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13 # Coupled protein quality control during nonsense mediated mRNA decay
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15 Alison J. Inglis*[1], Alina Guna*[2], Ángel Gálvez Merchán*[1], Akshaye Pal[1], Theodore K.
16 Esantsi[2,3], Heather R. Keys[2], Evgeni M. Frenkel[2], Robert Oania[1], Jonathan S. Weissman[2,3], and
17 Rebecca M. Voorhees[1†]
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34 * These authors contributed equally to this work
35
36 [1] Division of Biology and Biological Engineering, California Institute of Technology, 1200 E.
37 California Blvd, CA 91125, USA
38 [2] Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cam-
39 bridge, MA 02142, USA
40 [3] Howard Hughes Medical Institute, Massachusetts Institute of Technology; Cambridge, MA
41 02142, USA
42
43 [†] For correspondence: voorhees@caltech.edu

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44 **ABSTRACT**
45
46 Translation of mRNAs containing premature termination codons (PTCs) can result in truncated
47 protein products with deleterious effects. Nonsense-mediated decay (NMD) is a surveillance path-
48 way responsible for detecting and degrading PTC containing transcripts. While the molecular
49 mechanisms governing mRNA degradation have been extensively studied, the fate of the nascent
50 protein product remains largely uncharacterized. Here, we use a fluorescent reporter system in
51 mammalian cells to reveal a selective degradation pathway specifically targeting the protein prod-
52 uct of an NMD mRNA. We show that this process is post-translational, and dependent on an intact
53 ubiquitin proteasome system. To systematically uncover factors involved in NMD-linked protein
54 quality control, we conducted genome-wide flow cytometry-based screens. Our screens recovered
55 known NMD factors, and suggested a lack of dependence on the canonical ribosome-quality con-
56 trol (RQC) pathway. Finally, one of the strongest hits in our screens was the E3 ubiquitin ligase
57 CNOT4, a member of the CCR4-NOT complex, which is involved in initiating mRNA degrada-
58 tion. We show that CNOT4 is involved in NMD coupled protein degradation, and its role depends
59 on a functional RING ubiquitin ligase domain. Our results demonstrate the existence of a targeted
60 pathway for nascent protein degradation from PTC containing mRNAs, and provide a framework
61 for identifying and characterizing factors involved in this process.

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62 **INTRODUCTION**
63
64 Nonsense mediated mRNA decay (NMD) is a broadly conserved and essential surveillance
65 pathway that ensures the integrity of the transcriptome and regulates the levels of many cellular
66 mRNA transcripts. NMD was initially identified for its role in recognizing and degrading aberrant,
67 disease-causing mRNAs that contain a premature termination codon (PTC) within their open read-
68 ing frame (Chang & Kan, 1979; Losson & Lacroute, 1979; Maquat et al., 1981). When translated,
69 these mRNAs produce truncated proteins that can be aggregation-prone, develop gain of function
70 phenotypes, (Nonaka et al. 2009) or have dominant negative effects (Dietz et al., 1993; Hall &
71 Thein, 1994; Kugler et al., 1995; Thein et al., 1990). NMD thus plays a critical role in maintaining
72 cellular proteostasis by preventing expression of these potentially deleterious truncated proteins.
73 Further, one third of genetic disorders (Mort et al., 2008), including muscular dystrophy (Kerr et
74 al., 2001) and cystic fibrosis (O'Sullivan, 2014) and many cancers (Anczuków et al., 2008; Karam
75 et al., 2008; Perrin-Vidoz et al., 2002; Reddy et al., 1995; Ware et al., 2006) are the result of PTC-
76 causing mutations that lead to recognition and degradation of the resulting mRNAs by NMD.
77 In addition to its role in transcriptome maintenance, NMD also regulates the levels of
78 roughly 10% of endogenous transcripts, facilitating rapid and flexible changes in gene expression
79 in response to environmental and developmental stimuli (He et al., 2003; Lelivelt & Culbertson,
80 1999; Rehwinkel et al., 2005). NMD thus plays a fundamental role in diverse, but physiologically
81 essential processes including regulating the temporal expression of proteins during the cell cycle
82 (Choe et al., 2014); degrading PTC-containing transcripts produced by somatic recombination dur-
83 ing immune system development (Bruce & Wilkinson, 2003); and suppressing viral gene expres-
84 sion as a component of the innate immune response (Balistreri et al., 2014; Ramage et al., 2015).
85 While there are no definitive rules as to what defines an NMD substrate, the composition
86 of protein factors that decorate the 3'UTR of an mRNA seem to either promote or prevent its
87 degradation via NMD (Behm-Ansmant et al., 2007; Singh et al., 2008). For example, the position-
88 ing of poly(A) binding protein (PABP) adjacent to the termination codon has been shown to be
89 protective (Silva et al., 2008), while unusual physical features such as upstream open reading
90 frames (uORFs) and long 3'UTRs are established cues for degradation by NMD (Behm-Ansmant
91 et al., 2007; Mendell et al., 2004; Singh et al., 2008). It has also been observed that the many
92 apparently 'normal' transcripts that are regulated by NMD have lower codon optimality and a
93 higher rate of out-of-frame translation (Celik et al., 2017). However, the best characterized trigger
94 for recognition by NMD is the presence of an intron downstream of a stop codon, which is com-
95 monly the result of genetic mutations or defects in alternative splicing (Shoemaker & Green,
96 2012). Splicing of these introns results in the deposition of an exon-junction complex (EJC) 24
97 nucleotides upstream of the splice site, which is retained upon packaging and export to the cyto-
98 plasm (Ballut et al., 2005; Hoskins & Moore, 2012; le Hir et al., 2000, 2001). Because the majority
99 of endogenous stop codons are localized within the last exon of protein coding genes, EJCs are
100 typically removed during translational elongation (Dostie & Dreyfuss, 2002). The persistence of
101 an EJC downstream of a stop codon is thus a characteristic of a PTC-containing mRNA and results
102 in robust recognition by the NMD pathway (Gehring et al., 2003; Palacios et al., 2004).
103 Translation termination in the presence of a downstream EJC triggers NMD through a net-
104 work of interactions between the core NMD factors UPF1, UPF2 and UPF3B; the downstream
105 EJC; and the translational termination factors including eRF1 and eRF3 (Chamieh et al., 2008;
106 Czaplinski et al., 1998; Kim et al., 2001; le Hir et al., 2001). Phosphorylation of UPF1 by SMG1
107 recruits a suite of RNA decay machinery to decap (DCP2) (Cho et al., 2009; Lai et al., 2012),

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108 deadenylate (CCR4-NOT) (Loh et al., 2013), cleave (SMG6) (Eberle et al. 2009; Huntzinger et al.
109 2008), and ultimately degrade the associated mRNA.
110 Like other mRNA surveillance pathways, NMD substrates are recognized and targeted for
111 degradation co-translationally (Belgrader et al., 1993; J. Wang et al., 2002; Zhang & Maquat,
112 1997), resulting in the synthesis of a potentially aberrant nascent polypeptide chain. Pathways such
113 as no-go and non-stop mRNA decay rely on a coordinated protein quality control pathway, known
114 as ribosome associated quality control (RQC) to both rescue the ribosome and concomitantly target
115 the nascent protein for degradation (Doma & Parker, 2006; Frischmeyer et al., 2002; Juszkiewicz
116 et al., 2018; van Hoof et al., 2002). In both cases, a terminally stalled ribosome or a collided di-
117 ribosome triggers ribosome splitting (Becker et al., 2011; Pisareva et al., 2011; Shao et al., 2015,
118 2016; Shoemaker & Green, 2012) and nascent chain ubiquitination by the E3 ligase LTN1 (facil-
119 itated by NEMF, TAE2, and P97) (Brandman et al., 2012; Defenouillère et al., 2013; Lyumkis et
120 al., 2014; Shao et al., 2013, 2015; Verma et al., 2013). The ubiquitinated nascent chain is then
121 released from the ribosome by the endonuclease ANKZF1 (Vms1 in yeast) for degradation by the
122 proteasome (Rendón et al., 2018; Verma et al., 2018).
123 Given the potential dominant negative and proteotoxic effects of even small amounts of a
124 truncated NMD substrate, it has been suggested that a similar protein quality control pathway may
125 exist to recognize and degrade nascent proteins that result from translation of NMD mRNAs. In-
126 deed, proteins produced from PTC-containing mRNAs are less stable than those from normal tran-
127 scripts (Kuroha, Tatematsu, and Inada 2009; Kuroha et al. 2013; Pradhan et al. 2021; Udy and
128 Bradley 2021). However, these observations are largely based on comparison of truncated products
129 with longer, potentially more stable polypeptides, making it difficult to distinguish NMD linked
130 protein degradation from general cellular quality control mechanisms. Recent studies more directly
131 test this using a full-length protein product, but have not defined the mechanism of its targeting
132 and degradation, nor directly identified a role for the ubiquitin-proteasome pathway. Furthermore,
133 though it has been postulated that components of the RQC are involved in turnover of nascent
134 NMD substrates (Arribere & Fire, 2018), the factors required for this process have not been sys-
135 tematically investigated. Because NMD is triggered at a stop codon unlike no-go and non-stop
136 decay, a putative NMD-coupled protein quality control pathway may require a fundamentally dif-
137 ferent strategy to initiate nascent protein degradation.
138 Here we describe a reporter system that we have used to definitively define and character-
139 ize a coupled protein quality control branch of NMD. We demonstrate that in addition to triggering
140 mRNA degradation, NMD concomitantly coordinates degradation of the nascent polypeptide via
141 the ubiquitin-proteasome pathway. Using this reporter system, we systematically identify factors
142 required for NMD-coupled protein degradation, which are distinct from the canonical rescue fac-
143 tors of the RQC. Characterization of a coupled protein-degradation branch of NMD represents a
144 new facet of our understanding of how the cell ensures the integrity and composition of its prote-
145 ome, and sheds further light on the interplay between mRNA and protein quality control.
146
147 **RESULTS**
148
149 **A reporter strategy to decouple mRNA and protein quality control in NMD**
150 To identify a putative NMD-linked protein quality control pathway, we developed a reporter
151 system that sought to uncouple mRNA and protein quality control during NMD. The reporter con-
152 sists of a single open reading frame expressing GFP and RFP, separated by a viral 2A sequence
153 that causes peptide skipping (Y. Wang et al., 2015) (Fig. 1A, sFig. 1A). A robust example of an

