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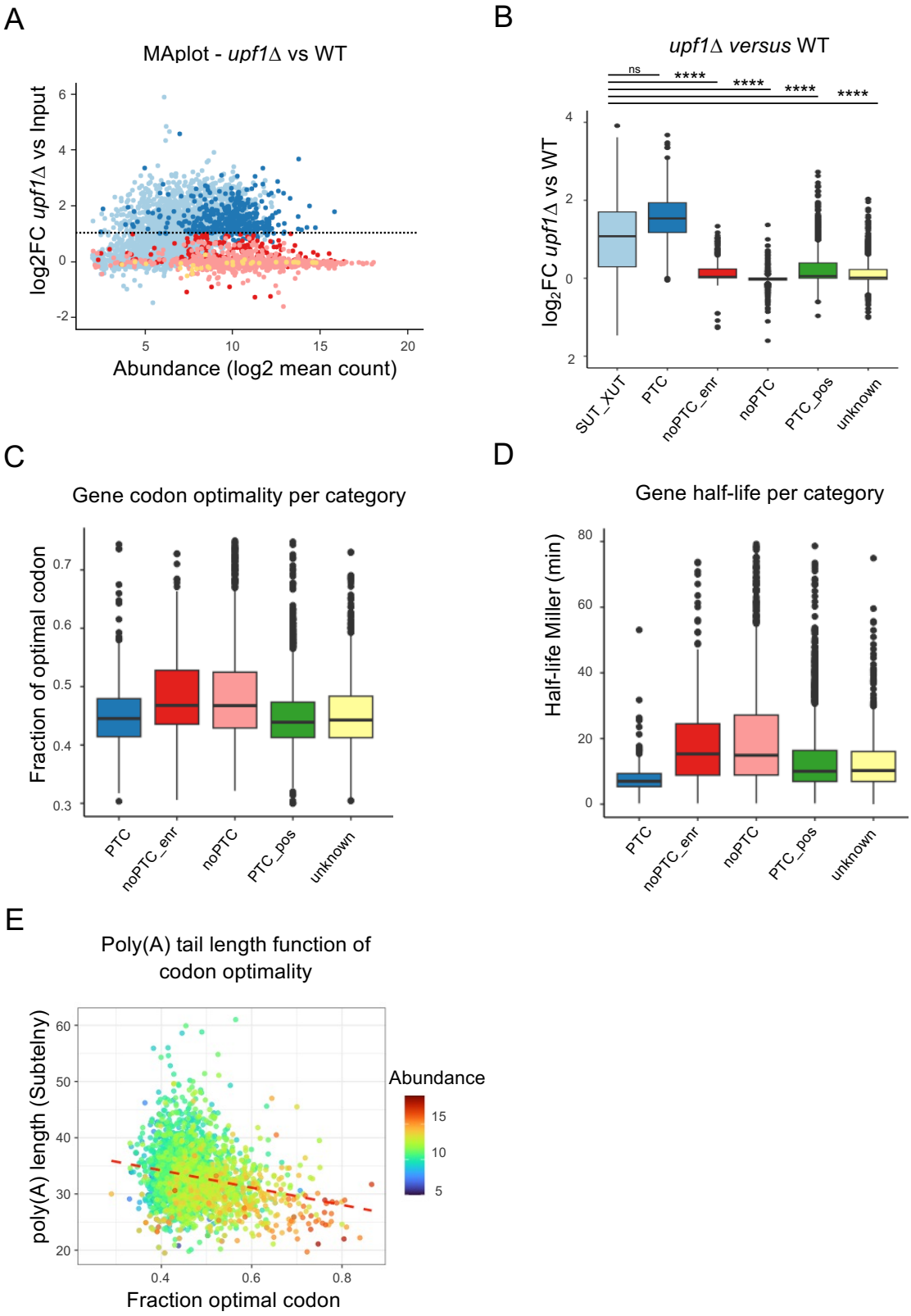
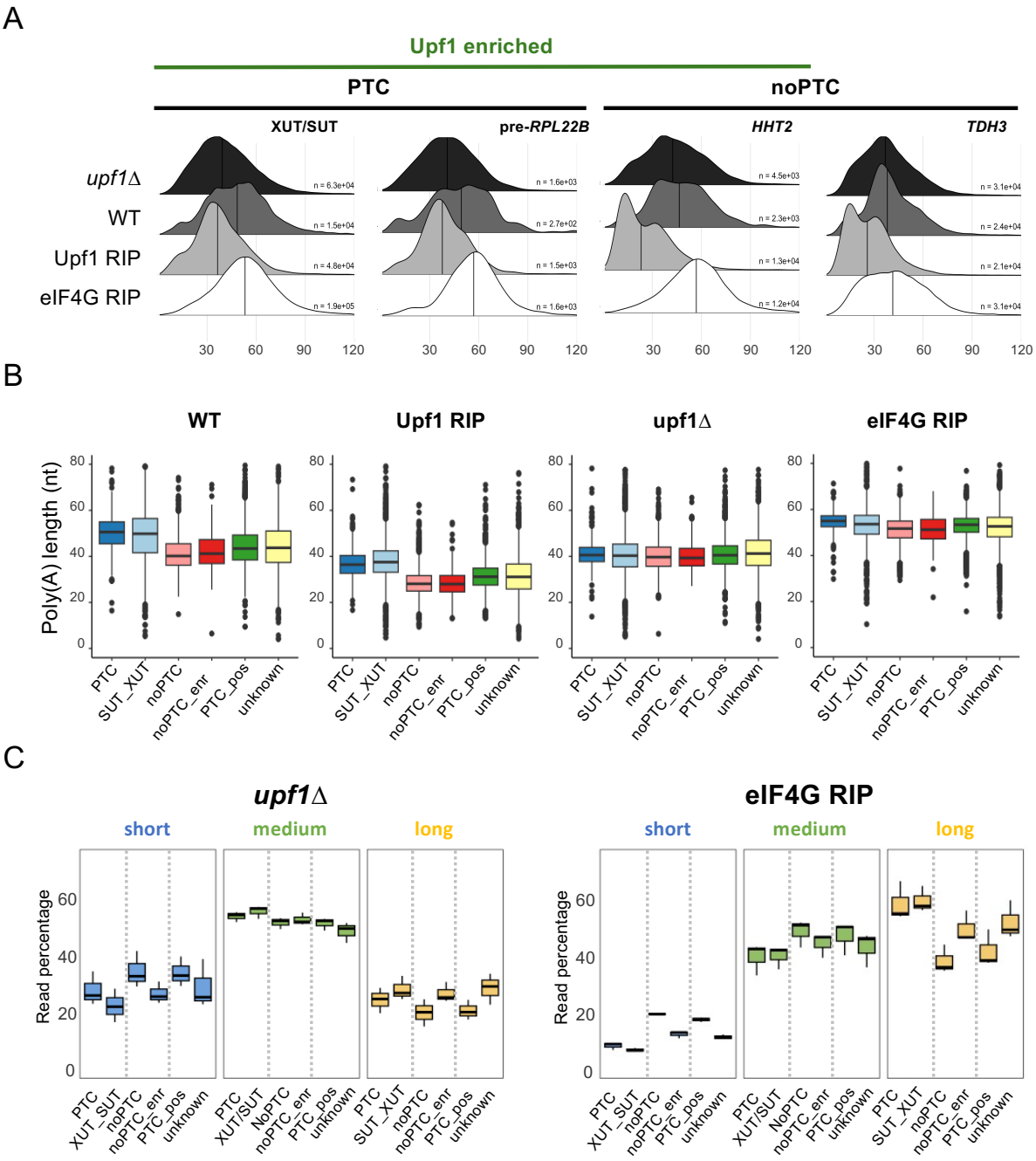


