Research article

Dev Reprod 2024;28(2):47-54
https://doi.org/10.12717/DR.2024.28.2.47

Development & Reproduction

ISSN 2465-9525 (Print)
ISSN 2465-9541 (Online)

Yeast Small Ubiquitin-Like Modifier (SUMO) Protease Ulp2 is Involved in RNA Splicing

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Received: March 15, 2024
Revised: April 25, 2024
Accepted: May 20, 2024

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Conflict of interests
The authors declare no potential conflict of interest.

Abstract

In eukaryotes, RNA splicing, an essential biological process, is crucial for precise gene expression. Inaccurate RNA splicing can cause aberrant mRNA production, disrupting protein synthesis. To regulate splicing efficiency, some splicing factors are reported to undergo Ubiquitin-like Modifier (SUMO)ylation. Our data indicate that in *Saccharomyces cerevisiae*, the SUMO protease, Ulp2, is involved in splicing. In the *ulp2Δ* mutant, some ribosomal protein (RP) transcripts exhibited a significant increase in the levels of intron-containing pre-mRNA because of improper splicing. Moreover, we confirmed Ulp2 protein binding to the intronic regions of RP genes. These findings highlight a critical Ulp2 role in RP transcript splicing.

Keywords: Ulp2, RNA splicing, Ribosomal proteins, Intron, Post-transcriptional modification

INTRODUCTION

Splicing, which involves excising introns from pre-mRNA and then ligating the exons (De Conti et al., 2013), occurs in a wide range of eukaryotes, including yeast, and is mediated by the spliceosome, a complex assembly of multiple snRNPs and proteins (Karijolich & Yu, 2010). Splicing is crucial for the production of mature mRNA, which is translated into proteins, and for the maintenance of protein functional diversity (Singh, 2002). Consequently, splicing aberrations can be significantly detrimental to cellular function (Choi et al., 2023; Lee et al., 2023).

The distinct sequence motifs, 5' splice site, branch site, and 3' splice site, mark intron–exon junctions. The branch site, which is positioned slightly closer to the intron's 3' end, is typically followed by a polypyrimidine tract, with the branch being centered around an adenine nucleotide (Xie et al., 2023). A conserved GU sequence characterizes the 5' splice site, and an AG sequence marks the 3' splice site (Kitamura-Abe et al., 2004).

Compared with other eukaryotes, yeast has fewer intron-containing genes (Stajich et al., 2007), and most, such as ribosomal protein (RP) genes, contain a single intron and are often highly expressed. However, intron numbers generally increase with increasing organism complexity. For instance, approximately 10% of all human genes contain exons (Sakharkar et al., 2004). Therefore, splicing must

Acknowledgements
This study was supported by the National Research Foundation of Korea (NRF) grant funded by the South Korea government (MSIT) [RS-2023-00212894], Learning & Academic research institution for Master's · Ph.D. students, and Postdocs (LAMP) Program of the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education [RS-2023-00301914], Korea Institute for Advancement of Technology funded by the Ministry of Trade, Industry and Energy [P0025489], and Korean Environment Industry & Technology Institute (KEITI) through Core Technology Development Project for Environmental Diseases Prevention and Management funded by Korean Ministry of Environment (MOE) (no. 2022003310001).

Authors' contributions
Conceptualization: Ryu HY.
Formal analysis: Park JM, Ryu HY.
Data analysis: Choi S, Choi J.
Writing-review & editing: Park JM, Choi S, Choi DK, Lee HS, Cho DH, Choi J, Ryu HY.

Ethics approval
This article does not require IRB/IACUC approval because there are no human and animal participants.

be tightly regulated.

In the budding yeast, *Saccharomyces cerevisiae*, Small Ubiquitin-like Modifier (SUMO) proteins are conjugated to target proteins through the sequential action of E1 activating enzymes, E2 conjugating enzymes, and E3 ligases (Melchior, 2000). This post-translational modification plays pivotal roles in various cellular processes, including transcriptional regulation, DNA repair, translation, and cell cycle progression (Choi et al., 2021; Ryu and Hochstrasser, 2021; Ryu, 2022). A subset of SUMO conjugates undergoes deSUMOylation catalyzed by the proteases, Ulp1 and Ulp2 (Ryu et al., 2019).