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154 endogenous NMD substrate is the β-globin gene with a nonsense mutation at codon 39, which
155 results in a premature stop codon followed by an intron (Jing et al., 1998). We therefore reasoned
156 that positioning the first intron of the human β-globin gene into the 3'UTR of our reporter after
157 the stop codon would also lead to its recognition as an NMD substrate, as has been previously
158 reported (Chu et al., 2021; Lykke-Andersen et al., 2000; Pereverzev et al., 2015). We confirmed
159 that the exogenous β-globin intron is efficiently spliced (sFig.1B), and observed that the mRNA
160 levels of the NMD reporter were ~5-fold lower than a matched non-NMD control (Fig. 1B). We
161 found that the GFP fluorescence of the NMD reporter and control correlated with their respective
162 mRNA levels, as directly measured by qPCR, suggesting that GFP fluorescence can be used as a
163 proxy for transcript levels (sFig. 1C). Finally, knockdown of the core NMD factor UPF1 specifi-
164 cally increased the GFP fluorescence of the NMD reporter (sFig. 1F), but had no effect on the
165 matched control. We therefore concluded that our fluorescent reporter is recognized and degraded
166 in an NMD-dependent manner.
167 Recognition of our reporter as an NMD substrate, and subsequent mRNA decay, is a pre-
168 requisite for establishing whether there is an additional pathway dedicated to nascent protein deg-
169 radation. To address this, our reporter has two important physical features. First, it can be used to
170 deconvolute post-transcriptional versus post-translational effects on reporter fluorescence. Upon
171 translation, the GFP is released by the 2A sequence while the RFP remains tethered to the ribosome
172 until the termination codon, where NMD is initiated by interaction between the downstream EJC
173 and the ribosome. We reasoned that if there is an NMD-coupled pathway that triggers degradation
174 of the nascent polypeptide, it would thus act only on the RFP but not the released GFP, resulting
175 in a reduction in the RFP:GFP ratio in comparison to a matched control. In contrast, if NMD
176 functions only in mRNA degradation, we would expect a decrease in both the RFP and GFP levels
177 but would observe no change in the RFP:GFP ratio. Second, these reporters can specifically dis-
178 tinguish nascent protein degradation by a coupled protein quality control pathway from non-spe-
179 cific recognition by general cellular quality control machinery. Canonical NMD substrates contain
180 PTCs that result in translation of a truncated protein, which may be misfolded and thus recognized
181 and degraded by non-specific cytosolic quality control pathways (Popp & Maquat, 2013). By in-
182 stead using an intact RFP moiety that is recognized as an NMD substrate only because of an intron
183 in its 3'UTR, any destabilization of RFP must result from a coordinated event that occurs prior to
184 its release from the ribosome.
185 Indeed, using flow cytometry, we observed a decrease in RFP:GFP fluorescence for an
186 NMD substrate compared to a matched control (Fig. 1C). Addition of a second β-globin intron to
187 the 3'UTR (Hoek et al., 2019) resulted in a larger decrease in both the mRNA levels and RFP:GFP
188 fluorescence, suggesting the two effects may be tightly coordinated (Hoek et al. 2019). While this
189 decrease in RFP:GFP levels was consistent with NMD-dependent protein quality control, we
190 sought to exclude several alternative models that could also account for this observation. First, we
191 swapped the order of the RFP and GFP to rule out that differential maturation and/or turnover rates
192 of the fluorophores could explain the decrease in RFP:GFP ratio (sFig. 1D) (Amrani et al., 2004;
193 Balleza et al., 2018). Second, we considered whether the decrease in RFP:GFP ratio could be the
194 result of NMD-dependent deadenylation and 3' to 5' exonuclease degradation of the reporter
195 mRNA (Chen & Shyu, 2003; Mitchell & Tollervey, 2003; Takahashi et al., 2003). However, we
196 detected no change in the relative mRNA levels of the RFP and GFP coding regions of the NMD
197 substrate (Fig. 1B), confirming that the effect must occur post-transcriptionally.
198 Finally, we addressed two related possibilities: whether slow translational termination, char-
199 acteristic of NMD substrates (Amrani et al. 2004), or SMG6-dependent endonucleolytic cleavage

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200 of the mRNA at the stop codon could explain the RFP:GFP ratio decrease (Eberle et al. 2009). The
201 former would result in increased dwell time of the ribosome at the stop codon when the ~30 C-
202 terminal residues of RFP remain occluded in the ribosomal exit tunnel and could prevent RFP
203 fluorescence. The latter would lead to production of full-length GFP but truncated RFP, and would
204 be consistent with models proposed for putative NMD-coupled protein quality control in *C. ele-*
205 *gans* (Arribere & Fire, 2018). However, appending a flexible linker to the C-terminus of RFP to
206 ensure it is fully emerged from the ribosome at the stop codon did not affect the RFP:GFP ratio
207 (Fig. 1D). Conversely, scrambling the 2A sequence, such that both the GFP and RFP are tethered
208 to the ribosome at the stop codon, abolished the ratio difference (Fig. 1E). Together these data
209 exclude that the NMD-dependent decrease in RFP:GFP ratio is due to changes in translation rate,
210 processivity, peptide release, endonucleolytic cleavage, or preferential 3'-5' degradation.
211
212 **NMD-dependent protein degradation occurs via the ubiquitin proteasome pathway.**
213 Having established that an NMD-dependent decrease in RFP fluorescence occurs post-
214 translationally, we tested whether inhibition of the ubiquitin-proteasome pathway could rescue the
215 observed phenotype. We found that both the proteasome inhibitor MG132 and the E1 ubiquitin-
216 activating enzyme inhibitor MLN7243 specifically increased the RFP:GFP ratio of the NMD re-
217 porter (Fig. 2A; sFig. 2A). Importantly, this increase was due to an effect on RFP and not GFP
218 (Fig. 2B), consistent with the model that NMD-dependent protein degradation acts post-transla-
219 tionally and selectively toward the polypeptide associated with the ribosome at the PTC.
220 To confirm that the observed changes in fluorescence reflect changes at the protein level,
221 we directly tested for stabilization of RFP upon E1 enzyme inhibition by Western blotting (sFig.
222 2B). The absence of truncated RFP would be consistent with a model in which NMD-dependent
223 protein quality control is initiated at the stop codon. Finally, we directly observed a marked in-
224 crease in ubiquitination of nascent NMD substrates compared to a matched control, excluding
225 potential indirect effects of ubiquitin-proteasome pathway inhibition (Fig. 2C). Therefore, we con-
226 cluded that in addition to its well-characterized role in mRNA degradation, NMD also triggers
227 degradation of nascent proteins via the ubiquitin proteasome pathway.
228
229 **Identification of factors required for NMD-coupled protein quality control**
230 Using our characterized reporter, we systematically identified factors required for the pro-
231 tein degradation arm of NMD using a fluorescence-activated cell sorting (FACS) based CRISPR
232 interference (CRISPRi) (Horlbeck et al., 2016) and CRISPR knockout (CRISPR-KO) screen. We
233 reasoned that the knockdown screen would enable study of essential proteins, including the core
234 NMD factors UPF1 and UPF2 (Hart et al., 2017). Conversely, the knockout screen would identify
235 factors that require near-complete depletion to induce a measurable phenotype, which can lead to
236 false negatives in CRISPRi screens (Rosenbluh et al., 2017). To do this, we engineered two K562
237 human cell lines that expressed the NMD2 reporter either alone or with the CRISPRi silencing
238 machinery (Gilbert et al., 2014). We transduced the CRISPRi cell line with a single guide RNA
239 (sgRNA) library targeting all known protein-coding open reading frames as previously described
240 (hCRISPRi-v2) (Horlbeck et al., 2016). For the knockout screen, we used a novel 100,000 element
241 library that targets all protein encoding genes (~5 sgRNA/gene), which we used to simultaneously
242 deliver both the genome wide sgRNA library and cas9 (see methods).
243 We hypothesized that depletion of factors required for NMD-coupled protein quality control
244 would stabilize RFP, thereby increasing the RFP:GFP ratio. However, depletion of factors that
245 impede NMD-coupled protein quality control would further decrease the RFP:GFP ratio. For the

