The stability and quality of metazoan mRNAs are under microRNA (miRNA)-mediated and nonsense-mediated control. Although UPF1, a core mediator of nonsense-mediated mRNA decay (NMD), mediates the decay of target mRNA in a 3′UTR-length-dependent manner, the detailed mechanism remains unclear. Here, we suggest that 3′UTR-length-dependent mRNA decay is not mediated by nonsense mRNAs but rather by miRNAs that downregulate target mRNAs via Ago-associated UPF1/SMG7. Global analyses of mRNAs in response to UPF1 RNA interference in miRNA-deficient cells reveal that 3′UTR-length-dependent mRNA decay by UPF1 requires canonical miRNA targeting. The destabilization of miRNA targets is accomplished by the combination of Ago2 and UPF1/SMG7, which may recruit the CCR4-NOT deadenylase complex. Indeed, loss of the SMG7-deadenylase complex interaction increases the levels of transcripts regulated by UPF1-SMG7. This UPF1/SMG7-dependent miRNA-mediated mRNA decay pathway may enable miRNA targeting to become more predictable and expand the miRNA-mRNA regulatory network.
The 3′ untranslated regions (UTRs) of messenger RNAs (mRNAs) are core noncoding structures that control the stability, localization, and translation of mature mRNAs at the posttranscriptional level. The 3′ UTRs of tissue-specific genes vary in length across different tissues more than those of housekeeping genes. Changes made to 3′ UTRs by alternative polyadenylation often lead to the inclusion and exclusion of cis-acting elements that interact with diverse RNA-binding proteins (RBPs) on 3′ UTRs, thus altering the stability, localization, and translational regulation of corresponding mRNAs.

The most widely studied cis-acting elements in the 3′ UTRs, which are microRNA (miRNA) recognition elements (MREs) that interact with miRNA-loaded Argonaute (Ago), regulate the stability and translation of target mRNAs. 3′ UTR shortening by alternative polyadenylation excludes MREs, resulting in the avoidance of miRNA targeting; however, 3′ UTR lengthening leads to the inclusion of more MREs, resulting in further downregulation of target genes. The global shortening of 3′ UTRs has been observed in highly proliferating cells, activated T cells, and cancer cells, and the corresponding mRNAs are generally derepressed by avoiding miRNA targeting. In contrast, globally progressive lengthening of 3′ UTRs is observed during brain development, and extended 3′ UTRs embed thousands of conserved MREs in mammals, strengthening miRNA-mediated gene regulation.

In addition to these cis-acting element-specific mechanisms, lengthening of the 3′ UTR may influence the stability of mRNAs through the nonsense-mediated mRNA decay (NMD) pathway. NMD is typically initiated by an aberrant stop codon at least 50–55 nt upstream of the last exon junction, known as a premature stop codon (PTC). Because this process is transduced by the exon junction complex (EJC) downstream of the PTC, it is also known as EJC-dependent NMD. EJC-independent NMD has also been reported to destabilize some mRNAs with a normal stop codon by modulating the distance between the poly(A)-binding protein (PABP) complex and the stop codon. Hence, the lengthening of 3′ UTRs can either trigger EJC-dependent NMD by introducing downstream exon junctions (deJEs) into the extended region, changing a normal stop codon to a PTC, or inducing EJC-independent NMD. EJC-dependent NMD targets are generally decayed through three distinct UPF1-dependent pathways after PTC recognition: (i) SMG6-mediated endoribonuclease cleavage near the PTC, (ii) SMG5-mediated decapping, and (iii) SMG7-mediated deadenylation of targets. However, the detailed mechanism of EJC-independent NMD in the regulation of long 3′ UTR transcripts remains unknown.

Previous studies have shown that UPF1 in association with 3′ UTRs senses the length of 3′ UTRs to potentiate mRNA decay. Global analysis of UPF1-dependent mRNAs (UMD) using UPF1 RNA interference (RNAi) and cross-linking immunoprecipitation (IP) followed by sequencing (CLIP-seq) has revealed that thousands of mRNAs are modulated in response to UPF1 knockdown and that hundreds of mRNAs with no annotated deJEs respond to UPF1 knockdown as the length of the 3′ UTR increases. Motif analysis in the 3′ UTR of target mRNAs revealed a high-GC context with a CUG-rich motif, which is required for UPF1-dependent mRNA decay. This 3′ UTR length-dependent regulation of UMD may expand the range of post-transcriptional gene regulation, although the specific mechanism and whether the regulation occurs through EJC-dependent or EJC-independent NMD are largely unclear.

In this study, we demonstrate that the 3′ UTR length-dependent regulation of UPF1-dependent mRNA decay occurs through EJC-independent but miRNA-dependent regulation. This UPF1- and miRNA-dependent mRNA decay represses Ago2-UPF1-binding targets, which do not require TNRC6, suggesting that this mRNA decay pathway can be considered an alternative miRNA targeting pathway.

**Results**

**UPF1-dependent, EJC-independent mRNA decay.** Although early studies have reported that a considerable fraction of mRNAs responsive to UPF1 depletion are dependent on the 3′ UTR length, the question of whether UPF1-responsive mRNAs are degraded in an EJC-independent manner has repeatedly been asked. To address this unanswered question, we sought to filter EJC-dependent NMD target genes out and reanalyze the genes remaining (considered deJ-free genes) to determine whether they continued to exhibit responses in UPF1-depleted cells (Fig. 1a; for additional details, see “Filtration of EJC-dependent NMD targets” in the Methods section). For deJ-free genes, the changes in the expression of the genes binned based on their 3′ UTR lengths were reinvestigated using publicly available RNA-seq data from UPF1-depleted human cell lines and mES cells. If a gene had multiple transcripts (mRNAs), the transcript with the longest 3′ UTR was selected as the representative mRNA of the gene to measure the 3′ UTR length. The bin sizes of the 3′ UTR lengths were chosen to include an equal number of genes in each bin. Of the genes, 48.0–53.4% for HeLa cells, 37.1% for K562 cells, and 42.0% for mES cells were derepressed by a greater than log2 fold-change (0.2) in response to UPF1 knockdown (Supplementary Table 1). As a result, the 3′ UTR-length-dependent expression changes in response to UPF1 knockdown remained significant in mES, HeLa, and K562 cells (Fig. 1b, c and Supplementary Fig. 1a, b) and were not dependent on the bin sizes of the 3′ UTR lengths (Supplementary Fig. 1c).

