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The upstream open reading frame of cyclin-dependent kinase inhibitor 1A mRNA negatively regulates translation of the downstream main open reading frame

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ABSTRACT

The first round of translation occurs on mRNAs bound by nuclear cap-binding complex (CBC), which is composed of nuclear cap-binding protein 80 and 20 (CBP80/20). During this round of translation, aberrant mRNAs are recognized and downregulated in abundance by nonsense-mediated mRNA decay (NMD), which is one of the mRNA quality control mechanisms. Here, our microarray analysis reveals that the level of cyclin-dependent kinase inhibitor 1A (CDKN1A; also known as Waf1/p21) mRNAs increases in cells depleted of cellular NMD factors. Intriguingly, CDKN1A mRNA contains an upstream open reading frame (uORF), which is a NMD-inducing feature. Using chimeric reporter constructs, we find that the uORF of CDKN1A mRNA negatively modulates translation of the main downstream ORF. These findings provide biological insights into the possible role of NMD in diverse biological pathways mediated by CDKN1A.

1. Introduction

The generation of mature messenger RNA (mRNA) requires coordinate processes in the nucleus [1]. During transcription, a cap structure is generated at the 5′-end of the pre-mRNA. Nuclear cap-binding complex (CBC), which is composed of cap-binding protein 80 and 20 (CBP80/20), binds to the cap structure. The pre-mRNAs are then processed into mRNA as a consequence of a splicing event in which introns are removed, the neighboring exons are connected, and the exon junction complex (EJC) is deposited onto each exon–exon junction [1]. The fully and properly processed mRNAs undergo export through the nuclear pore complex toward the cytoplasm.

During export, the CBP80/20 at the 5′-end of mRNA recruits translation initiation factors including eukaryotic translation initiation factor (eIF) 3 and CBP80/20-dependent translation initiation factor (CTIF), which direct the first (or pioneer) round of translation or CBP80/20-dependent translation [2–6]. During this round of translation, transcripts harboring premature termination codons (PTCs) are recognized and degraded by nonsense-mediated mRNA decay (NMD), which is the best-characterized mRNA quality control mechanism in eukaryotes [2,4,6–8].

In addition to abnormal transcripts harboring PTCs, cellular transcripts can also be subject to NMD, as long as they contain EJCs downstream of the terminating ribosome on a PTC. Therefore, the NMD-inducing features in cellular transcripts can be categorized as follows: (i) the presence of upstream open reading frame (uORF) in the 5′ untranslated region (5′UTR), (ii) the presence of intron in the 3′UTR, (iii) selenocysteine-encoding transcripts, (iv) a frame-shift generated by alternative splicing, and (v) retro-transposon [9–13].

Cyclin-dependent kinase (CDK) inhibitor 1A (CDKN1A; also known as Waf1/p21) is a well-known CDK inhibitor [14,15]. CDKN1A is involved in various biological pathways including cell-cycle regulation, DNA damage repair, DNA replication, and senescence [16–18]. At least four alternatively spliced variants of CDKN1A mRNA have been reported in the NCBI nucleotide sequence database, producing the identical polypeptides [19,20].

It has been established that the translation of major ORF is affected by the regulation of the translation of uORF [21–24]. For instance, in *Drosophila*, the translation of msl-2 mRNA is regulated by Sex-lethal protein (SXL), which is crucial for X-chromosomal dosage compensation. Sxl protein binds downstream of uORF to inhibit the translation of the major ORF and, as a consequence, it provides higher efficiency of translation initiation of uORF [21].

Here, we show that cellular CDKN1A mRNA is a *bona fide* NMD substrate. Using chimeric reporter constructs, we also show that the uORF of CDKN1A mRNA inhibits the translation of the main downstream ORF. Our findings indicate a possible role for NMD in diverse pathways mediated by CDKN1A.

2. Materials and methods

2.1. Plasmid construction

The details for plasmid construction are provided in Supplementary information.

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2.2. Microarray analysis

Microarray analysis was conducted by Macrogen (Korea) as previously described [25]. In brief, biotin-labeled complementary RNAs were hybridized to human-8 expression bead array (24 K human gene chips; Illumina, USA). Hybridized chips were scanned in an Illumina bead array reader confocal scanner (Illumina). The scanned images were analyzed with Gene Expression BeadStudio v3.1.3 (Illumina).

