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Nuclear UPF1 Is Associated with Chromatin for Transcription-Coupled RNA Surveillance

Dawon Hong, Taeyoung Park, and Sunjoo Jeong*

Graduate Department of Bioconvergence Science and Technology, Dankook University, Yongin 16892, Korea
*Correspondence: sjsj@dankook.ac.kr
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mRNA quality is controlled by multiple RNA surveillance machineries to reduce errors during gene expression processes in eukaryotic cells. Nonsense-mediated mRNA decay (NMD) is a well-characterized mechanism that degrades error-containing transcripts during translation. The ATP-dependent RNA helicase up-frameshift 1 (UPF1) is a key player in NMD that is mostly prevalent in the cytoplasm. However, recent studies on UPF1-RNA interaction suggest more comprehensive roles of UPF1 on diverse forms of target transcripts. Here we used subcellular fractionation and immunofluorescence to understand such complex functions of UPF1. We demonstrated that UPF1 can be localized to the nucleus and predominantly associated with the chromatin. Moreover, we showed that UPF1 associates more strongly with the chromatin when the transcription elongation and translation inhibitors were used. These findings suggest a novel role of UPF1 in transcription elongation-coupled RNA machinery in the chromatin, as well as in translation-coupled NMD in the cytoplasm. Thus, we propose that cytoplasmic UPF1-centric RNA surveillance mechanism could be extended further up to the chromatin-associated UPF1 and co-transcriptional RNA surveillance. Our findings could provide the mechanistic insights on extensive regulatory roles of UPF1 for many cellular RNAs.

Keywords: nonsense-mediated mRNA decay, nuclear localization, RNA surveillance, transcription, UPF1

INTRODUCTION

Eukaryotic gene expression is regulated from nuclear transcription to cytoplasmic translation by a highly interconnected ribonucleoprotein (RNP) network (Maniatis and Reed, 2002). During transcription, nascent transcripts are assembled as RNPs in the chromatin and are continually remodeled as mRNPs up to their final destination in the cytoplasm (Moore, 2005; Muhlemann and Jensen, 2012). To accurately transmit genomic information to the proteome and reduce significant errors in the transcriptome, eukaryotic cells utilize many RNA surveillance machineries for RNA quality control at multiple steps (Manning and Cooper, 2017; Muhlemann and Jensen, 2012). The most well-characterized RNA surveillance mechanism is the translation-coupled mRNA decay process called nonsense-mediated mRNA decay (NMD), in which premature termination codon (PTC)-containing transcripts are subjected to degradation in the cytoplasm (Ahn et al., 2013; Karousis et al., 2016; Kurosaki and Maquat, 2016). To maintain cellular homeostasis in response to physiological states, transcription events are likely linked to the cytoplasmic surveillance machinery; however, whether NMD factors are involved in this process is unknown.

Up-frameshift 1 (UPF1) is a key protein component of the NMD machinery that is mostly found in the cytoplasm and plays a critical role in promoting mRNA decay (Maquat and Gong, 2009; Nicholson et al., 2010). UPF1 is an ATP-dependent RNA helicase with a central helicase domain comprising two flexible RecA domains and an ATP-binding site (Apple-

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quist et al., 1997; Chakrabarti et al., 2011; Chawla et al., 2011; Karousis et al., 2016; Pal et al., 2001). The helicase domain binds to single-stranded RNA and DNA and slowly but processively translocates along the nucleic acid to unwind the long double-stranded structures (Karousis et al., 2016). Thus, the biochemical activity of UPF1 is well suited to play multiple functions in the DNA and RNA metabolism (Isken and Maquat, 2008).