Supplementary Figure S1. Characteristics of transcripts per category, related to Figure 1

(A) MAplot representing the log2FC between *upf1*Δ and WT as a function of abundance (A = log2 mean count in *upf1*Δ or in Upf1-RIP and WT respectively). Each transcript type is represented as follow: XUT/SUT (light blue), PTC (dark blue), noPTC-Upf1enr (red), noPTC (pink), and mitochondrial transcripts as a control (yellow). Dotted lines indicate a 2-fold threshold (log2FC = 1).
(B) Boxplots comparing *upf1*Δ vs WT log2FC for mentioned categories of transcripts. Pvalues (Tukey HSD test) : ns = p> 0.05, * = p<0,05, ** = p<0,01, *** = p<0,001 and **** = p<0,0001. Detailed pvalues of all Figures are listed Table S2.
(C) Boxplot representing the fraction of optimal codon per gene and per category of transcript, determined according to Pechmann and Frydman, 2013.
(D) Boxplot representing the transcript half-lives (in minute, according to Miller *et al.*, 2011) per category.
(E) Scatter plot representing the relationship between the fraction of optimal codon per gene (abscissa, determined from Pechmann and Frydman, 2013) function of the poly(A) tail length in a WT strain at steady state (ordinate, from Subtelny *et al.*, 2014). Each point represents an individual transcript, colored according to its abundance as indicated by the color gradient. The dashed red line represents the linear regression trend line (Pearson's correlation coefficient, r = -0.219 p value < $2.2x10^{-16}$).