The microarray data obtained from HeLa cells depleted of CTIF were deposited in the National Center for Biotechnology Information Gene Expression Omnibus web-based data repository (series ID: GSE37538). The microarray results obtained from HeLa cells depleted of Upf1 or PNRC2 were recently reported [26] and retrieved from GEO repository (Series ID: GSE26781) for comparison.

2.3. Cell culture and transfections

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) containing 10% fetal bovine serum (Lonza) and 1% penicillin/streptomycin (Lonza).

For plasmid transfection, HeLa cells were transiently co-transfected with 0.5 μg RLuc reporter plasmid and 1.5 μg FLuc reference plasmid using Lipofectamine 2000 (Invitrogen, USA). One day after transfection, total-cell RNA and proteins were purified using TRIzol (Invitrogen) and passive lysis buffer (Promega), respectively.

For small interfering RNA (siRNA) transfection, HeLa cells were transiently transfected with 100 nM *in vitro*-synthesized siRNA (Invitrogen) using Oligofectamine (Invitrogen). The following siRNA sequences were used in this study: 5′-r(GAUGCAGUUCCGCUCCAUU)d(TT)-3′ for Upf1, 5′-r(GGCUUUUGUCCCAGCCAUC)d(TT)-3′ for Upf2, 5′-r(GGAGAAGCGAGUAACCCUG)d(TT)-3′ for Upf3X, 5′-r(GAAGUGGAGAUCGCACACA)d(TT)-3′ for CTIF, 5′-r(AGUUGGAAUUCUAGCUUAU)d(TT)-3′ for PNRC2, 5′-r(UCCAGCCUUCAACAGAGCG)d(TT)-3′ for Y14, 5′-r(CGAGCAAUCAAGCAGAUCA)d(TT)-3′ for eIF4AIII, and 5′-r(ACAAUCCUGAUCAGAAACC)d(TT)-3′ for nonspecific negative control, respectively, as previously reported [27–30].

2.4. RT-PCR using α-[^{32}P]-dATP and specific oligonucleotides

RT-PCR using specific oligonucleotides and α-[^{32}P]-dATP (PerkinElmer NEN) was performed as described previously [6,28]. The [^{32}P]-labeled PCR products were separated by 5% acrylamide gel, visualized by PhosphorImaging (BAS-2500; Fuji Photo Film Co.), and then quantitated by Multigauge (Fuji Photo Film Co.). The oligonucleotides used in our study are provided in Supplementary information.

2.5. Determination of mRNA half-life

HeLa cells were transiently transfected with the indicated siRNAs. Three days later, cells were treated with 100 μg/ml 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) to inhibit transcription. Total-cell RNAs were purified at the indicated time points and analyzed by RT-PCR using specific oligonucleotides and α-[^{32}P]-dATP. The each level of endogenous CDKN1A mRNA variant 4 was normalized to endogenous GAPDH mRNA. The normalized levels of endogenous CDKN1A mRNA variant 4 were then plotted as a function of time after DRB treatment. The linear trend lines were obtained using the Excel program.

2.6. Western blotting

The protein samples were analyzed by 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to the Hybond ECL nitrocellulose (Amersham). The indicated proteins were detected using specific antibody: α-Upf1 (a gift from L.E. Maquat), α-CTIF [5], α-PNRC2 [28], α-Upf2 [26], α-eIF4AIII [26], α-Y14 (Abnova), and α-β-actin (Sigma–Aldrich). Antibody against human Upf3X was raised in rabbits using the synthetic peptide CKRDRIRNKDRPA (Peptron, Korea).

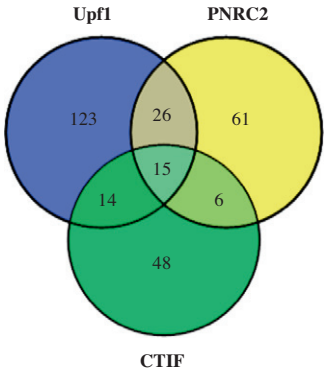
2.7. Dual luciferase assays

Proteins were purified using passive lysis buffer (Promega) and analyzed using the Dual Luciferase assay kit (Promega) according to the manufacturer's instructions.

