Proteomic Analysis Reveals Branch-specific Regulation of the Unfolded Protein Response by Nonsense-mediated mRNA Decay*

Jana Sieber‡§, Christian Hauer‡§¶, Madhuri Bhuvanagiri‡§, Stefan Leicht¶, Jeroen Krijgsveldd¶, Gabriele Neu-Yilik‡§, Matthias W. Hentze¶¶, and Andreas E. Kulozik‡§

Nonsense-mediated mRNA decay (NMD) has originally been described as a surveillance mechanism to inhibit the expression of mRNAs with truncated open reading frames (ORFs) and to contribute to the fidelity of gene expression. It is now recognized that NMD also controls the expression of physiological genes with “intact” mRNA. Stress can decrease NMD efficiency and thus increase the mRNA levels of physiological NMD targets. As stress can also inhibit translation, the net outcome for shaping the proteome is difficult to predict. We have thus analyzed de novo protein synthesis in response to NMD inhibition or the induction of mild endoplasmic reticulum (ER) stress by treatment of cells with the reducing agent dithiotreitol (DTT). For this purpose, we combined pulsed azidohomoalanine (AHA) and stable isotope labeling by amino acids in cell culture (SILAC). Labeled proteins were purified by click chemistry-based covalent coupling to agarose beads, trypsinized, fractionated, and analyzed by mass spectrometry (MS). We find that mild ER stress up-regulates the de novo synthesis of components of all three branches of the unfolded protein response (PERK, IRE1 and ATF6) without increasing eIF2α phosphorylation or impairing of protein translation. In contrast, inhibition of NMD induces de novo protein synthesis of downstream targets of the PERK and IRE1 pathways, whereas we could not detect regulation of ATF6-responsive genes. These data thus support a model that implicates a positive feedback loop of ER stress inhibiting NMD efficiency which further promotes the ER stress response in a branch-specific manner. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.054056, 1584–1597, 2016.

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Nonsense-mediated mRNA decay (NMD) is the major surveillance mechanism of the cell to recognize and degrade faulty mRNA molecules. NMD is translation- and splicing-dependent, and activated by a premature termination codon (PTC) that can be introduced into mRNAs by DNA mutations or RNA processing defects (1, 2). The translation of such transcripts may otherwise cause the biosynthesis of truncated and potentially harmful proteins in the cell is thus limited (3). PTCs are recognized during translational termination by the “SURF” complex. This complex consists of the essential NMD factor UPF1, its kinase SMG1 as well as the eukaryotic release factors eRF1 and eRF3 and interacts with the exon-junction complex (EJC). If the EJC resides at least 50 nucleotides downstream of the PTC (4), phosphorylation of UPF1 by SMG1 will trigger a sequence of events that will ultimately lead to fast decay of the mRNA molecule (5). In addition, NMD can also be activated by a very long 3’UTR, even in the absence of 3’UTR introns and downstream EJCs (6, 7).

Previous studies have shown that many physiological mRNAs are regulated by NMD in addition to PTC-containing transcripts (8–14). Interestingly, many of these genes are involved in stress response pathways, such as amino acid homeostasis (9) and the integrated stress response following hypoxia or endoplasmic reticulum (ER) stress (15–19). In addition to its surveillance function, NMD might therefore play a regulatory role in these signaling pathways.

When misfolded proteins accumulate in the ER they are bound by the molecular chaperone bone-inducing protein...
used, and a linear model to the data, an empirical Bayes moderated reversed in biological duplicates. Statistical analyses were performed for both treatments and enabled better comparability. SILAC labels were control siRNA-transfected plates could serve as negative control for activated in a similar manner as PERK, the translation of the transcription factor ATF4 (21). IRE1 is (20). In addition, phosphorylated eIF2α as a consequence, reduces the global protein translation rate of stress and how NMD may thus contribute to the less efficient protein translation of a stressed cell.

It is an important question, therefore, whether regulated NMD plays a major role in shaping the proteome under conditions of stress and how NMD may thus contribute to the stress response. Although it has been shown that some NMD target mRNAs can be translated into functional proteins (25–28), global data addressing the translation of NMD target mRNAs are still missing. Here, we have monitored the expression of global de novo protein synthesis following NMD inhibition and stress induction to determine the contribution of NMD on the cellular stress response.

