Attenuation of nonsense-mediated mRNA decay facilitates the response to chemotherapeutics

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Nonsense-mediated mRNA decay (NMD) limits the production of aberrant mRNAs containing a premature termination codon and also controls the levels of endogenous transcripts. Here we show that when human cells are treated with clinically used chemotherapeutic compounds, NMD activity declines partly as a result of the proteolytic production of a dominant-interfering form of the key NMD factor UPF1. Production of cleaved UPF1 functions to upregulate genes involved in the response to apoptotic stresses. The biological consequence is the promotion of cell death. Combined exposure of cells to a small-molecule inhibitor of NMD, NMDI-1, and the chemotherapeutic doxorubicin leads to enhanced cell death, while inhibiting UPF1 cleavage protects cells from doxorubicin challenge. We propose a model to explain why the expression levels of genes producing mRNAs of diverse structure that encode proteins of diverse function are under the purview of NMD.
An estimated approximately one-third of inherited diseases are the result of premature termination codon (PTC) acquisition. Nonsense-mediated mRNA decay (NMD) is a conserved mRNA-quality-control pathway deployed by cells to eliminate mRNAs containing a PTC. Because proteins produced by PTC-containing mRNAs may have deleterious consequences, selection and destruction of these mRNAs by NMD maintains cellular homeostasis. Less well understood, but equally important, is the role of NMD in maintaining and regulating the levels of endogenous, non-mutated transcripts. These transcripts are of heterogeneous structure and encode proteins of heterogeneous function, yet they have the unifying feature that disrupting NMD elicits their upregulation. How NMD-mediated changes in the levels of these transcripts are integrated into cellular physiology is unclear for all but a few situations. During muscle cell differentiation in humans and rodents, NMD efficiency is downregulated, while the efficiency of a competing pathway, Staufen-mediated mRNA decay, is upregulated. These changes tailor the mRNA pool to favour the expression of promyogenic factors. During hypoxia, the efficiency of NMD is inhibited, promoting tumorigenesis. Additional stresses that cause cell death fail to be translated, with NMD's death verdicts being the result of incompletely characterized responses.

Results

NMD activity is blunted during doxorubicin treatment. We examined the stability of a panel of known NMD target mRNAs in human MCF7 breast cancer cells during doxorubicin treatment. Pretreatment with doxorubicin (5 μM) resulted in significant increases in the half-lives of PANK2, TSTD2 and NAT9 mRNAs but not β-actin mRNA after actinomycin D-mediated transcriptional arrest (Fig. 1a), indicating a decline in NMD activity. To support this, we measured the level of each mRNA relative to the level of the pre-mRNA from which it derives as a function of time after doxorubicin treatment to control for transcriptional effects. An increase in the mRNA/pre-mRNA ratio (a metric used to distinguish a subset of direct NMD targets from those that are not in UPF1-ablated HeLa cells) may not reliably distinguish NMD targets from those that are not during doxorubicin treatment, because global transcriptional shut-down may inflate this number. However, a decrease in this ratio would rule out the possibility that NMD activity is blunted. Consistent with our half-life data, the mRNA/pre-mRNA ratio, as assessed using reverse transcription (RT)–quantitative PCR (qPCR), increases for all three transcripts (none of which is known to be stress regulated), in response to doxorubicin (Supplementary Fig. 1a). The mRNA/pre-mRNA ratios for three additional known NMD-targeted transcripts, CDKN1A, GADD45a and GADD45b, were also significantly increased by 5 h of doxorubicin treatment. As in HeLa cells, the ratio of CDKN1A, GADD45a and GADD45b mRNAs to their corresponding pre-mRNAs remained constant, indicating that these mRNAs are indeed NMD targets in MCF7 cells (Supplementary Fig. 1b). Decreases in pre-mRNA levels cannot account for the increased mRNA/pre-mRNA ratio since, even in the most extreme example (CDKN1A RNA), at 5 h the mRNA/pre-mRNA ratio increased ~5.7-fold relative to 0 h, while the pre-mRNA level decreased only ~3.2-fold.

To further corroborate the inhibition of NMD during doxorubicin treatment, we transfected MCF7 cells with the previously described β-globin (β-Gl) NMD reporter plasmid encoding either β-Gl Norm transcripts that lack a PTC, or β-Gl Ter transcripts that harbour a PTC at position 39. Cells were cotransfected with a plasmid encoding the mouse urinary protein (MUP) transcript to control for variations in transfection efficiency and RNA recovery and, 24 h later, were exposed to...
Figure 1 | NMD is inhibited during doxorubicin treatment. (a) mRNA decay assays in MCF7 cells. MCF7 cells either were (red) or were not (black) pretreated with 5 μM doxorubicin for 1 h before addition of 3 μg ml⁻¹ actinomycin D to halt transcription. Cells were collected at the indicated times after actinomycin D addition. Levels of the indicated NMD-targeted mRNAs were assessed by RT-qPCR, normalized to 18S rRNA levels and displayed as a percentage of the levels at t = 0 h. Error bars = s.e.m., n = 4 independent biological quadruplicates. (b) Human β-Gl mRNA half-life studies in HeLa Tet-off cells. HeLa Tet-off cells were transfected with plasmids encoding human β-Gl Norm mRNA and MUP mRNA or β-Gl 39 Ter mRNA and MUP mRNA. β-Gl Norm and β-Gl 39 Ter mRNA transcription occurs under the agency of the non-stress-responsive Tet-off promoter. Cells were either pretreated with nothing (top), 50 μM doxorubicin for 1 h (middle) or 50 μg ml⁻¹ puromycin for 3 h (bottom) before transcriptional shut-off with 2 μg ml⁻¹ doxycycline. Cell aliquots were removed at the indicated ‘chase’ time points, and RT-qPCR was used to assess the remaining levels of β-Gl Norm and β-Gl 39 Ter mRNAs, each after normalization to MUP mRNA. (c) Western blots of lysates of MCF7 cells from (Supplementary Fig. 1a) (blots derive from and are representative of the three biological replicates in Supplementary Fig. 1a that had been exposed to doxorubicin (5 μM) for the indicated times. GAPDH levels serve as loading controls. Threefold serial dilutions (wedge) reveal the dynamic range of analysis. α, anti; 1-416, UPF1 amino acids; CP, cleavage product. (d) As in (c), but cells were exposed to a 10-fold higher concentration of doxorubicin and analysed at earlier time points. Representative of two biological replicates.
doxorubicin (5 μM). After 5 h of doxorubicin treatment, the level of β-Gl Ter mRNA increased from ~65 to ~85% of the level of β-Gl Norm mRNA. These measurements occur on the backdrop of global RNA degradation at later time points28,30, accounting for why the normalized ratio of β-Gl Ter mRNA to β-Gl Norm mRNA is not elevated at later time points.