2.8. Statistics

Two-tailed, equal-sample variance Student's t-tests were used to determine statistical significance of differences between the data sets. Differences of P value (P) < 0.01 or P < 0.05 were considered significant.

A



B

Gene name	Upf1 siRNA	PNRC2 siRNA	CTIF siRNA	NMD-inducing feature
CDKN1A	6.08	2.38	8.04	uORF, alternative splicing
C16orf28	4.64	4.31	2.17	uORF, alternative splicing
ISG20L1	4.58	3.00	3.16	uORF
KLF2	3.61	7.75	2.66	unknown
HMOX1	3.42	5.98	2.05	uORF
C1orf41	3.12	3.91	3.68	unknown
COMMD2	2.88	2.54	3.34	unknown
OASL	2.76	2.47	2.34	unknown
CAPZA2	2.56	2.67	2.95	unknown
IARS	2.50	2.88	2.60	uORF
IFIT3	2.30	2.43	2.75	uORF
SFRS1	2.27	2.27	2.96	unknown
COMMD7	2.26	2.10	2.86	uORF
JUN	2.25	2.83	2.52	uORF
YRDC	2.08	2.09	3.17	unknown

Fig. 1. Microarray analysis of mRNAs purified from HeLa cells depleted of Upf1, PNRC2 or CTIF. HeLa cells were transiently transfected with 100 nM Control siRNA, Upf1 siRNA, PNRC2 siRNA or CTIF siRNA. After 3 days, total RNAs were purified and analyzed by microarray. Transcripts that were commonly upregulated by at least 2-fold change in two independent microarray analyses were considered differentially expressed. The microarray results obtained from HeLa cells depleted of Upf1 or PNRC2 were recently reported [26] and retrieved from the GEO repository (Series ID: GSE26781). (A) Venn diagrams showing the number of transcripts upregulated by at least 2-fold upon downregulation of Upf1, PNRC2, or CTIF. (B) A list of commonly upregulated transcripts by at least 2-fold upon downregulation of Upf1, PNRC2, or CTIF. The table includes the fold-change of the level of each mRNA upon downregulation of indicated protein and NMD-inducing features.

3. Results

3.1. A subset of cellular transcripts is commonly up-regulated when Upf1, PNRC2, or CTIF is down-regulated

In our recent microarray studies to search for cellular NMD substrates [26], we analyzed quantitative changes of 24,000 cellular transcripts in HeLa cells that were depleted of either Upf1 or PNRC2, both of which are involved in mammalian NMD [28,31–33]. The microarray results showed that 178 and 108 transcripts were upregulated in abundance by at least 2-fold upon downregulation of Upf1 and PNRC2, respectively (Fig. 1A). Among them, 41 transcripts were commonly upregulated by at least 2-fold (Fig. 1A). The simple interpretation of these results is that approximately 23% of endogenous NMD substrates require PNRC2.

To increase the stringency for the search of the NMD substrates, we carried out additional microarray analysis using total mRNAs purified from HeLa cells depleted of CTIF. It should be noted that NMD occurs during translation of CBP80/20-bound mRNAs [2,4,6,34]. In particular, our group has reported that translation of CBP80/20-bound mRNAs preferentially requires a cellular factor, CTIF, rather than eIF4GI/II [5].

The upregulated transcripts upon CTIF downregulation were compared with those upon Upf1 or PNRC2 downregulation. The results showed that 83 transcripts were upregulated by at least 2-fold upon CTIF downregulation. Among them, 15 transcripts were