Supplementary Figure S2

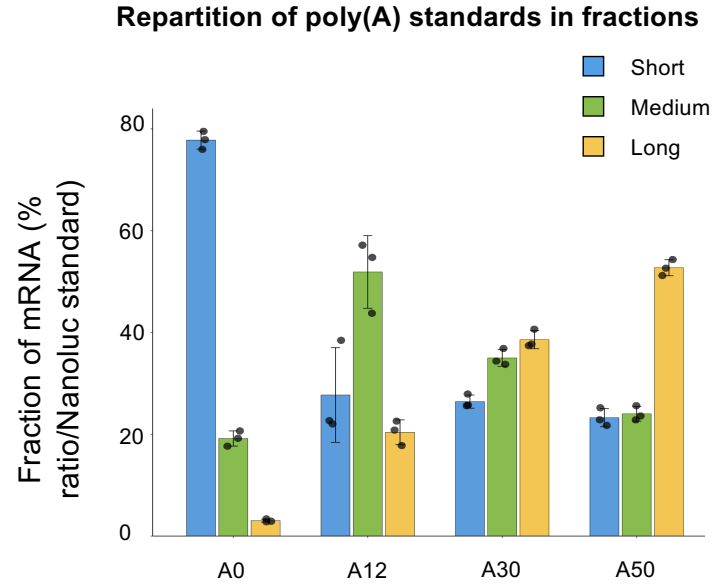


Supplementary Figure S2. Detailed characteristics of poly(A) tails per category, related to Figure 3

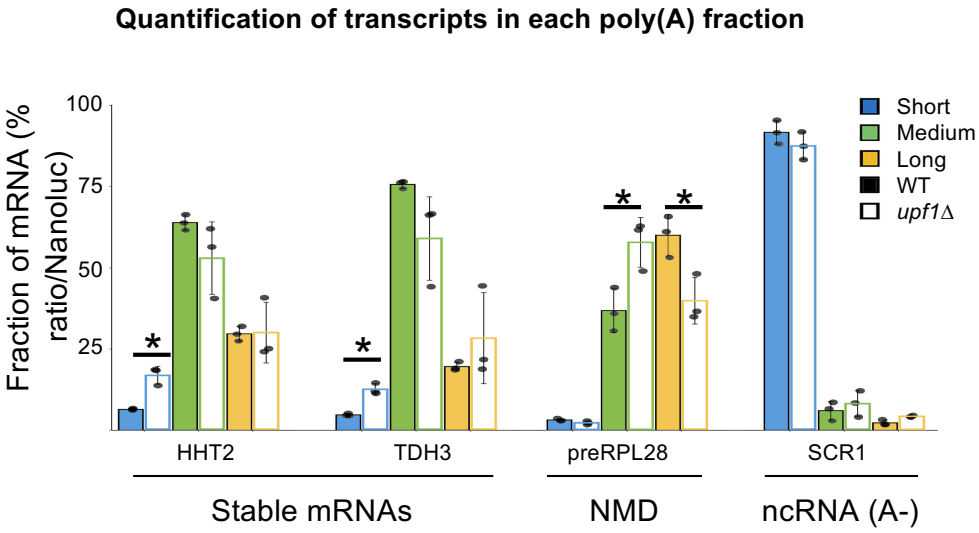
(A) Ridge plot representing the poly(A) tail length distribution across representative examples in WT (Input) and *upf1*Δ total RNA, as well as Upf1-RIP and eIF4G2-RIP samples. XUT/SUT and *pre-RPL22B* are representative examples of PTC-containing NMD targets, HHT2 is a representative example of noPTC-enr, and TDH3 is a representative example of the noPTC category. Upf1-enriched target are mentioned (green line) while PTC/noPTC categories are indicated in black.
(B) Boxplots showing the proportion of poly(A) tail length in short, medium and long bins across *upf1*Δ and eIF4G-RIP samples. The analysis shows the different sub-categories: PTC (dark blue), SUT/XUT (blue), noPTC (pink), noPTC-enr (red), PTC_poss (green), and unknown (yellow).
(C) Boxplots displaying the proportion of poly(A) tail length in bins (short, medium and long) across *upf1*Δ and eIF4G RIP samples.