We performed additional mRNA decay assays using a previously described human cervical carcinoma Hela cell Tet-off cell system47 to halt the synthesis of human β-Gl Norm mRNA or β-Gl Ter mRNA and subsequently measured the remaining levels of each mRNA relative to the level of MUP mRNA after doxycycline addition. We used this system because the Tet-off promoter that controls the production of β-Gl Norm mRNA or β-Gl Ter mRNA is not stress responsive and HeLa cells, like MCF7 cells, are devoid of erythroid cell-specific β-Gl mRNA. Without doxorubicin, the level of β-Gl Ter mRNA declined to ~50% of its starting level by ~180 min of doxycycline addition, in agreement with reported values49, while the level of β-Gl Norm mRNA did not decrease during this time (Fig. 1c, top). In contrast, pretreatment of cells with doxorubicin for 1 h before doxycycline addition eliminated the selective decay of β-Gl Ter mRNA; the half-lives of both β-Gl Norm mRNA and β-Gl Ter mRNA exceeded the chase period (Fig. 1b; middle). Doxorubicin treatment mirrored the effect of the translational inhibitor puromycin, which is known to inhibit NMD (Fig. 1b; bottom). From all results, we conclude that NMD activity is attenuated during doxorubicin treatment.

We next examined biochemical changes to the key NMD factor, UPF1, that correlate with doxorubicin treatment (Fig. 1c). We exposed MCF7 cells to doxorubicin (5 μM) for varying amounts of time and analysed cell lysates using western blotting and an in-house generated polyclonal rabbit serum raised against the N-terminal 416 amino acids of human UPF1. To eliminate post-lysis proteolysis, lysates were generated in the presence of post-lysis proteolysis, lysates were generated in the presence of a protease inhibitor cocktail supplemented with N-ethylmaleimide at levels (50 μg/ml−1) known to alkylate the most active viral cytosine proteases. In addition to full-length UPF1, two additional bands of greater mobility were resolved by 5 h of doxorubicin treatment (Fig. 1c). Phosphorylation of UPF1 at both its N- and C termini is a key feature that differentiates UPF1-bound NMD targets destined for degradation from those that are not7,15,41,42. Western blotting using a monoclonal antibody recognizing limited cleavage of poly (ADP-ribose) polymerase (PARP), a cytosolic protein not known to cleave during apoptosis, but cleavage early during apoptotic progression and cleavage conservation across species indicate functional relevance33.

To examine the timing of UPF1 CP generation, we treated Hela cells with the clinically used topoisomerase inhibitor etoposide (ETP). ETP induced generation of a UPF1 CP before full cleavage of cleaved initiator caspase 9 (CASP9) and cleaved executioner CASP3 (Fig. 2b). Generation of the UPF1 CP before full CASP9 and CASP3 cleavage is recapitulated in human embryonic kidney (HEK)293T cells during CHX treatment (Supplementary Fig. 2a). We examined the effects of other apoptotic inducers on UPF1 CP generation. Treatment of HEK293T cells with staurosporine also yielded two UPF1 CPs, before full cleavage of CASP9 and CASP3 (Supplementary Fig. 2b). Exposure of the human Daudi B-lymphoblast cell line to either tumour necrosis factor-α or doxorubicin led to the generation of the UPF1 CP before maximal PARP cleavage (Supplementary Fig. 2c). Staurosporine-challenged Jurkat T cells also yielded a UPF1 CP before maximal cleavage of CASP3 or PARP (Supplementary Fig. 2d).

To probe whether generation of the UPF1 CP is evolutionarily conserved, we exposed mouse C2C12 myoblasts to CHX or ETP, both of which generated a UPF1 CP before maximal CASP3 cleavage (Fig. 2c). Exposure of canine (MDCK), bovine (MDBK) and Chinese hamster (CHO) cells to staurosporine led to UPF1 CP production (Supplementary Fig. 2e–g). Likewise, exposure of African Green monkey (COS-7) cells to staurosporine or doxorubicin yielded a UPF1 CP (Supplementary Fig. 2h). UPF1 CP levels varied drastically across cell lines, likely for three reasons: (i) in non-human cells, we cannot assess how efficiently our treatments elicited apoptosis because antibodies to human PARP, cleaved human CASP9 and cleaved human CASP3 do not cross-react; (ii) our anti-UPF1 antiserum was raised against the first 416 amino acids of human UPF1 and may exhibit reduced cross-reactivity to non-human UPF1 CP; and (iii) as a result of cleavage at the N terminus (see below), the human UPF1 CP exhibits less than one-third the immunoreactivity of full-length human UPF1 with our UPF1 antiserum. Notwithstanding this, UPF1 CP generation is an early event that is evolutionarily conserved, indicating that UPF1 cleavage may play a role in the cellular response to apoptotic induction.

Mapping UPF1 hydrolysis. To probe whether caspases are involved in UPF1 CP generation, we preincubated HEK293T cells with a panel of caspase inhibitors followed by exposure to CHX (Fig. 3a). Cells treated with each caspase inhibitor showed drastically reduced UPF1 CP levels, with Z-DEVd-fmk and Z-VAD-fmk lowering the level of UPF1 CP to nearly undetectable. Thus, caspases, and/or alternative proteases44 activated downstream of caspases, are involved in UPF1 CP production. We exposed a HeLa cell line stably expressing N-terminally tagged FLAG-UPF1 (ref. 45) to CHX. While anti-FLAG immunoblots failed to reveal any UPF1 CP even after long exposure, anti-UPF1 immunoblots using antiserum raised against amino acids 1–416 (Fig. 3b) yielded detectible UPF1 CP (Fig. 3c), indicating that cleavage occurs within the first 416 amino acids of UPF1 so as to eliminate the FLAG epitope but preserve partial immunoreactivity with our UPF1 antiserum.