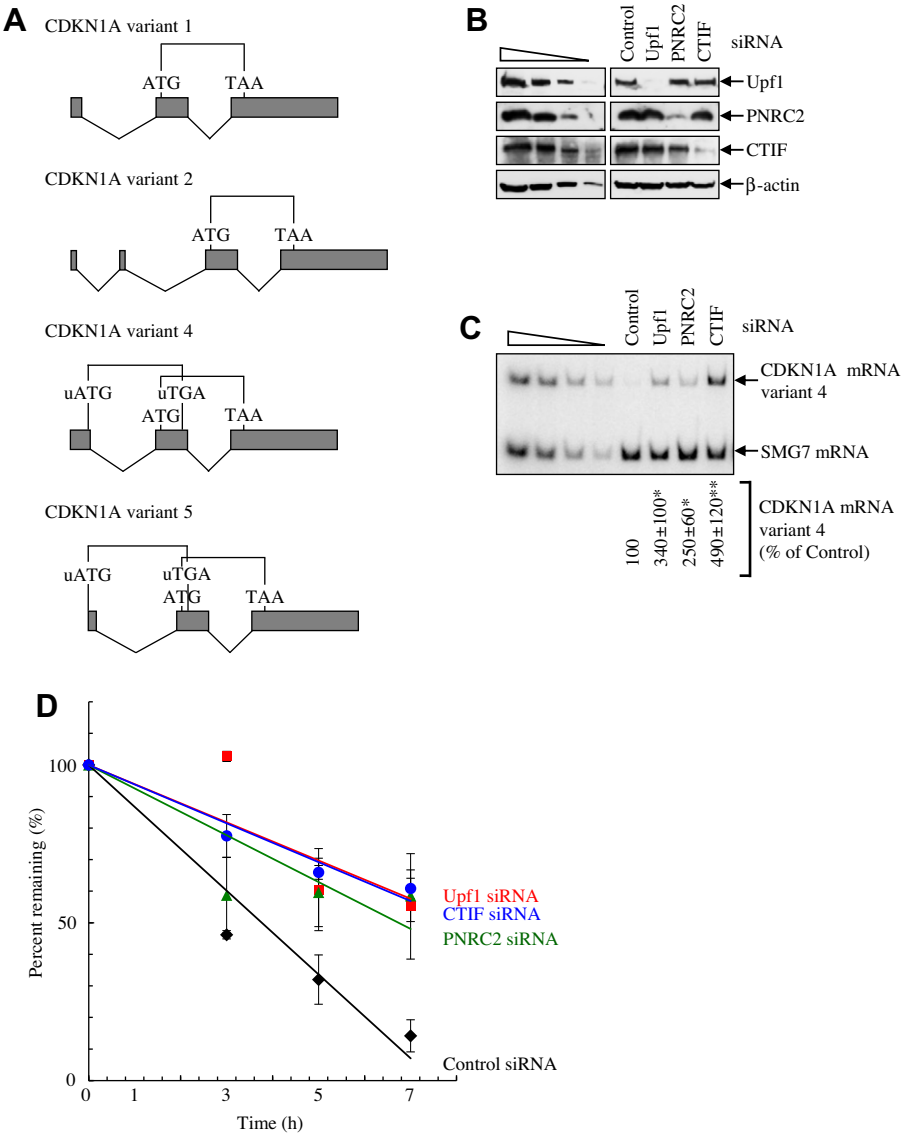


Fig. 2. CDKN1A mRNA is a *bona fide* NMD substrate. (A) Schematic representations of CDKN1A mRNA variants. CDKN1A mRNA variants 4 and 5 contain a single uORF and a single downstream main ORF. On the other hand, CDKN1A mRNA variants 1 and 2 contain a single main ORF and lacks uORF. (B and C) HeLa cells were transiently transfected with the indicated siRNAs. After 3 days, total-cell RNAs and proteins were purified. (B) Western blotting demonstrating specific downregulation. To demonstrate that the Western blotting under our conditions was semi-quantitative, 3-fold serial dilutions of total-cell extracts were loaded in the four left-most lanes. (C) RT-PCR of endogenous CDKN1A mRNA variant 4. The level of endogenous CDKN1A mRNA variant 4 was normalized to the level of endogenous SMG7 mRNA, which served as a control. The normalized level of CDKN1A mRNA in the presence of Control siRNA was set to 100%. The data represent the mean and standard deviation of at least three independently performed transfections, RNA purifications, and RT-PCRs. $^{**}P < 0.01$; $^{*}P < 0.05$. (D) Half-life of endogenous CDKN1A mRNA variant 4. HeLa cells were transiently transfected with the indicated siRNAs. Three days later, cells were treated with 100 μg/ml DRB. The levels of endogenous CDKN1A mRNA variant 4, which were normalized to endogenous GAPDH mRNA, were plotted as a function of time after DRB treatment. The data represent the mean and standard deviation of at least two independently performed transfections and RT-PCRs.