MATERIALS AND METHODS

Experimental Design and Statistical Rationale—The pulsed azido-homoalanine (AHA) and stable isotope labeling by amino acids in cell culture (SILAC) experiments were carried out in biological duplicates. The same negative control cells were used for the normalization of the UPF1 depletion and the ER stress induction. HeLa cells were cultured as separate batches for 4 passages until the experiment was performed. Each batch served as biological duplicate. For the experiment, two plates of each batch were transfected with control siRNA and one plate was transfected with siRNA against UPF1. During the labeling time, the cells of one of the two control siRNA-transfected plates were incubated with (dithiothreitol) DTT, the other one with the equivalent volume of solvent (H2O). In this way, the H2O-incubated, control siRNA-transfected plates could serve as negative control for both treatments and enabled better comparability. SILAC labels were reversed in biological duplicates. Statistical analyses were performed using the Limma package in R/Bioconductor (29, 30). After fitting a linear model to the data, an empirical Bayes moderated t test was used, and p values were adjusted for multiple testing with the Benjamini-Hochberg method. For all other experiments two to six independent biological replicates were assayed and statistical analysis was performed as indicated in the figure legends.

Cell culture, Transfections, and Treatments—Stable HeLa cells constitutively expressing the NMD reporter construct Renilla-HBB NS39 (25) from a single copy integration site were cultured in complete DMEM ( Gibco/Thermo Fisher Scientific, Carlsbad, CA), supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin (P/S) with 150 µg/ml Hygromycin B to maintain the stably transfected NMD reporter. Hygromycin B was omitted in experiments. For DTT treatment, cells were seeded at 2 × 10^6 cells/well into six-well plates. For analysis of the ATF4 and ATF6 response elements 1.3 × 10^6 HeLa cells/well were seeded in 6-cm dishes and transfected with 40 pmol of either siUPF1 or AllStars control siRNA (Qiagen, Hilden, Germany). Two days later cells were again transfected with 40 pmol siRNA and with 650 ng of the reporter plasmid pGL4.39[lu2Cp/ATF6-RE/Hygro] or pGL4[lu2Cp/ATF4-RE/Hygro] (Promega, Madison, WI), 100 ng of pCI-neo-Renilla as normalization control and 100 ng of pCI-neo-YFP as transfection control using jetPRIME™ (Polyplus, Illkirch, France) according to the manufacturer’s protocol. The next day, cells were treated with 1.5 mM DTT or H2O for 6 h. For the pulsed SILAC experiment cells were divided into two batches at passage 6 (P6) and kept separately for the remaining experiment as biological duplicates. At P10 1.2 × 10^7 cells were seeded into 15-cm dishes. The next day cells were transfected with 300 pmol of either siUPF1 or siLuc (31) and 46 µl Oligofectamine (Life Technology Invitrogen/Thermo Fisher Scientific, Carlsbad, CA) in 15 ml Opti-MEM ( Gibco/Thermo Fisher Scientific, Carlsbad, CA) free of serum and antibiotics. After 4 h 15 ml DMEM containing 20% FCS and 2% P/S was added. The next day, cells from one 15 cm dish were split into 10-cm dishes at a density of 2 × 10^6 cells/dish and kept in DMEM containing 10% FCS and 1% P/S.

Pulse Labeling of Cells with AHA and SILAC—Two days after siRNA transfection cells were washed twice with PBS and pre-incubated in DMEM free of methionine, arginine and lysine, and containing 10% dialyzed FCS (Sigma, Munich, Germany) and 1% l-glutamine for 30 min to deplete them of methionine, arginine and lysine. Then the medium was replaced by SILAC medium containing 0.1 mm l-AHA (AnaSpec, Inc., Fremont, CA) and either 84 µg/ml [13C6,15N2]l-arginine and 146 µg/ml [13C6,15N4]l-lysine (heavy) or 84 µg/ml [4,4,5,5-D4]l-lysine (intermediate) (Cambridge Isotope Laboratories, Inc., Tewksbury, MA), and 1.5 mm DTT or H2O. Labels were reversed in biological duplicates. After 6 h cells were washed in PBS, put on ice and scraped into ice-cold PBS. 20% of the cells were lysed in RLT buffer (Qiagen RNeasy Mini) for RNA extraction and stored at −80 °C. 2% of the cells were taken for measurements of luciferase activity. These and the remaining cells were used for the pulsed SILAC (pSILAC) analysis were frozen separately as dry cell pellets.

Enrichment of Newly Synthesized Proteins and On-bead Digestion—Newly synthesized proteins were enriched using the Click-IT Protein Enrichment Kit (Invitrogen C10416) applying the vendor’s protocol with slight modifications as described before (32). Briefly, after washing 100 µl agarose resin slurry with 900 µl water, the protein extract was diluted in 250 µl urea buffer, and catalyst solution was incubated for 16–20 h at room temperature. The resin was washed with 900 µl water, 0.5 ml SDS buffer and 0.5 µl 1 M DTT at 70 °C for 15 min. After removing the supernatant 3.7 mg iodoacetamide (Bio-Rad) was added and incubated for 30 min. Then the resin was washed with 20 ml of SDS buffer, 20 ml of 8 M urea in 100 mm Tris, pH 8, 20 ml of 20% isopropanol and 20 ml of 20% acetonitrile, followed by proteolysis by 0.5 µg trypsin (Promega) in digestion buffer (100 mm Tris, pH 8, 2 ml 0.1% CaCl2 and 10% acetonitrile).