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246 CRISPRi screen, after eight days of knockdown, we sorted cells with high and low RFP:GFP ratios
247 via FACS, and identified sgRNAs enriched in those cells by deep sequencing. For the knockout
248 screen we isolated cells with perturbed RFP:GFP ratios on days eight, ten and twelve post infection
249 of the CRISPR-KO library. We postulated that essential genes would be better represented at the
250 earlier time points before their depletion becomes lethal, while factors that require complete de-
251 pletion and/or have longer half-lives would be detected at later time points.
252 In both the knockdown and knockout screens, we find substantial differences between the
253 hits identified here and those reported from earlier NMD RNA-degradation screens (Alexandrov
254 et al., 2017; Baird et al., 2018; Sun et al., 2011; Zinshteyn et al., 2021), suggesting our reporter is
255 indeed specific to the protein quality control branch of NMD. However, we also identified several
256 splicing and core NMD factors as effectors of the RFP:GFP ratio. For example, we found that the
257 core component of the EJC, CASC3 (Gerbracht et al., 2020) is required for NMD-coupled protein
258 degradation (Fig. 3B, 3C). Furthermore, depletion of several known NMD factors—UPF1, UPF2,
259 UPF3b, SMG6—increased the RFP:GFP ratio of our NMD-reporter. Together, these results sug-
260 gest a single, shared recognition step for both the mRNA and protein quality control branches of
261 NMD, which requires recognition of an intact EJC downstream of the stop codon via interactions
262 between the canonical NMD factors and the ribosome.
263 At day eight of the knockout screen, we found that several essential factors required for 5'
264 to 3' mRNA degradation were enriched in the population of cells with lower RFP:GFP fluores-
265 cence (Fig. 3C). In both the knockdown and knockout screen, we found that depletion of the E3
266 ubiquitin ligase CNOT4 increased the RFP:GFP ratio of the reporter, suggesting a potential role
267 in NMD-coupled protein quality control.
268

269 **NMD-coupled protein quality control is not mediated by canonical RQC factors**

270 Notably absent in both the knockdown and knockout screen were canonical components of
271 the RQC pathway, suggesting that NMD substrates may rely on an alternative strategy for nascent
272 protein degradation. Because the CRISPRi screen was performed using the same strategy and con-
273 ditions as earlier reporter screens for non-stop decay—including the same cell type, sgRNA li-
274 brary, and sampling time point—the screens are directly comparable (Hickey et al., 2020). While
275 depletion of RQC factors including pelota and the E3 ubiquitin ligase LTN1 were identified in the
276 non-stop reporter screen, neither are significant hits for NMD-dependent protein degradation in
277 our system (Fig. 4A, 4B). We directly verified that LTN1 knockdown has no effect on our NMD
278 reporter, though had a marked effect on the fluorescence ratio of an established non-stop decay
279 substrate (Fig. 4C). We therefore concluded that NMD-coupled protein degradation is mediated
280 by a new, uncharacterized set of factors (Chu et al., 2021).
281

282 **Factors required for NMD-coupled protein quality control**

283 Hits from the FACS based reporter screens were validated using an arrayed screen with a
284 matched non-NMD control. These data confirmed that knockdown of the splicing factor CASC3
285 increased both the GFP levels and the RFP:GFP ratio of our NMD reporter (Fig. 5A). Knockdown
286 of the 5'decapping enzyme DCP1A also increased GFP levels, but decreased the RFP:GFP ratio.
287 Having observed that the nascent protein is directly ubiquitinated and degraded by the pro-
288 teasome (Fig 2), we were particularly interested in identifying an E3 ubiquitin ligase responsible
289 for targeting the NMD-linked nascent chain for degradation. The core NMD factor UPF1 is an E3
290 ubiquitin RING ligase (Takahashi et al., 2008) and thus would be well-positioned to mediate nas-
291 cent chain degradation during NMD. Previous studies have demonstrated that UPF1 stimulates

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292 proteasomal degradation of proteins expressed from NMD-targeted mRNA transcripts in yeast,
293 with reporter stability significantly increased in *upf1* knockout strains; however, the mechanism
294 underlying this phenotype is unclear and a direct role in nascent chain ubiquitination by UPF1 was
295 not shown (Kuroha et al., 2009). UPF1 was identified as a weak hit in our CRISPRi screen (Fig.
296 3B), and its depletion resulted in a shift in the RFP:GFP ratio of the NMD reporter (sFig. 1F).
297 However, rescue of UPF1 knockdown with a RING mutant that disrupts binding with E2 ubiquitin-
298 conjugating enzymes (Feng et al., 2017) phenocopied wild-type UPF1 in restoring both the GFP
299 levels and RFP:GFP ratio of our NMD reporter (sFig. 3). This result would be inconsistent with a
300 role for the RING domain of UPF1 in ubiquitination of the nascent protein, and suggests that the
301 involvement of UPF1 may instead be upstream of the protein degradation branch.
302 In addition to UPF1, we identified four additional E3 ubiquitin ligases in either the knock-
303 down and knockout screen (KEAP1, MYLIP, CBLL1, and TRIM25). However, the only ligase
304 identified in both screens was the RING ligase CNOT4 (Fig 3B, 3C). CNOT4 is a conserved com-
305 ponent of the multi-subunit CCR4-NOT complex, which regulates eukaryotic gene expression by
306 deadenylation, i.e. processive shortening of mRNA poly(A) tails (Collart, 2016; Yamashita et al.,
307 2005). CNOT4 is not a core structural component of the CCR4-NOT complex, is not required for
308 deadenylation, and in mammals a population of CNOT4 exists independently from the rest of the
309 CCR4-NOT complex (Jeske et al., 2006; Lau et al., 2009). Indeed, other members of the CCR4-
310 NOT complex were not identified as significant hits in either the knockdown or knockout NMD
311 screens (sFig. 4A). However, the matched CRISPRi screen for non-stop decay identified CNOT1
312 as a robust hit, verifying the efficacy of the sgRNAs (Hickey et al, 2021; sFig. 4B). Further,
313 CNOT4 contains an N-terminal RING ligase domain (Albert et al., 2002; Hanzawa et al., 2001),
314 as well as a conserved RNA-binding motif and zinc finger domain (Fig. 6A) (Inada and Makino
315 2014; Panasenko 2014). These data together suggest an independent role for CNOT4 in NMD-
316 linked nascent protein degradation.
317 To validate the results of the screens, we first used small interfering RNA (siRNA) to de-
318 plete CNOT4, and observed a modest, but reproducible increase in the RFP:GFP ratio of our NMD
319 reporter (Fig. 6B). Exogenous expression of wildtype CNOT4 specifically rescued the RFP:GFP
320 ratio of the NMD reporter but had no effect on a matched control (Fig. 6B, sFig. 4C), excluding
321 off-target effects. We then tested whether the rescue was dependent on the E3 ligase activity of
322 CNOT4. For this, we generated two mutant constructs: (i) a mutation to the catalytic residues of
323 the CNOT4 RING ligase based on sequence alignments with other RING-containing E3 ligases;
324 and (ii) a mutant that disrupts binding between CNOT4 and its cognate E3 enzyme (Albert et al.,
325 2002). Neither the catalytic mutant nor the E2 mutant were able to rescue the RFP:GFP ratio.
326 Indeed, in the case of the E2 mutant we observed a small but reproducible dominant negative
327 effect. To exclude off-target effects, we confirmed that depletion of CNOT4, and overexpression
328 of the wild type or mutant protein did not affect our non-NMD control reporter. Together these
329 data suggest that CNOT4 is specifically involved in degradation of nascent NMD protein products,
330 in a manner dependent on its E3 ligase activity.
331
332 ## DISCUSSION
333
334 Recognition of an NMD-substrate occurs co-translationally, necessarily resulting in the
335 production of a nascent, potentially cytotoxic polypeptide chain. NMD typically reduces the
336 mRNA level of its substrates between 2-50 fold, depending on the transcript and function of the
337 resulting protein product: a reduction that may not be sufficient to maintain the proteostasis of the