commonly upregulated by at least 2-fold upon Upf1, PNRC2, or CTIF downregulation, implicating these transcripts as strong candidates for NMD substrates (Fig. 1). Among the 15 commonly upregulated transcripts, eight transcripts had uORF in the 5′UTR. Two of the eight transcripts had both an uORF in the 5′UTR and a PTC generated by frameshift due to alternative splicing (Fig. 1B). The remaining seven transcripts did not have any known NMD features. In support of our analysis, the COMM domain containing 7 (COMMD7) mRNA and isoleucine-tRNA synthetase (IARS) mRNA among 15 transcripts, both of which contain uORF as NMD-inducing feature [10], were previously demonstrated as *bona fide* cellular NMD substrates [28]. In addition, c-JUN mRNA and krüppel-like factor 2 (KLF2) mRNA were recently identified to be targets for Staufen1-mediated mRNA decay (SMD), which is another mRNA decay pathway that shares NMD factors, Upf1 and PNRC2 [26,27]. All together, these results suggest that our microarray analyses used for searching for NMD substrates are sufficiently efficient.

3.2. Level of CDKN1A mRNA increases when NMD is compromised

An uORF is one of the NMD-inducing features [10]. To elucidate a molecular mechanism by which uORF in the 5′UTR regulates the stability of NMD substrates, we used CDKN1A mRNA because CDKN1A mRNA contains uORF and was the most strongly upregulated transcript among the 15 transcripts in our microarray analysis (Fig. 1). First, the microarray results in Fig. 1 were confirmed by RT-PCR using α-[^{32}P]-dATP and specific oligonucleotides. To this end, HeLa cells were depleted of Upf1, CTIF, PNRC2 using siRNAs (Supplementary Fig. S1A–D). We also included downregulation of Upf3X, Y14, Upf2, or eIF4AIII (Supplementary Fig. S1E and F), all of which are constituents of EJC [2,7,8,29,30]. The RT-PCR results using specific oligonucleotides, which anneal to all variants of CDKN1A mRNAs (see below), showed that the level of endogenous CDKN1A mRNA increased by 4.6–6-, 14.9-, 5.5-, 4.8-, 2.1-, 2.0-, and 2.1-fold upon downreg-