Peptide Fractionation by High-pH Reversed Phase Chromatography—Peptide fractionation was performed via High pH reversed-phase chromatography on an Agilent 1260 HPLC system using an XBridge BEH C18 column (1 × 100 mm, 3.5 µm, 130 Å, Waters,
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Milford, MA). Elution was performed at a flow rate of 0.1 ml/min using a gradient of mobile phase A (20 mM ammonium formate, pH 10) and B (acetonitrile), from 1% to 35.7% over 61 min. Fractions were collected every 2 min across the entire gradient length and concatenated into 10 final samples as discussed previously (33). Fractions were dried in a SpeedVac centrifuge and reconstituted in 0.1% formic acid prior to mass spectrometry (MS) analysis.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)—Peptides were separated using a nanoAcquity UPLC system (Waters) fitted with a trapping (nanoAcquity Symmetry C18, 5 μm, 180 μm × 20 mm) and an analytical column (nanoAcquity BEH C18, 1.7 μm, 75 μm × 200 mm). The outlet of the analytical column was coupled directly to a linear trap quadrupole (LTQ) OrbitrapVelos or OrbitrapVelos Pro (Thermo Fisher Scientific) using a Proxeon nanospray source. The samples were loaded with a constant flow of solvent A (0.1% formic acid) at 15 μl/min and were eluted at a constant flow of 0.3 μl/min. During the elution step, the percentage of solvent B (0.1% formic acid in acetonitrile) increased in a linear fashion from 3% to 25% in 110 min, followed by an increase to 40% in 4 min, and an increase to 85% in 1 min. The peptides were introduced into the mass spectrometer by a Pico-Tip Emitter 360. Only multiply charged (2+, 3+) peptides with an FDR determined by the Benjamini-Hochberg method. If not stated otherwise, protein identifications were performed using the Limma package in R/Bioconductor (29, 30).

Spectra of quantified single peptide identifications are published as supplementary files (Single Peptides Spectra). After fitting a linear model to the data, an empirical Bayes moderated t test was used, and peptide masses were selected for MS/MS. The dynamic exclusion list was re-established. Average protein ratios were reported if they could be quantified in both replicates each on the basis of at least two ratio counts. For protein quantification, a minimum of two ratio counts was set and the “Requantify” and “match between runs” functions were enabled. Average protein ratios were reported if they could be quantified in both replicates each on the basis of at least two ratio counts. Spectra of quantified single peptide identifications are published as supplementary files (Single Peptides Spectra).

Biosatistical Analysis of Proteomics Data—Statistical analyses were performed using the Limma package in R/Bioconductor (29, 30). After fitting a linear model to the data, an empirical Bayes moderated t test was used, and p values were FDR-adjusted for multiple testing with the Benjamin-Hochberg method. If not stated otherwise, proteins with an FDR < 0.05 were considered to be differentially expressed. Prior to statistical tests of the DTT data set, values with high variation between replicates were removed if the difference of the log2-fold change between the biological replicates was greater than the 10-fold average standard deviation of 0.245. For the siUPF1 depletion data set, we considered all quantified proteins.

Correlations between replicates were calculated in R using Pearson correlation. Heatmaps were created in R/Bioconductor using the pheatmap package. 3′UTR length analysis of the principal transcripts was done manually using Ensembl database, assembly GRCh38,p2 (37).

Gene ontology enrichment analysis was performed using AmiGO 1.8 and the GO database (release date 2015–01–11, www.geneontology.org).

Quantitative RNA Analysis—RNA was isolated using the RNasy Mini Kit from Qiagen. 500 ng total RNA were reverse transcribed following manufacturer’s instructions for RevertAid H minus reverse transcriptase (Thermo Scientific) using oligo-dT primers. The RT-PCR was performed on a StepOnePlus™ machine (Applied Biosystems/Thermo Scientific, Foster City, CA), using Absolute™ SYBR green mix (Thermo scientific, AB-1185). The primers used in this study were:

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| SC35C | GCCGTTATTTGGACACAGATGTA | GCTGCTACACAGGCTG |
| PTGS2 | ATCACAGGTGGTCAATGCC | AGGGACATGAGGAGG |
| TGM2 | GGCGAGAGAGAGGAAAGAGC | TGCAGCTAGGGAGCTT |
| RPL3 | GCCATTGTGGGCTACGTG | CTTCCAGGAGCAGAGGGA |
| RPL5 | TGCACATTGGACCTTACAG | GTACCCGCATTCTTTG |
| RCC1 | CTCCTCAGGTTTGGCAGC | TTTATTGGGGCTACAG |