UPF1 has been reported to display nuclear functions such as genome stability, telomere maintenance, cell cycle regulation, and DNA replication and repair (Azzalin and Lingner, 2006; Chawla et al., 2011; Fiorini et al., 2015; Mendell et al., 2002). These studies have suggested that UPF1 can localize to the nucleus and play distinct roles in DNA metabolism (Nicholson et al., 2010; Varsally and Brogna, 2012). Moreover, separable roles of UPF1 have been proposed in altered splicing or nonsense-associated alternative splicing, implying it as a shuttling protein (Hwang and Kim, 2013; Mendell et al., 2002). In addition, recent global analyses have demonstrated that UPF1 can bind to large number of transcripts with diverse architecture, error-containing decay targets, and/or regulatory RNAs (Hurt et al., 2013; Kim and Maquat, 2019; Kishor et al., 2019; Lee et al., 2015; Wang et al., 2014). Based on such different targets of UPF1, this protein is likely to play more comprehensive roles in RNA metabolism than previously known. However, the intracellular localization of UPF1 has not been extensively analyzed and cytoplasmic role is mostly studied (Atkin et al., 1995; Serin et al., 2001).

Here, we revisited the intracellular localization of UPF1 by cell biological and biochemical approaches and demonstrate its localization in the nucleus as well as in the cytoplasm. Particularly, nuclear UPF1 is highly enriched in chromatin, as shown by subcellular fractionation and immunofluorescence analyses. In addition, the perturbation of transcription initiation and elongation differentially affects the intranuclear localization of UPF1. These data propose a role of UPF1 in transcription elongation-coupled processes in the chromatin. Furthermore, a cycloheximide treatment accumulates UPF1 more strongly to chromatin, implying that chromatin-associated UPF1 acts as a linker to translation-coupled NMD in the cytoplasm. These data suggest that UPF1 orchestrates transcription- and translation-coupled RNA networks that are pertinent to the UPF1-centric view of the RNA surveillance machinery.

MATERIALS AND METHODS

Cell culture and inhibitor treatment

HeLa cells (cervical carcinoma) were cultured in DMEM high glucose medium (Hyclone, USA) supplemented with 10% fetal bovine serum and 1% antibiotics (Hyclone) at 37°C in 5% CO_2 incubator. Embryonic kidney epithelial HEK293 cells were cultured in MEM medium (Hyclone). Cells were treated with 10 μg/ml Actinomycin D (Sigma, USA), 1 μM Flavopiridol (Sigma) and 10 μg/ml Cycloheximide (Sigma) for 15 min at 37°C.

Western blot analysis

Cells were lysed in RIPA lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). Fifteen microgram of proteins were loaded and separated by 8% (for UPF1 proteins) or 12% SDS-PAGE gel, then transferred to PVDF membrane (Millipore, USA). The following antibodies were used: Anti-UPF1 antibody-A (Cell Signaling Technology, USA), anti-UPF1 antibody-B (a gift from Jens Lykke-Andersen), anti-SRSF1 (Thermo Fisher Scientific, USA), anti-Lamin B1 (Abcam, UK), anti-α-tubulin (Abcam) and anti-Histone H3 (Abcam) antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs (Thermo Fisher Scientific) were used as secondary antibodies.

Immunofluorescence

HeLa cells were grown on coverslips, rinsed in phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS for 10 min at 4°C, and permeabilized with methanol for 5 min at 4°C. Permeabilized cells were rinsed three times in PBS supplemented with 0.5% Tween-20 and blocked with blocking solution (5% BSA in PBS, pH 7.4) for 1 h. Cells were rinsed three times in PBS and incubated with primary antibodies against UPF1 (Cell Signaling Technology) in humidified chamber overnight at 4°C. After rinsing three times in PBS supplemented with 0.5% Tween-20, cover slips were incubated with the Alexa Fluor 568 secondary antibody (1:1,000; Life Technologies, USA) for 1 h at room temperature. Cells were washed 3 × 5 min with PBS and mounted onto glass slides using VECTASHIELD mounting medium with DAPI (4',6-diamidino-2-phenylinodole) for nuclear staining. All images were taken and processed using confocal fluorescence microscopy (Fluoview, FV300; Olympus, USA).