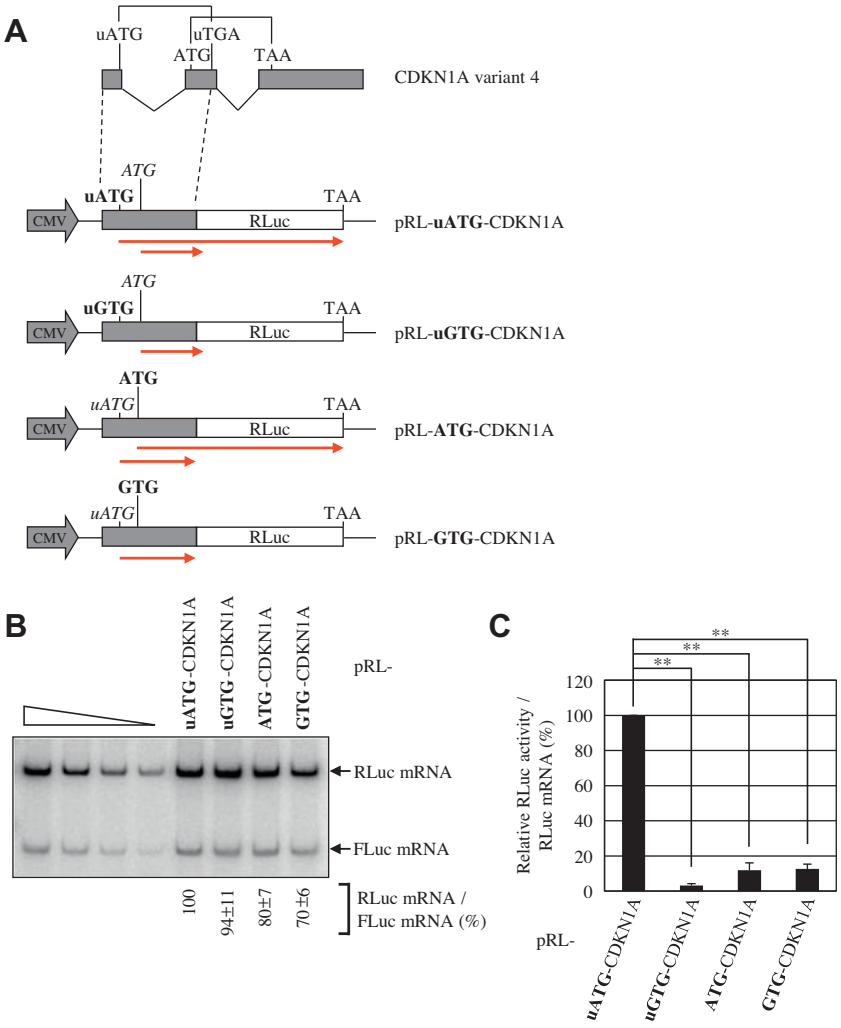


Fig. 3. An uORF of CDKN1A mRNA is efficiently translated in the reporter system. (A) Schematic representations of the RLuc reporter constructs, in which the sequences including both 5′UTR and uORF of CDKN1A variant 4 were fused either in-frame or out-of-frame to a RLuc cDNA. The expression of the downstream main ORF (RLuc ORF) is dependent on in-frame translation initiation codon (uATG or ATG). The in-frame and out-of-frame codons are shown in bold and italic, respectively. Arrows below each construct specify the expected uORF and ORF within each construct. CMV, CMV promoter; ATG, translation initiation codon in the main ORF; uATG, translation initiation codon in the uORF; TAA, translation termination codon; uTGA, translation termination codon in the uORF; RLuc, *Renilla* luciferase. (B and C) HeLa cells were transiently co-transfected with one of reporter constructs expressing RLuc and a reference plasmid, pFL-CMV expressing FLuc, which serves to control variations. One day after transfection, total-cell RNAs and proteins were analyzed by RT-PCR and dual luciferase assay. (B) RT-PCRs of RLuc mRNAs and FLuc mRNAs. The level of RLuc mRNA was normalized to the level of FLuc mRNA. The normalized level of RLuc mRNA obtained from cells transfected with pRL-**uATG**-CDKN1A was set to 100%. The data represent the mean and standard deviation of at least three independently performed transfections, RNA purifications, and RT-PCRs. (C) Translation yield of each CDKN1A reporters. Translation efficiency was calculated by comparing the relative ratio of RLuc activity to FLuc activity, which was normalized by the relative ratio of RLuc mRNA to FLuc mRNA. The normalized level of RLuc activity in pRL-**uATG**-CDKN1A was defined as 100%. $^{**}P < 0.01$.

ulation of Upf1, CTIF, PNRC2, Upf3X, Y14, Upf2, and eIF4AIII, respectively. All these results suggest that CDKN1A mRNA is a cellular NMD substrate.

3.3. CDKN1A mRNA is a bona fide NMD substrate

Four alternatively spliced variants of CDKN1A mRNA have been reported in the NCBI nucleotide sequence database [19,20]. Among them, CDKN1A mRNA variant 4 (NM_001220778.1) and variant 5 (NM_001220777.1) contain an uORF in the 5′UTR, which is a NMD-inducing feature [10]. On the other hand, CDKN1A mRNA variant 1 (NM_000389.4) and variant 2 (NM_078467.2) did not contain an uORF (Fig. 2A). Based on the presence of uORF, we speculated that CDKN1A mRNA variants 4 and 5, but not 1 and 2, are targeted for NMD. As expected, the levels of endogenous CDKN1A mRNA variant 4 increased by 2.5- to 4.9-fold upon downregulation of Upf1, PNRC2, or CTIF (Fig. 2B, C). We also tried to test the levels of other variants of endogenous CDKN1A mRNA variants. However, we failed to amplify endogenous CDKN1A mRNA variants, 1, 2, and 5 under our conditions (data now shown), probably due to the weak alternative splicing. More importantly, the half-life of CDKN1A mRNA variant 4 was significantly increased upon downregulation of Upf1, PNRC2, or CTIF (Fig. 2D). All together, our findings indicate that CDKN1A mRNA variant 4 is a *bona fide* cellular NMD substrate.