Multiple apoptotic insults cause UPF1 hydrolysis. We characterized the upper UPF1 CP because we observed that it was consistently generated by an array of treatments in many cell lines (see below). We verified that this band derives from cellular UPF1 rather than a protein that fortuitously cross-reacts with our polyclonal anti-UPF1 serum by using short interfering RNA (siRNA) to reduce the level of UPF1 in HeLa cells to <10% of normal and subsequently exposing cells to cycloheximide (CHX) to induce apoptosis (Fig. 2a). In addition to halting protein synthesis, CHX causes apoptosis via incompletely understood mechanisms45. siRNA treatment reduced the levels of both full-
Inventories of in vivo apoptotic cleavage events indicate that cleavage specificity in living cells is determined chiefly by an aspartic acid residue at the P1 position; P4–P2 residues contribute far less to specificity in cells than is indicated by in vitro-derived peptide-based substrate profiles\textsuperscript{32,33}. Accordingly, we focused our attention solely on aspartic acid (D) residues in human UPF1 and interrogated residues D27, D37 and D75 near the UPF1 N terminus by mutating each to asparagine (N). Full-length wild-type (WT) UPF1 and, separately, each variant was expressed bearing an N-terminal MYC-tag and a C-terminal FLAG-tag in HeLa cells at a level equal to endogenous UPF1, and cells were subsequently challenged with CHX.

For UPF1 WT, UPF1 D27N and UPF1 D75N, the UPF1 CP was generated at approximately one-third the level of uncleaved UPF1, as judged using an anti-FLAG immunoblot (Supplementary Fig. 3a). Both the UPF1 CP and uncleaved UPF1 retained the C-terminal FLAG tag, allowing unambiguous assessment of the ratio of UPF1 CP to full-length UPF1. UPF1 D37N yielded no UPF1 CP, indicating that the amide bond after D37 is the site of hydrolysis (that is, D37 is the P1 residue).

We generated HeLa cells stably expressing one copy of retrovirally introduced MYC-UPF1-FLAG WT or MYC-UPF1-FLAG D37N transgene. Each protein was expressed at 2.7-fold the level of endogenous UPF1 (Fig. 3d). In these cell lines, the D37N mutation abolished UPF1 CP generation in response to CHX and doxorubicin (Fig. 3d). MCF7 cells stably transduced with MYC-UPF1-FLAG WT also generated the UPF1 CP at approximately one-third the level of uncleaved UPF1 in response to doxorubicin, and the UPF1 CP matched the molecular weight of a UPF1 fragment encompassing residues 38–1118 (Supplementary Fig. 3b). We cannot detect the N-terminal 37 amino-acid fragment released on cleavage, likely either for technical reasons or because this fragment is unstable. Having established that one cleavage event occurs after position 37, we examined the conservation of surrounding amino acids by aligning UPF1 sequences from multiple species using ClustalX (Supplementary Fig. 3c). The putative consensus cleavage site EFTD is completely conserved in human, bovine, mouse and *Xenopus laevis* UPF1—it deviates in chicken UPF1 at a single amino acid (where D is G)—and harbours T at the P2 residue, consistent with the high frequency of S and T residues at P4.

**Figure 2 | UPF1 CP production is an early and conserved event.** (a) Western blots of lysates of HeLa cells (*Homo sapiens*) transfected with 100 nM of either control (Ctrl) siRNA or UPF1 siRNA and, 48 h later, exposed to CHX (300 μg ml\(^{-1}\)) for 3 h. (b) Western blots of lysates of HeLa cells (*Homo sapiens*) exposed to CHX (100 or 300 μg ml\(^{-1}\)) for either 3 or 5 h, or to ETP (44 μM) for 6 h, incubated in fresh medium and withdrawn from ETP at the indicated times. (c) Essentially as in b except C2C12 myoblasts (*Mus musculus*) were analysed. CHX concentrations were 100 or 300 μg ml\(^{-1}\), and a 5 h pulse of ETP was used at 100 μM. Note that at least two apoptotic inducers were used for each cell line, and at least two cell lines were tested with each apoptotic inducer.
P3 and P2 residues in cellular apoptotic protein cleavage sites.32. Previously confirmed caspase substrates also bear similar cleavage sites: protein kinase C ζ is cleaved after EETD46, and the NF-kB p65/RelA subunit is cleaved after VFTD47.

We characterized which caspase(s) are sufficient to cleave UPF1 in vitro by treating immunoprecipitated samples of full-length MYC-UPF1-FLAG WT or the non-cleavable MYC-UPF1-FLAG D37N variant with recombinant caspases (Supplementary...
Fig. 3d). CASP3 and CASP7 cleaved MYC-UPF1-FLAG WT but not MYC-UPF1-FLAG D37N into a fragment with the same molecular weight as a Δ37-UPF1-FLAG variant lacking the N-terminal MYC-tag and first 37 residues of MYC-UPF1-FLAG WT (recapitulating the mapped UPF1 CP). This is consistent with our observation that Z-DEVD-fmk and Z-VAD-fmk blunt UPF1 CP production (Fig. 3a).

UPF1 CP is not functional in NMD. What might cleavage at D37 in human UPF1 accomplish? Both serine 10 (S10) and threonine 28 (T28) are phosphorylated by the NMD-associated kinase SMG1 (ref. 18), and phosphorylation is critical for NMD7,18,19,41,42. Cleavage would cause a loss of these phosphorylation sites and, indeed, experimental truncation of the first 35 amino acids in Arabidopsis thaliana UPF1 (causing loss of three phosphorylation sites) eliminates its NMD activity and causes it to act dominantly negative48. A previously described deletion of the N-terminal 63 amino acids of human UPF1 (dNT) causes loss of NMD activity and dominant-negative behaviour, as does mutation of the threonine 28 phosphorylation site to alanine15,49.

We assayed the NMD activity of exogenously expressed UPF1 proteins without endogenous UPF1. We depleted endogenous UPF1 levels in HEK293T cells to <10% of normal using siRNA and subsequently transiently introduced one of several siRNA-resistant UPF1 expression vectors: MYC-UPF1-FLAG WT; MYC-UPF1-FLAG D37N; Δ37-UPF1-FLAG; MYC-UPF1-FLAG TEV (described later); MYC-UPF1 dNT15, or MYC-UPF1 R843C, which abolishes UPF1 helicase activity50. Transfections included either a ‘Norm’ or a ‘Ter’ plasmid set to assess NMD activity. The ‘Norm’ set consists of the β-Gl Norm reporter plasmid, the MUP reference plasmid, and a T-cell receptor (TCR)β-based reporter plasmid. This TCRβ-based reporter plasmid contains a bidirectional promoter driving synthesis of an HA-Cerulean fluorescent protein and, in the opposite orientation, a 3′/C2 linker and FLAG epitope of mCherry transcript an EJC-mediated NMD substrate (Fig. 4a). Each variant was expressed at a level equivalent to endogenous UPF1 as assessed by comparing anti-UPF1(1–416) linker and FLAG epitope of mCherry transcript an EJC-mediated NMD substrate (Fig. 4a). Each variant was expressed at a level equivalent to endogenous UPF1 as assessed by comparing anti-UPF1(1–416) with our observation that Z-DEVD-fmk and Z-VAD-fmk blunt UPF1 CP production (Fig. 3a).