Subcellular fractionation

To prepare total cell extracts, cells (2×10^7 cells/ml) were lysed in RIPA lysis buffer. To isolate nuclear extracts, the cells were washed once with PBS and resuspended in the fractionation buffer (1 M HEPES-KOH [pH 7.5], 1 M KCl, 1 M $MgCl_2$, 100 mM DTT, 10% NP-40, protease inhibitors, 80 U/ml RNase inhibitor). Cell lysates were centrifuged at 1,000*g* for 5 min, and the supernatant (cytoplasmic fraction) was separated from the nuclear pellet. The nuclear pellets were resuspended in nuclear buffer (1 M Tris-HCl [pH 7.5], 5 M NaCl, 0.5 M EDTA, 20% Triton X-100, 10% Na-deoxycholate, protease inhibitors).

Chromatin isolation

To isolate chromatin, cells were resuspended (2×10^7 cells/ml) in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, and protease inhibitor). Triton X-100 (0.1%) was added, and the cells were incubated for 5 min on ice. Nuclei were collected in pellet 1 (P1) by low-speed centrifugation (4 min, 1,300*g*, 4°C). The supernatant (S1) was further clarified by high-speed centrifugation (15 min, 20,000*g*, 4°C) to remove cell debris and insoluble aggregates. Nuclei were washed once in buffer A, and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors cocktail). Insoluble chromatin was collected by centrifugation (4 min, 1,700*g*, 4°C), washed once in buffer B, and centrifuged again under the same conditions. To disrupt DNA, the chromatin and nucleoplasm frac-

tions were sonicated with an ultrasonic processor (VCX130; Sonics & Materials, USA) at 30% amplitude, for 30 s total (10 s ON and 20 s OFF). All three fractions were separately centrifuged at 20,000*g* for 5 min.

Statistical analysis

The paired Student's *t*-test was used to analyze data for statistically significant differences. Differences with a P value < 0.05 were considered statistically significant.

RESULTS

Endogenous UPF1 is localized in the nucleus and is associated with the chromatin

It is commonly assumed that abundant cytoplasmic UPF1 is recruited to nuclear originated PTC-containing mRNPs and degrades target RNAs during translation (Fig. 1A). To analyze the location of human UPF1 proteins, HeLa and HEK293 cells were fractionated to the nuclear and cytoplasmic fractions. As expected, UPF1 was mostly found in the cytoplasmic fraction as previously reported (Applequist et al., 1997; Lykke-Andersen et al., 2000), but a substantial portion of UPF1 was also found in the nuclear fraction (Fig. 1B). Two different antibodies were used for western blot analysis, revealing common and prominent localization of UPF1 in the nucleus. Subcellular fractionation was confirmed using nuclear (Lamin B1) or cytoplasmic (α-tubulin) markers (Fig. 1B). To clearly demonstrate the intracellular locations of UPF1, confocal microscopy was used for immunofluorescence staining in HeLa cells. Quantification of the UPF1 fluorescence signal clearly in-