3.4. An uORF of CDKN1A mRNA variant 4 is efficiently translated in artificially designed reporter construct

Considering that NMD is coupled to translation [2,4–6], it is most likely that uORFs of CDKN1A mRNA variants 4 and 5 are translated efficiently enough to trigger efficient NMD. To test this possibility, we constructed several chimeric reporter plasmids, in which an uORF sequence of CDKN1A variant 4 spanned from uATG (a translation initiation codon in the uORF) to immediately upstream of uTGA (a translation termination codon in the uORF; this region that partially overlapped with 5′-end of the main ORF) was fused to *Renilla* luciferase (RLuc) cDNA (Fig. 3A). In the constructs, the expression of RLuc protein would be dependent on in-frame translation initiation codon (uATG or ATG). For clarity, codons, which are in-frame with the ORF of RLuc cDNA, are depicted in bold. On the other hand, codons, which are out-of-frame with the ORF of RLuc cDNA, are depicted in italic (Fig. 3A).

To monitor the expression of RLuc proteins from reporter plasmids, HeLa cells were transiently co-transfected with one of reporter plasmids and reference plasmid expressing firefly luciferase (FLuc), which controls the variations of transfections, RNA purifications, and dual luciferase assay.

RT-PCR using α-[^{32}P]-dATP demonstrated comparable expression of reporter mRNA (Fig. 3B). Next, translation efficiency of each reporter mRNA was determined by comparing the relative ratio of

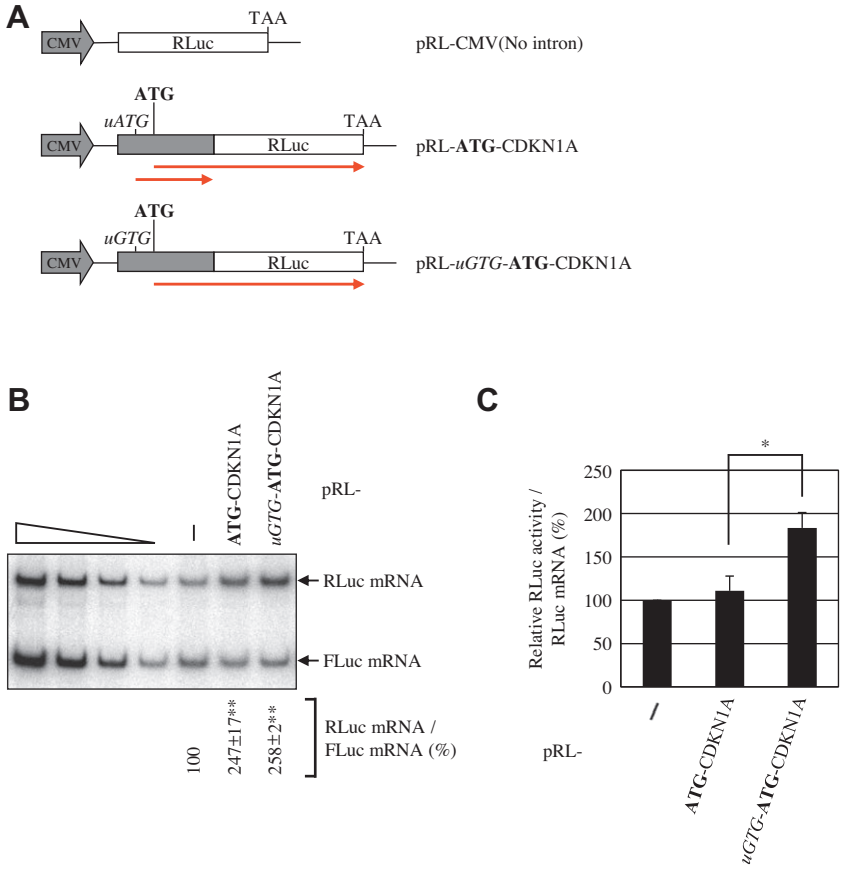


Fig. 4. Translation of uORF inhibits a translation of downstream main ORF in CDKN1A mRNA. (A) Schematic representations of the CDKN1A reporter constructs expressing RLuc protein. The in-frame and out-of-frame codons are shown in bold and italic, respectively. Arrows below each construct specify the expected uORF and ORF within each construct. (B and C) HeLa cells were transiently co-transfected with either pRL-CMV(No intron), pRL-**ATG**-CDKN1A, or pRL-*uGTG*-**ATG**-CDKN1A, and a reference plasmid, pFL-CMV expressing FLuc, which serves to control variations. One day after transfection, total-cell RNAs and proteins were analyzed by RT-PCR and dual luciferase assay. (B) RT-PCRs of RLuc mRNAs and FLuc mRNAs. As in Fig. 3B, except that the normalized level of RLuc mRNA obtained from cells transfected with pRL-CMV(No intron) was set to 100%. The data represent the mean and standard deviation of at least three independently performed transfections, RNA purifications, and RT-PCRs. (C) Translation yield of each CDKN1A reporters. As in Fig. 3C, except that the normalized relative level of RLuc activity in pRL-CMV(No intron) was defined as 100%. $^{**}P < 0.01$; $^{*}P < 0.05$.