To confirm the results in our system, RNA-seq experiments in siRNA for UPF1 (siUPF1)- and control siRNA (siControl)-treated HeLa cells were performed. Using the RNA-seq data and the pipeline shown in Fig. 1a, we compiled 7117 deJ-free genes, 3419 (48.0%) of which were derepressed by a greater than log2 fold change (0.2) upon the downregulation of UPF1 (Supplementary Table 1), while 18.3% were downregulated by a less than log2 fold change (~0.2). The 3′ UTR length-dependent regulation of genes was similar in our experiment (Fig. 1d). Taken together, these results indicate that UMD does not result from the EJC-dependent NMD pathway but rather from the EJC-independent NMD pathway in mammals.

The 3′ UTRs of UMD targets commonly contain MREs with CUG-rich content. The lengthening or shortening of mRNA 3′ UTRs allows corresponding transcripts to contain more or less cis-acting elements by chance, leading to further regulation. Thus, we hypothesized that AU-rich elements (AREs; AUUA), GU-rich elements (GReS; UGUUUGUUGU), and/or MREs are involved in regulating UMD targets, as they are comparatively more prevalent than others in the 3′ UTRs. To identify the probable cis-acting elements, motif-enrichment analysis was performed with all 7-mers of the 3′ UTR sequences of 3419 UMD targets showing a log, fold-change ≥ 0.2 against dinucleotide-shuffled 3′ UTR sequences (P < 0.005; Fisher’s exact test). Highly ranked 7-mers generally showed a high GC content (Fig. 2a). GC-rich sequences tend to contain more stable secondary structures, which are preferred binding sites of UPF1 (Supplementary Fig. 2a). The UMD targets contained significantly more CUG motifs and extended 7-mers, CUG[AG][AG][AG] (Fig. 2a), as previously described.

Among the significantly enriched 7-mers, 15.2% were 7-mer MREs of 682 human miRNA families (Supplementary Data 1), with significantly higher levels than there would be by chance
UMD requires miRNA-mediated gene regulation. The enrichment of CUG triplet nucleotides in miRNA 7-mer sites raised the question of whether UMD is related to miRNA-mediated gene regulation. Changes in the expression of the dEJ-free miRNAs with 7-mer sites of the 50 most abundant miRNA families in HeLa and mES cells (Supplementary Table 2) were measured between siUPF1-transfected and siControl-transfected cells and compared to those of 3′UTR-length-controlled genes with random 7-mer sites (Supplementary Fig. 3a; see “Generation of random controls and statistical test” in the Methods section for more details). The miRNA targets with 7-mer sites were significantly more derepressed than were controls in both public and private datasets (Supplementary Fig. 3b; see “Generation of random controls and statistical test” in the Methods section for more details). The miRNA targets with 7-mer sites were significantly more derepressed than were controls in both public and private datasets (Supplementary Fig. 3b; see “Generation of random controls and statistical test” in the Methods section for more details). The miRNA targets with 7-mer sites were significantly more derepressed than were controls in both public and private datasets (Supplementary Fig. 3b; see “Generation of random controls and statistical test” in the Methods section for more details).
our RNA-seq data (Fig. 3a; P ≤ 0.014 for all three; one sample t test), although the level of miRNA families was not significantly changed in siUPF1-transfected cells (Supplementary Fig. 3b). Even for the 10 and 30 most abundant miRNA families in HeLa cells, the targets containing the sites were significantly more derepressed than in the controls (Fig. 3a), suggesting that miRNA-mediated gene regulation is likely involved in UMD.

We then examined whether the derepression of UMD targets in response to UPF1 knockdown was diminished in the absence of miRNAs. HeLa cells were transfected with siUPFI, siRNA for Dicer1 (siDicer1), siUPFI/siDicer1, or siControl, and the level of UMD targets was assessed by RNA-seq. Western blot analysis indicated that UPF1 and Dicer1 were efficiently downregulated compared to the endogenous protein Calnexin (Fig. 3b). Indeed, the depletion (Supplementary Fig. 3c) or knockout38,39 of Dicer1 led to the global downregulation of miRNAs, resulting in the derepression of miRNA targets (Supplementary Fig. 3d)38,40. Changes in the expression of deJ-free genes in siUPF1-treated cells were compared to those in siControl-treated cells with a Dicer1-depleted background, and the dysregulation of UMD targets and their 3′ UTR length-dependent repression disappeared in cells with the Dicer1-depleted background (Fig. 3c, d).

We next predicted that the number of MREs could explain the effect of the UMD targets according to their 3′ UTR lengths under UPF1-depleted conditions because the 7-mer sites of miRNAs were increased by chance as the 3′ UTR lengths under UPF1-depleted conditions were longer than controls (Fig. 3a). To test this hypothesis, changes in the expression of deJ-free genes with different numbers of 7-mer sites were compared to controls with random sites. 3′ UTR length-dependent regulation resulted from the increase in MREs (Fig. 3f and Supplementary Fig. 3e, f), which depended on the presence of Dicer1 (Fig. 3g), suggesting that UMD requires miRNA targeting. In fact, the derepression of UMD targets by UPF1 depletion was explained by a computational model (wContext + + model of TargetScan 7.041) of miRNA targeting (Supplementary Fig. 3g, h, i).

UMD is activated via miRNA seed-type targeting. Because of the requirement for miRNA targeting in UMD, we evaluated how UPF1 and Ago2 function together to determine the fate of
mRNAs by analyzing public UPF1 and Ago2 CLIP-seq data\(^{42,43}\). As previously reported\(^{31}\), UPF1- and Ago2-binding sites significantly overlapped compared to randomized controls in the 3′ UTR (Supplementary Fig. 4a, b; P < 5.36 x 10\(^{-6}\) for HeLa cells; P < 9.52 x 10\(^{-7}\) for mES cells; Fisher’s exact test). They appeared to be proximally colocalized with each other (Supplementary Fig. 4c, d). To examine whether colocalization of Ago2 and UPF1 on miRNA target sites is necessary for miRNA-dependent UMD, we analyzed changes in expression of the targets with Ago2-UPF1 overlapping sites. The changes in the expression of the targets with an Ago2-UPF1 overlapping site (for the 50 most abundant miRNAs) were marginally greater than those of the targets with a single Ago2 site (Supplementary Fig. 4c; P = 0.07; Wilcoxon’s rank-sum test).