Comparing the levels of Δ37-UPF1-FLAG and MYC-UPF1 dNT with the level of MYC-UPF1-FLAG WT in immunoblots using the UPF1 a.a. 1–416, antiserum revealed a greater than threefold loss in immunoreactivity despite expression at equivalent levels (as assessed using anti-FLAG and anti-MYC immunoblots; Fig. 4b). Comparing the level of β-Gl Ter mRNA with the level of β-Gl Norm mRNA revealed that Δ37-UPF1-FLAG is unable to promote NMD whereas MYC-UPF1-FLAG WT, MYC-UPF1-FLAG D37N and MYC-UPF1-FLAG TEV can: the β-Gl Ter mRNA level was ~2.4-fold higher in Δ37-UPF1-FLAG transfectants than in MYC-UPF1-FLAG WT transfectants (Fig. 4c). MYC-UPF1 dNT and MYC-UPF1 R843C were non-functional, yielding β-Gl Ter mRNA levels ~2.6- and ~4-fold higher than in MYC-UPF1-FLAG WT transfectants. These results confirmed the mCherry-TCRβ reporters (Fig. 4d). The anti-FLAG immunoblot of 3 × FLAG-mCherry protein (normalized to HA-Cerulean protein, whose level is unaffected by changes in NMD) revealed that the NMD substrate produces approximately twofold more protein in cells expressing non-functional Δ37-UPF1-FLAG, MYC-UPF1 dNT or MYC-UPF1 R843C relative to cells expressing MYC-UPF1-FLAG WT (Fig. 4b). Thus, UPF1 CP fails to support NMD.

UPF1 CP is a dominant-interfering protein. Could the UPF1 CP play a dominant-interfering role in suppressing NMD even at stoichiometric levels relative to uncleaved UPF1? We challenged HeLa cell UPF1 function by introducing increasing amounts of plasmid DNA to express increasing but substoichiometric amounts of Δ37-UPF1-FLAG or, as a control, MYC-UPF1-FLAG WT; in parallel, we introduced empty vector DNA (0) as an additional control (Fig. 5a). These transfections included the ‘Norm’ or ‘Ter’ plasmid sets. While the flexible linker and FLAG epitope of Δ37-UPF1-FLAG limit its complete resolution from endogenous UPF1 (Fig. 5a), the level of Δ37-UPF1-FLAG can be compared with the level of MYC-UPF1-FLAG WT in anti-FLAG blots, and since MYC-UPF1-FLAG WT is cleanly resolved from endogenous UPF1 in anti-UPF1(1–416) blots, it is possible to determine the levels of Δ37-UPF1-FLAG relative to endogenous UPF1 (Fig. 5a). Levels of the β-Gl Ter NMD substrate revealed that, relative to transfections employing empty vector, increasing amounts of the Δ37-UPF1-FLAG elicited an increase in the level of β-Gl Ter mRNA (Fig. 5b). Δ37-UPF1-FLAG expression at approximately one-third the level of endogenous UPF1 (Fig. 5a) yielded an ~2.4-fold higher β-Gl Ter mRNA level than in transfections employing empty vector (Fig. 5b, red arrow). Δ37-UPF1-FLAG expression increased the level of the NMD substrate mCherry-TCRβ + JC intron mRNA ~2.3-fold (Fig. 5c; in the fourth sample, the decrease to ~1.5-fold and large error bars are likely due to experimental noise since the levels of β-Gl Ter mRNA continue to increase with increasing Δ37-UPF1-FLAG levels). The amount of 3×FLAG-mCherry that derived from mCherry-TCRβ + JC intron mRNA increased with increasing yet substoichiometric amounts of Δ37-UPF1-FLAG (Fig. 5a). Almost no changes were observed in the level of mCherry-TCRβ + JC intron mRNA or its product protein when endogenous UPF1 was challenged with increasing but substoichiometric levels of MYC-UPF1-FLAG WT (Fig. 5a,c). We confirmed that the challenge of endogenous UPF1 in HEK293T cells with increasing amounts of Δ37-UPF1-FLAG, relative to empty vector control, increased β-Gl Ter mRNA levels, whereas MYC-UPF1-FLAG WT had no such effect (Supplementary Fig. 4a,b).

We sought to rule out the trivial explanation for the lack of Δ37-UPF1-FLAG function in NMD, that is, that the truncated protein is misfolded, by characterizing the composition of the RNP containing either MYC-UPF1-FLAG WT or Δ37-UPF1-FLAG. HEK293T cells were depleted of endogenous UPF1 using siRNA, and either MYC-UPF1-FLAG WT or Δ37-UPF1-FLAG was expressed at a level equivalent to the normal level of endogenous UPF1 (Fig. 6). MYC-UPF1-FLAG WT or Δ37-UPF1-FLAG complexes were immunoprecipitated from lysates using anti-FLAG resin, each immunoprecipitate was divided in half and one half was incubated with bovine serum albumin, while the other half was incubated with RnaSe ONE to identify protein–protein interactions that are stabilized by RNA.

Immunoblotting using antibodies directed against phosphorylated-S1089 (p-S1089) or p-S1116 in UPF1 revealed slightly enhanced phosphorylation of Δ37-UPF1-FLAG relative to MYC-UPF1-FLAG WT (Fig. 6). Accumulation of C-terminal phosphates is a feature of ATPase-deficient UPF1 variants that cannot support NMD3,15,21,42. Equivalent levels of EJC components UPF2, UPF3X and MLN51, the cap-binding protein CBP80 and the poly(A)-binding protein (PABP)C1 were co-immunoprecipitated with both UPF1 variants (Fig. 6). Δ37-UPF1-FLAG retrieved slightly increased levels of SMG5 and SMG7 relative to MYC-UPF1-
FLAG WT in RNase-insensitive interactions (Fig. 6). Equivalent levels of SMG6 were retrieved in a partially RNase-sensitive interaction (Fig. 6). SMG6 association with a region outside of the UPF1 N terminus is consistent with several recent reports. Our results indicate that gross misfolding of the UPF1 CP cannot explain its non-functional and dominant-interfering behaviour.

