The DNA damage response and RNA Polymerase II regulator Def1 has posttranscriptional functions in the cytoplasm

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Abstract:

Yeast Def1 mediates RNA polymerase II degradation and transcription elongation during stress. Def1 is predominantly cytoplasmic, and DNA damage signals cause its proteolytic processing, liberating its N-terminus to enter the nucleus. Cytoplasmic functions for this abundant protein have not been identified. Proximity-labeling (BioID) experiments indicate that Def1 binds to an array of proteins involved in posttranscriptional control and translation of mRNAs. Deleting *DEF1* reduces both mRNA synthesis and decay rates, indicating transcript buffering in the mutant. Directly tethering Def1 to a reporter mRNA suppressed expression, suggesting that Def1 directly regulates mRNAs. Surprisingly, we found that Def1 interacts with polyribosomes, which requires its ubiquitin-binding domain located in its N-terminus. The binding of Def1 to ribosomes requires the ubiquitylation of eS7a (Rsp7A) in the small subunit by the Not4 protein in the Ccr4-Not complex. Not4 ubiquitylation of the ribosome regulates translation quality control and co-translational mRNA decay. The polyglutamine-rich unstructured C-terminus of Def1 is required for its interaction with decay and translation factors, suggesting that Def1 acts as a ubiquitin-dependent scaffold to link translation status to mRNA decay. Thus, we have identified a novel function for this transcription and DNA damage response factor in posttranscriptional regulation in the cytoplasm and establish Def1 as a master regulator of gene expression, functioning during transcription, mRNA decay, and translation.

Introduction:

Controlling gene expression and maintaining genomic integrity are intertwined and require coordinating multiple processes in the cytoplasm and the nucleus. One type of coordination is the phenomenon called gene expression buffering, whereby changes in mRNA synthesis in the nucleus are compensated by changes in mRNA decay in the cytoplasm(SUN *et al.* 2012; HAIMOVICH *et al.* 2013a; TIMMERS AND TORA 2018). Sometimes, a factor initially identified for a function in gene expression in one cellular compartment is later found to participate in other control steps in another. For example, complexes and proteins that were known to affect transcription in the nucleus were later found to control posttranscriptional events in the cytoplasm (HAIMOVICH *et al.* 2013a; HAIMOVICH *et al.* 2013b; REESE 2013; SUN *et al.* 2013). These types of proteins are attractive candidates to explore how different stages in gene expression are coregulated. How coordination is achieved is unclear, but it may involve either the shuttling of these regulators between the nucleus and cytoplasm or indirectly through feedback loops.

DNA damaging agents shut down multiple steps in gene expression, impacting transcription, mRNA decay, and translation. One response to damaged DNA is the ubiquitylation and degradation of the large subunit of RNA Polymerase II (RNAPII), Rpb1 (WILSON *et al.* 2013a; STEURER AND MARTEIJN 2017; AKINNIYI AND REESE 2021; REESE 2023). An essential protein in this process is yeast DEgradation Factor 1 (DEF1), first discovered as a protein co-purifying with the chromatin-bound transcription-coupled repair protein Rad26 (WOUDSTRA *et al.* 2002). Def1 binds arrested RNAPII and acts as a scaffold to initiate the recruitment of ubiquitin ligases and other factors used to degrade RNAPII(SOMESH *et al.* 2005; WILSON *et al.* 2013b; AKINNIYI AND REESE 2021). Def1 was found to undergo DNA damage or transcription stress-dependent proteasome-mediated proteolytic processing in the cytoplasm(WILSON *et al.* 2013b). The processing separates the N-terminus of the protein from the unstructured, polyglutamine-rich region located in the C-terminus that retains the protein in the cytoplasm. Def1 has nuclear functions, such as regulating TFIIH and transcription initiation, elongation, telomere maintenance, and multiple DNA repair pathways (AKINNIYI AND REESE 2021). Although much attention has been given to Def1's nuclear functions, it is a very abundant cytoplasmic protein. A

major question is if Def1 has cytoplasmic functions or if its retention in this compartment only prevents it from interfering with RNAPII transcription in the nucleus under non-stress conditions. A hint of the former hypothesis is proximity labeling (BioID) experiments found that subunits of Ccr4-Not deadenylase complex interact with Def1 and that the presumed human homology of Def1, UBPA2/2L, binds multiple cytoplasmic RNA binding proteins (YOUN *et al.* 2018; PFANNENSTEIN J 2024).