Recent studies indicate that for efficient pre-mRNA splicing, SUMOylation is significantly involved in spliceosomal protein regulation (Pozzi et al., 2017). For example, SUMOylation at specific sites, such as Lys-289 and Lys-559 on PRP3, a component of the U4/U6-U5 snRNP complex, is critical. Mutations that disrupt these SUMOylation sites are reported to impede recruitment to the active spliceosome, highlighting the importance of precise SUMOylation for RNA processing dynamics. *In vitro*, recombinant SENP1, a SUMO protease, is reported to diminish pre-mRNA splicing efficiency (Pozzi et al., 2017). Moreover, in the human cell line, HeLa, mRNA splicing-related proteins are known to undergo endogenous polySUMOylation (Bruderer et al., 2011). Here, we investigated the role of Ulp2 protein, a protease involved in SUMOylation regulation, in RNA splicing.

MATERIALS AND METHODS

1. Yeast strains and growth conditions

Table 1 shows the yeast strains used in this study. To generate HYS536, the *snt309Δ::KanMX4* cassette was PCR-amplified using *SNT309* KO primers and HYS418. The amplified products were used to transform MHY500, followed by transformant selection on YPD+G418. The cells were grown at 30℃ in a YPD medium with appropriate supplements. RNA was isolated from cells (one OD 600 equivalent) grown to the mid-exponential phase.

2. RNA-seq data re-analysis

The RNA-seq dataset, GSE121898, from Gene Expression Omnibus, was used to re-analyze *ulp2Δ*'s differentially expressed introns compared with the wild-type (WT) (Ryu et al., 2018). Volcano plots were drawn using R studio's ggplot2 package.

3. ChIP-seq data re-analysis

The ChIP-seq dataset, GSE130623 (Ryu et al., 2019), from Gene Expression Omnibus, was

Table 1. Yeast strains

Strain	Genotype	Source
HYS114 (WT)	MATa *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2*	This study
MHY1379	MATa *his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 ulp2Δ::HIS3* YCplac33-*ULP2*	(Li & Hochstrasser, 2000)
HYS418	MATa *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 snt309Δ::KanMX4*	TAP tag library
HYS536 (*snt309Δ*)	MATa *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 snt309Δ::KanMX4*	This study
HYS93 (*ubc9-1*)	MATa *his3-Δ200 leu2-3,112::LEU2::ubc9-1 ura3-52 lys2-801 trp1-1 gal2 ubc9Δ::TRP1*	This study
HYS90 (*ulp1ts*)	MATa *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ulp1Δ::HIS3* yCplac22-*ulp1ts(3-33)*	This study
HYS183 (*ulp2Δ*)	MATa *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ulp2Δ::HIS3*	This study
MHY7863	MATa *his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 ULP2-6xGly-3xFlag::HIS3MX6*	(Ryu et al., 2016)

WT, wild-type.

https://doi.org/10.12717/DR.2024.28.2.47

used for the re-analysis of Ulp2-Flag enrichment at RP introns. ChIP-seq tracks were viewed using the Integrative Genomics Viewer (https://igv.org/) (Liu et al., 2024).

4. Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from samples using an APure™ total RNA kit (AP Bio, Brooklyn, NY, USA). Next, 1 mg of RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Table 2 shows the sequences of the oligonucleotides used for qPCR. For qPCR, to determine the expression of the RP gene, *RPL31B*, cDNA was diluted at 1:100. All qPCR reactions were done in technical triplicate, and relative RNA levels were determined using the comparative Ct (ΔΔCt) method (Schmittgen & Livak, 2008; Ryu et al., 2020b).

5. Statistical analyses

RT-PCR analyses were done four times. Splicing efficiency was compared using a Student two-tailed *t*-test. Data are presented as mean±SEM. $p<0.05$ indicates statistically significant differences.

6. Data analysis

Gene ontology (GO) analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID). GO data were filtered by EASE score, a modified Fisher's exact *p*-value used on the DAVID database, with an EASE score of <0.1. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted using the YeastErichr database, and the data were filtered based on a Fisher's exact test with an adjusted *p*-value of <0.05 and a combined score.