We characterized the binding of MYC-UPF1-FLAG WT, Δ37-UPF1-FLAG and MYC-UPF1-FLAG D37N to PTC-containing mRNAs relative to their PTC-free counterparts by transfecting cells expressing equivalent levels of each UPF1 variant with a combination of plasmids encoding β-Gl Ter mRNA and MUP mRNA, or separately, plasmids encoding the β-Gl Norm mRNA and MUP mRNA. We measured the binding of each variant to β-Gl Ter mRNA and to its PTC-free counterpart (Supplementary Fig. 5) in immunoprecipitates. As previously reported, MYC-UPF1-FLAG WT retrieved ~26-fold higher levels of the
PTC-containing mRNA when adjusted for expression levels and MYC-UPF1-FLAG D37N did likewise. Like the non-functional dNT UPF1 variant as well as a non-functional 4SA variant lacking four phosphorylation sites, Δ37-UPF1-FLAG also retrieved β-Gl Ter mRNA relative to β-Gl Norm mRNA with an efficiency that was comparable to that of MYC-UPF1-FLAG WT and MYC-UPF1-FLAG D37N (~36-fold enrichment), despite being non-functional (Figs 4 and 5). We conclude that, like the dNT variant, Δ37-UPF1-FLAG is not misfolded—it can bind to the same complement of proteins as WT UPF1 and is enriched on a PTC-bearing transcript. Rather, a defect in the NMD cycle after RNA binding occurs.

**UPF1 cleavage upregulates genes involved in apoptosis.** What is the physiological relevance of UPF1 cleavage and the attenuation of NMD for cells exposed to chemotherapeutics that cause apoptosis? We interrogated the 91 genes upregulated on UPF1 downregulation in Mendell et al. using the online DAVID gene ontology tool to cluster genes by function. We found a cluster (11 genes) under ‘positive regulation of programmed cell death’ (P = 2.3E - 4; Supplementary Data 1) as well as a group of genes belonging to ‘p53 signalling pathway’ (four genes) and ‘regulation of cell cycle’ (five genes). Results from Viegas et al. showed a cluster (20 genes) under ‘positive regulation of programmed cell death’ (P = 7.4E - 8; Supplementary Data 1). DAVID analysis of results from Cho et al. also yielded genes in ‘positive regulation of programmed cell death’ (10 genes) and ‘regulation of cell cycle’ (five genes). Indeed, NMD targets that we previously analysed (Supplementary Fig. 1a) include CDKN1A mRNA, which encodes the classical cell cycle inhibitory protein p21, and GADD45β and GADD45α mRNAs, which produce proteins involved in cell cycle arrest that also transiently upregulate CASP3 and CASP7 to promote apoptosis. Thus, we hypothesized that among the transcripts upregulated on NMD attenuation is a group that the cell can exploit in response to apoptotic inducers.

We generated stably transduced HeLa cell lines bearing either empty vector or a fully functional MYC-UPF1-FLAG TEV allele that harbours the tobacco etch virus (TEV) protease cleavage site substituted into the D37 position (Fig. 4). Since TEV protease has no cleavage sites in the mammalian proteome, transfection with a plasmid encoding two complementary MYC-tagged TEV protease fragments expressed from a bidirectional promoter allows MYC-UPF1-FLAG TEV to be specifically cleaved in living cells in the absence of apoptotic inducers (Fig. 7a). To identify changes in cellular mRNAs that are due UPF1 CP production, we performed RNA-Seq on the empty vector cell line and the MYC-UPF1-FLAG TEV cell line, both in the presence or absence of TEV expression, with the assumption that we would uncover direct and indirect NMD targets.

We note that abundance changes in either class of targets may have important effects on cellular physiology. To control for differences in the cellular responses to plasmid identity and transfection, we normalized the changes in mRNA abundance of the MYC-UPF1-FLAG TEV cell line with and without TEV protease to changes in the empty vector cell line with and without TEV protease. We recovered upregulation of mRNAs for CDKN1A (~3-fold), GADD45α (~3.7-fold) and GADD45β (4.7-fold; Supplementary Data 2). By expressing increasing amounts of MYC-UPF1-FLAG WT or Δ37-UPF1-FLAG in either HeLa (Fig. 7b) or MCF7 (Fig. 7d) cells and measuring the resultant changes in mRNA abundance (Fig 7c, e), we verified the upregulation of a subset of additional genes, each of which has individual literature-documented roles in promoting cell cycle arrest or apoptosis when its expression is increased (Table 1). While we cannot explain the decrease observed for most mRNA ratios at the highest level of Δ37-UPF1-FLAG in HeLa cells (but not in MCF7 cells), we note that this expression level is greater than that observed for the CP in doxorubicin-treated cells.

To support these observations, we transfected HeLa cells with substoichiometric amounts of either MYC-UPF1-FLAG WT or an equivalent amount of Δ37-UPF1-FLAG (Supplementary Fig. 6), treated cells 48 h later with 5,6-dichloro-1-β-D-ribofuranosyl-1H-benzimidazole (DRB), and analysed the half-lives of endogenous NMD targets after DRB-mediated translational arrest. In contrast to GAPDH and β-actin mRNAs, there were noted increases in the stability of GAS5 ncRNA as well as GADD45α, BAK1 and BCL3 mRNAs. Stability of ARF1 and SERPINE1 mRNAs (two Staufen-mediated mRNA decay targets) were unaffected (Supplementary Fig. 6). Thus, UPF1 CP can partially attenuate NMD levels at substoichiometric amounts. Each of the genes verified from the RNA-seq data can individually promote either cell cycle arrest or apoptosis (Table 1) and thus may be exploited by cells in response to chemotherapeutic treatment.

**Modulation of NMD activity affects doxorubicin sensitivity.** Two testable hypotheses follow from the observation that generating UPF1 CP in the absence of chemotherapy augments the expression of genes involved in apoptotic progression. First, inhibiting UPF1 CP production should slow the cell death response to doxorubicin. Second, inhibiting NMD through exogenous induction of UPF1 CP or small-molecule treatment should promote doxorubicin-mediated cell death.