Supplementary Figure S3

A



B



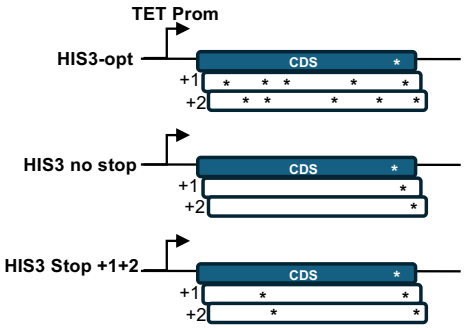
Supplementary Figure S3. Relative quantification of poly(A) subfractions during oligodT fractionation, related to Figure 5

(A) Barplot reporting the relative proportion of each poly(A) tail length in short (S), medium (M), and long (L) fractions of Nanoluciferase mRNA standards with calibrated poly(A) tail lengths (A0, A12, A30 and A50 - see also STAR methods). Data were obtained by RT-qPCR, and show the mean of three biological replicates, black dots represent the value for each replicate, error bar represent the standard deviation (SD).

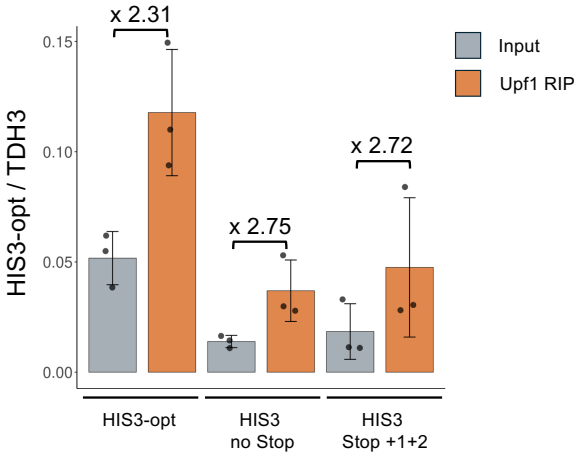
(B) Barplot reporting the relative proportion of poly(A) tail length in each fractions short (blue), medium (green) and long (yellow) of *ScR1* (deadenylated control), *preRPL28* (NMD control), *HHT2* (non-NMD*) and *TDH3* (non-NMD) transcripts, in wild-type (filled-bar) and *upf1*Δ strains (outlined-bar). Data show the mean of three biological replicates, black dots represent the value for each replicate, error bar represent the SD. * (significant P-values T.test.) : (*preRPL28* – Medium) p = 0.02401; (*preRPL28* – Long) p = 0.02279; (*HHT2* – Short) p = 0.02124; (*TDH3* – Short) p = 0.01165.