To experimentally validate the effect of the Ago2–UPF1 interaction on the UMD targets, we first examined miR-24-3p targets with 7-mer sites because miR-24-3p was found to be one of the ten most abundant miRNA families and their 7-mer sites were significantly enriched in the UMD targets (Fig. 2a). Of the miR-24-3p targets upregulated (a log\(_2\) fold-change greater than 0.3) in UPF1-knockdown cells compared to control cells and greater than those in Dicer1-depleted cells (yellow-colored shades in Fig. 4a), 78 with a wContext + + score less than –0.3 were considered confident miRNA-dependent UMD targets (green-colored circles in Fig. 4a). Among the 78 targets, nine UPF1-dependent miR-24-3p targets (DNAL4, ATAD2B, RPS19BP1, PEA15, CRAT, ELL, PAK4, DBNDD1, and ZNF740) were selected using the following criteria in order of priority: (1) targets more derepressed in UPF1-knockdown cells than in control cells; (2) targets with miR-24-3p 8-mer or 7m8 sites but no sites for the other nine most abundant miRNAs; and (3) a representative target in each 3′ UTR length bin. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) using the lysate of HeLa cells transfected with siUPF1 was carried out for the nine selected targets, eight of which were consistently derepressed in the cells (Fig. 4b). We then examined whether the selected targets were actual miRNA targets via 7-mer site libraries, and thus the targets are likely to be actual miRNA targets

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**Fig. 3** UMD regulation requires miRNAs. **a** The mean changes in expression (log\(_2\) scale) of dEJ-free mRNAs embedding 7-mer target sites of random controls (gray), the 10 (blue), 30 (purple), and 50 (red) most abundant miRNA families under siUPF1-treated conditions are shown. The P value was calculated by comparing the mean change in the expression of the targets to those in the 1000 random control cohorts (see “Generation of random controls and statistical tests”) using a one sample t test. The same analyses were performed for our data (left), Wang et al.’s data (middle), and Hurt et al.’s data (right). The error bars indicate the s.e.m. for the changes for the targets. **b** HeLa cells were transfected with siUPF1, siDicer1, or both siUPF1 and siDicer1. The downregulation of endogenous UPF1 and/or Dicer1 was confirmed by western blotting. Calnexin served as a loading control. **c** CDF graphs of changes in the expression of dEJ-free mRNAs between siUPF1-treated and siControl-treated cells under Dicer1-depleted conditions are shown across different 3′ UTR length bins. Otherwise, as in **b**. **d** Mean changes in the expression of dEJ-free mRNAs with 7-mer target sites between siUPF1-treated and siControl-treated cells under Dicer1-depleted conditions. Otherwise, as in **a**. **e** Mean numbers of 7-mer miRNA target sites in the 3′ UTRs of dEJ-free miRNAs are shown across different 3′ UTR length bins. The sites were detected in the major 3′ UTR isofrom, as evident by our previous 3′-seq data profile from HeLa (green) and mES (orange) cells\(^{11}\). **f** Mean changes in expression of dEJ-free mRNAs in siUPF1-treated cells against siControl-treated cells are shown across the different 7-mer site numbers of the ten most abundant miRNA families (red) and different random 7-mer sites (gray). Asterisks denote groups whose number of sites significantly different from that of the controls (’’’P < 0.001; K-S test). **g** Mean changes in expression between dEJ-free mRNAs of siUPF1-treated and siControl-treated cells under Dicer1-depleted conditions are shown for the different 7-mer site numbers. Otherwise, as in **f**
effects of the inhibitor, suggesting that decay of the targets was regulated through the 7-mer site of miR-24-3p (Fig. 4d). To verify that UMD is activated via miR-24-3p-mediated targeting, we examined the effects of UPF1 knockdown under miR-24-3p-depleted conditions (Fig. 4e). RT-qPCR results demonstrated that changes in the expression of miR-24-3p targets, except for CRAT mRNA, showed greater derepression in the presence of miR-24-3p (treated with miR-inhibitor-control) than in the absence of miR-24-3p (treated with miR-24-3p inhibitor) in response to UPF1 knockdown (Fig. 4e; Supplementary Fig. 4f); this is presumably because impaired miRNA targeting diminishes the effect of UMD. The above results were also observed for another miRNA, miR-26a lacking a CAG motif, one of the ten most abundant miRNA families, as depicted in Fig. 2 (Supplementary Fig. 4g–k). Taken together, our findings suggest that UMD functions via miRNA seed-type targeting.