To test the first hypothesis, we utilized HeLa cell lines stably expressing MYC-UPF1-FLAG WT or non-cleavable MYC-UPF1-FLAG D37N (Figs 3d, 4b and 8a), both of which support NMD. Each was expressed at ~2.7-fold above the level of endogenous UPF1 and, more importantly, at levels identical to one another (Fig. 8a). We exposed these cell lines to a range of doxorubicin
Figure 5 | UPF1 CP dominantly interferes with NMD at substoichiometric levels. (a) Western blots of lysates of HeLa cells transiently expressing increasing amounts of MYC-UPF1-FLAG WT or ∆37-UPF1-FLAG together with either the 'Norm' plasmid set (producing β-Gl Norm mRNA, 3 × FLAG-mCherry-TCRβ JC intron mRNA, and MUP mRNA) or the 'Ter' plasmid set (producing β-Gl Ter mRNA, 3 × FLAG-mCherry-TCRβ + JC intron mRNA and MUP mRNA). Control experiments used empty vector (θ) in place of a UPF1 variant. Anti(α)-FLAG immunoblot allows unambiguous comparison of MYC-UPF1-FLAG WT and ∆37-UPF1-FLAG levels: ∆37-UPF1-FLAG exhibits ~one-third the immunoreactivity of full-length UPF1 with anti-UPF1(1–416) antiserum. Blots derive from (and are representative of) the triplicate samples analysed in b,c. (b) RT-qPCR, where the level of β-Gl Ter mRNA was first normalized to the level of MUP mRNA, and subsequently normalized to the empty-vector (θ) control (defined as 100%). The red arrow denotes the level of ∆37-UPF1-FLAG (based on a) that is comparable to the physiological level of UPF1 CP. (c) RT-qPCR, where the level of 3 × FLAG-mCherry-TCRβ JC intron mRNA or 3 × FLAG-mCherry-TCRβ + JC intron mRNA was first normalized to the level of MUP mRNA, and subsequently normalized to the empty-vector (θ) control (defined as 100%). The red arrow denotes the level of ∆37-UPF1-FLAG (based on a) that is comparable to the physiological level of UPF1 CP. Error bars = s.e.m., * = P < 0.05 relative to no UPF1 variant (empty vector) sample using the Student’s t-test. n = 3 independent biological replicates.
concentrations and assessed cell viability after 16 h using an assay that detects ATP generation by living cells. At a sublethal doxorubicin concentration (0.5 μM), no statistically significant difference in viability was detected. However, as doxorubicin toxicity increased, the MYC-UPF1-FLAG D37N cell line showed increased resistance to death relative to the MYC-UPF1-FLAG WT cell line, reaching a maximum of ~2.2-fold greater survival.

With the first hypothesis verified, we next transiently expressed either MYC-UPF1-FLAG WT or Δ37-UPF1-FLAG in HeLa cells (Supplementary Fig. 7a) or MCF7 cells (Supplementary Fig. 7c) and challenged transfectants with doxorubicin. Although we observed statistically significant increases in sensitivity for Δ37-UPF1-FLAG transfectants relative to MYC-UPF1-FLAG WT transfectants, the effect was mild in both cell types.
Figure 7 | Generation of UPF1 CP in the absence of doxorubicin upregulates genes that promote the apoptotic response to doxorubicin. (a) Western blot of lysates of HeLa cells retrovirally transduced with empty vector (0) or the MYC-UPF1-FLAG TEV construct bearing a substitution of the TEV protease cleavage site (ENLYFQS) at D37, and subsequently transiently transfected with empty vector (0) or a vector expressing both halves of TEV protease (each half is MYC-epitope tagged). Both full-length MYC-UPF1-FLAG TEV and the resultant TEV-generated CP retain the C-terminal FLAG. Blots derive from (and are representative of) the triplicate samples analysed in Supplementary Data 2. (b) HeLa cells were transfected as in Fig. 5a, but without NMD reporter sets. Blots derive from (and are representative of) the triplicate samples analysed in c. (c) RT-qPCR of mRNA from cells in b where the levels of the indicated transcripts in each transfecnt were first normalized to the level of GAPDH mRNA, and the ratio of normalized transcript levels in the Δ37-UPF1-FLAG transfectant to the normalized transcript levels in cells expressing an equivalent amount of MYC-UPF1-FLAG WT are displayed. The normalized transcript levels in the MYC-UPF1-FLAG WT sample for each amount of transfected plasmid is set at 100%. (d) As in b but using MCF7 cells. Blots derive from (and are representative of) the triplicate samples analysed in e. (e) As in c but using mRNA from MCF7 cells in d. Error bars = s.e.m., * = P < 0.05 for Δ37-UPF1-FLAG sample relative to MYC-UPF1-FLAG WT sample using the Student’s t-test. n = 3 independent biological replicates.

(Supplementary Fig. 7b,d), likely because the toxic effects of lipofection obscure differences between the two transfected cell populations and limit the dynamic range of the assay.

Therefore, we utilized a small-molecule inhibitor of NMD, NMDI-1 (Fig. 8c), that interferes with the interaction between UPF1 and SMG5 (refs 57,58). Application of NMDI-1 and the consequential attenuation of NMD may more fully replicate the complete inhibition of NMD mediated by the UPF1 CP generation explored here as well as UPF1 dephosphorylation and the generation of additional UPF1 CPs seen with doxorubicin (Fig. 1c). NMDI-1 is effective in HeLa cells, raising the levels of β-Gl Ter mRNA and another NMD target—a PTC-bearing glutathione peroxidase 1 mRNA—by ~3.8-fold and ~1.6-fold, respectively, at a concentration of 10 μM, which was used in subsequent experiments (Supplementary Fig. 8a,b).

Since NMDI-1 had no effect in MCF7 cells (Supplementary Fig. 8c,d), we focused on HeLa cells. Because of results indicating that the efficacy of combination small-molecule treatments is affected by both drug order and timing59, we considered three treatment regimens. First, we challenged HeLa cells with various concentrations of doxorubicin alone. Second, we continuously co-incubated cells for 16 h with doxorubicin and NMDI-1. Third, we applied a transient pulse of NMDI-1 for 8 h, washed cells to incubate cells for 16 h with doxorubicin and NMDI-1. Third, we applied a transient pulse of NMDI-1 for 8 h, washed cells to remove NMDI-1 and then applied doxorubicin. At sublethal doses of doxorubicin (0.5 μM), none of the treatments significantly affected viability (Fig. 8d), in accordance with previous observations58. Confirming our hypothesis that inhibiting NMD should increase sensitivity to doxorubicin, continuous co-treatment with NMDI-1 led to statistically significant decreases in cell viability relative to doxorubicin treatment alone (Fig. 8d, blue histograms). Transient pretreatment with NMDI-1 led to an even more pronounced effect and up to an ~2.5-fold reduction in cell viability at 50 μM doxorubicin relative to doxorubicin alone (Fig. 8d, red
histograms), despite the total time of exposure to NMDI-1 being half of that in the co-treatment regimen. Thus, inhibiting NMD promotes doxorubicin-mediated cell death, and conversely, inhibiting UPF1 CP generation obscures this effect.