Luciferase Assay and Western blotting—For analysis of Renilla luciferase activity cell pellets were lysed in Passive Lysis Buffer (Promega). Dual-Luciferase assay (Promega) was performed according to manufacturer’s instructions to analyze the activity of the firefly luciferase reporter gene. For Western blot analysis cells were either lysed in RIPA buffer containing Protease Inhibitor Mixture cOmplete (Roche Diagnostics, Mannheim, Germany) or directly in 2× SDS sample buffer (for phospho-eIF2α detection). Ten to twenty micrograms of total lysate were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking in 5% milk or BSA, membranes were probed with antibodies against hUPF1 (Bethyl Laboratories, Montgomery, TX, A300–036A), BiP (Cell Signaling Technology, Danvers, MA, #3177), eIF2α (Cell Signaling, #2103), phospho-eIF2α (Cell Signaling, #3978), tubulin (Sigma, T5168) or actin (Sigma, A1978).

Analysis of Global Protein Translation by Metabolic Labeling—HeLa cells were seeded in six-well plates at 2 × 10^5 cells per well and kept in complete DMEM. The next day, cells were treated with either DTT or cycloheximide (CHX) for 5.5 h. Then, complete DMEM was replaced with depletion medium (DMEM without methionine and cysteine, 10% dialyzed FCS, 1%L-glutamine, DTT or CHX) for 5.5 h. Then, complete DMEM was replaced with depletion medium (DMEM without methionine and cysteine, 10% dialyzed FCS, 1%L-glutamine, DTT or CHX) for 30 min to deplete cells of internal stores of methionine and cysteine. Newly translated proteins were then labeled for 30 min with 35S Easy Tag Express (20 μCi/ml, Perkin Elmer, Waltham, MA) in the presence of DTT or CHX (full treatment time 6.5 h). Cells were washed twice in PBS, lysed in ice-cold RIPA buffer and centrifuged at 16,000 × g, 4 °C. For quantification of 35S incorporation 10 μl supernatant were spotted onto a glass microfiber filter (Whatman, Little Chalfont, UK) and dried. Proteins were precipitated with ice-cold 15% TCA for 30 min, washed once with ice-cold 15% TCA and twice with ice-cold 100% ethanol, and dried. Radioactivity was then determined by scintillation counting. Scintillation counts were normalized with protein concentration. For autoradiography analysis, 10 μg of lysate were separated on a 12% SDS gel and radioactive signals were quantified by phosphor imaging in a FLA-3000 fluorescent image.
NMD and increased activity of the Renilla-HBB NS39 NMD. DTT treatment robustly inhibited G and translated, resulting in a measurable increase of Renilla is impaired the reporter mRNA becomes stabilized, enriched, and efficiently degrades the reporter mRNA. If NMD efficiency HeLa cell line. Under normal conditions NMD is highly active constitutively expressed from a single copy integration in our molecular duplicates. Newly synthesized proteins containing AHA were covalently bound to alkyne-coupled agarose beads after 6 h incubation. Isotope labels were switched in biosynthetic designs. Although we exclusively detected proteins which were labeled during a relatively short period (6 h), the transcriptome studies detected RNAs which accumulated up to 96 h after UPF1 depletion (9, 11, 14). We chose this relatively short labeling time to ensure comparability between the UPF1 depletion and the ER stress experiment and to avoid confounding of data interpretation by protein degradation. Moreover, the transcriptome studies did not differentiate between protein-coding and non-coding transcripts. The fold change of the RNA is the sum of all (coding and noncoding) transcripts of a gene, whereas the proteomics approach detected proteins translated only from the subset of protein-coding transcripts. Furthermore, protein synthesis is a highly regulated process in the cell (39–41) and not every protein-coding gene product (GABARAPL1, STC2, IFRD1, PEA15, ARFRP1) were found to be up-regulated in all five experi-
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**FIG. 1.** Low dose of DTT induces ER stress, but only mildly inhibits protein translation. 

_A_, Experimental overview of the metabolic labeling experiments to analyze protein translation efficiency. 

_B_, Quantification of $^{35}$S incorporation after 6 h of DTT or CHX treatment. Cellular lysate was spotted onto glass fiber membranes, dried, precipitated with 15% TCA and $^{35}$S incorporation was quantified by scintillation counting and normalized to the total protein concentration. Bars represent mean ± S.D. Statistical analysis was done by one-sample, two-tailed t test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001). 

_C_, Representative autoradiograph of $^{35}$S-labeled proteins and the Coomassie stain of the same gel. 

_D_, Phosphorylation of eIF2α and induction of BiP after DTT and arsenite treatment for 6 h was analyzed by Western blotting. 