**UMD functions through the CUG motif on MREs.** To assess the role of CUG motifs in the miRNA-mediated silencing of UMD targets, changes in the expression of deJi-free genes with 7-mer sites harboring the CUG motif of the 50 most abundant miRNA families were compared to those of genes with neither 7-mer sites nor CUG motifs, and to those with both but not overlapping in their 3′UTRs. Although targets containing only the CUG motif were slightly derepressed
compared to those with neither sites nor CUG motifs (Fig. 5a; $P < 0.02$; K–S test), expression of UMD target genes with 7-mer sites with an embedded CUG motif responded more significantly than did those with only a CUG motif (Fig. 5a; $P < 4.95 \times 10^{-15}$; K–S test) or those with both in the 3′ UTRs (not CUG-embedded) with UPF1 knockdown ($P < 1.27 \times 10^{-8}$; K–S test). However, the effects of CUG embedded in 7-mer sites disappeared under Dicer1-depleted conditions (Fig. 5b). To validate the effect of UPF1-binding CUG motifs embedded in 7-mer sites of UMD targets, we chose two transcripts, PEA15
we mutated the 7-mer site with the remaining CUG (CUG possibility of the UPF1-independent function via the CUG motif, 
miR-24-3p, the UPF1-independent function via the CUG motif 
depends on the CUG motif embedded in the 7-mer site of miR-
recovered by downregulation of UPF1, indicating that UMD 
reduced the level of Mut-A reporter transcripts, which were not 
tary Fig. 5, bottom) and downregulating UPF1 (Fig. 5h) 
but not to WT reporter transcripts, was introduced in the 
mimic, which is capable of binding to Mut-A reporter transcripts 
CUG motif embedded in the 7-mer site, the miR-24-3p mutant 
binding sites in the 3′ region was mutated to UCA (Mut-A), which prevents miR-24-3p 
from binding to the 3′ region of the reporter constructs. As 
expected, the miR-24-3p pseudo-mimic reduced the levels of 
wild-type (WT) reporter transcripts but not those of mutant 
(Mut-A) transcripts (Fig. 5f; Supplementary Fig. 5, top). In 
contrast, introducing a miR-24-3p inhibitor (Fig. 5g; Supplementary 
Fig. 5, bottom) and downregulating UPF1 (Fig. 5h) 
derpressed the levels of WT reporter transcripts but not those of 
muts of Mut-A (Mut-A). To examine whether UMD depends on 
the CUG motif embedded in the 7-mer site, the miR-24-3p mutant mimic, which is capable of binding to Mut-A reporter transcripts 
but not to WT reporter transcripts, was introduced in the presence or absence of siUPF1 (Fig. 5i). The mutant mimic reduced the level of Mut-A reporter transcripts, which were not 
recovered by downregulation of UPF1, indicating that UMD 
depends on the CUG motif embedded in the 7-mer site of miR-
24-3p. Because a miRNA inhibitor may not completely remove 
miR-24-3p, the UPF1-independent function via the CUG motif might also explain our RT-qPCR results. To exclude the 
possibility of the UPF1-independent function via the CUG motif, we 
mutated the 7-mer site with the remaining CUG (CUGAGCC 
to CUGCCAG) in the reporter constructs (Mut-B). The level of the 
Mut-B transcripts was not affected by downregulating UPF1 
(Fig. 5j), suggesting that UMD triggers mRNA decay via miRNA-
mediated gene regulation in a CUG-motif-dependent manner 
and that the cooperative roles of Ago2 and UPF1 may be 
necessary for UMD.

UPF1 helicase activity, SMG6, and SMG5 are not related to 
UMD. We next asked how the Ago2–UPF1 axis mediates UMD. 
UPF1 functions as an indispensable helicase for NMD and 
removes NMD complexes after the cleavage of mRNA by 
SMG6[46,47]. Based on this, we hypothesized that helicase activity 
could enhance miRNA targeting by either increasing the target 
accessibility of structured sites in the 3′ UTR or by cleaving 
mRNA targets with SMG6. To test this hypothesis, we ectopically 
expressed a UPF1 helicase mutant (R843C) in HeLa cells after 
downregulating endogenous UPF1 by RNAi. Western blot anal-
ysis indicated that the expression levels of UPF1-WT and UPF1-
R843C were comparable, while that of endogenous UPF1 was low 
after UPF1-WT or UPF1-R843C transfection (Supplementary 
Fig. 6a). RNA-seq of WT and UPF1-R843C mutant HeLa cells 
was performed, and changes in expression of dEJ-free genes were 
examined. In addition, the changes in expression were examined 
using publicly available RNA-seq data from mES cells treated 
with siRNA for SMG6 (siSMG6) [48]. Changes in expression, 
however, were not observed in mRNAs with different 3′ UTR 
lengths in cells expressing UPF1-R843C (Supplementary Fig. 6b) 
or in siSMG6-treated mES cells (Supplementary Fig. 6c), indicat-
ing that the miRNA-mediated mRNA decay enhanced by 
UPF1 resulted from neither improved target accessibility nor the 
direct cleavage of targets by SMG6.

Phosphorylated UPF1 also interacts with SMG5 and SMG7, 
which control the deadenylation and decapping of NMD targets, 
respectively[28,49]. We thus examined whether SMG5 and/or SMG7 
are involved in UMD. For this, we examined changes in dEJ-free 
gene expression depending on their 3′ UTR length using publicly 
available microarray data profiled from HeLa cells treated with 
siRNA for SMG5 (siSMG5) or siRNA for SMG7 (siSMG7) [26]; 
siSMG5 treatment did not affect expression of these genes 
(Supplementary Fig. 6d), whereas the results of siSMG7 
resembled those of siUPF1 (Fig. 6a). The results of siSMG7 were 
also confirmed in other publicly available RNA-seq data for 
SMG7-depleted cell lines [50], for which different siSMG7 sequences 
were employed (Supplementary Fig. 6e).

SMG7 is considerably responsible for UMD. To further examine the relationship between SMG7 and UMD targeting, we 
performed RNA-seq with total RNAs isolated from the lysates of 
scrambled siRNA-treated and siSMG7-treated HeLa cells and 
observed gradual derepression according to 3′ UTR length (Fig. 6b); 
(P < 0.258 × 10−12, K-S test) and significantly enriched 7-
mer MREs and CUG-embedded motifs in the 3′ UTRs of SMG7-
dependent targets (Fig. 6c), similar to UPF1-dependent targets. 
Analysis of UMD targets with CUG-embedded 7-mer sites (Fig. 6b) 
or significant 7-mer sites (Supplementary Fig. 6f) revealed that the changes in expression were explained mainly by 
targeting the ten most abundant miRNA families (Fig. 6d) and 
correlated with the number of 7-mer sites (Fig. 6e), although the 
magnitude of the changes in expression was lower than that 
under UPF1 knockdown. In fact, the change in expression of the 
UMD targets with significant 7-mer sites in siSMG7-treated cells 
reached approximately 40% of that in siUPF1-treated cells 
(Figs. 3a, 6d, and Supplementary Fig. 6g).