RLuc activity to FLuc activity, which was normalized by the relative ratio of RLuc mRNA to FLuc mRNA (Fig. 3C). The results revealed that **uATG**-CDKN1A mRNA was efficiently translated by 31-fold more than **uGTG**-CDKN1A mRNA in which uATG was replaced by uGTG. Unexpectedly, **uATG**-CDKN1A mRNA was efficiently translated by 8-fold more than **ATG**-CDKN1A mRNA. In addition, **ATG**-CDKN1A mRNA and **GTG**-CDKN1A mRNA, in which ATG in the main ORF was substituted to GTG, showed a comparable level of RLuc expression. All these results suggest that, whereas the main ORF is translated inefficiently, uORF of CDKN1A mRNA variant 4 is translated efficiently.

3.5. Translation of downstream main ORF is negatively regulated by translation of uORF in CDKN1A mRNA

It has been well known that uORFs modulate the translatability of downstream ORF [21,22]. Considering that uORF of CDKN1A mRNAs is more efficiently translated than the main ORF (Fig. 3C), we speculated that the translatability of uORF in CDKN1A mRNA may regulate translation efficiency of the downstream main ORF. To test this idea, an additional reporter plasmid was constructed (Fig. 4A). Whereas the original **ATG**-CDKN1A mRNA contained a functional uORF, *uGTG*-**ATG**-CDKN1A mRNA contained a nonfunctional uORF, because uATG was changed to uGTG.

The results of RT-PCR and dual luciferase assay revealed that, whereas comparable amounts of **ATG**-CDKN1A mRNA and *uGTG*-**ATG**-CDKN1A mRNA were expressed (Fig. 4B), *uGTG*-**ATG**-CDKN1A mRNA was more efficiently translated (by approximately 1.7-fold more) than **ATG**-CDKN1A mRNA (Fig. 4C). These results suggest that the uORF of CDKN1A mRNA variant 4 is translated, resulting in the inhibition of the translation of downstream main ORF. Since CDKN1A mRNA is a *bona fide* NMD substrate (Fig. 2), it is speculated this inhibitory effect modulates NMD efficiency.

4. Discussion

Here we show that under the normal conditions, uORFs of CDKN1A mRNA variant 4 and probably variant 5, both of which contain an uORF at 5′UTR, are translated efficiently enough to induce both mRNA destabilization via NMD (Figs. 1 and 2) and translational silencing of the downstream main ORF (Figs. 3 and 4). Under stressful conditions where overall translation is compromised, however, a translation yield of uORF would be decreased, consequently enhancing mRNA stability and derepressing the translation of the downstream main ORF. Alternatively, when certain microRNA anneals to the 3′UTR of CDKN1A mRNA, the translation of uORF would be silenced [25]. As a result, CDKN1A mRNA would escape NMD and the translation of the downstream main ORF would be increased. The resultant induction of level of CDKN1A protein may contribute to a variety of biological events including cell-cycle regulation, DNA damage repair, DNA replication, and senescence [16–18].

Emerging cases where alternative splicing events exploit NMD to control the gene expression post-transcriptionally in response to physiological changes have been reported [35–37]. For instance, several splicing factors including SR protein and polypyrimidine tract-binding protein (PTB) take advantage of gene regulation mediated by alternative splicing and NMD for maintaining autoregulatory negative feed-back loops [35,36,38]. The same negative feedback may apply to the regulation of CDKN1A expression. Maintaining CDKN1A protein at a constant level using negative feedback may provide the cells with the ability to resist to environmental or physiological stress. Future studies should address the molecular mechanism by which the regulation of CDKN1A gene expression by alternative splicing and NMD is connected to biological functions involved in CDKN1A.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.135.

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