_E_, Induction of the ATF6-responsive element (ATF6-RE) after DTT incubation. HeLa cells were transfected with a reporter plasmid containing the ATF6-RE fused to a firefly luciferase open reading frame and treated with DTT for 6 h. Expression of firefly was assessed by a luciferase assay. 

_F_, NMD reporter construct to monitor NMD efficiency. The open reading frame of Renilla luciferase (white) is fused in frame to a human β-globin (HBB) minigene (dark gray) bearing a nonsense mutation on position 39 (NS39). Ter depicts the natural termination codon of HBB. The expression of the reporter construct is controlled by a CMV promoter (light gray). 

_G_, Depletion of UPF1 or ER stress causes inhibition of NMD. HeLa cells stably expressing an NMD reporter construct (Renilla-HBB NS39) were transfected with siUPF1 or a control siRNA. In addition one of the two siCtrl-transfected wells was treated with 1.5 mM DTT. After 6 h cells were harvested and the expression of Renilla-HBB NS39 was measured by luciferase assay. Bars show mean of n = 4, ± S.D.
ments, all of which contain either an upstream open reading frame (uORF, whose termination codon might trigger NMD), a long 3’UTR or both (Table I). The identified peptides are evenly distributed over the full length of the principal isoform in most cases, indicating that we quantified mostly full-length proteins. Supporting the previously published model of an auto-regulatory NMD feedback loop (14, 44), we also detect up-regulation of the known NMD factors UPF2, UPF3B, SMG5 and SMG7 at the protein level (supplemental Table S1). The overlap between the transcriptomic and the proteomic data sets shows that a subset of up-regulated mRNA is translated and likely contributes to altered cellular function.

**NMD-inducing Characteristics in Identified UPF1 Targets—** Known NMD-inducing properties include the deposition of an EJC within the 3’UTR at least 50 nucleotides downstream of the termination codon (1, 2, 45), the presence of an uORF in the 5’UTR (46), or a long 3’UTR (>1kb) (6, 7). We thus analyzed the mRNAs encoding the 30 most up-regulated proteins (1.8–2.8-fold) for the presence of one or more of these NMD-inducing features. The 30 most downregulated (1.8–3.2-fold) as well as the 30 most steadily expressed (‘least regulated’) proteins served as control groups. The mRNAs encoding the most up-regulated proteins show a highly significant enrichment for 3’UTR length >1kb (Fig. 5A). In this group, twice as many principal transcripts harbor a 3’UTR longer than 1 kb (22/30, $p = 0.0098$ and 9/30, $p = 0.017$, respectively). Furthermore, the 3’UTRs of the principal transcripts of the up-regulated proteins are significantly longer than the 3’UTRs of the other groups (median of 1325 nt versus 559 nt ($p = 0.0183$) and 690 nt ($p = 0.0057$), respectively) (Fig. 5C). We validated this analysis by reanalyzing previously published RNA-seq data (11), which confirmed the marked difference in the number of translated mRNAs with long 3’UTRs and also in the length of the 3’UTRs for up- and downregulated proteins (Fig. 5B and 5D). By contrast, neither the presence of uORFs nor of annotated 3’UTR introns is significantly enriched in the mRNAs that encode NMD-induced proteins (supplemental Fig. S1), albeit there is a trend of uORF-containing transcripts to be more common for the up-regulated group. We thus conclude that a long 3’UTR is a key NMD-inducing property of translated physiological, NMD-regulated transcripts.

**Identification of De Novo Synthesized Proteins in Response to ER Stress—** NMD efficiency can be affected by endoplasmic reticulum (ER) stress (16–19), and a regulatory feedback loop between NMD and the unfolded protein response has been proposed (16, 17). Hence, we were interested to determine the overlap of newly synthesized proteins in response to ER stress and NMD inhibition by UPF1 depletion. We performed combined AHA and SILAC labeling followed by LC-MS/MS analysis of enriched proteins for cells subjected to mild ER stress. For better comparability of the two pulsed SILAC experiments (i.e. siUPF1 transfection and ER stress induction) these were performed in parallel and the same negative control was used for both treatments. We treated control siRNA-transfected cells with 1.5 mM DTT or the solvent for 6 h and used BiP as a marker of ER-stress induction, which was strongly induced in DTT-treated cells (Fig. 6A). In parallel, the NMD reporter was up-regulated 3.5-fold at the protein level and endogenous NMD target mRNAs were also up-regulated 2–4-fold by ER stress induction with DTT (Fig. 6B and 6C). The LC-MS/MS analysis identified 5201 proteins that could be quantified in both biological replicates and whose expression changes were normally distributed (Fig. 6D and 6E). We found 99 of these to be significantly regulated (FDR < 0.05), with 58 up- and 41 down-regulated proteins. As
expected, ER stress response proteins (GO:0034976) were highly enriched in the up-regulated group (26-fold, \( p / H11005 \) 8 /H11003 10 /H11002 09) and all quantified UPR proteins were also induced (Fig. 7A and 7C). The NMD factors UPF1, UPF2, UPF3B and the EJC component Y14 are among the downregulated proteins (supplemental Fig. S3) offering a possible explanation for the inhibition of NMD by ER stress.