RESULTS

1. The identification and functional categorization of intron-containing genes in budding yeast

Unlike other eukaryotes, yeast exhibits a relatively modest number of intron-containing genes. Here, using the *Saccharomyces* Genome Database to determine the number of yeast genes with introns, we identified 348 budding yeast intron-bearing genes (Fig. 1A). The genes were categorized into the ribosomal and non-ribosomal groups, with further classification into the large and small ribosomal subunit genes, and 25% of the intron-containing genes were confirmed to be RP genes. Next, we used GO and KEGG pathway analyses to determine the functional categories of intron-containing genes (Fig. 1B–C). These analyses revealed that RP genes essential for translation were significantly abundant, suggesting that during post-transcriptional modification, many RP gene

Table 2. List of oligonucleotides

Name	Sequence
RPL31B pre-mRNA forward	ATTTCTCTGTGTTCTGCGATCGAT
RPL31B pre-mRNA reverse	AGCGCCATTATAGTGTAAACGTGAG
RPL31B total mRNA forward	AAAAGAGGTGTTAAGGGTGTTGAATAC
RPL31B total mRNA reverse	ACGGTTTGTAGACCCTTAGCAGAG
RPL31B mature mRNA forward	GCACAAAAGACTACATGGTGTCAGTT
RPL31B mature mRNA reverse	CAATTCTGGGGCTAGACGGAC
SNT309 KO forward	TCCTTTGTTGAGGGCAGAATACA
SNT309 KO reverse	CAGAGGTCCAAAGGCTGAAGAA

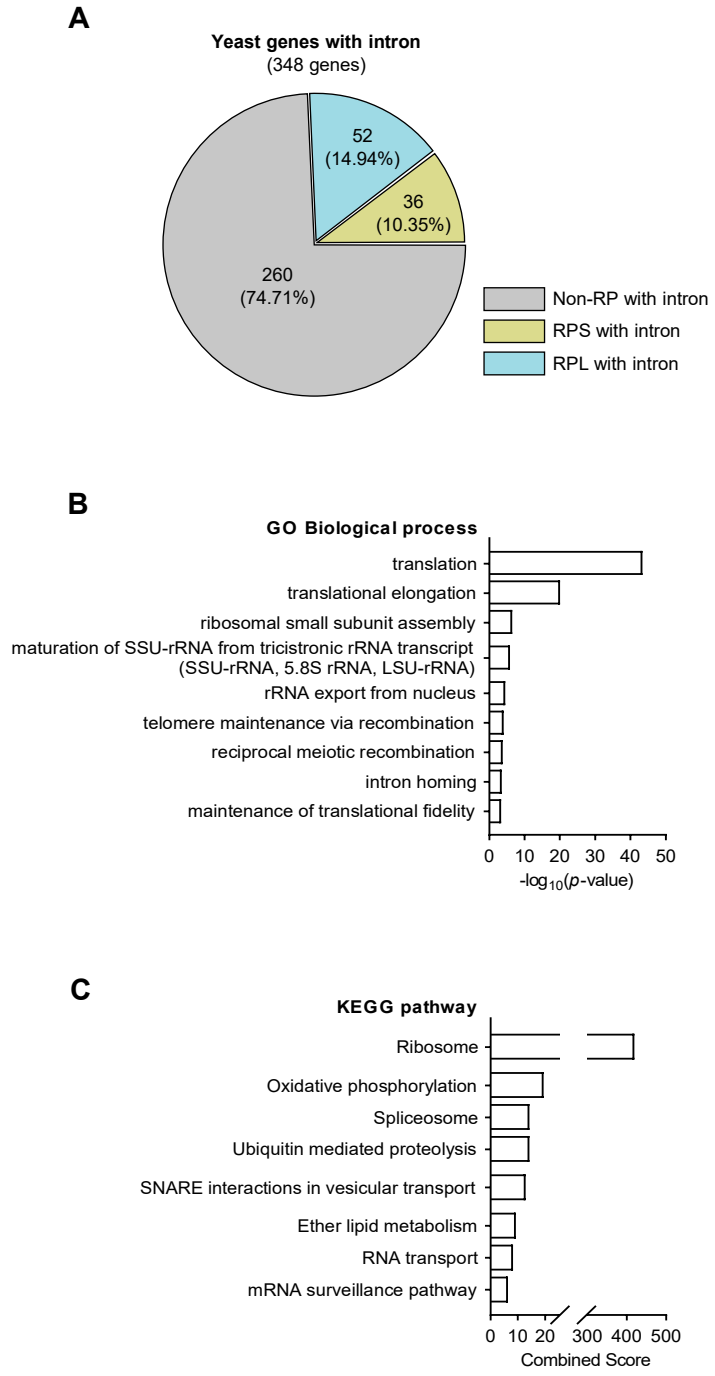

Fig. 1. Analysis of intron-containing budding yeast genes. (A) The percentage of intron-containing genes in *Saccharomyces cerevisiae* was determined using the *Saccharomyces* genome database. (B,C) The characteristics of intron-containing genes were evaluated using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. RP, ribosomal protein.

intronic regions are accurately regulated through splicing.