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338 cell. As such, there has long been speculation as to whether NMD leverages an additional, post-
339 translational pathway to directly target these nascent proteins for degradation (Chu et al., 2021;
340 Kuroha et al., 2009, 2013; Pradhan et al., 2021; Udy & Bradley, 2021).
341 There are two plausible strategies by which protein degradation of NMD nascent chain
342 occurs. Since many NMD substrates are truncated and thus likely to misfold, they expose hydro-
343 phobic degrons that will be recognized by general cytosolic quality control machinery. However,
344 this type of uncoordinated clearance strategy would risk the cell's exposure to transient dominant
345 negative or gain-of-function activity of these truncated or aberrant proteins. In contrast, a coordi-
346 nated protein quality control pathway that co-translationally initiates protein degradation prior to
347 dissociation from the ribosome would be more consistent with other mRNA surveillance pathways.
348 Indeed, tight coupling of quality control to biogenesis is a strategy used throughout biology to
349 ensure robust and efficient clearance of mRNA and protein products that fail during their matura-
350 tion (Rodrigo-Brenni & Hegde, 2012).
351 In the case of NMD, the lack of a robust in vitro reconstitution system; the difficulty of
352 deconvoluting post-transcriptional versus post-translational effects on expression of NMD sub-
353 strates; and the putative contribution of generalized quality control in turnover of the classical
354 truncated NMD substrates has made it difficult to definitively identify this type of coordinated
355 pathway. Using a fluorescent reporter strategy that addresses several of these technical challenges,
356 we demonstrated that in mammals, NMD relies on a coupled protein quality control branch to
357 concomitantly target the nascent protein for degradation via the ubiquitin proteasome pathway.
358
359 **A coupled protein quality control branch of NMD**
360 We propose the following working model for protein quality control during NMD in mam-
361 mals (Fig. 7). As the ribosome reaches the stop codon during translational elongation, the protein
362 composition of the downstream mRNA serves as the primary cue for initiating NMD. At this point,
363 the nascent polypeptide remains tethered to the ribosome via the peptidyl tRNA. We postulate that
364 the early recognition steps between the mRNA and protein quality control branches of NMD are
365 shared, and rely on core NMD factors such as UPF1, UPF2, UPF3b, and CASC3. We postulate
366 that NMD-coupled quality control is thus initiated through the canonical pathway for recognition
367 of PTC-containing mRNAs that involves binding between the ribosome, NMD factors, and the
368 downstream EJC (Gerbracht et al., 2020; Chamieh et al., 2008; Czaplinski et al., 1998; Kim et al.,
369 2001; le Hir et al., 2001). However, because our screens were designed to specifically query factors
370 required for NMD-coupled protein quality control, we find substantial differences between hits
371 identified here and those reported from earlier NMD RNA-degradation screens (Alexandrov et al.,
372 2017; Baird et al., 2018; Sun et al., 2011; Zinshteyn et al., 2021). This discrepancy suggests that
373 following recognition of an NMD substrate, the mRNA and protein quality control pathways di-
374 verge, relying on distinct sets of factors to target and degrade either the mRNA or nascent protein.
375 We favor a model in which degradation of the nascent polypeptide is initiated prior to its
376 release from the ribosome, as is common to other mRNA surveillance pathways and would mini-
377 mize potential exposure of an aberrant protein to the cytosol. Consistent with this model we (i)
378 found that only the nascent polypeptide tethered to the ribosome at the stop codon is subjected to
379 NMD-coupled degradation (Fig. 1D, Fig. 2B); and (ii) we observe an NMD-specific destabiliza-
380 tion of an intact, folded protein compared to a matched control. We therefore concluded that the
381 nascent protein must be somehow 'marked' for degradation prior to its dissociation from the ribo-
382 some.

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383 Following ubiquitination of the nascent protein, it can then be safely released into the cy-
384 tosol for degradation by the proteasome. In contrast to non-stop and no-go mRNA decay where
385 the primary cue for protein quality control is ribosome stalling (Brandman & Hegde, 2016), NMD
386 is initiated at a stop codon and thus may utilize the typical strategy for nascent protein release and
387 ribosome recycling. Because termination at PTCs occurs more slowly than at a canonical stop
388 codon (Amrani et al. 2004), this additional window may be critical to allow tagging of the nascent
389 chain for degradation prior to its release from the ribosome. However, we cannot formally exclude
390 the possibility that this occurs simultaneously or immediately following translational termination,
391 but prior to dissociation of the nascent chain.
392
393 **A potential role for the RQC pathway in NMD-coupled protein quality control**
394 Several non-mutually exclusive models have been proposed for how to coordinate ubiqui-
395 tination of the nascent protein chain prior to release. Experiments in *Drosophila* and *C. elegans*
396 have suggested that at least in some systems, NMD and non-stop decay may be coupled, and levels
397 of some mRNAs and their associated protein products are regulated by both pathways (Arribere
398 & Fire, 2018; Hashimoto et al., 2017). A forward genetic screen in *C. elegans* further identified
399 the canonical RQC factor Pelo (the functional ortholog of dom34/Pelota) as required for repression
400 of an NMD reporter. Based on these and other experiments, the authors proposed a model whereby
401 quality control by NMD in initiated by endonucleolytic cleavage of the mRNA upstream of the
402 stop codon by SMG6. Translation of the resulting truncated mRNA would result in stalling of
403 subsequent ribosomes at its 3' end, triggering further repression at both the mRNA and protein
404 level by the non-stop decay and RQC pathways (Arribere & Fire, 2018).
405 If a similar mechanism was occurring in mammalian cells, post-translational degradation
406 of NMD substrates would depend on the canonical RQC factors including the E3 ubiquitin ligase
407 LTN1, and the ribosome rescue factors pelota and HBS1. However, the majority of RQC factors
408 were not significant hits in either of our screens, though were identified in an earlier non-stop
409 decay screen performed using matched conditions (Hickey et al., 2020). Further, depletion of
410 LTN1 directly did not affect our NMD reporter under conditions that robustly stabilized a non-
411 stop decay substrate (Fig. 4C). These results suggest that at least for the class of NMD substrates
412 represented by our reporter, NMD-coupled protein degradation does not rely on the canonical RQC
413 pathway. Together these data suggest a functional separation of nonsense and non-stop decay in
414 mammals, as was observed in *S. cerevisiae* (Arribere & Fire, 2018) and is consistent with the
415 distinct molecular players identified by NMD versus non-stop mRNA decay screens (e.g. Hodgkin
416 et al., 1989; Leeds et al., 1991; Pulak & Anderson, 1993; Wilson et al., 2007).
417
418 **Direct ubiquitination of the nascent NMD polypeptide**
419 The simpler model for NMD-coupled protein degradation is the direct recruitment of an
420 E3 ligase that ubiquitinates the nascent chain while it remains tethered to the ribosome. Earlier
421 studies have suggested that UPF1, a RING domain E3 ubiquitin ligase and core NMD factor that
422 interacts with both the ribosome and eukaryotic release factors, could carry out this role. UPF1
423 knockdown has been shown to stabilize protein products produced from NMD substrates mRNAs
424 (Kuroha, Tatematsu, and Inada 2009; Kuroha et al. 2013; Feng, Jagannathan, and Bradley 2017;
425 Park et al. 2020; Kadlec et al. 2006; Takahashi et al. 2008). Consistent with these reports, UPF1
426 was identified in our knockdown screen, and depletion of UPF1 stabilized both the mRNA and
427 protein levels of our NMD reporter. However, we found that point mutations to UPF1 that specif-
428 ically affect its ability to recruit its E2 ubiquitin-conjugating enzyme while leaving its ribosome-