**NMD Inhibition Causes Increased Synthesis of ER Stress Proteins Regulated by the PERK/IRE1 Branch of the UPR**—It has been postulated on the basis of transcriptome data that ER stress and NMD are linked via a positive feedback loop, in that ER stress not only impairs NMD efficiency but that this inhibition will in turn further activate the UPR (16, 17). We tested this hypothesis by comparing the expression change of 22 ER stress proteins, which are regulated by all three branches of ER stress response and were quantified by mass spectrometry following DTT treatment. The synthesis of all of these proteins was up-regulated by ER stress with the exception of calnexin whose expression did not change (Fig. 7A).

Twenty of these proteins could also be quantified following NMD inhibition by UPF1 depletion. Interestingly, all proteins up-regulated by NMD inhibition have been recognized as targets of either the PERK or the IRE1 pathway (TXNIP, STC2, PTGS2, ASNS, PRNP, ATF3; (47–54)), and all of their principal transcripts harbor NMD-inducing features (Table II). In contrast, we did not detect up-regulation of proteins known to be activated by the ATF6 branch. Those were either downregulated (HERP) or did not respond to inhibition of NMD (BiP, SEL1L, DNAJC3, DNAJB11, GRP94, Calreticulin, PDIA4; (22, 23)) (Fig. 7B and 7C). This is consistent with the observation

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**Fig. 3. Identification of de novo synthesized UPF1 target proteins.** HeLa cells stably expressing the NMD reporter Renilla-HBB NS39 were transfected with UPF1 siRNA or a control siRNA. Two days after transfection DMEM was replaced with methionine, arginine and lysine-free DMEM containing AHA and isotope-labeled arginine and lysine. Isotope labels were reversed in biological duplicates. Cells were harvested after 6 h of incubation. NMD inhibition was controlled by expression analysis of the NMD reporter and known endogenous NMD targets. The remaining cell lysate was used for enrichment of de novo synthesized proteins and subsequent analysis by LC-MS/MS. A, Luciferase assay reveals a twofold up-regulation of Renilla-HBB NS39 on protein level upon UPF1 depletion. B, RT-qPCR analysis of endogenous NMD target RNAs. RPL13A serves a non-NMD target control. UPF1 mRNA was downregulated to 40% by siUPF1. C, 4898 proteins were identified and quantified in two biological replicates. Proteins that were regulated with statistical significance (FDR < 0.05) are shown in red. UPF1 is the most downregulated protein (down to 27%). R = Pearson correlation coefficient. D, Distribution of the average change of expression after UPF1 depletion.
that ATF6 itself is downregulated in response to UPF1 depletion (Fig. 7A and 7C). These data thus indicate that ER stress and NMD inhibition are linked by the PERK and the IRE1 pathways, but not by the ATF6 branch of the UPR.

We next asked whether NMD inhibition up-regulates stress response proteins by activating the ER stress response signaling, including upstream ER stress regulators such as ATF4, or whether these ER stress-response proteins are directly induced by NMD inhibition because their mRNAs are NMD targets. To test whether the PERK-responsive genes TXNIP, STC2, and PTGS2 are direct NMD targets, we inhibited NMD by incubation with cycloheximide, isolated RNA and analyzed

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**Table I**

Transcripts identified in all global screens for UPF1 targets contain NMD-inducing features

| Gene       | Principal isoform | Identified protein | Log2-fold change after siUpf1 depletion |
|------------|-------------------|-------------------|----------------------------------------|
|            | 3’UTR length | uORF | pSILAC | Mendell (9) | Viegas (12) | Tani (11) | Yepiskoposyan (14) |
| GABARAPL1  | 1278             | yes   | N-term. peptides | 1.18 | 2.15 | 2.29 | 2.42 | 1.11 |
| STC2       | 3134             | yes   | full-length | 0.97 | 2.54 | 1.77 | 3.18 | 1.60 |
| IFRD1      | 673              | yes   | full-length | 0.63 | 1.81 | 1.45 | 2.44 | 1.78 |
| PEA15      | 1890             | yes   | full-length | 0.54 | 2.01 | 2.65 | 2.21 | 1.39 |
| ARFRP1     | 987              | no    | full-length | 0.51 | 2.66 | 1.15 | 1.87 | 0.92 |