2. The effects of Ulp2 deficiency on splicing regulation in yeast

Because recent studies indicate that spliceosomal protein SUMOylation regulates splicing (Pozzi et al., 2017), we investigated how SUMOylation deregulation in a *ULP2*-deficient strain impacts

https://doi.org/10.12717/DR.2024.28.2.47

splicing. Next, we used a previously reported RNA-seq WT and *ulp2Δ* mutant dataset (GSE121898) to re-identify differentially expressed introns in the intron-containing genes as depicted in Fig. 1 (Fig. 2A). When compared with the WT, this analysis identified significant changes in RNA transcript levels in the *ulp2Δ* mutants' intronic regions (Fig. 2B). Notably, some RP transcripts had more than a twofold expression increase in *ulp2Δ* mutants' intronic regions (Table 3). Among some RP genes, we observed an increase in the levels of RNA transcripts that retained introns within the entire set of intron-containing RPs (Fig. 2C), suggesting that *ULP2* deletion can dysregulate RNA splicing.

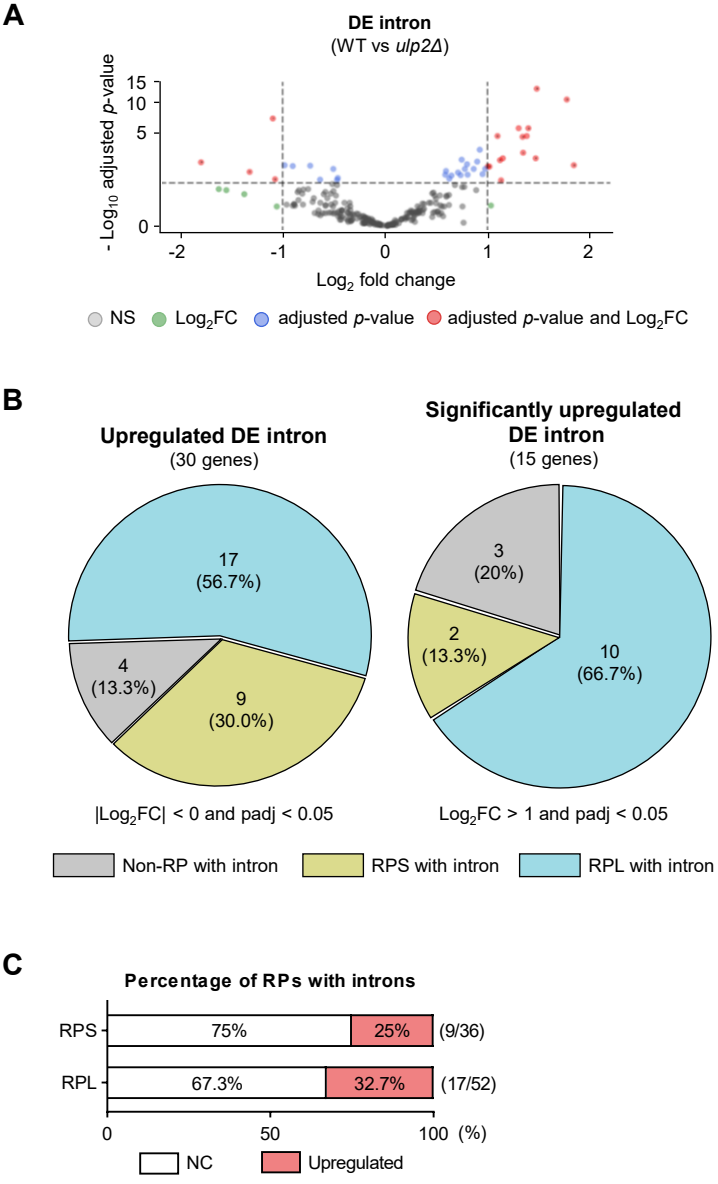

Fig. 2. *ULP2* deletion impaired splicing. (A) An RNA-seq data volcano plot of the differentially expressed exons. In the *ulp2Δ* strain, 320 differentially expressed introns were classified based on $|\log_2$ fold-change$|>1$ and $p<0.05$. (B) The ratio of upregulated RNA intron-containing transcripts in *ulp2Δ*. Left: $|\log_2$ fold-change$|>0$, right: $|\log_2$ fold-change$|>1$. (C) A bar graph of the upregulated intron-containing RP RNA transcripts in *ulp2Δ* vs WT. RP, ribosomal protein.

Table 3. Predicted splicing defect genes in *ulp2Δ* vs WT

Type	Gene name	Description	Log2FC	*P*-adj.
Non-RPs	*BUD25*	Protein involved in bud-site selection	1.844	0.005873694
	COX5B	Subunit Vb of cytochrome c oxidase	1.133	0.038536579
	UBC4	Ubiquitin-conjugating enzyme (E2)	1.471	0.002046848
RPs	*RPS10A*	Ribosomal protein of the small subunit	1.098	2.19389E-05
	RPS30A		1.481	9.33249E-14
	RPL2B	Ribosomal protein of the large subunit	1.003	0.007109459
	RPL6B		1.384	2.19389E-05
	RPL17A		1.123	0.002761582
	RPL17B		1.019	0.007109459
	RPL22A		1.775	3.95807E-11