Supplementary Figure S4

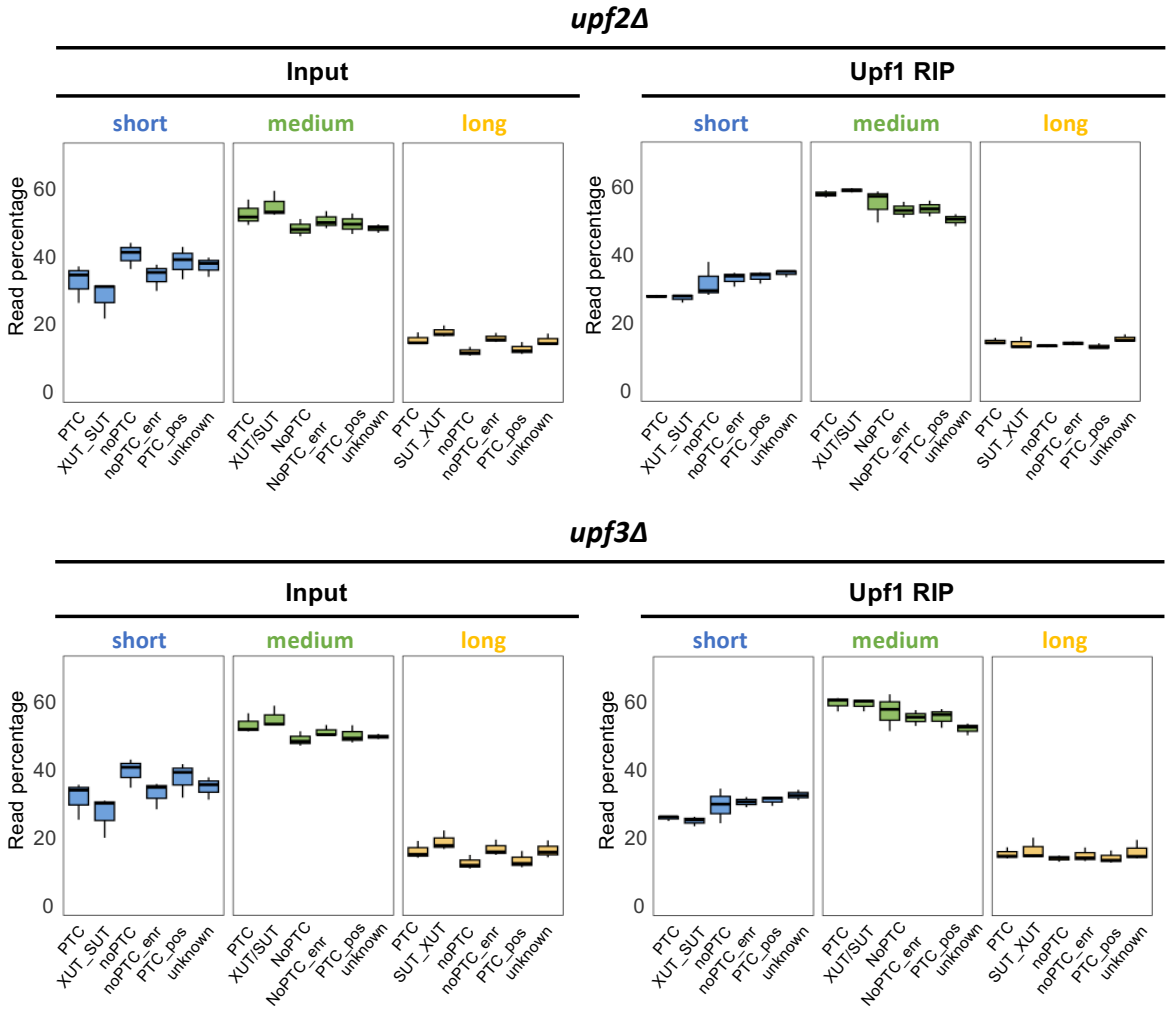
A



B



C



Supplementary Figure S4. Effect of Upf2 or Upf3 depletion on Upf1-associated transcripts, related to Figure 6

(A) Schematic representation of the reporters: *HIS3-opt* ("normal"), *HIS3* no stop (no stop in frame +1 or +2), and *HIS3* Stop +1+2 (with a stop in frame +1 and +2). Stop codons are indicated by stars.

(B) Relative quantification of *HIS3-opt* in Upf1-RIP versus WT input, normalized by *TDH3* (non-enriched control). Data show the mean of three biological replicates, black dots represent the value of each replicate, error bar represent the SD.

(C) Boxplot displaying the proportion of poly(A) tail length in bins short, medium and long across Upf1-RIP and Input RNAs for *upf2*Δ or *upf3*Δ strains, comparing indicated transcript categories as in Figure2B.