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Fig. 4. High correlation of expression changes of NMD-regulated genes identified by both the pSILAC screen and previously reported global RNA screens after UPF1 depletion. A, Heatmap comparing the log2-fold change of expression of UPF1 targets on protein (pSILAC) or RNA level (9, 11, 12, 14). Only genes are shown that have been identified in the pSILAC screen and at least 2 RNA screens. B–E, Candidate genes that have been identified by our pSILAC approach and by 3 out of 4 independent RNA screens show a high correlation of expression regulation. R = Pearson correlation coefficient.
steady-state RNA levels by RT-qPCR. All mRNAs tested were up-regulated 4 to 10-fold, consistent with them being directly regulated by NMD (Fig. 7D). To address the alternative possibility that these responses are elicited by UPR signaling as a consequence of NMD inhibition, we tested whether depletion of UPF1 activates the ATF4 or ATF6 pathway. We transiently transfected reporter plasmids containing a firefly luciferase expression cassette bearing an ATF4 or ATF6 response element into UPF1-depleted or control HeLa cells. We co-transfected a Renilla luciferase plasmid as normalization control. Although DTT treatment activates the ATF4 and ATF6-responsive promoters as expected, UPF1 depletion alone does not affect firefly luciferase activity (Fig. 7E and 7F). The expression of BiP is also not affected by depletion of UPF1 (Fig. 6A, 7B, and 7C), indicating that NMD inhibition is not sufficient to activate the whole UPR signaling cascade. We conclude that the absence of UPF1 and the resulting inhibition of NMD do not detectably potentiate the activity of the UPR key regulators ATF4 and ATF6, and that TXNIP, STC2, and PTGS2 appear to be directly regulated by NMD at the RNA level.

**DISCUSSION**

This work describes NMD as a regulated rather than a constitutive process. NMD inhibition by stress increases the expression of physiological NMD targets that contribute to the stress response. Our data implicate long 3'UTRs as the major...
NMD-inducing feature of endogenous NMD target mRNAs that are subsequently translated. The data reported here also show that NMD inhibition induces an ER stress response specifically through the PERK and IRE1 pathways, but not through the ATF6 pathway.

Previous transcriptomic studies have identified physiological NMD target mRNAs after depletion of UPF1, many of which encode proteins involved in the stress response (9–14). It is important to note that, in general, transcript levels correlate poorly with the abundance of proteins, although correlation is better for differentially expressed mRNAs (55–57). It is therefore biologically highly relevant to determine to what extent endogenous NMD target mRNAs are indeed translated into functional proteins. Answering this question is of particular importance because stress can cause global inhibition of translation which would prevent the expression of proteins encoded by NMD target mRNAs (58). In a previous proteomic study NMD inhibition did not result in an up-regulation of proteins encoded by NMD target mRNAs (59). However, the authors of this study employed 2D difference gel electrophoresis (2D-DiGE) multi-gel study with protein identification by LC-MS/MS, which is a method with a bias for high abundant proteins and proteins with low abundance might have escaped the analysis (60).

Here, we have conducted a global analysis of de novo protein synthesis monitoring the effects of stress and NMD inhibition on the proteome. Because only newly synthesized, labeled proteins are quantified, the sensitivity to detect changes in the proteome is strongly increased. As expected, we identified many previously described endogenous NMD targets among the up-regulated proteins following UPF1 depletion. We note that the changes of de novo protein synthesis tended to be quantitatively moderate, similar to the subtle differential protein expression observed for other regulated and biologically highly relevant pathways (61, 62).
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A

B

C

D

E

F

G
Cells were treated with 1.5 mM DTT or H2O for 6 h, harvested and luciferase activity was analyzed with a Dual-Glo luciferase™ assay. Bars luciferase controlled by an ATF4 or ATF6-response element. A plasmid encoding Renilla luciferase was co-transfected as normalization control.

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In the experimental system employed here, we used conditions of mild ER stress that nevertheless resulted in a robust induction of the unfolded protein response. The link between the induction of ER stress and NMD inhibition was indicated by the induction of de novo protein synthesis of endogenous NMD targets by DTT treatment. These data support the hypothesis that endogenous NMD targets contribute to the cellular stress response and potentially to recovery (9, 15). Our analysis further revealed a branch-specific regulation of UPR factors by NMD inhibition, as only PERK and IRE1-responsive proteins were up-regulated but none of the ATF6-responsive proteins. Because the activated proteins TXNIP, PTGS2, STC2, ASNS and PRNP were all detected as full-length proteins in the LC-MS/MS analysis, they should be fully functional.