	RPL26A		1.343	2.6809E-05
	RPL31B		1.349	0.000783984
	RPL33A		1.305	2.66727E-06
	RPL37A		1.400	2.66727E-06
	RPL40B		1.150	0.002046848

RP, ribosomal protein; WT, wild-type.

3. Ulp2 mediates ribosomal protein transcript splicing efficiency

To confirm if the differences observed from the RNA-seq results are actually because of Ulp2 binding, we investigated Ulp2 protein binding to each RP intron using a previously reported ChIP-seq dataset (GSE130623) based on Ulp2-Flag. This analysis confirmed that Ulp2 is highly bound to RP intronic regions (Fig. 3A). Based on these results, to examine *RPL31B*'s splicing efficiency using SUMO pathway mutants, we designed RT-PCR primers divided into three parts (Fig. 3B). By designing primers within the *RPL31B*'s intron, we could measure pre-mRNA amounts, and primers targeting the two exons that are joined through splicing allowed the assessment of mature mRNA levels. As a control, *SNT309*, an mRNA-splicing factor was knocked out (Albulescu et al., 2012). For SUMO pathway mutants, we impaired SUMO-E2 conjugating enzyme function with *ubc9-1*, and functionally impaired or deleted SUMO proteases with *ulp1ts* and *ulp2Δ*. RNA was then extracted from each strain, followed by RT-PCR analysis of the splicing efficiency of *RPL31B* transcripts (Fig. 3C). This analysis revealed that although pre-mRNA levels were not as high as in the *snt309Δ* mutant, they were more than twice higher than in the WT, indicating that Ulp2 is required for splicing of some RP transcript.

DISCUSSION

Although recent studies indicate that SUMOylation regulates various RNA metabolism stages that are associated with tumorigenesis and cancer progression (Ryu et al., 2020a; Cao et al., 2023), it is unclear if the SUMO proteases that regulate SUMOylation directly impact RNA splicing. Here, we show that the Ulp2 protein affects the splicing process. These study's results suggest that Ulp2 protein can regulate several SUMOylated proteins involved in splicing, which may affect splicing efficiency. Additionally, we show that during post-transcriptional modification, ribosomes, which