To experimentally examine the functional interaction of UPF1 
and SMG7 in UMD, we first evaluated miR-24-3p targets with 7-
mer sites, which were derepressed in both siUPF1- and siSMG7-
treated cells. Of the 1488 miR-24-3p targets with 7-mer sites in 
their 3′ UTRs, 301 were depressed by more than a 0.2 log2 fold-
change in siSMG7-treated cells. Indeed, 187 of the targets were 
UPF1- and SMG7-dependent miR-24-3p targets. Of the nine 
UPF1-dependent miR-24-3p targets depicted in Fig. 4, eight 
were UPF1- and SMG7-dependent miR-24-3p targets, and the 
remaining one (ZNF740) was the only UPF1-dependent miR-24-
3p target (Fig. 6f). To verify these candidates, the levels of 
candidates, including the SMG7-dependent miR-24-3p target 
HPCAL1, upon the depletion of UPF1 and/or SMG7 were 
(1882 nt of 3′ UTR) and RPS19BP1 (395 nt of 3′ UTR), which 
contain putative miR-24-3p-binding site(s), and showed a greater 
response to UPF1 depletion (Fig. 5c, d). The putative miR-24-3p-
binding sites in the 3′ UTR were inserted into the reporter 
constructs (Fig. 5e). Furthermore, the CUG motif in the seed 
region was mutated to UCA (Mut-A), which prevents miR-24-3p 
from binding to the 3′ UTR of the reporter constructs. As 
expected, the miR-24-3p pseudo-mimic reduced the levels of 
mut-A (Mut-A) transcripts (Fig. 5f; Supplementary Fig. 5, top). In 
contrast, introducing a miR-24-3p inhibitor (Fig. 5g; Supplementary 
Fig. 5, bottom) and downregulating UPF1 (Fig. 5h) 
derpressed the levels of WT reporter transcripts but not those of 
muts of Mut-A (Mut-A). To examine whether UMD depends on 
the CUG motif embedded in the 7-mer site, the miR-24-3p mutant mimic, which is capable of binding to Mut-A reporter transcripts 
but not to WT reporter transcripts, was introduced in the presence or absence of siUPF1 (Fig. 5i). The mutant mimic reduced the level of Mut-A reporter transcripts, which were not 
recovered by downregulation of UPF1, indicating that UMD 
depends on the CUG motif embedded in the 7-mer site of miR-
24-3p. Because a miRNA inhibitor may not completely remove 
miR-24-3p, the UPF1-independent function via the CUG motif might also explain our RT-qPCR results. To exclude the 
possibility of the UPF1-independent function via the CUG motif, we 
mutated the 7-mer site with the remaining CUG (CUGAGCC 
to CUGCCAG) in the reporter constructs (Mut-B). The level of the 
Mut-B transcripts was not affected by downregulating UPF1 
(Fig. 5j), suggesting that UMD triggers mRNA decay via miRNA-
mediated gene regulation in a CUG-motif-dependent manner 
and that the cooperative roles of Ago2 and UPF1 may be 
necessary for UMD.
were calculated from independent experiments. (* replicate experiments was
SMG7 in HeLa cells were depleted by siRNA-mediated downregulation. RT-qPCR was performed to examine the ef-
mRNA was normalized to that of
each transcript.
selected miRNA-dependent UMD target candidates in Fig.4a and nine SMG7-dependent targets. The values in parentheses are the lengths of the 3
Fig. 3a.
Mean changes in expression of a random control (gray), dEJ-free mRNAs with 7-mer sites (blue), targets with CUG-embedded 7-mer sites (green), or
mRNAs (solid lines) and targets with CUG-embedded 7-mer sites of the 50 most abundant miRNA families (dotted lines) in siSMG7-treated and
Targets with CUG-embedded 7-mer sites
CDFs for changes in the expression of dEJ-free mRNAs in microarray data prepared
from siSMG7-treated and siControl-treated cells are shown. Otherwise, as in Fig.1b
Mean changes in expression of dEJ-free mRNAs in SMG7-knockdown versus control cells are shown over different numbers of miRNA sites (red)
...nificant differences (** P<0.01; *** P<0.001; K-S test). Otherwise, as in Fig. 3f. f Venn diagram of nine selected miRNA-dependent UMD target candidates in Fig. 4a and nine SMG7-dependent targets. The values in parentheses are the lengths of the 3'UTR in each transcript. “No site” indicates that the mRNAs did not contain any putative miRNA sites of the 50 most abundant miRNAs. g Endogenous UPF1 and SMG7 in HeLa cells were depleted by siRNA-mediated downregulation. RT-qPCR was performed to examine the efficiency of downregulation. The level of
mRNA was normalized to that of GAPDH mRNA. h As in g, however, the levels of transcripts listed in f were examined by RT-qPCR. Mean values and s.e.m. were calculated from independent experiments. (* P<0.05; unpaired Student’s t test); ns not significant. The minimum number of independent biological replicate experiments was n = 9 in g, h.

quantified by RT-qPCR. The levels of endogenous UPF1 mRNA and SMG7 mRNA were effectively downregulated to approxi-
mately 30% (Fig. 6g). RT-qPCR of SMG7-depleted HeLa cell lysates indicated that eight SMG7-dependent miR-24-3p targets (including HPCAL1) were consistently derepressed in nine
candidate genes (Fig. 6h). These results were verified by miR-
26a targets, indicating that the levels of FAM98A and HTATIP2 mRNA increased upon SMG7 depletion (Supplementary Fig. 6h).
Considering these findings, SMG7 may play a role in miRNA-
mediated UMD.
UPF1-SMG7 forms a complex with Ago2. To determine whether the UPF1-SMG7 complex is associated with the Ago2 complex, HeLa cells were transiently transfected with FLAG-Ago2 or empty vector containing FLAG-tag. Cell lysates were subjected to IP with anti-FLAG antibodies in the presence or absence of RNaseA (Fig. 7a). Western blot analysis of the IP inputs and eluates indicated that FLAG-Ago2 was efficiently and comparably immunoprecipitated by anti-FLAG antibodies. The amounts of Dicer1 that coimmunoprecipitated with FLAG-Ago2 were comparable, whereas coimmunoprecipitated PABP was observed only in the absence of RNaseA treatment, indicating that RNA was efficiently degraded. As expected, UPF1 and SMG7 coimmunoprecipitated with FLAG-Ago2 in a partially RNA-independent manner, suggesting that UPF1-SMG7 could form a complex with Ago2. To determine whether SMG7 joins the Ago2 complex depending on UPF1, HeLa cells were transiently
transfected with FLAG-Ago2 one day after treatment with siControl or siUPF1 (Fig. 7b). UPF1 was efficiently downregulated, while other proteins were unchanged by transfection with siUPF1. In contrast with the co-IP of SMG7 with FLAG-Ago2 in the presence of UPF1, the level of SMG7 was not detectable with FLAG-Ago2 in UPF1-depleted cells, indicating that SMG7 forms a complex with Ago2 by interacting with UPF1. Furthermore, NOT1 and NOT3, which are components of the deadenylase complex, were communoprecipitated with FLAG-Ago2, and the amounts of coimmunoprecipitated NOT1 and NOT3 were reduced by the downregulation of UPF1. To show whether the interaction of SMG7 and the deadenylase complex is involved in UMD, we employed a FLAG-tagged SMG7 mutant (FLAG-SMG7 ΔPC), which failed to interact with the deadenylase complex28 (Supplementary Fig. 7a). The RT-qPCR results indicated that the loss of SMG7 interaction with the deadenylase complex stabilized the miR-24-3p targets (except ATAD2B) and was regulated by UPF1, but did not stabilize the negative controls (Supplementary Fig. 7b). These observations provide insight into the unknown possible mechanism of UMD, i.e., UPF1/SMG7-dependent miRNA targeting.