**Discussion**

Here we observe that NMD activity is blunted during chemotherapeutic treatments (doxorubicin, staurosporine and so on) that ultimately cause apoptosis. During treatment with doxorubicin and other clinically relevant small molecules (for example, ETP), one or more UPF1 CPs are produced. The UPF1 CP that we have mapped to a region encompassing UPF1 amino acids 38–1118 acts to inhibit NMD in a dominant-interfering manner. This UPF1 CP is generated, the more PTC-containing reporter fusions, that is, at substoichiometric levels relative to cellular mRNAs. Ectopic expression of this UPF1 CP, as well as additional UPF1 CPs (Fig. 1c,d), and changes to UPF1 phosphorylation status (Fig. 1c) all likely contribute to inhibition of NMD and upregulation of cell cycle inhibitory and apoptosis-promoting transcripts seen during doxorubicin treatment (Figs 1a and 7). Small-molecule-mediated inhibition of NMD may provide an improved therapeutic strategy when delivered in combination with cytotoxic agents already in clinical use (Fig. 8d).

Transient pretreatment with NMDI-1 before addition of doxorubicin leads to enhanced cell death relative to either doxorubicin alone or to continuous co-treatment of NMDI-1 and doxorubicin. This suggests one model for NMD involvement in enabling the establishment of different cellular states by sculpting the mRNA milieu (Fig. 8e). Transcription produces mRNAs that are (red) or are not (blue) NMD targets, or are indirect NMD targets (black). NMD activity drives NMD-sensitive transcripts that are either not allowed into the pool of translated mRNAs or allowed at only low levels. Here we show that NMD activity is under the purview of the cell: NMD activity is tuned by changes to the mRNA milieu (Fig. 8). The sum effect of these changes to the mRNA pool alters the cellular state to one that is competent to respond (via death) to the insult that elicited the inhibition of NMD (doxorubicin). Such a model explains why transient pretreatment with NMDI-1 before application of doxorubicin is a more effective treatment regimen than mere doxorubicin alone or to continuous co-treatment of NMDI-1 and doxorubicin. The combined effects of this UPF1 CP, as well as additional UPF1 CPs (Fig. 1c,d), and changes to UPF1 phosphorylation status (Fig. 1c) all likely contribute to inhibition of NMD and upregulation of cell cycle inhibitory and apoptosis-promoting transcripts seen during doxorubicin treatment (Figs 1a and 7). Small-molecule-mediated inhibition of NMD may provide an improved therapeutic strategy when delivered in combination with cytotoxic agents already in clinical use (Fig. 8d).

Which transcripts are direct NMD targets or indirect NMD targets may be an academic distinction to the cell. Clearly, inhibiting NMD is able to change the mRNA milieu to promote physiological consequences (Fig. 8). The sum effect of these changes to the mRNA pool alters the cellular state to one that is competent to respond (via death) to the insult that elicited the inhibition of NMD (doxorubicin). Such a model explains why transient pretreatment with NMDI-1 before application of doxorubicin is a more effective treatment regimen than mere doxorubicin alone or to continuous co-treatment of NMDI-1 and doxorubicin. The combined effects of this UPF1 CP, as well as additional UPF1 CPs (Fig. 1c,d), and changes to UPF1 phosphorylation status (Fig. 1c) all likely contribute to inhibition of NMD and upregulation of cell cycle inhibitory and apoptosis-promoting transcripts seen during doxorubicin treatment (Figs 1a and 7). Small-molecule-mediated inhibition of NMD may provide an improved therapeutic strategy when delivered in combination with cytotoxic agents already in clinical use (Fig. 8d).

**Methods**

**Reagents.** Doxorubicin (Sigma; D1515), CHX (Sigma; C4859), ETP (Sigma; E1383), staurosporine (EMD; 569397), caspase inhibitors (EMD; set IV 80510–354), human tumour necrosis factor-α (Invitrogen; PHC3015), N-ethyl maleimide (NEM; Sigma; E3876), DRB (Sigma; D9196) and doxycycline (Sigma; D3072) were used at the concentrations and times indicated in figures and figure legends. NMDI-1 was a generous gift from David Bedwell57. Active recombinant Caspase 3 and 7 were from PromoKine. HeLa Tet-off cells were obtained from Clontech.

**Cell culture and transfections.** All cell lines were cultivated in DMEM (Gibco) containing 1% fetal bovine serum (Gibco) with the exception of Jurkat and Daudi cell lines which were cultured in RPMI containing 10% fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin (Gibco).

| Gene name | Fold-increase | Potential NMD-inducing feature | Comments on cell cycle inhibition | Comments on apoptotic progression | References |
|-----------|---------------|-------------------------------|----------------------------------|----------------------------------|------------|
| GADD45α   | 3.77          | Unknown                        | Induces G2/M arrest               | Promotes Bim translocation to mitochondria | 63,64      |
| GADD45β   | 4.72          | Intrin in 3’ UTR >55 nt downstream of termination codon | —                                | Promotes p38 interaction with Rb | 65         |
| BAK1      | 2.68          | uORFs                          | —                                | Promotes cytochrome c release by antagonizing Bcl2 | 66,67      |
| GAS5      | 1.58          | ncRNA in polysomes             | Ectopic expression causes growth arrest | —                                | 56,67      |
| DAP5      | 1.26          | uORFs                          | —                                | Ectopic expression causes apoptosis | 68         |
| DUSP2     | 2             | Unknown                        | —                                | Overexpression increases susceptibility to apoptosis | 69         |
| BCL3      | 2.9           | uORF in one splice variant     | —                                | Promotes apoptosis in multiple myeloma cells | 70         |

ncRNA, non-coding RNA; uORF, upstream open reading frame; UTR, untranslated region.

“Table 1 | NMD targets identified from RNA-seq data in Fig. 7.”

[44x339]more UPF1 CP is generated, the more PTC-containing reporter fusions, that is, at substoichiometric levels relative to cellular mRNAs. Ectopic expression of this UPF1 CP, as well as additional UPF1 CPs (Fig. 1c,d), and changes to UPF1 phosphorylation status (Fig. 1c) all likely contribute to inhibition of NMD and upregulation of cell cycle inhibitory and apoptosis-promoting transcripts seen during doxorubicin treatment (Figs 1a and 7). Small-molecule-mediated inhibition of NMD may provide an improved therapeutic strategy when delivered in combination with cytotoxic agents already in clinical use (Fig. 8d).
cells, which were grown in RPMI-1640 (Gibco) with 10% fetal bovine serum. HeLa and MCF7 cells (ATCC) were transfected with plasmid DNA using Lipofectamine LTX (Invitrogen), and HEK293T cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen). Transfections using siRNA employed RNAi MAX (Invitrogen) according to the manufacturer’s directions, with the exception of Fig. 2a, which employed Oligofectamine (Invitrogen). Cells were plated in antibiotic-free medium for a minimum of 24 h before transfection and harvested 48 h after plasmid transfections or 72 h after siRNA transfections.