Ccr4-Not was first identified as a transcriptional regulator in yeast, but it is now known to function at many points of gene control, including transcription, mRNA deadenylation, translation quality control and protein ubiquitylation(MILLER AND REESE 2012; COLLART 2016; CHALABI HAGKARIM AND GRAND 2020). The complex is the major mRNA deadenylase that initiates degradation by polyA tail removal. We have shown that Ccr4-Not in the nucleus promotes transcription elongation and is essential for the DNA-damage-dependent destruction of the large subunit of RNA polymerase II, Rpb1, a function shared with Def1(KRUK *et al.* 2011; REESE 2013; JIANG *et al.* 2019). Although Ccr4-Not's Not4 subunit is an RING domain ubiquitin ligase, it does not directly modify Rpb1. Instead, it functions by recruiting the HECT domain E3 ligase Rsp5 and the E2 enzyme Ubc4. Rsp5 ubiquitylation of Rpb1 recruits Def1 to polymerase. The collection of substrates modified by Not4 is not known. One of the first proteins identified to be ubiquitylated by Not4 is eS7a (Rsp7a), a protein in the small subunit of the ribosome(PANASENKO AND COLLART 2012; PANASENKO 2014; BUSCHAUER *et al.* 2020; ALLEN *et al.* 2021). Here, it functions during translation quality control. It was later discovered that the Ccr4-Not complex plays a role in co-translational degradation of poorly translated mRNAs, such as those rich in non-optimal codons. Arrest of the ribosome over a non-optimal codon in the A-site of the ribosome leads to the evacuation of the tRNA in the E-site, which is then filled by the N-terminus of the Not5 subunit of the complex. Then, Not4 ubiquitylates eS7a, and Ccr4-Not's deadenylase activity degrades the transcript(BUSCHAUER *et al.* 2020). Although the Not4-dependent ubiquitylation of the small subunit is associated with translation quality control and co-translational decay, precisely what recognizes this modification and initiates these downstream events is unclear.

Here, we describe the cytoplasmic functions of yeast Def1. We demonstrate that Def1 binds many cytoplasmic proteins involved in posttranscriptional control of mRNAs, such as those involved in decay and translation. The loss of Def1 reduces mRNA decay rates globally, and a tethering reporter assay suggests that Def1 directly represses mRNA expression. We find that Def1 is associated with polyribosomes, which is dependent on its ubiquitin-binding domain and Not4-dependent ubiquitylation of the small subunit of the ribosome. We present evidence that suggests the C-terminus of Def1 recruits translation control and mRNA decay factors to the ribosome. Thus, we have identified a novel function for Def1 and uncovered a previously unknown consequence of ribosome protein ubiquitylation. Drawing parallels to this cytoplasmic function and how it regulates Rpb1 degradation during DNA damage, we propose that Def1 is an important ubiquitin-dependent scaffold protein that regulates multiple stages of gene expression.

METHODS

Yeast strains and plasmids

Strains and plasmids used in this work are contained in Supplemental Tables 1 and 2, respectively. Gene deletions were produced by homologous recombination using PCR-generated cassettes (BRACHMANN *et al.* 1998). CUE domain mutants of *DEF1* were constructed using CRISPR-Cas9 gene editing as described (LAUGHERY *et al.* 2015). GFP fusion of Def1 were constructed as described in a previous publication (LONGTINE *et al.* 1998). Genotypes of mutants were screened by PCR of genomic DNA and sequencing of PCR fragments (gene editing).