UMD targeting appear not to require TNRC6A/C. We then examined whether UPF1/SMG7-dependent miRNA targeting requires the GW182 complex, which interacts with the canonical CCR4/NOT pathway. Thus, we first downregulated two major paralogs of GW182, TNRC6A/C, using siRNA. Western blot analysis showed that endogenous TNRC6A/C were efficiently downregulated (Fig. 7c). The RT-qPCR results indicated that the TNRC6A/C-dependent and UPF1-independent transcripts were upregulated by TNRC6A/C depletion (Fig. 7d). Then, we downregulated UPF1 and/or TNRC6A/C using siRNA (siTNRC6A/C; Supplementary Table 6) and examined changes in the expression of UPF1-SMG7-dependent mir-24-3p targets, a UPF1-only-dependent miR-24-3p target and nontarget compared to control cells transfected with scrambled siRNA (Fig. 7e). Interestingly, no examined genes, including the UPF1-dependent miRNA targets, were significantly changed by the downregulation of TNRC6, except for PEA15 and CRAT, which were slightly increased; these targets were derepressed by UPF1 depletion, except for ATAD2B, CRAT, and PAK4 (Fig. 7e). These results suggest that the UPF1/SMG7-dependent miRNA targeting pathway might function in a TNRC6A/C-independent manner.

To validate whether UPF1-dependent miRNA targets function independently of TNRC6A/C, changes in expression of dEJ-free genes by UPF1 knockdown were compared with those of the control under TNRC6A/C-depleted background conditions. The 3′UTR length dependency of dEJ-free genes was also observed by additional UPF1 knockdown under TNRC6A/C-depleted conditions (Fig. 7f), and changes in expression of UMD targets were mostly attributed to miRNA targeting (Supplementary Fig. 7c). In contrast, 3′UTR length-dependent changes in expression of dEJ-free genes were not observed by downregulating TNRC6A/C in a UPF1-depleted background (Fig. 7g), and the changes in expression of miRNA targets were smaller than those of the targets in a UPF1-knockdown cells with the siTNRC6A/C background (Supplementary Fig. 7c, d). miRNA targets with multiple sites, however, were more derepressed in the absence of TNRC6 as the number of sites increased (Supplementary Fig. 7e, f). RT-qPCR using TNRC6A/C-depleted HeLa cells was performed to quantify the miRNA levels of UPF1-SMG7-dependent miR-26a targets, UPF1-dependent targets, and non-targets compared to those in siControl-transfected cells (Supplementary Fig. 7g). As expected, none of the genes examined, including UMD targets, were significantly changed by the downregulation of TNRC6A/C, except for GREB1L and HUWE1, which were increased slightly (Supplementary Fig. 7g).

In addition, the responses of UPF1/SMG7-dependent miRNA targets with respect to siUPF1 treatment were more effectively explained by the wContext + + score sum of miRNAs in cells with a TNRC6A/C-depleted background (Fig. 7j), and UPF1/SMG7-dependent miRNA targets had a greater impact by ectopically expressing miRNAs in HeLa cells (Fig. 7i), but not siTNRC6A/C in cells with an UPF1-depleted background (Supplementary Fig. 8). Taken together, these results indicate that UMD targeting occurs through a distinct pathway involving the Ago2/UPF1/S MG7 axis rather than the canonical Ago2-TNRC6 miRNA targeting pathway, and miRNA target prediction can be more predictable through UMD targeting.

Discussion
In this study, we propose a UPF1/SMG7-dependent miRNA-mediated mRNA decay pathway, which appeared to be the major molecular mechanism of UMD, through experimental and computational analyses in mammalian cells. The proposed miRNA targeting pathway indicates the presence of an alternative regulatory layer in miRNA-mediated gene silencing. UPF1-dependent miRNA targeting via the CUG motif may explain the evolutionarily conserved relationship between miRNA-mediated mRNA decay and the NMD pathway.

Although the series of RNAi experiments for UPF1 and SMG7 supported the existence of UMD targeting, the results of our RNAi experiments could suffer from the issue of off-target effects. However, even after excluding all off-targets with 6-mer
seed sites, our results and conclusions were not changed (Supplementary Fig. 9a, b), and repeated experiments using antisense oligonucleotides (ASOs), which depend on RNaseH cleavage, confirmed what we observed in our RNAi experiments (Supplementary Fig. 9c, d). These results suggest that our findings are not the result of off-target effects of siRNAs.

Both the candidate experiments and global analysis of gene expression changes revealed that UPF1-dependent miRNA targeting is mediated by the UPF1–SMG7 interaction. Although our IP results suggest interactions between Ago2 and UPF1, we cannot completely exclude the possibility that Ago2 indirectly interacts with UPF1 because the interaction may result from the proximal interaction of two proteins via either undigested RNA or other mediators. In addition, although the Ago2 and SMG7 interaction appears to be dependent on UPF1, the UPF1–SMG7 pathway can partly explain UPF1-dependent miRNA targeting (Fig. 6). Approximately 40% of the mean change in the expression of the miRNA targets (for the 50 most abundant miRNA families) under UPF1-depleted conditions, compared to that of the random control, was mediated by the UPF1–SMG7 pathway (Figs. 3a, 6d). This finding could indicate the presence of a mediator other than SMG7 or technical limitations, including limitations in knockdown efficiency or experimental variations.

