shown are means ± SEM of four separate experiments. (D) Coexpression of siRNA targeted to SMG-8 and HTRA1 resulted in the suppression of the phosphorylation status of Smad2 or Smad3. Fb, fibroblasts.
Fig. S6. Basal level of mRNA abundance in NMD-suppressed Ullrich disease fibroblasts (A) and CARASIL fibroblasts (B). Values shown are means ± SEM of four separate experiments.

Fig. S7. Effect of prolonged knockdown of SMG-8 on the induction of ER stress. (A) Stable cell line generated by transfection of plasmid-expressed shRNA targeting SMG-8 into a human skeletal muscle cell line knocked down SMG-8 protein expression and resulted in NMD suppression (decrease in Upf1 phosphorylation). (B) Stable SMG-8–knocked down cells did not demonstrate up-regulation of ER stress-related proteins (eIF2α phosphorylation, ATF4, GRP78).
Fig. S8. Sequence comparison of human MAGOH and MAGOHB with Mago-nashi proteins of other species. (A) Alignment of MAGOH and MAGOHB cDNAs. A single siRNA target sequence for both genes is indicated by a yellow box. (B) Alignment of Mago-nashi proteins.