Growth spot assay

Strains were grown at 30˚C to saturation in 5 ml of the appropriate medium. Serial dilutions were prepared in sterile distilled water, and diluted samples were spotted on plates containing the appropriate media and supplements. Plates were incubated at 30˚C for 24 hr or more, as indicated in the respective figures.

Auxin-inducible degron rapid depletion

Auxin-inducible degron (AID) strains were generated using plasmid constructs as previously used and described (SHETTY *et al.* 2019). Cells were grown to mid-log (OD600~0.5-0.6) phase in 1% yeast extract, 2% bacto-peptone, 2% dextrose supplemented with 20 ug/ml adenine sulfate (YPAD). Auxin (Indole-3-acetic acid, IAA) was added to a concentration of 500 uM for 2 hr. To monitor protein depletion, 3.5 OD600 cells were harvested by centrifuging at 3000 rpm for 5 minutes and then washed with sterile distilled water. Proteins were isolated by TCA protein extraction.

Trichloroacetic acid (TCA) lysate preparation and Western blotting

Approximately 5 OD600 cells were harvested by centrifugation, washed in cold sterile distilled water, and resuspended in 250 ul 20% TCA. Three hundred microliters of glass beads were added, and cells were bead-beaten on a foam-head vortexer for 20 min. 500 ul 5% TCA was added, and lysates were vortexed for 10sec. Lysates were recovered from beads and centrifuged at 10000 rpm for 5 min at 4˚C. Pellets were rinsed with 1 ml ice-cold 0.25M Tris pH 7.4, re-centrifuged and the pellet was resuspended in 120 ul 0.15 M Tris-HCl pH 9. 60 ul 3X SDS-PAGE buffer was added, and samples were boiled for 7min. Samples were centrifuged at top speed for 10 min, and supernatants were stored at -20˚C till needed. Samples were separated on either SDS-PAGE or Tris-acetate gradient gels, and proteins were transferred unto 0.1 uM (small MW proteins) or 0.45um nitrocellulose membrane using a semi-dry transfer apparatus. Membranes were ponceau-stained (0.1% Ponceau S, 2% acetic acid), imaged and de-stained. Membranes were blocked in 5% milk in TBST 50mM Tris-HCl, pH 7.5, 0.15M NaCl, 0.1mM EDTA, and 0.1% Tween-20) for at least 1hr and then probed with the primary antibody in 2% or 4% milk for at least 2hr. Antibodies to Not1, Not4, Taf14 and Dhh1 are described in another publication (PFANNENSTEIN J 2024). Antibodies to Def1 were raised in rabbits to a 6HIS-Def1 (1-500) fusion. Commercial primary antibodies used are Anti-GFP (UBPbio, Y1031), Anti-FLAG M2 (Millipore Sigma) and anti-HA, (HA.11, Biolegend). Signals were detected by fluorescence or chemiluminescence (ECL) and imaged on X-ray film or a BioRad ChemiDoc-MP imaging system.