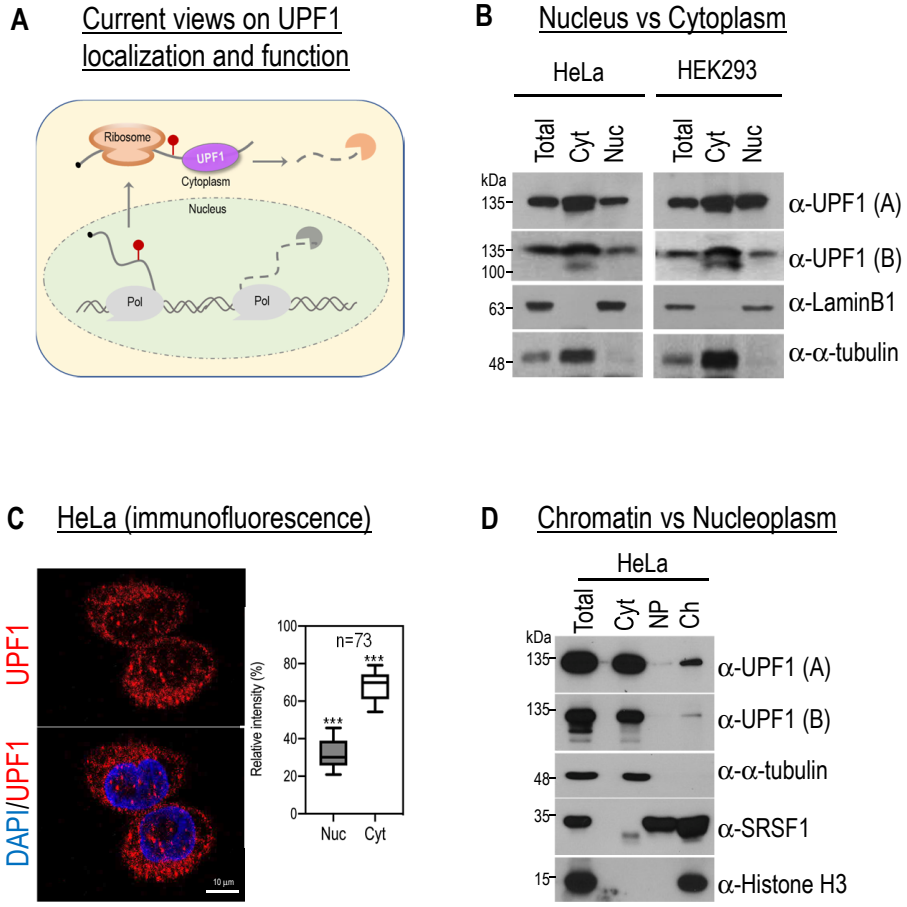

Fig. 1. UPF1 is localized in the nucleus and is associated with chromatin. (A) Current views on nuclear and cytoplasmic RNA surveillance and the localization of UPF1. When a transcript contains a PTC (red dot), it is degraded by NMD in which UPF1 plays a key role. (B) Intracellular localization of UPF1, as determined by cellular fractionation and western blot analysis. Nuclear (Nuc) and cytoplasmic (Cyt) extracts were prepared from HeLa and HEK293 cells and two different UPF1 antibodies (A and B) were used. Lamin B1 was used as a marker for nuclei and α-tubulin for the cytoplasm. (C) Localization of UPF1, as determined by immunofluorescence microscopy. A representative fluorescence image of HeLa cells is shown with anti-UPF1 antibody staining and DAPI for nuclei staining. Cells were counted (n = 73) and the percentages of Nuc and Cyt UPF1 are presented as a graph. (n = 73; ***P < 0.001; Student's *t*-test). (D) HeLa cells were subjected to biochemical fractionation, as described in the Materials and Methods section. Subcellular distribution of UPF1 using anti-UPF1 for western blot detection. The markers showing enrichment of α-tubulin in the cytoplasm (Cyt), Histone H3 in chromatin (Ch), and SRSF1 in the nucleoplasm (NP), and chromatin (Ch).

dicated that ~70% of UPF1 is localized in the cytoplasm, but 30% of UPF1 is persistently localized in the nucleus (Fig. 1C, Supplementary Fig. S1). Intriguingly, nuclear-localized UPF1 is visible as dots or puncta, suggesting its presence on chromatin and/or nuclear envelope-associated locations. Because human UPF1 protein could be involved in safeguarding the stability of the genome, we next investigated whether UPF1 is directly associated with chromatin (Brugiolo et al., 2017). To our surprise, most of UPF1 was strongly associated to the chromatin fraction but no significant amount of UPF1 was found in the nucleoplasmic fraction (Fig. 1D). To demonstrate the quality and equal loading of the fraction, antibodies against α-tubulin, Histone H3 and SRSF1 were used as markers. Of note, SRSF1 can be used as the nucleoplasmic and chromatin markers based on the phosphorylation status. It appears as two discrete bands in SDS-PAGE, which correspond to a slowly migrating hyperphosphorylated species and a fast migrating hypophosphorylated species, respectively (Aubol et al., 2017; 2018; Robichaud and Sonenberg, 2017). Taken together, we found that the nuclear localized UPF1 is mostly associated with chromatin, in addition to the cytoplasmic localized protein.