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429 binding and helicase domains intact, did not have any effect on the protein-degradation phenotype
430 of our reporter. We therefore concluded that UPF1 is required for NMD-coupled protein quality
431 control, but plays a role that does not depend on its E3 ubiquitin ligase activity. To reconcile these
432 results with previous studies, we propose that UPF1 is involved in the early recognition steps of
433 NMD substrates, which affects both the mRNA and protein degradation branches of NMD. How-
434 ever, our data are inconsistent with a direct role for UPF1 in ubiquitination of the nascent poly-
435 peptide.
436
437 **A potential role for the E3 ubiquitin ligase CNOT4 in NMD-coupled protein quality control**
438 One of the most striking hits in both our knockdown and knockout screen was the E3 ubiq-
439 uitin ligase CNOT4. CNOT4 is a component of the CCR4-NOT complex, a conserved multi-sub-
440 unit complex that plays a broad role in gene regulation primarily through its deadenylase activity.
441 In NMD, the CCR4-NOT complex is recruited to transcripts through interactions between SMG7
442 and the CCR4-NOT subunit POP2, where it promotes deadenylation and the subsequent 3'-5'
443 degradation of the mRNA (Loh et al., 2013). CNOT4 is found in all eukaryotes, but is not a core
444 structural component of the complex: in human cells it is known to cycle on and off, and its deple-
445 tion does not destabilize other complex components (Jeske et al. 2006; Lau et al. 2009). CNOT4
446 was not identified as a significant hit in earlier screens querying NMD-mRNA levels (Alexandrov
447 et al., 2017; Baird et al., 2018; Sun et al., 2011; Zinshteyn et al., 2021), suggesting its function is
448 specific to the protein degradation branch of NMD.
449 Consistent with this model, we find that depletion of CNOT4 increases the RFP:GFP ratio
450 of our NMD reporter by preferentially stabilizing the RFP levels, suggesting it does not markedly
451 effect mRNA transcript levels. Though knockdown of CNOT4 had a reproducible effect in multi-
452 ple cell types, the phenotype of its depletion on our NMD reporter is modest. This may be due to
453 several contributing factors. For example, it is clear that most protein quality control pathways are
454 highly redundant, making it challenging to observe large effects as a result of a single genetic
455 perturbation (Rodrigo-Brenni & Hegde, 2012; Zavodszky et al., 2021). Indeed, when we generated
456 a full null mutant, we observed compensation for loss of CNOT4, suggesting that there may be
457 other E3 ubiquitin ligases that may be at least partially redundant. The phenotypes observed in our
458 acute knockdown experiments are in-line with those previously reported in other systems. Because
459 our model suggests CNOT4 may act catalytically in NMD-coupled protein quality control, very
460 efficient depletion may be required to observe marked phenotypes.
461 However, the modest, but reproducible effect of CNOT4 depletion is highly specific to
462 NMD substrates. Despite its reported role in proteasome maturation and assembly (Panasenko &
463 Collart, 2011), CNOT4 depletion does not affect the fluorescence of our matched non-NMD re-
464 porter. We therefore concluded that CNOT4 plays a specific role in NMD-coupled protein degra-
465 dation which cannot be explained by global changes to protein turnover rates. The role of CNOT4
466 in NMD-coupled protein degradation further appears to be distinct from its role in the CCR4-NOT
467 complex, because CNOT1 was not a significant hit in our NMD screen, though was identified in
468 an earlier matched CRISPRi screen for non-stop decay (Hickey et al., 2020) . Finally, we demon-
469 strate that both the E3 ligase activity of CNOT4, and its ability to bind to cognate E2 conjugating
470 enzymes, is required for its role in NMD-coupled nascent protein degradation.
471 The domain architecture of CNOT4 would be consistent with a putative role in ribosome
472 binding, given the presence of both a conserved RNA binding and zinc finger domains. Further,
473 earlier work has implicated CNOT4 in protein quality control of non-stop mRNA decay substrates,
474 as Not4p (the yeast ortholog of CNOT4) knockout, but not depletion of other CCR4-NOT

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475 components, stabilized truncated proteins produced from non-stop mRNAs (Dimitrova et al.,
476 2009). The mechanistic role of CNOT4 in protein quality control of non-stop and NMD substrates
477 in diverse eukaryotic systems thus represents an important area for future study.
478

479 **Implications of nascent protein degradation in proteostasis**

480 The identification of a tightly coupled protein degradation branch of NMD has several
481 immediate implications. Most notably, destabilization at the post-translational level will increase
482 the suppression of NMD substrates. Though we find the effects of NMD-coupled protein degra-
483 dation on our reporters to be modest (~2-fold), in the context of the cell or an organism, this addi-
484 tional level of regulation may be critical to prevent deleterious or off-target effects. Effects on
485 these fluorescent reporters, which are both over-expressed and in which phenotypes require deg-
486 radation of the remarkably stable RFP moiety, may also underestimate the true effect size on an
487 endogenous substrate.

488 There are numerous physiologically relevant examples where NMD's role in transcriptome
489 regulation, and subsequent production of potentially aberrant proteins, require stringent clearance
490 of the nascent product. During histone production, synthesis must be tightly regulated in a manner
491 coupled to the progression of the cell cycle, and the production of even small amounts of down-
492 regulated proteins could be problematic. Our results also have implications for viral infection. Co-
493 translational protein degradation is thought to be a key source of peptides for MHC presentation
494 (Balistreri et al., 2014; Fontaine et al., 2018; Wada et al., 2018; Yewdell & Nicchitta, 2006), with
495 viral messages often targeted by NMD (Balistreri et al., 2014; Fontaine et al., 2018; Wada et al.,
496 2018). Factors such as CNOT4 could mediate this process, promoting the immunological presen-
497 tation of these peptides.

498 Finally, NMD plays an important role in a wide range of genetic diseases: over one third
499 of all human genetic disorders are caused by PTC-creating mutations, including muscular dystro-
500 phy and cystic fibrosis. While generally protective, for numerous disease-causing mutations the
501 NMD pathway contributes to pathogenesis by suppressing expression of partially functional mu-
502 tant proteins (~11% of mutations that cause human disease (Mort et al., 2008)). The characteriza-
503 tion of a second, parallel branch of NMD and the factors involved in NMD-coupled protein quality
504 control therefore represent novel targets for the therapeutic treatment of human disease.
505
506
507

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841
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856 **Supplementary Materials:**

857 Materials and Methods

858 Figs. S1-S4

859 Supplementary Tables 1-3

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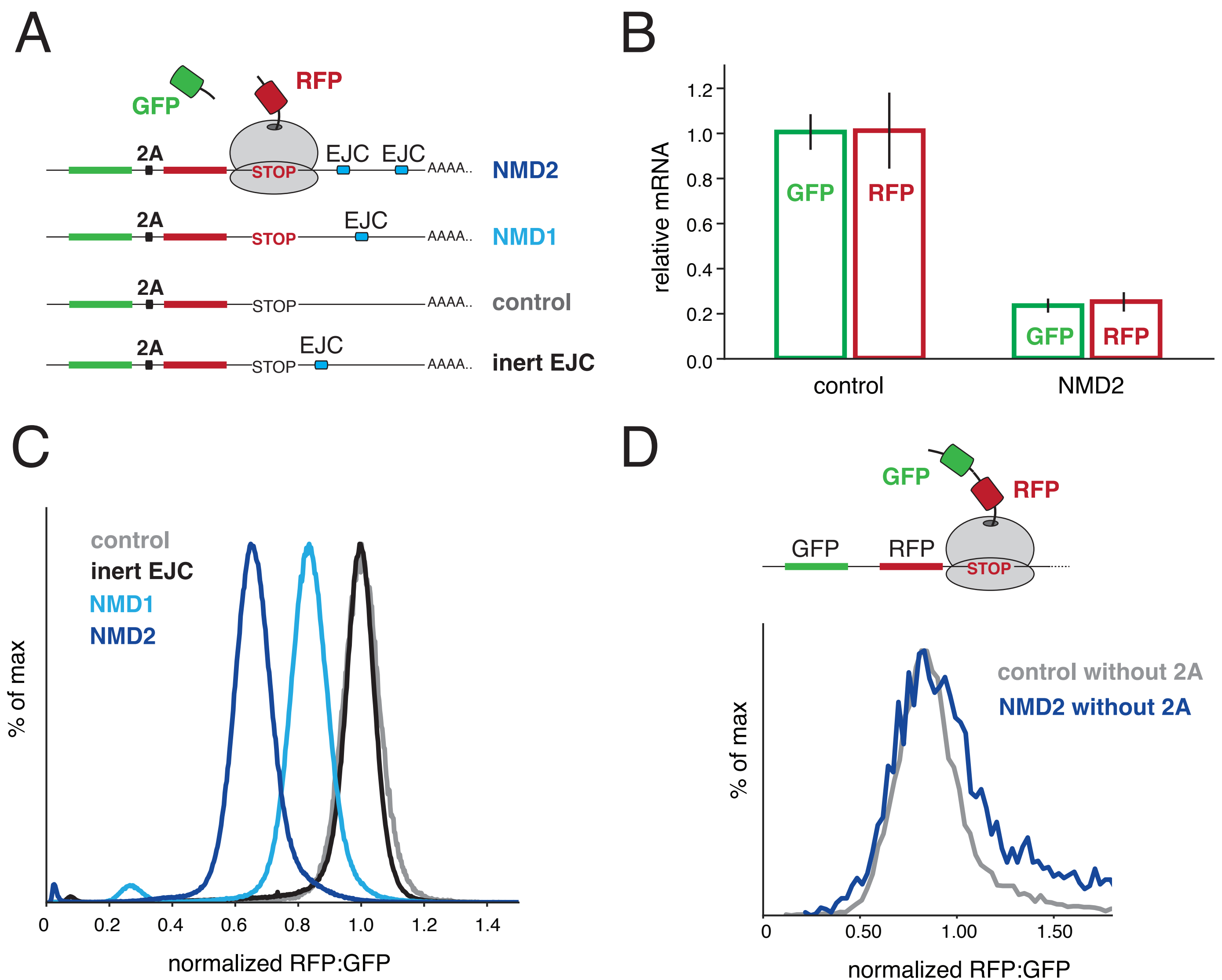


**Figure 1. Destabilization of nascent proteins from PTC-containing mRNAs.**
(A) Schematic of the reporter strategy used to decouple protein and mRNA degradation in NMD. GFP and RFP are encoded in a single open reading frame separated by a viral 2A sequence. Positioning an intron within the 3' UTR results in deposition of an exon junction complex (EJC) upon splicing, trigger-ing NMD when compared to a matched control (stop codon depicted in red). Either one or two in-trons derived from the β-globin gene are inserted after the stop codon (NMD1 and NMD2 respectively). To control for the documented stimulation in translation that results from the presence of an EJC (Nott et al., 2004), we creat-ed a reporter in which the intron was positioned twelve nucleotides after the stop codon, a distance insuffi-cient for recognition as an NMD substrate (inert EJC) (Nagy & Maquat, 1998). (B) Stable cell lines expressing either the control or the reporter was induced with doxycycline for 24 hours and the total mRNA was then purified. Relative mRNA levels were determined by RT-qPCR using primers that anneal to the very 5' region of the GFP and 3' region of the RFP open reading frames. The results were normalized to the control and the standard deviation from three independent experiments is displayed. (C) Stable cell lines for the indicated report-ers were analyzed by flow cytometry. The ratio of RFP:GFP fluorescence, as normalized to the control reporter is depicted as a histogram. (D) The NMD2 and control reporters in which the 2A sequence was scrambled, resulting in tethering of both GFP and RFP to the ribosome at the stop codon were analyzed as in (C).