https://doi.org/10.12717/DR.2024.28.2.47

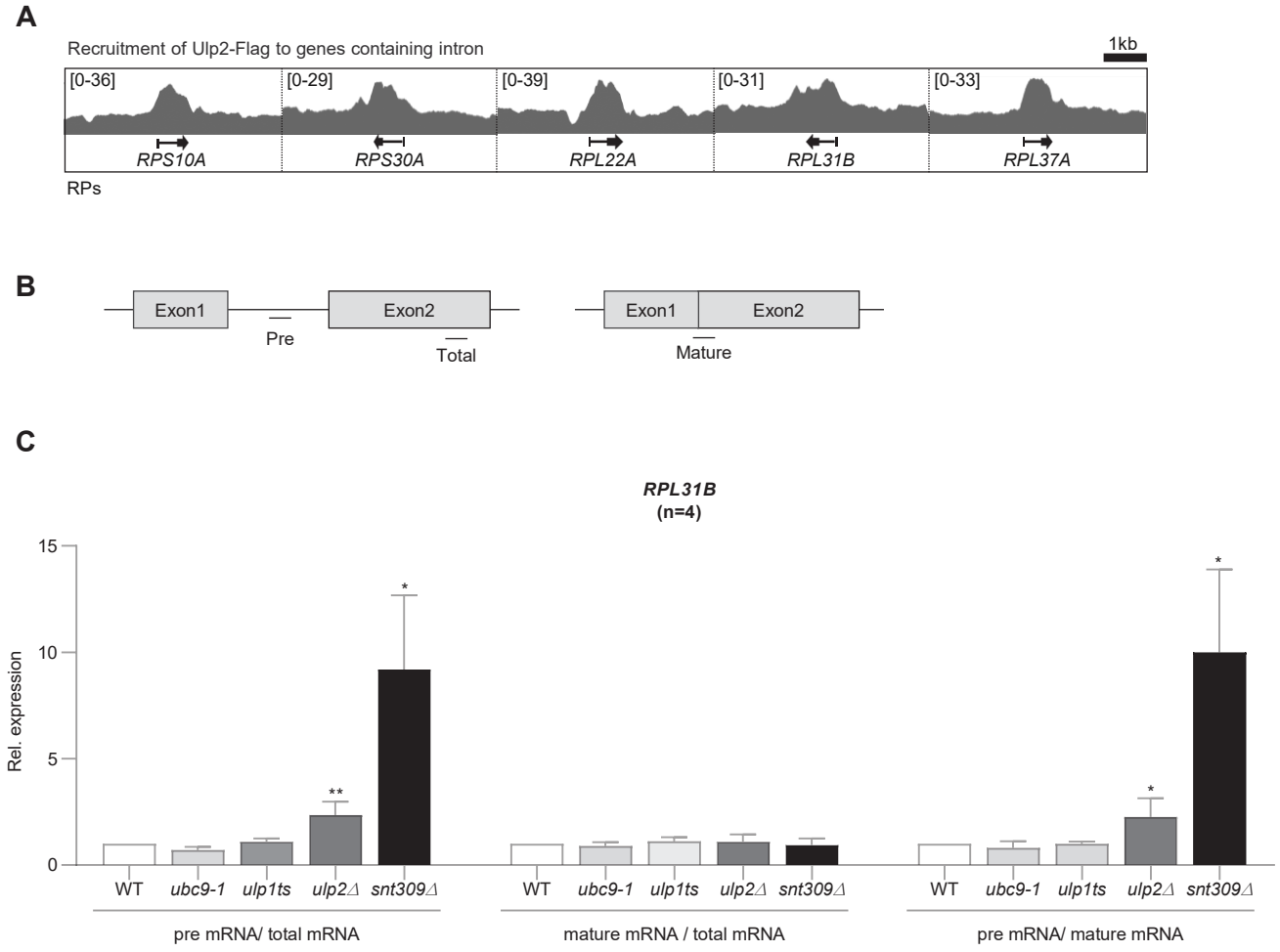

Fig. 3. Ulp2 is involved in pre-mRNA splicing. (A) Ulp2-ChIP-seq data analysis using the integrative genomics viewer revealed significant Ulp2 binding to the intronic regions of intron-containing ribosomal protein genes. (B) The locations of *RPL31B*'s RT-PCR primer pairs are shown. (C) qRT-PCR analysis of the ribosomal protein gene, *RPL31B*, in Small Ubiquitin-like Modifier (SUMO) pathway mutants. Error bars indicate the standard deviation of four independent RNA preparations. Asterisks represent statistically significant differences based on pairwise comparisons between each SUMO pathway mutant and WT (HYS114) using a two-tailed Student t-test (* and **) indicate $p<0.05$ and <0.01, respectively.

play a crucial role in protein synthesis, can be partially regulated by the Ulp2 protein. In future studies, we will aim to provide mechanistic insights into Ulp2 function in post-transcriptional modification.

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