CAG triplet nucleotides were overrepresented in the seed region of both highly expressed and low-abundance miRNAs in the cells of humans and other animals, including flies and worms (Fig. 2c and Supplementary Tables 3–5), suggesting that miRNAs nonrandomly possessed CAG triplet nucleotides in the seed regions and that miRNAs embedding the CAG motif were preferentially selected for targeting UMD targets during evolution. This finding also suggests that the UPF1-dependent, TNRC6-independent miRNA targeting pathway via the CUG motif does not occur in certain cell types but is a form of general, evolutionarily conserved gene regulation. Interestingly, the CUG motif was preferentially observed in UPF1-dependent targets and thought to be within the UPF1-binding motif. Because CAG triplet nucleotides in the seed regions of miRNAs are on the inside of Ago2, UPF1, and Ago2 cannot structurally bind to the CUG motif at the same time, suggesting that miRNA-loaded Ago2 transiently binds to UPF1 on the CUG motif in MREs, in turn allowing Ago2 to directly bind to the MREs (Fig. 7). However, it is not clear why UPF1 does not block AGO binding to a site. These questions should be further investigated.

Methods

Data sources. NCBI RefSeq gene annotation (hg19: Aug-22-2011, mm9: Feb-15-2014) was used throughout the analyses. To precisely measure the 3'UTR length and expression level of miRNAs, the end of the 3'UTRs was updated with the major form profile from 3'P-seq of HeLa and mES cells. RNA-seq data for UPF1-knockdown and WT cells were downloaded from GSE63091 and DRR006622 for HeLa cells, from GSE88140 and GSE88083 for K562 cells, and from GSE41785 for mES cells. CLIP-seq data for WT UPF1 in HeLa cells were from GSE47976 and in mES cells were from GSE41785. Raw CLIP-seq data for WT Ago2 of HeLa cells were from GSE43666, and the processed CLIP-seq data of mES cells were from GSE25310. 3P-seq data for HeLa cells and mES cells were from GSE52531. sRNA-seq data for HeLa cells were from GSE22068, and those for mES cells from GSE20384.

Statistical tests. All statistical tests were performed using one-tailed tests in R packages unless otherwise specified. For all CDF and miRNA targeting analyses, K–S tests and one-sample t-tests were performed unless otherwise specified. For enrichment analyses, Fisher's exact tests were carried out.

Filtration of EJC-dependent NMD targets. For this, we first removed known EJC-dependent NMD target genes and all genes that included any transcript with potential PTGs in the annotation (NCBI RefSeq: see “Data Source” in the Methods section for additional details). Because the annotations are far from complete and often lack aberrantly spliced transcripts of a gene, we additionally excluded genes with aberrantly spliced transcripts possibly including dEJ (defined as exon-exon junction after 50 nucleotides downstream of a stop codon) by assembling transcripts from the RNA-seq data of UPFI-depleted cells using our computational pipeline. Filtering of dEJ-free genes and filtering genes with nonproductive splicing forms (see “Methods” in the Section Methods). In addition, we removed all the siRNA off-targets (for UPF1, GFP, and the scrambled siRNAs) that included 7-mer sites in the 3'UTR (see "Filtering siRNA off-targets" in the Methods section for additional details), and the remaining targets were considered dEJ-free genes (7732 for HeLa cells; 8,130 for mouse embryonic stem [mES] cells), which were used in ensuing analyses.

dEJ-free genes. All analyses started with RefSeq genes that transcribe nonspurious miRNAs with 5'UTR ≥ 25 nt, CDS ≥ 200 nt, and 3' UTR ≥ 50 nt. The annotated (known) NMD target genes (if any transcript was an annotated NMD target) were first excluded from the RefSeq genes. All potential EJC-dependent NMD target genes with at least one authentic AGO average putative dEJ (excluding an exon–intron junction signal downstream of a stop codon), were then excluded. dEJs can be generated via a cryptic splicing event in the 3'UTR, as indicated by putative splicing signals (GU-AG with an inner distance greater than 60 nt) or by RNA-seq-supported exon junctions in the 3'UTR, and via nonproductive splicing patterns under UPF1-depleted conditions (see “Filtering genes with nonproductive splicing forms” in the Methods section for more details). Because dEJs might be in unannotated or unassessed transcripts, particularly in NMD-responding RNAs, all potential dEJs in all-annotated and newly assembled transcripts were further examined by identifying putative nonproductive splicing forms in control and UPF1-depleted cells. The NMD classifier20 detected EJC-dependent NMD transcripts with exon inclusion/exclusion or changes in exon start/ends that produced dEJs. Thus, any gene with at least one EJC-dependent NMD-sensitive transcript from a nonproductive splicing form was filtered out. To reduce the random variability in expression fold-changes, genes with fewer than three fragments per million mapped reads in the RNA-seq data of UPF1-knockdown cells were excluded, and to avoid the divide-by-zero error, those showing no expression in control cells were excluded. In the next step, the putative off-targets of siRNAs against UPF1 and GFP (for ref. 35–36) and scrambled siRNA (our experiment) were filtered out (see “Filtering siRNA off-targets” in the Material and Methods section for more details).

Cumulative distribution function (CDF) analysis. For both RNA-seq and microarray data, mRNA expression log fold-changes between siRNA-treated and control cells were converted into z-scores, filtered for genes with nonproductive splicing forms within the shortest 3'UTR bin (50–394 nt). The distribution of the values was transformed to a CDF to evaluate the statistical significance of the difference
among groups from different 3′ UTR bins using R function, ecfv (version 3.1.2). Statistical significance was tested by the K–S test.

**7-mer enrichment analysis.** All possible 7-mers, including CCUG[AG][AG][AG] and miR-7 7-mer sites (7-mer-m8 (7m8), and 7-mer-A1(7A1)), were extracted from the 3′ UTR of UMD targets, and the count of each 7-mer was compared to that of the 7-mer extracted from number-matched dinucleotide shuffled sequences of the 3′ UTRs. The P value for 7-mer enrichment was estimated by Fisher’s exact test. miRNA 7-mer sites, significantly enriched in UPR1-knockdown cells (P < 0.005), were considered significant sites. 7-mers that were more significant than the most significant miRNA 7-mer site (miR-16-5p) were subjected to sequence logo analysis.