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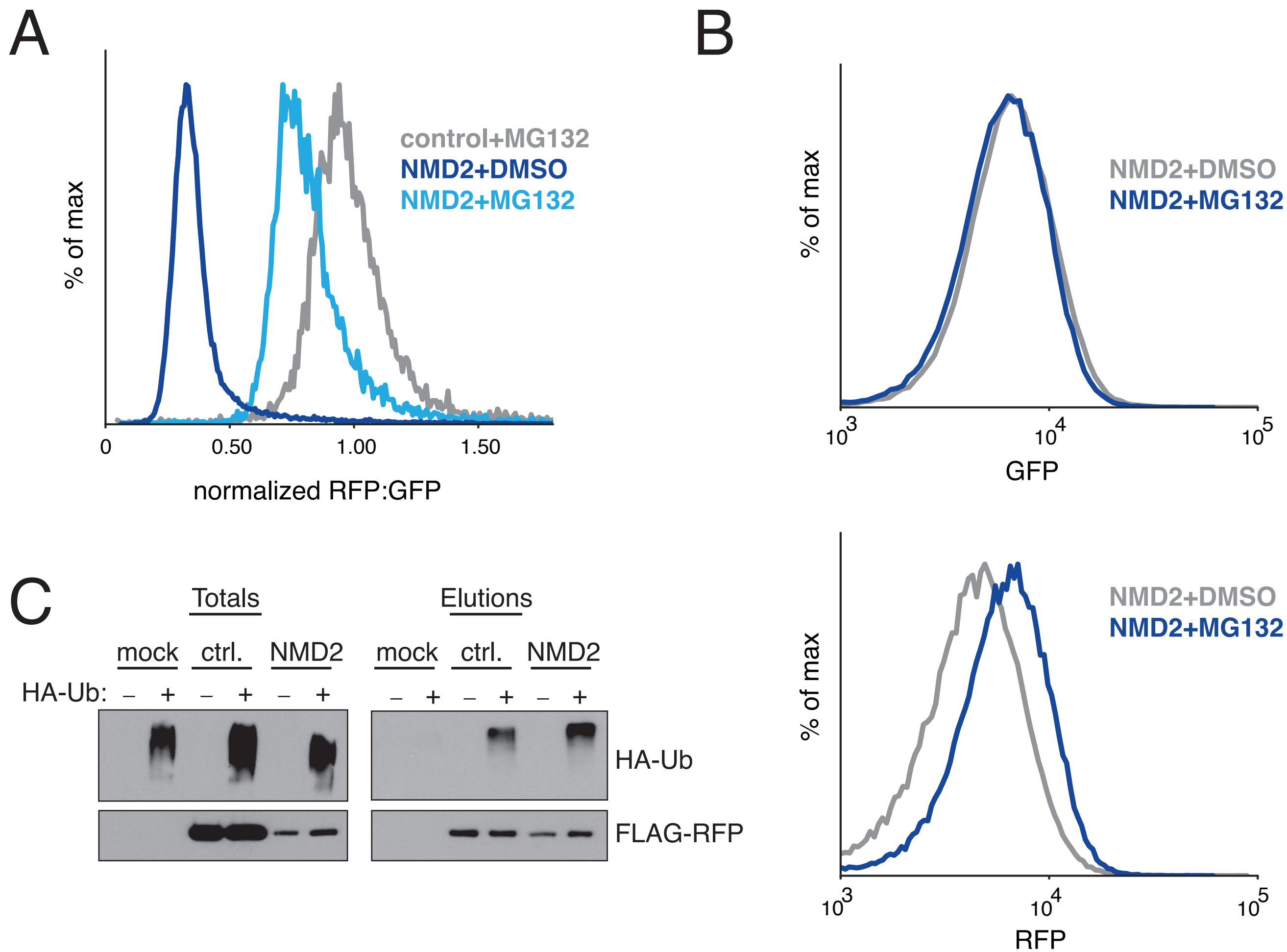


**Figure 2. NMD-dependent protein degradation occurs via the ubiquitin proteasome pathway.** (A) Flow cytometry analysis of HEK293T cells transiently transfected with either the control or NMD2 reporter (Fig. 1A) and treated with the proteasome inhibitor MG132 or DMSO. (B) As in (A) using stable K562 cell lines expressing an inducible NMD2 reporter treated with either MG132 or DMSO. Shown are the GFP (top) and RFP (bottom) channels for the indicated condi-tions displayed as a histogram. (C) HEK-293T cells were transiently transfected with either the control or NMD2 reporter (modified to incorporate a 3xFLAG tag at the N-terminus of RFP) in the presence of HA-tagged ubiquitin (HA-Ub). To stabilize ubiquitinated species, cells were treated with MG132 prior to lysis, and RFP was immunoprecipitated with anti-FLAG resin. Ubiquitinated species were detected by Western blotting for HA-Ub.

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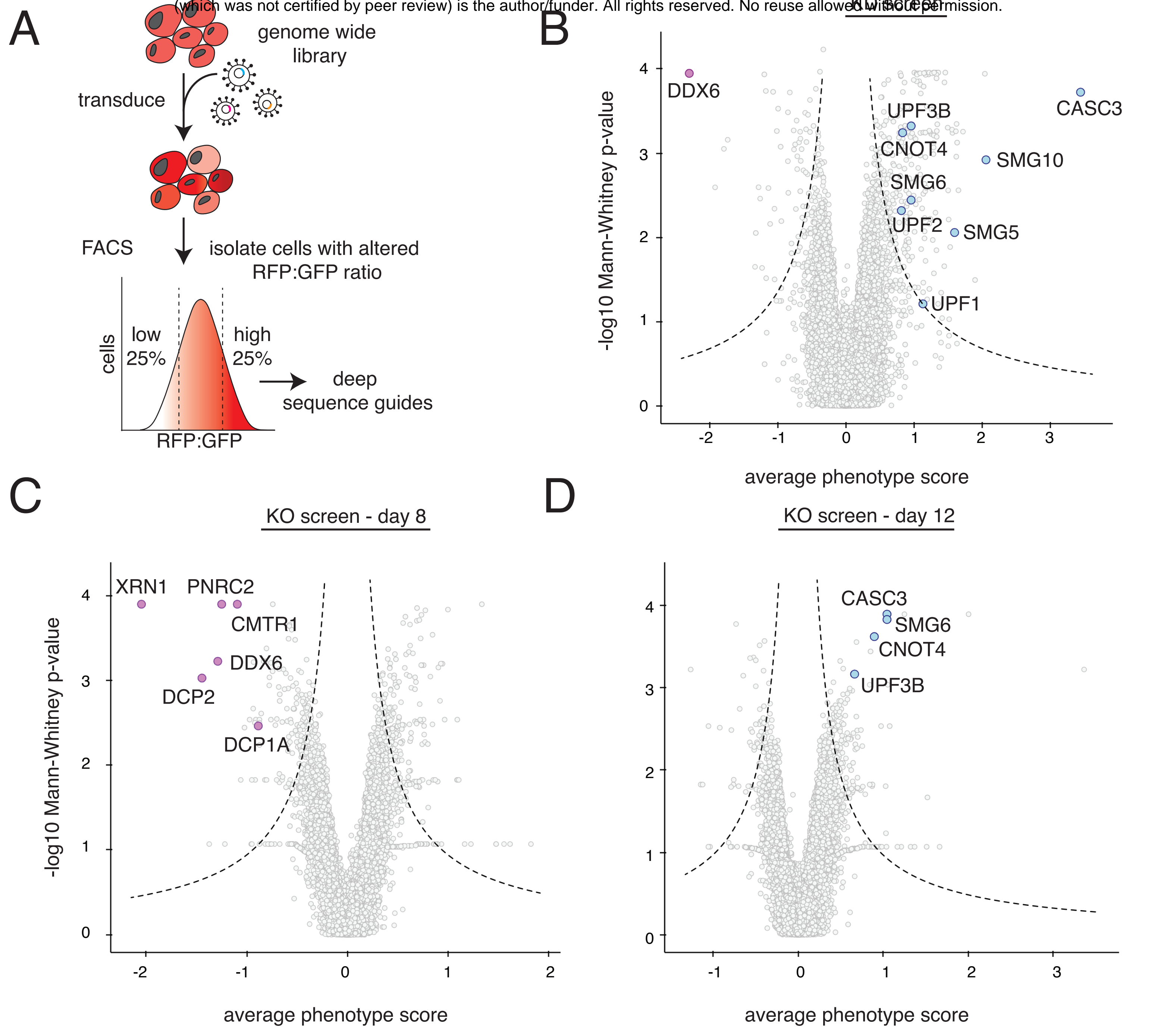