BioID

Turbo ID (TID) was fused to the C-terminus of Def1, and truncation derivatives, using PCR-derived cassettes from pJR106 as described in (PFANNENSTEIN J 2024). Free TID was expressed from the CHA1 promoter, and this strain was used as the control. Cells were grown to OD600□0.4 and 0.7, respectively, in synthetic complete media + 2% dextrose. Biotin was added to a final concentration of 1 uM for 3 hr. Cells were harvested (OD600=1-1.2) and washed twice with ice-cold STE-containing protease inhibitors (0.5 mM PMSF, 1 mM Benzamidine-HCl). Cells were resuspended in SA buffer (20 mM HEPES-KOH pH 7.5, 10 mM MgOAc, 0.4 M NaCl, 2 mM EGTA, 10% glycerol) with protease inhibitors (0.5ug/ml leupeptin, 1ug/ml aproptinin, 1 mM benzamidine-HCl, 0.5 mM PMSF, 1 mM DTT). 300ul glass beads were added and cells were bead-beaten on a Beadbuster (Highmark Scientific) at 4 cycles, 30 seconds on, 1 min off. Nucleic acid digestion was carried out by incubation with benzonase for 15min. Lysates were spun twice at 14k rpm for a total of 3min. The extract (supernatant) was recovered and protein concentration was determined using Bradford assay. 1 mg of protein was diluted into 1 ml, and SDS was added to a final concentration of 0.1%. 50 ug protein equivalent of the SDS-treated cell extracts was saved as input sample. Streptavidin magnetic beads (Pierce, Thermofisher, #88817) were washed thrice in wash buffer 1 (0.1% Triton X-100 in SA buffer) and resuspended at a final concentration of 10mg/ml, then used to pull down biotinylated proteins by incubating 25ul washed beads with 950ul cell extracts overnight at 4□C. Beads were washed at 4□C twice with SA wash buffer 1 (0.1% Triton X-100 in SA buffer) for 1min and 10min respectively and collected on magnet, then washed once in wash buffer 2 (20mM HEPES-OH pH 7.5, 10 mM MgOAc, 1.5 M NaCl, 2mM EGTA, 0.1% Triton X-100, 10% glycerol, 1mM DTT, protease inhibitors) for 10min, once in wash buffer 3 (2M urea, 100mM tris pH 8, 0.2% SDS) for 10min, once in wash buffer 4 (50mM HEPESS pH 7.5, 0.5M NaCl, 0.1% sodium deoxycholate, 1% triton x-100, 1mM EDTA) for 10min, and once in wash buffer 5 (20m M Tris pH 8, 250 mM LiCl, 0.5% NP-40, 1 mM EDTA, 0.5% sodium deoxycholate) for 10min. For quality control a portion (10%) of the beads were eluted in 1X SDS-PAGE (containing 3.3 mM biotin, 15 mM tris pH 8 and 15 mM NaCl) by boiling for 7 min and collected by incubating beads on magnet. A fraction of that was analyzed

by blotting using ultra-Streptavidin-horseradish peroxidase conjugates, SA-HRP (ThermoFisher). Streptavidin pulled-down proteins were digested on the beads with trypsin-digested after alkylation with iodoacetamide. Purified peptides were labeled with TMTpro 16plex reagents (ThermoFisher, #A44520) per the manufacturer's recommended protocol. Details are found in (KULKARNI *et al.* 2024).

RT-qPCR

RNA samples were quantified on Nanodrop and run on 1% agarose gel to confirm the quality. 1 ug of RNA was treated with 1U DNaseI for 30 min at 37˚C followed by quenching with 5 mM EDTA at 65˚C for 10 min. RevertAid RT kit (FERK1622) was used for cDNA preparation according to the manufacturer's protocol using a mix of random and oligo dT primers. Serial dilutions of cDNA were used to set up qPCR reactions. PCR reactions were performed with PerfeCTa SYBR green SuperMix and using an Agilent Real-Time machine. The data was analyzed using the Agilent AriaMx software and Excel.

Total RNA-seq for steady state mRNA levels

Cells were grown in YPAD and harvested at log phase (OD600~0.8-1). Cells were washed with nuclease-free water and resuspended in AE-buffer (50m sodium acetate pH 5.3, 10 mM EDTA). Cells were spiked with S. pombe cells at a ratio of 14:1. RNA was isolated using AE-phenol chloroform extraction, followed by overnight ethanol precipitation with sodium acetate. RNA samples were washed with 80% ethanol and resuspended in 150 uL nuclease-free water. RNA was quantified on Nanodrop, and the quality was verified by analysis on a Tapestation. RNA samples with RIN scores >9.0 were used.