Supplementary Table 1
Primer Table

Oligonucleotide	Sequence	Description
LA83	CCGCTCTAACCGAAAAGGAAG	tCyc_fw, Cyc1 cloning and DNA probe - for HIS3opt qPCR
LA84	GGGACCTAGACTTCAGGTTG	tCyc_rv, Cyc1 cloning and DNA probe - for HIS3opt qPCR
CS887	CCATCTCACTGTTGAGACGG	RPL28intron forward for qPCR
CS888	CTCAGTTTGCGATGGAAGAG	RPL28intron reverse for qPCR
CS1400	GCGAGCTCCGCGGCCGCGTTTTTTTTTTT	ePAT primer for all
CS1401	GCGAGCTCCGCGGCCGCGTTTTTTTTTTTV N	TVN-PAT for all (size control, 12A)
CS1402	GCGAGCTCCGCGGCCGCG	ePAT primer for all - step 2
CS1455	CGTTTACAGACATAATTGCGGG	HHT2 for ePAT
GB798	GGTAACATCATCCCATCCTCC	TDH3 forward for qPCR
GB799	CAAGACCTTACCGACAGCC	TDH3 reverse for qPCR
GB987	CAATTATCCGACTGATATGTGC	SCR1 forward for qPCR
GB998	GGTAGTTCTGGGTCCTTAG	SCR1 reverse for qPCR
GB1402	GCCAGGTACTGTTGCCTTGAG	HHT2 forward for qPCR
GB1403	GTCGGTCTTGAAATCTTGAGCG	HHT2 reverse for qPCR
GB1659	ATGGTTTTTACTTTAGAAGATTTTG	Nanoluc all forward for qPCR
GB1679	TAATACGACTCACTATAGGGGCTTGCCGGT TATAAAATTACG	HHT2 probe reverse with T7 for Northern
GB1690	TCTATGCATTTAGACTGGGG	HHT2 forward for Northern
GB1728	GTTGAAAACCTGTTTTACTAACG	RPL28 Intron for ePAT
GB1729	AAACTTGGTCTGACCGTGAT	Nanoluc forward index 1 for qPCR - A12
GB1730	AAACTTGGTCTGACACATCG	Nanoluc forward index 2 for qPCR - A30
GB1731	AAACTTGGTCTGACGCCTAA	Nanoluc forward index 3 for qPCR - A50
GB1732	AAACTTGGTCTGACTGGTCA	Nanoluc forward index 4 for qPCR - A0
GB1733	GAAGAAACACCACCTTGTTC	Nanoluc reverse qPCR - All and each index
GB1734	CGTCTCAGCCGGTAAAGGTC	RPL28 exon for ePAT
GB1761	CCCATTGGACGAAGCTTTGTC	HIS3noSTOP and HIS3+1+2 for qPCR
GB1762	CGACAACAGCGTATGGTCTG	HIS3noSTOP and HIS3+1+2 for qPCR

Supplementary Table 2

Statistical Comparisons and p-values

Figure Number	Category comparison	Sample comparison	Test Performed	p-value	Significance level
1E	All	**Upf1-RIP vs Input**	Anova	<2e-16	***
	PTC vs SUT_XUT		Tukey_HSD	0.7129882	ns
	unknown vs PTC_pos			0.7661451	ns
	All others			<10-7	***
3B	All	**Short poly(A)**	Anova	6.85e-05	***
	Upf1 RIP-Input		Tukey_HSD	0.0005144	**
	Upf1 RIP-Tif4632 RIP			0.0000515	***
	upf1_del-Tif4632 RIP			0.0035811	**
	upf1_del-Upf1 RIP			0.0093503	**
	All	**Medium poly(A)**	Anova	0.0486	*
	Tif4632 RIP-Input		Tukey_HSD	0.0433833	*
	All	**Long poly(A)**	Anova	4.11e-05	***
	Tif4632 RIP-Input		Tukey_HSD	0.0020115	**
	Upf1 RIP-Input			0.0052971	**
	Upf1 RIP-Tif4632 RIP			0.0000257	***
	upf1_del-Tif4632 RIP			0.0005051	***
	upf1_del-Upf1 RIP			0.0295042	*
5C	HHT2	**WT vs *upf1*Δ**	T-test	0.02124	*
	TDH3			0.01165	*
	RPL28premRNA			0.09388	ns
6C	All	**Short poly(A)**	Anova	0.000463	***
	Input_Δupf2-Input		Tukey_HSD	0.0054273	**
	Input_Δupf3-Input			0.0103408	*
	RIP_Upf1-Input			0.0002976	***
	RIP_Upf1_Δupf2-RIP_Upf1			0.0321670	*
	RIP_Upf1_Δupf3-RIP_Upf1			0.0080369	**
	upf1del-RIP_Upf1			0.0171608	*
	All others			>0.05	ns
	All	**Medium poly(A)**	Anova	0.00667	**
	RIP_Upf1_Δupf2-RIP_Upf1		Tukey_HSD	0.0323948	*
	RIP_Upf1_Δupf3-RIP_Upf1			0.0079720	**
	All	**Long poly(A)**	Anova	9.31e-06	***
	Input_Δupf2-Input		Tukey_HSD	0.0001308	***
	Input_Δupf3-Input			0.0002259	***
	RIP_Upf1-Input			0.0000127	***
	RIP_Upf1_Δupf2-Input			0.0002749	***
	RIP_Upf1_Δupf3-Input			0.0005434	***
	upf1del-Input_Δupf2			0.0060493	**
	upf1del-Input_Δupf3			0.0112666	*
	upf1del-RIP_Upf1			0.0003901	***
	upf1del-RIP_Upf1_Δupf2			0.0140438	*
	upf1del-RIP_Upf1_Δupf3			0.0296784	*
S1A	All	***upf1*Δ vs Input**	Anova	<2e-16	***
	unknown vs No_PTC_enr		Tukey_HSD	0.9854782	ns
	PTC_pos vs No_PTC_enr			0.0040005	***
	unknown vs PTC_pos			0.0000196	***