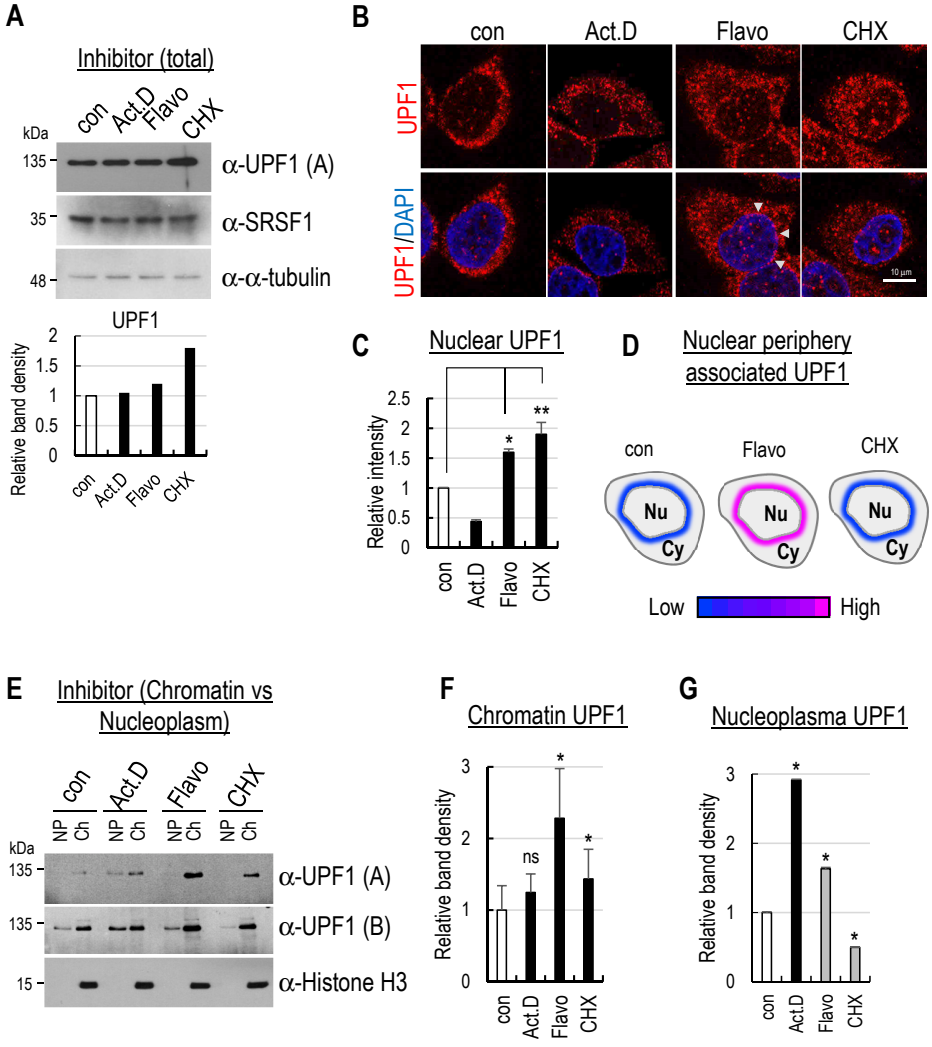

Fig. 2. UPF1 functions in transcription-coupled RNA surveillance. (A) Western blot analysis of HeLa whole-cell lysates after 15 min of incubation with actinomycin D (Act.D; 10 μg/ml), flavopiridol (Flavo; 1 μM), and cycloheximide (CHX; 10 μg/ml). con, control. The protein bands were quantified by ImageJ. (B) Representative immunofluorescence images of methanol-fixed HeLa cells after 15 min of incubation with Act.D (10 μg/ml), Flavo (1 μM), and CHX (10 μg/ml). The cells were incubated with anti-UPF1 antibodies and the nuclei were visualized with DAPI (scale bar = 10 μm). Nuclear periphery accumulated UPF1 is marked with white arrows. (C) Fluorescence intensity of nuclear UPF1. The asterisks indicate statistically significant differences between samples (n = 50; $*P < 0.05$; $**P < 0.01$; Student's *t*-test). (D) The intensity map shows UPF1 localized at the nuclear periphery site. Low intensity is indicated in blue, and higher intensity is indicated in pink. (E) Cells were treated with inhibitors and then were fractionated into cytoplasmic, nucleoplasm (NP), and chromatin (Ch) extracts, followed by western blot analysis using the indicated antibodies. (F) Quantification of chromatin-associated UPF1. ns, not significant. (G) Quantification of nucleoplasm-associated UPF1.

UPF1 is related to transcription elongation-coupled processes

Given the prominent association of UPF1 with chromatin, we tested whether UPF1 is involved in co-transcriptional events. HeLa cells were treated with the transcription initiation inhibitor actinomycin D (Act.D) and the elongation inhibitor flavopiridol (Flavo), which inhibits the transition of Pol II from pausing to productive elongation as a CDK9 inhibitor (Steurer et al., 2018). Acute inhibition of transcription did not change the total level of UPF1, as shown by western blot analysis (Fig. 2A). However, the nuclear level of UPF1 was dramatically changed by