RNA metabolic labeling studies

The procedure is a derivative of that used in a previous publication (FRANCETTE AND ARNDT 2024). *Saccharomyces cerevisiae* cells growing in YPD were treated at log phase (OD_{600}~0.8-1) for 6-, 9-, 12-, 24- and 90-min with 0.65mg/ml 4-thio-uracil. Thirty OD_{600} cultures were transferred to 2 volumes ice-cold methanol on dry ice to halt 4tU

incorporation. Cells were harvested by centrifugation at 3000 rpm for 3 min, and resuspended in AE-buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA). The *S. cerevisiae* cells were spiked with pre-labeled *S. pombe* cells at a ratio of 1:14. *S. pombe* spike-in were generated by growing cells in YPD to log phase (OD_{600}~0.8) and treated with 50 uM 4tU for 12min. 250 OD_{600} culture was split and transferred into 2 volumes ice-cold methanol. Cells were harvested and resuspended in cold YPD at a final density of 5 OD_{600}/ml. Cells were frozen at -80°C till needed.

RNA was isolated using AE-phenol, then chloroform, followed by overnight ethanol precipitation with sodium acetate. RNA samples were resuspended in 150uL nuclease-free water. RNA was subjected to biotinylation (120 ug RNA, 20 mM HEPES pH 7.4, 12 ug MTSEA biotin-XX (Biotium Co) in 600ul final volume for 30 min at room temperature. Free MTS-biotin reagent was removed by PCIAA-extraction and precipitation with NaCl and isopropanol. Dynabead MyOne streptavidin C1 beads were washed twice in high salt wash buffer, HSWB, (100 mM Tris-HCl pH 7.4, 10 mM EDTA, 1M NaCl) and blocked with blocking buffer (100 mM Tris-HCl ph7.4, 10 mM EDTA, 1M NaCl, 40 ng/ul glycogen), then resuspended in HSWB. 80 ug of biotinylated RNA was incubated at 65°C for 7 min and streptavidin pull down was performed on 80ug equivalent of preheated RNA using 100 ul blocked beads, washed thrice with HSWB, and eluted twice in 25 ul elution buffer (100 mM DTT, 10 mM EDTA, 1M NaCl, 0.05% Tween-20). The RNA was concentrated, quantified on a Qubit instrument and used for library preparation.

Library preparation and NextGen sequencing

Total RNA was depleted of rRNA using the Qiagen FastSelect (#334215)kit. Libraries for steady-state RNA and RATE-seq samples were prepared using the Illumina Total Stranded library prep kit and the Illumina UDI indexes (#20040529). The sequencing libraries were analyzed on TapeStation-4150 at the Genomics Core Facility, Penn State University, USA and were quantified using NEBNext® Library Quant Kit for Illumina®. Libraries were sequenced on the Illumina NextSeq 2000. Illumina P2 100 cycle reagent cartridge #EC1591461-EC11 was used for paired-end sequencing. Reads were mapped to the sacCer3 genome using the ENCODE 3 pipeline. Spike-in reads

were mapped to the S. pombe genome (https://www.encodeproject.org/pipelines/ENCPL002LPE/). Star was used to map the reads, and RSEM-1.2.28 to get estimated counts and TPMs (Genes: Saccharomyces_cerevisiae.R64-1-1.106.ucsc.gtf). DESeq2 was used to determine the differential expression of genes(LOVE *et al.* 2014). The truncated expected counts from the RSEM output were used as input for DESeq2. Genes with <15 reads across samples were filtered out. The p_{adj} value of 0.01 was used as a threshold.

RATE-seq data modeling

RNA reads obtained from Deseq2 were used for nonlinear regression modelling to obtain decay rates in *R* using the *nls.table* function. The model for RNA decay is (NEYMOTIN *et al.* 2014):

$$Y_t = Y_{eq}\left(1 - e^{-(\propto_{RNA} - \propto_{growth})(t - t_d)}\right)$$

Where

Y_t = Amount of labelled transcript at time *t*

Y_{eq} = Abundance of labelled transcript at steady state

t = labeling time

t_d = delay time

RNA decay rate constant, $\propto = \left(\propto_{RNA} - \propto_{growth}\right)$