	No_PTC vs No_PTC_enr			0.0000001	***
	All other			<10-7	***
S1B	All	**WT**	Anova	<2e-16	***
	PTC_pos vs PTC		Tukey_HSD	0.9442642	ns
	unknown vs PTC			0.6208114	ns
	unknown vs PTC_pos			0.7679930	ns
	No_PTC vs No_PTC_enr			0.9885980	ns
	All other			<10-7	***
S1C	All	**WT**	Anova	<2e-16	***
	PTC_pos vs PTC		Tukey_HSD	0.9999997	ns
	unknown vs PTC			0.9438146	ns
	unknown vs PTC_pos			0.7411271	ns
	No_PTC vs No_PTC_enr			0.3270383	ns
	All other			<10-7	***
S2B	All	**WT**	Anova	<2e-16	***
	SUT_XUT-NMD		Tukey_HSD	0.9579658	ns
	unknown-NMD_possible			0.4881714	ns
	non_NMD*-non_NMD			0.6010138	ns
	All other			<10-7	***
	All	**Upf1_RIP**	Anova	<2e-16	***
	SUT_XUT-NMD		Tukey_HSD	0.0225963	*
	unknown-NMD_possible			0.4408504	ns
	non_NMD*-non_NMD			0.9940636	ns
	All other			<10-7	***
	All	**upf1Δ**	Anova	1.36e-11	***
	non_NMD-NMD		Tukey_HSD	0.0213614	*
	non_NMD*-NMD			0.0265282	*
	non_NMD-NMD_possible			0.0000620	***
	non_NMD*-NMD_possible			0.0072717	**
	unknown-NMD_possible			0.0215096	*
	SUT_XUT-non_NMD			0.0054339	**
	unknown-non_NMD			<10-7	***
	SUT_XUT-non_NMD*			0.0436739	*
	unknown-non_NMD*			0.0000051	***
	unknown-SUT_XUT			0.0015113	**
	all others			>0.05	ns
	All	**Tif4632-RIP**	Anova	<2e-16	***
	SUT_XUT-NMD_possible		Tukey_HSD	0.9998352	ns
	unknown-NMD_possible			0.0292741	*
	non_NMD*-non_NMD			0.9982048	ns