transcription inhibitors as shown by immunofluorescence (Fig. 2B). Transcription initiation blockade (Act.D) drastically decreased the nuclear UPF1 protein level, but elongation blockade (Flavo) significantly increased the nuclear level (Fig. 2C). Surprisingly, careful image inspection of immunofluorescence micrographs revealed marked accumulation of UPF1 in the nuclear periphery (likely at the nuclear envelope or envelop-associated chromatin) by Flavo treatment (Fig. 2D). Indeed, subnuclear fractionation more convincingly demonstrated that UPF1 was strongly associated with chromatin when transcription elongation is blocked by Flavo treatment (Figs. 2E and 2F). Because transcription elongation is coupled with many co-transcriptional processes, such as the splicing of pre-mRNAs, export of mRNAs, and possibly RNA surveillance, UPF1 is likely involved in such processes. Intriguingly, most chromatin-bound UPF1 is redistributed to the nucleoplasm by a short pulse of Act.D (Figs. 2E and 2G). Because the amount and time of Act.D treatment are low (10 μg/ml, 15 min), the release of UPF1 into the nucleoplasm may be related to the reduced efficiency of transcription-coupled RNA surveillance (Fig. 2G).

Protein translation and mRNA surveillance pathways are interrelated in the cytoplasm, as exemplified by NMD. So, we also analyzed the UPF1 localization by translation blocker (CHX) treatment (Fig. 2A). Immunofluorescence clearly showed more prominent accumulation of UPF1 in the nucleus with larger sized puncta, compared with the control cells (Fig. 2B). Similar to Flavo-treated cells, CHX-treated cells showed marked accumulation of UPF1 in the nucleus, especially in the chromatin (Figs. 2E and 2F). Taken together, inhibition of transcription elongation and translation markedly accumulates UPF1 in the chromatin. So, we propose the critical role of UPF1 in the transcription-coupled RNA surveillance and the path of mRNP to cytoplasmic RNA machinery.