**Figure 3. Systematic characterization of factors required for NMD-coupled protein quality control.**
(A) Schematic of the workflow used to carry-out the FACS-based reporter screens to identify factors involved in NMD-linked nascent chain degradation. K562 cells reporter cell lines contained a tet-inducible NMD2 reporter and were infected with either a whole-genome CRISPRi sgRNA library or a CRISPR-KO library. Reporter expression was induced with doxycycline for 24 hours prior to cell sorting. Cells were sorted based on ratiometric changes in RFP relative to GFP, and the sgRNA expressed in those cells were identified using deep sequencing. The CRISPR knockout screen was sorted on days 8, 10 and 12 post library infection to account for drop out of essential genes. The CRISPRi screen was sorted on day 8. (B) Volcano plot of the RFP:GFP stabilization phenotype (log2 for the three strongest sgRNAs) and Mann-Whitney p values from the genome-wide CRISPRi screen. Genes falling outside the dashed lines are statisti-cally significant. Each gray point represents a gene. Notable hits causing an increase in the RFP to GFP ratio are shown in light blue and include known NMD factors (UPF1, UPF2, UPF3B, SMG5, SMG6. SMG10) and the E3 ligase CNOT4. DDX6, a known suppressor of NMD, which causes a lower RFP to GFP ratio, is shown in purple. (C) Volcano plot as in (B) from the ge-nome-wide CRISPR knock-out screen sorted at an early time point, prior to essential gene drop out. In purple are highlighted factors that cause a decrease in RFP relative to GFP. These include genes involved in mRNA decapping (PNRC1, CMTR1, DCP1A, and DCP2), DDX6, and the 5'-3' exonuclease XRN1. (D) As in (C) but from day 12. In blue are shown known NMD factors (CASC3, SMG6, UPF3B) and the E3 ligase CNOT4.

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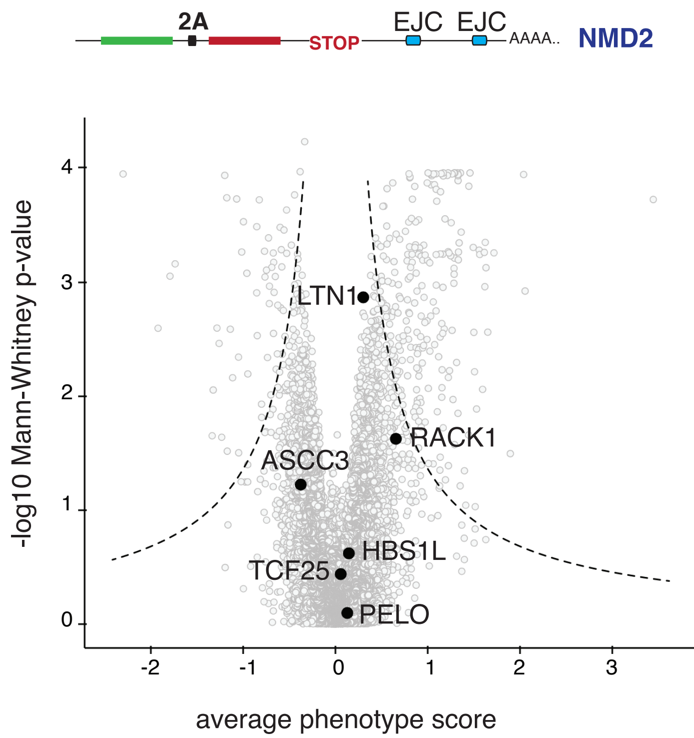


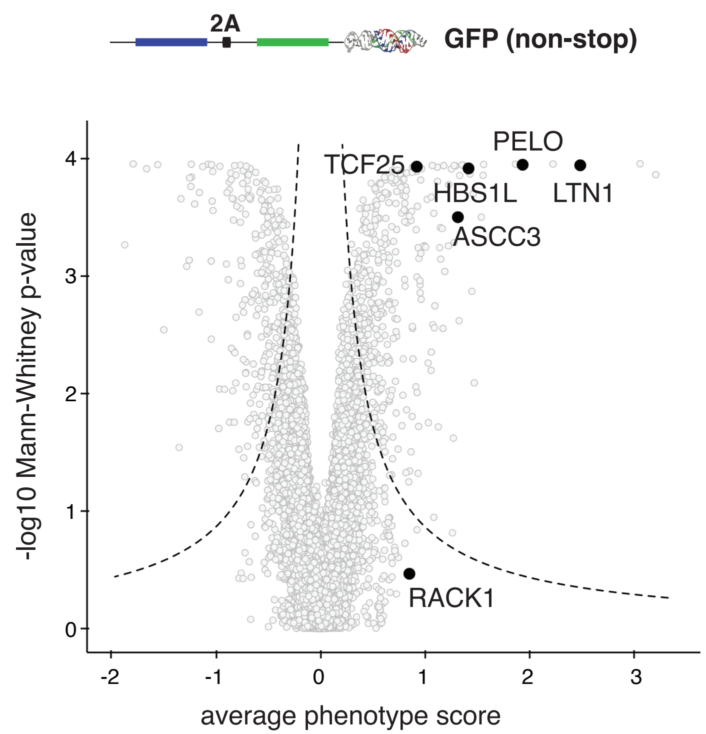


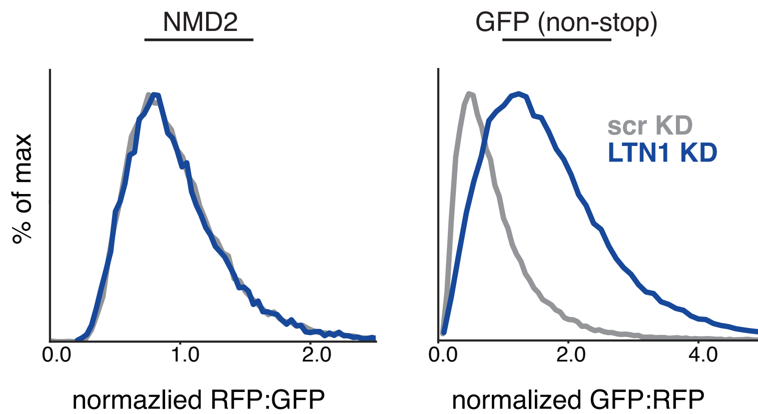


**Figure 4. NMD-linked protein degradation is not mediated by the canonical RQC pathway.**
(A) Volcano plot of the NMD2 reporter CRISPRi screen as in Fig 3A. Highlighted in black are factors involved in the canonical RQC. (B) For comparison, RQC factors are highlighted in black on a volcano plot for a earlier CRISPRi screen using a non-stop reporter conducted using identical conditions as in (A) (Hickey et al., 2020). (C) K562 cells containing CRISPRi machinery and either an inducible NMD2 reporter or a constitutively expressed bidirectional GFP non-stop reporter (as in Hickey, 2020) were infected with a sgRNA targeting the E3 ligase LTN1. The RFP to GFP ratio for NMD2, and the GFP to RFP ratio for the GFP non-stop reporter as determined by flow cytometry are displaced as a histogram.