**Western blotting.** Cells were lysed and the protein was isolated using hypotonic buffer that consists of 10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 2 mM EDTA, 0.5% α-Tubulin (α-FLAG)

![Western blot](image1)

![Western blot](image2)

**Figure 8 | Inhibiting UPF1 CP generation protects cells from doxorubicin challenge and inhibiting NMD promotes doxorubicin-induced cell death.**

(a) Western blots of HeLa cells stably expressing empty vector (ft) or equivalent amounts of MYC-UPF1-FLAG WT or MYC-UPF1-FLAG D37N. Blots derive from (and are representative of) four biological replicate samples used in b. (b) Cell lines from a were plated in 96-well opaque tissue-culture dishes (5,000 cells per well) and exposed to the indicated concentrations of doxorubicin for 16 h. Viable cells were quantitated using a Cell Titer Glo assay. Data are normalized to untreated cells for each cell line. Errors bar = s.e.m., * = P < 0.05 relative to MYC-UPF1-FLAG WT cell line samples using the Student’s t-test. n = 4 biological replicates. (c) Structure of NMDI-1. (d) HeLa cells were plated as in b and exposed to one of three treatments 24 h later: the indicated doxorubicin concentration is provided for 16 h either alone (grey histograms) or in the presence of 10 μM NMDI-1 (blue histograms). Alternatively, cells were preincubated with 10 μM NMDI-1 for 8 h, washed three times and incubated with the indicated concentrations of doxorubicin for 16 h in the absence of NMDI-1. Viable cells were quantitated as in b. Error bars = s.e.m., * = P < 0.05 relative to doxorubicin alone treatment using the Student’s t-test. n = 4 biological replicates. (e) Model implicating NMD modulation in establishing different cellular states (compare white box to the left and grey box to the right) by sculpting the mRNA milieu. Transcription produces mRNAs that are (red) or are not (blue) NMD targets (that is, are unregulated) or are indirect NMD targets (black). Normally, NMD is active, eliminating direct and indirect NMD targets from the mRNA milieu (left white box). However, NMD activity can be modulated by various perturbations. We show here that production of UPF1 CP(s) at substoichiometric levels downregulates NMD activity, causing direct NMD targets to enter the mRNA milieu, which secondarily causes upregulation of indirect NMD targets (right grey box). Together these changes sculpt the pool of mRNAs to one that is competent to rapidly respond to the stimulus that elicited the inhibition of NMD. Here we have shown that the stimulus (doxorubicin) attenuates NMD, facilitating an appropriate response (cell death).
phosphatase inhibitor cocktail (Roche) and 1 × protease inhibitor cocktail (Roche). After 10 min of incubation at 4 °C, NaCl was added to 150 mM, and lysates were cleared by centrifugation. Proteins were precipitated with polyacrylamide gel electrophoresis, transferred to Hybond ECL nitrocellulose (GE) and probed using antibody that recognizes one of the following: UFPI (1:4-16) (1:200), P-SUFI1 (1:116) (1:200), Millipore 07-1016, P-UFP1 SI089 (1:1000), Millipore 07-1015), Calnexin (1:200, Enzo Life Sciences A13886B0), UFP2 (1:200, Santa Cruz Biotechnology 516027), UFP3 and 3 (1:100; ref. 8), MLN51 (1:1000; Thermo Fisher Scientific; 5′-GAUGACCUUCUUCGCCUCAUUddT-3′).

**siRNA sequences.** siRNAs used were: Control siRNA #3 (Ambion) and UFP1 siRNA (Thermo Fisher Scientific; 5′-GAUGACGUUCUUCGCGCAUUddT3′).

**Immunoprecipitation and on-bead RNase digestion.** HEK293T cells were transfected as described in figure legends. Cells were lysed as described for western blotting. Input lysate protein concentrations were determined using the Bradford method (BioRad), equalized and preclreated twice with protein-A conjugate agarose (Roche) for 30 min with end-over-end rotation at 4 °C. Preclreated lysates were subjected to immunoprecipitation (IP) using anti-FLAG M2 Sepharose (Sigma) for 2 h at 4 °C, washed with lysis buffer supplemented to contain 0.1% Triton-X-100 and divided into two equal volumes. One-half of each IP was incubated with bovine serum albumin in RNase One (Promega) reaction buffer, and the other half was incubated with 1,000 U RNAse ONE (Promega) in reaction buffer for 30 min at 4 °C. Samples were washed three times with wash buffer and eluted using 3 × FLAG peptide (Sigma) according to the manufacturer’s directions.

**In vitro capso cleavage assays.** HEK293T cells were transfected with the indicated constructs. Cells were harvested 48 h later and anti-FLAG IP was performed as above, without RNase ONE digestion. Proteins were eluted with 3 × FLAG peptide. One microlitre of each immunoprecipitate was incubated for 5 h at 37 °C with 6 U of either Caspase 3 or Caspase 7 in caspase cleavage buffer (50 mM Tris-C176, NaCl was added to 150 mM, and protease inhibitor cocktail /C2 was used). Samples were washed three times with wash buffer and eluted using 3 × FLAG peptide (Sigma) according to the manufacturer’s directions.

**mRNA decay assays.** For mRNA decay assays using actinomycin D, MCC7 cells were plated at 60,000 cells well in 24-well dishes. After 24 h, cells either were or were not pretreated for 1 h with 5 μM doxorubicin before addition of 3 μg ml−1 actinomycin D (Sigma). Cells were harvested at the indicated time points (3 μg ml−1 doxorubicin-treated cells received doxorubicin during the chase period). For Tet-off assays, HeLa Tet-off cells (Clontech) were plated at 40,000 cells well in 24-well dishes. After 16 h, cells were transfected with the indicated plasmids in the presence of 1 μg/ml DTT.

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Author contributions
M.W.P. was responsible for the conception of the project. M.W.P. and L.E.M. designed the experiments, M.W.P. performed the experiments and M.W.P. and L.E.M. wrote the manuscript. L.E.M. provided resources.

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