ns	p > 0.05
*	p < 0.05
**	p < 0.01
***	p < 0.001

Supplementary Table 3
List of reagents and resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Sheep anti-digoxigenin-POD (poly), Fab fragments	Roche	Cat#11633716001
Mouse anti-FLAG M2 - HRP	Sigma-Aldrich	Cat#A8592
Rabbit Peroxidase Anti-Peroxidase soluble complex	Sigma-Aldrich	Cat#P1291
Bacterial strains		
NEB 10-beta competent E. coli	New England Biolabs	Cat#C3019H
Chemicals, peptides, and recombinant proteins		
Clarity Western ECL substrate	Bio-Rad	Cat#1705061
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad	Cat#1725271
TruSeq Stranded mRNA sequencing kit	Illumina	Cat#20020594
NextSeq500 flowcell	Illumina	Cat#20024906
*BamH*I	New England Biolabs	Cat#R0136S
PstI	New England Biolabs	Cat#R0140S
Oligo $d(T)_{25}$ magnetic beads	New England Biolabs	Cat# S1419S
Q5 high-fidelity DNA polymerase	New England Biolabs	Cat#M0491
DNA polymerase I, Large (Klenow) Fragment	New England Biolabs	Cat#M0212S
T4 DNA ligase	New England Biolabs	Cat#M0202T
Direct RNA Sequencing Kit	Oxford Nanopore	Cat#SQK-RNA002
RNasin ribonuclease inhibitor	Promega	Cat#N2511
cOmplete protease inhibitor mix (no EDTA)	Roche	Cat#11873580001
DIG RNA labelling kit (SP6/T7)	Roche	Cat#11175025910
Digoxigenin-11-dUTP	Roche	Cat#11093088910
Indole-3-acetic acid (IAA)	Sigma-Aldrich	Cat#I2886
Beta-estradiol	Sigma-Aldrich	Cat#E2758
Doxycycline	Sigma-Aldrich	Cat#D3447
Rabbit IgG	Sigma-Aldrich	Cat#I5006
DNase TURBO	ThermoFisher	Cat#AM2238
Phusion high-fidelity DNA polymerase	ThermoFisher	Cat#F530L
Superscript II reverse transcriptase	ThermoFisher	Cat#18064022
Superscript III reverse transcriptase	ThermoFisher	Cat#18080044
Dynabeads M-270 epoxy	ThermoFisher	Cat#14302D
Deposited data		
UPF1_RIP, WT and *upf1*Δ Truseq Illumina sequencing	GEO	GSE283053
UPF1-RIP, WT input, *upf1*Δ eIF4G2-RIP, UPF1-RIP in *upf2*Δ (input and RIP) and UPF1-RIP in *upf3*Δ (input and RIP) Nanopore Direct RNA sequencing	GEO	GSE284490
R scripts for filtering Nanopore DRS reads and generating R figures	Zenodo	10.5281/zenodo.14615891
Experimental models: Organisms/strains		
LMA1667 - BY4741 *nam7(upf1)*Δ::KANMX6	[1]	N/A
LMA2154 (BY4741) (*S. cerevisiae*) *MATa ura3*Δ0 *his3*Δ1 *leu2*Δ0 *met15*Δ0	[2]	N/A

LMA2194 - BY4741 + NAM7*(UPF1)*::HIS3MX6	[3]	N/A
LMA3730 – BY4741 + NAM7*(UPF1)*-HTP	This study	N/A
LMA3314 – BY4741 + TIF4632-TAP::HIS3MX6	[3]	N/A
LMA3682 – PB1623 (S. pombe) + *nam7(upf1)*-CRAC	This study	N/A
Oligonucleotides		
PCR primers	This study	Table_Primers
Recombinant DNA		
pCM189-NFLAG-HIS3-100 p1640 (TETO7-NFLAG-HIS3-100) (URA3)	[4]	N/A
pCM189-NFLAG-HIS3-100-NoSTOP p1803 (TETO7-NFLAG-HIS3-100) (URA3)	This study	N/A
pCM189-NFLAG-HIS3-100-STOP+1+2 p1804 (TETO7-NFLAG-HIS3-100) (URA3)	This study	N/A
Software and algorithms		
ImageJ (1.54)	National Institutes of Health (NIH) https://imagej.net/ij/	N/A
Image Lab Software (6.1)	Bio-Rad	12012931
CFX Maestro Software (qPCR)	Bio-Rad	12013758
STAR (2.7)	[5]	N/A
Integrated Genome Viewer (IGV, 2.11)	[6]	N/A
R (3.8-4.4)	(R Core Team, 2024) https://www.R-project.org/	N/A
RStudio	https://posit.co/products/open-source/rstudio/	N/A
Guppy	Oxford Nanopore	N/A
Minimap2 (2.1)	[7]	N/A
SAMtools (1.9)	[8]	N/A
Nanopolish (0.13.2)	[9]	N/A
Rscripts were deposited on Zenodo (see Deposited Data)	Zenodo	10.5281/zenodo.14615891
Other		
ChemiDoc XRS+ imaging device	Bio-Rad	N/A
Trans-Blot Turbo transfer system	Bio-Rad	Cat#1704150
Supported nitrocellulose membrane	Bio-Rad	Cat#1620094
Amersham Hybond-N+	Cytiva Life Sciences	Cat#RPN303B
NextSeq 500	Illumina	N/A
Gel and PCR clean-up	Macherey-Nagel	Cat#740609.250
MinION sequencing device	Oxford Nanopore	N/A
GridION sequencing device	Oxford Nanopore	N/A
NuPAGE 4–12% Bis-Tris electrophoresis gel	ThermoFisher	Cat#NP0323BOX

Supplementary references

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