The up-regulation of several ER stress-responsive proteins by NMD inhibition leads to the question of whether down-regulation of UPF1 may cause stress-independent enrichment of their transcripts (because they are direct NMD targets), or whether NMD inhibition causes ER stress which in turn would activate the UPR and finally lead to the increased expression of ER stress-responsive proteins. Our data support the interpretation that the identified genes are directly regulated by NMD, because inhibition of translation, and hence NMD, leads to a strong increase in the abundance of their transcripts. Moreover, UPR-responsive promoter elements and the UPR marker BiP are not activated by UPF1 depletion, indicating that the UPR is not activated per se. Our findings suggest that NMD contributes as a post-transcriptional mechanism to the regulatory network controlling the expression of this subset of PERK and IRE1-responsive genes. Although the UPR signaling cascades of PERK and IRE1 activate transcription of these genes, NMD controls the stability of their functional transcripts, probably via their long 3'UTRs. Interestingly, other post-transcriptional mechanisms such as binding of miRNAs or AU-rich element-binding proteins add further modularity to the regulation of these genes (49, 63).

Another explanation for the up-regulation of IRE1 responsive genes is provided by a recent study of Karam et al. (16). The authors identified IRE1 as a direct NMD target and postulate that NMD shapes the UPR by controlling the RNA stability of the key regulator IRE1. Although we did not detect IRE1 in our mass spectrometry analysis, our protein data of downstream UPR factors support and expand this model by assigning the NMD-regulated UPR factors specifically to the PERK and IRE1 branches of the UPR.

We did not observe an up-regulation of either ATF6 or ATF6 target genes on the protein level after UPF1 depletion, indicating a branch-specific regulation of UPR genes by NMD. In line with this, ATF6 mRNA was either not affected or destabilized after UPF1 depletion in previous studies (9, 11, 12, 14, 16). ATF6 mainly regulates the expression of molecular chaperones such as BiP or GRP94, whereas the target genes of the PERK and IRE1 branches have more diverse functions such as enzyme activity and therefore might benefit from a tighter control of expression (23).

Taken together, we suggest (1) that physiological NMD target mRNAs will be translated into functional proteins if NMD efficiency is downregulated, (2) that the cellular stress

| ER stress response gene | Principal transcript | Identified protein | ER stress activation by |
|------------------------|----------------------|-------------------|------------------------|
|                         |                      | PERK              | IRE1                  | ATF6                  |
| TXNIP                  | 1417                 | full-length       | (49)                  | (49)                  |
| PTGS2                  | 2541                 | full-length       | (48)                  | n.a.                  |
| STC2                   | 3134                 | full-length       | (52)                  | n.a.                  |
| PRNP                   | 1606                 | full-length       | (61)                  | (64)                  |
| ASNS                   | 142                  | full-length       | (51)                  | (52)                  |
| ATF3                   | 1202                 | full-length       | (47)                  | n.a.                  |

**TABLE II**

ER stress proteins upregulated by the inhibition of NMD (/ - is activated, X—is not activated, n.a.–not analysed)

**Fig. 7.** UPF1 depletion specifically up-regulates components of the PERK/IRE1 ER stress response pathways. A, B. Scatter plots showing the high correlation of the change of UPR protein expression between both biological replicates. Proteins marked with an asterisk were identified with an FDR < 0.05 by Benjamini-Hochberg analysis. A, Proteins involved in ER stress response or activated by ER stress are up-regulated by DTT treatment. B, UPF1 depletion causes up-regulation of PERK IRE1 pathways (green triangles, upper right quadrant) but not of ATF6-regulated proteins (orange rectangles). C, Average fold change of ER stress response proteins. Light bars - regulation after DTT treatment, dark bars - regulation after UPF1 knockdown. XBP1 and HYOU1 were detected in only one replicate of the UPF1 knockdown experiment and hence could not be quantified. D, HeLa cells stably transfected with either Renilla-HBB WT or NS39 were treated with 50 μg/ml CHX for 6 h, harvested and RNA was extracted and reverse transcribed. Expression of Renilla-HBB and putative endogenous NMD targets was analyzed by RT-qPCR. Bars represent means of 3 (HBB) or 6 (endogenous mRNAs) independent experiments, ± S.E. (E and F) HeLa cells were transfected with siRNA against UPF1 or a control siRNA and a reporter plasmid which encodes firefly luciferase controlled by an ATF4 or ATF6-response element. A plasmid encoding Renilla luciferase was co-transfected as normalization control. Cells were treated with 1.5 mM DTT or H2O for 6 h, harvested and luciferase activity was analyzed with a Dual-Glo luciferase™ assay. Bars represent means of 4 independent experiments, ± S.E. Statistical analysis of (D-F) was done by two-tailed t test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001) (G) Immunoblot analysis of the lysate used in (E) and (F) using specific antibodies against UPF1, BiP and β-Actin.
response involves NMD inhibition, and (3) that stabilized NMD target mRNAs belonging to the PERK and IRE1 pathways of the UPR are translated into proteins whereas target genes of the ATF6 branch do not appear to be detectably regulated by NMD.

Proteomics data—The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (64) via the PRIDE partner repository with the data set identifier PXD002648.

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