DISCUSSION

In this study, we demonstrated that significant portion of UPF1 is found in the nucleus, primarily in the chromatin. We also observed cytoplasmic localization of UPF1 as previously reported (Applequist et al., 1997; Atkin et al., 1995; Lykke-Andersen et al., 2000; Serin et al., 2001). Inhibitor treatment experiments suggested the role of UPF1 in transcription-coupled process in the chromatin, which can lead to translation-coupled surveillance in the cytoplasm (Fig. 3). Since protein interaction network analysis demonstrated that UPF1 can interact with proteins associated with RNA metabolism, chromatin-associated UPF1 is likely to be a component of nuclear RNA surveillance machinery (Varsally and Brogna, 2012). Notably, it was recently reported that UPF1 shuttles between the nucleus and cytoplasm and is associated with the Pol II transcription site in *Drosophila* cells (Singh et al., 2019). Our data agree with the findings of this report and suggest that UPF1 plays an important role in the transcription-coupled processes in mammalian cells.

Previous studies on the nuclear function of UPF1 were restricted to the aspect of DNA stability and telomere maintenance (Azzalin, 2012; Azzalin et al., 2007). Thus, our findings propose the novel role of chromatin-associated UPF1 related to transcription-coupled RNA surveillance. Considering the linear flow of genomic information from chromatin to cytoplasm, it still remained to understand whether chromatin-associated UPF1 is linked to NMD in the cytoplasm. To this end, we propose that the nuclear localized UPF1 can be viewed as a component of co-transcriptional RNA machinery in the chromatin and a linker to co-translational RNA surveillance in the cytoplasm (Fig. 3). It is also important to characterize which modified UPF1 is involved in nuclear events and whether such selective protein trafficking in and out of the nucleus affects transcriptional and translational surveillance in mammalian cells.

We utilized three different inhibitors for transcription initiation, elongation and translation. More comprehensive understanding of inhibitors is required to elucidate the transcription and translation-coupled functions of UPF1. For example, Act.

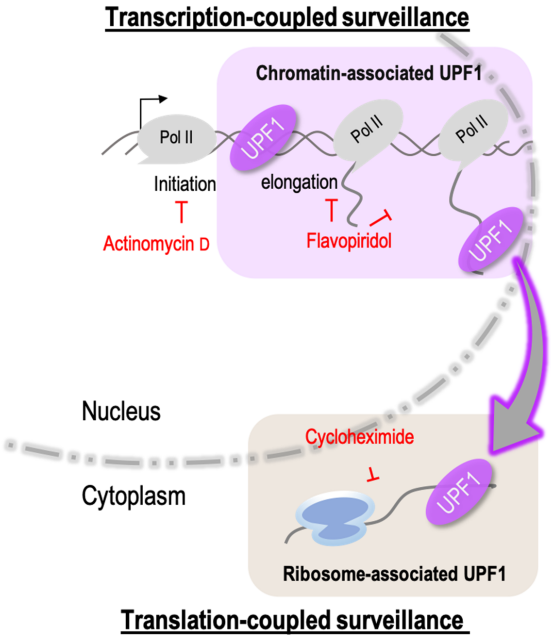

Fig. 3. Model for the UPF1-centric view of nuclear RNA surveillance. UPF1 is associated with chromatin in the nucleus. We suggest that UPF1 plays a role in transcription elongation-coupled RNA surveillance. In addition, nuclear RNA surveillance by UPF1 is also related to mRNA export. Therefore, we suggest that UPF1 is a master regulator from the transcription elongation step to translation-mediated RNA surveillance.

D inhibits RNA polymerase itself or transcription initiation, so transcribing Pol II may be required for UPF1's association with chromatin (Sobell, 1985). The possibility of Act.D-induced DNA damage could also be considered (Steurer et al., 2018). Translation inhibition by CHX is direct or indirect because CHX can induce stress and up-regulate the expression of NMD factors, such as UPF1 as shown here (Kamelgarn et al., 2018). Thus, more analytical approaches should be taken to understand the role of chromatin-associated UPF1. For example, chromatin immunoprecipitation and co-immunoprecipitation is underway to more clearly demonstrate the role of UPF1 as a transcription-coupled regulator.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

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ORCID

Taeyoung Park https://orcid.org/0000-0002-1266-925X
Sunjoo Jeong https://orcid.org/0000-0001-5519-5587

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