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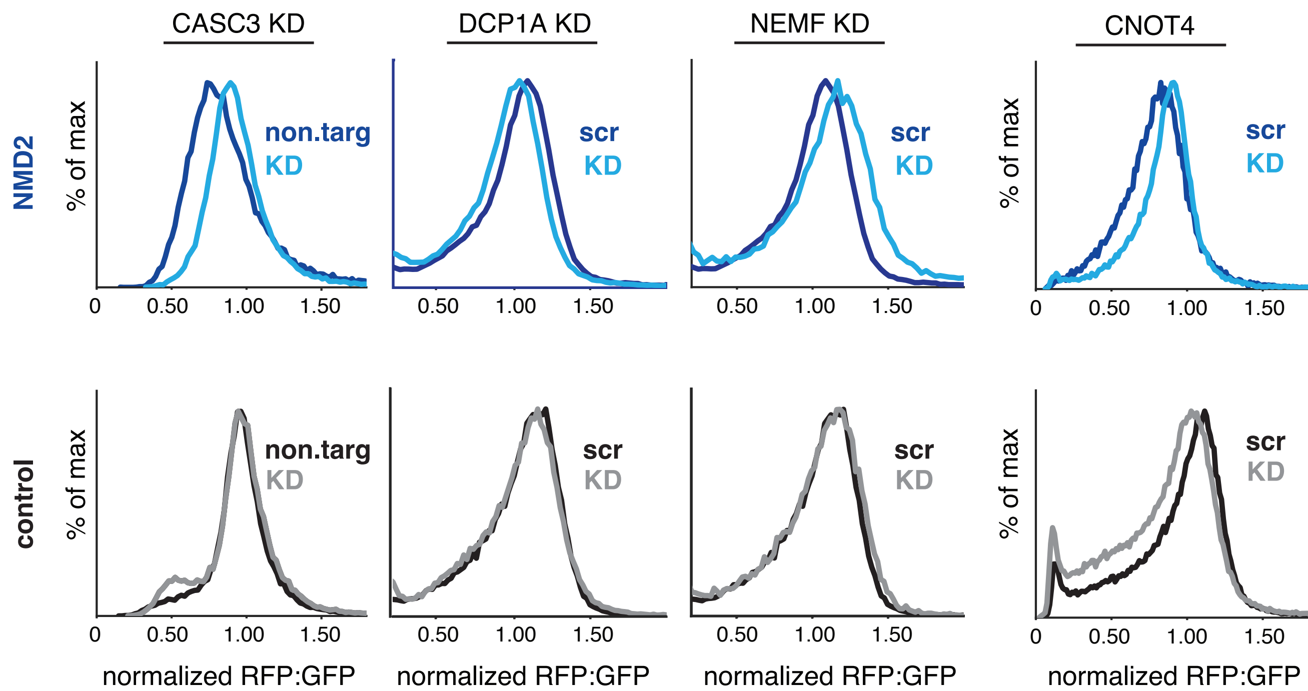


**Figure 5. Validation of factors involved in NMD-coupled protein quality control.**
Panel of validated hits from NMD2 screens. Factors of interest were depleted by either sgRNA in K562 cells, or by siRNA in HEK293T cells as indicated. Displayed are the RFP:GFP ratio for the NMD2 (top) and control (bottom) reporters as determined by flow cytometry. Data was normalized to the scrambled siRNA (scr) or the non-targeting guide controls (non.targ).

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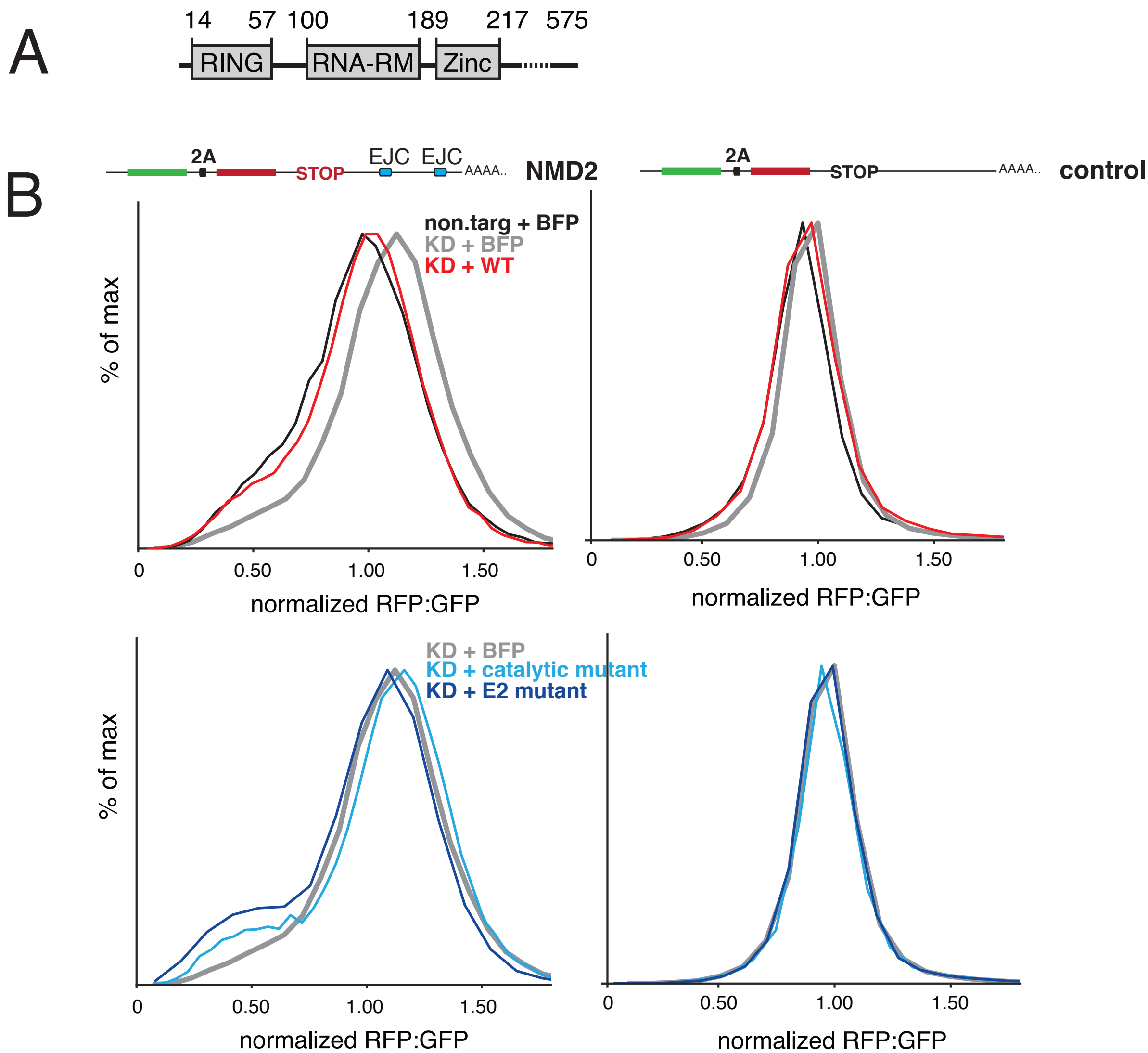


**Figure 6. A putative role for CNOT4 in NMD-coupled protein quality control.**
(A) Schematic of the E3 ubiquitin ligase CNOT4 domain architecture (RNA recognition motif=RNA-RM). (B) CNOT4 depletion was rescued by expression of either wild type or two CNOT4 mutants: (i) a catalytic mutant (C14A, C17A), predicted to disrupt the folding of the CNOT4 RING domain; or (ii) an E2 mutant (L16A, C17A, C33R) that disrupts binding of CNOT4 to its cognate E2 (Albert et al., 2002). BFP was co-expressed from the rescue plasmids to allow identification of cells expressing the CNOT4 wt or mutant proteins. Displayed is a histogram of RFP:GFP fluorescence of the NMD2 and control reporters in the indicated conditions in comparison to a mock control expressing BFP alone. For comparison, data for KD+BFP is displayed in both the top and bottom histograms.

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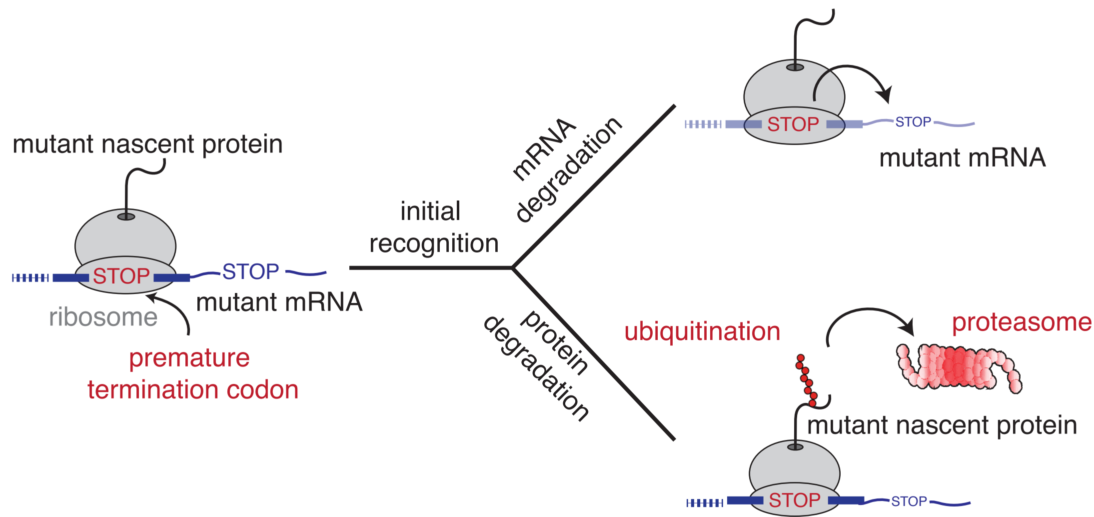


**Figure 7. Model for NMD-coupled protein quality control.**
When the ribosome reaches the stop codon, NMD substrates are recognized in a context-dependent manner. These early recognition steps initiate two parallel pathways that rely on distinct suites of factors to concomitantly degrade the mRNA and nascent protein. We postulate that NMD-coupled quality control results in ubiquitination of the nascent protein prior to its release from the ribosome where it subsequently degraded by the proteasome.