**Signal-to-noise ratio of triplet nucleotides (3-mers).** All possible triplet nucleotides were extracted from the 3′ UTR of UMD targets and from sequences reverse-complementary to miRNAs, and the observed count of each triplet nucleotide was compared to that (expected) of the triplet nucleotides extracted from number-matched dinucleotide shuffled sequences. The ratio of observed versus expected counts was regarded as the signal-to-noise ratio of each triplet nucleotide.

**miRNA target sites.** The expression values of miRNAs were downloaded from previous studies in HeLa and mES cells, and the 30 most abundant miRNA families in HeLa and mES cells, 8-mer (seed-pairing + base-pairing at 8th position + A at the position opposite to the 1st nucleotide of miRNA), 7m8 (seed-pairing + match at 8th position), 7A1 (seed-pairing + A at the position opposite to the 1st nucleotide of miRNA), 6-mer (seed-pairing), and offset-6-mer (base-pairing at 3 to 8th nucleotides of miRNA) sites were considered miRNA target sites. Of these sites, 7-mer sites, including 8-mer, 7m8, and 7A1 sites, were analyzed as effective target sites. For each miRNA, effective target sites were counted in the 3′ UTR and updated with a major 3′ UTR end, and the number of sites was correlated to the 3′ UTR length and mean expression fold-change in UPR1-knockdown cells.

**Generation of random controls and statistical tests.** To build random controls of DEI-free genes, 275 nontarget miRNAs lacking any 6-mer site for the 50 most abundant miRNAs were selected. Because the target miRNAs with 7-mer sites generally had longer 3′ UTRs than the nontarget miRNAs with no target sites, control of the 3′ UTR length is required for miRNA target expression analysis. To compare the changes in expression of the miRNA targets with those of the nontargets, nontargets that embedded 7-mer sites of number-matched random miRNAs were selected as random negative controls. The random miRNA sequences were generated by dinucleotide-shuffling of the 50 most abundant miRNAs using the Python random module. To test the statistical significance, 1000 random miRNA cohorts (each included 50 random miRNA sequences) and 1000 corresponding random control cohorts (each included random targets that embedded 7-mer sites of the random miRNAs) were generated. For each sample test, the mean values of the random control cohorts (that followed the normal distribution based on the central limit theorem) were compared to those of the targets to measure the P values.

**Expression changes of miRNA targets with effective 7-mer sites.** The mean changes in the expression of targets with effective 7-mer sites of miRNAs in UPP1–, Dicer–, and TNR6-cleavage knockdown cells were normalized to the levels of random controls. The mean changes in expression were sorted by the number of sites and wContext + + score.

**wContext + + score calculation.** All wContext + + scores were downloaded from TargetScan v7.1 Human and Mouse41. For each target, all wContext + + scores for 7-mer sites of miRNAs were summed. If sites of different miRNAs overlapped in the 3′ UTR, the site showing greater efficacy was considered.

**Cell culture.** HeLa cells (KCLB #1002) were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and cells were tested for mycoplasma. No additional cell authentication was performed. Cells were seeded one day before transfection with plasmid DNA, siRNA, or miRNA inhibitor/mimic using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After incubation, the cells were lysed with hypotonic buffer for western blotting or IP, as previously described29,60, where TRIZol reagent (Invitrogen) for RT-qPCR or RNA-seq. When indicated, FLAG-tagged Ago2, MYC-tagged UPF1 WT, or the helicase mutant R843C62 was transfected into HeLa cells. To silence the expression of endogenous DNA contamination. The RNA was reverse-transcribed using random hexamers (MacroGen, Seoul, Korea), and reverse-transcribed cDNA was amplified by RT-qPCR. PCR was performed using the primers listed in Supplementary Table 8.

**RT-qPCR.** Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s protocol. Next, RNA was further treated with RNase-free DNAse I (Promega, Madison, WI, USA) for 30 min at 37 °C to eliminate possible exogenous or endogenous DNA contamination. The RNA was reverse-transcribed using random hexamers (Macrogen, Seoul, Korea), and reverse-transcribed cDNA was amplified by RT-qPCR. PCR was performed using the primers listed in Supplementary Table 8.

**Quantification of miRNA.** miRNAs were quantified using QuantmiR (Systems Biosciences, Palo Alto, CA, USA) according to the manufacturer’s protocol. Briefly, cell lysates were added to purified total RNA using poly(A) polymerase and OligoT adaptor. RT-qPCR was performed using the primers listed in Supplementary Table 9.

**Plasmid construction.** To construct pmirGLO-PEA15-WT and pmirGLO-PEA15-Mut-A and pmirGLO-PEA15-Mut-B, the pmirGLO plasmid (Promega) was digested with Xhol and XbaI, and the digested vector fragment was ligated to a PCR product digested with the same enzymes. The PCR product was obtained by annealing three primers named PEA15-5′, PEA15-middle-WT or PEA15-middle-Mut-A or PEA15-middle-Mut-B, and PEA15-3′, where PEA15-middle-Mut contained mutated sequences, as indicated in Fig. 5e; the sequences were amplified using two primers named PEA15-XhoI-F and PEA15-XbaI-R. Similarly, pmirGLO-RPS19BP1-F and pmirGLO-RPS19BP1-R were constructed. Similarly, the miR-24-3p-binding sites in the 3′UTRs of the DNAL4, ATAD2B, ELL, PAK4, DBNDD1, and ZNF740 genes were cloned into reporter constructs. All primer sequences are provided in Supplementary Table 10.

To construct pFLAG-SMGG-7WT or mutant (ΔPC) plasmids, which cannot bind to the deadenylase complex, the pFLAG plasmid (Sigma) was digested with Gln and KpnI, and the digested vector fragment was ligated to a PCR product digested with the same enzyme. The PCR product was obtained using pEFPPN1-SmG7 as a template and two primers, SMG7-WT-1-Clal-F and SMG7-WT-327-KpnI-R (for WT) and SMG7 Mut-1876-KpnI-R (for a mutant, ΔPC).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions

J.W.H. and J.-W.N. conceived and devised the study. J.Y.P. and N.R.A. performed all the experiments. J.-W.S. and S.J.P. performed the bioinformatics and statistical analysis. J.W.H. and J.-W.N. supervised the research and wrote the paper together with J.-W.S.

Additional information

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