α_{RNA} = RNA decay rate

α_{growth} = Growth rate = *ln2/doubling time* = 0.069 (WT) or 0.048

Synthesis rate was estimated in Excel from decay rate constant using the formula:

$$Synthesis\ rate = \propto\ [mRNA]$$

Where mRNA abundance estimated from steady state read counts assuming a total of 60000 mRNA molecules per cell (ZENKLUSEN *et al.* 2008).

mRNA half-life was estimated in Excel from decay rate constant using the formula:

$$Halflife = \frac{ln2}{\propto}$$

Polysome profiling

Fifty ml of mid-log-phase cultures were treated with 100ug/ml cycloheximide for 10min. Cells were harvested by centrifugation at 4000 rpm for 5 min. Lysate preparation was carried out as previously described (PANASENKO AND COLLART 2012; PREISSLER *et al.* 2015). Cells were resuspended in 500 ul polysome buffer with cycloheximide and protease inhibitors (20mM HEPES-OH pH 8, 10 mM $MgCl_2$, 50 mM KCl, 1% triton X-100, 1 mM DTT, 100 ug/ml CHX, 1 mM PMSF, 0.5 ug/ml leupeptin, 1 ug/ml pepstatin) followed by addition of 500 ul glass beads. Cells were broken by shaking for 18 min on a vortex mixer at 4˚C (3 cycles of 6 min bead-beating and 5 min of cooling on ice). Lysates were centrifuged for 10 min at 14,000 rpm and the protein concentration of the clarified supernatant was quantified by Bradford assay. Extracts were at -80°C until use. Continuous 10-50% sucrose density gradients (SDG) were prepared on GradientMaster (Biocomp) and stored at 4°C for at least 1hr. 1 mg of protein equivalent of lysate was layered onto the SGD and centrifuged at 39k rpm for 2hr30min in a SW41 (Beckman Coulter) rotor. SDG was fractionated on a Brandel fractionator and the OD254 of peaks were recorded by *PeakChart 3*. Samples used for Western blotting were prepared by precipitation with 10% TCA in the presence of 10 or 20 ug of BSA overnight at 4°C. The precipitated proteins were collected by centrifugation at 14k rpm for 10 min and the pellet was washed with 100% acetone. Proteins were solubilized in 1X SDS-PAGE loading buffer and boiled for 5min. Western blotting was performed as described above.

mRNA decay and transcription reporter assays

The MS2-MCP mRNA reporter assay was conducted as described in a previous publication (SWEET *et al.* 2012). Plasmids expressing MCP fusions with Def1, or fragments of the Def1 coding sequence, were constructed from gene synthesis and PCR-generated fragments using inPhusion assembly (Takara Bio) (Supplemental Table 2). MCP fusion protein expression plasmids were co-transformed with the reporter vector pJC428 into W303 cells (Supplemental table 1). Cells were grown in triplicate in -uracil/-leucine dropout media containing 2% galactose to an OD_{600} of 1.0. Each sample

was split into two, one used for protein extraction, and the other for RNA extraction. Expression levels of the MCP fusion proteins were verified using anti-FLAG antibodies and the levels of reporter GFP protein using anti-GFP antibodies. To calculate the levels of GFP protein, a serial dilution standard curve of an extract was included on the blots. Loading was normalized to Taf14, which served as a loading control. GFP mRNA was measured by qPCR and normalized to *ACT1* mRNA.

The Beta-galactosidase transcription reporter assay was conducted by co-transforming W303 cells with pSH18-34 and plasmids expressing LexA fusion proteins with Def1 (Supplemental Table 2). Cells were grown in triplicate in -uracil/-leucine dropout media containing 2% dextrose to an OD_{600} of 1.0. Protein extraction and beta-galactosidase assays were conducted as described in another publication (ROSE AND BOTSTEIN 1983).

Results:

Def1 affects steady state RNA levels and RNA kinetic rates

Def1 regulates elongation, especially removing arrested RNA polymerase II during transcription stress. Although Def1 mediates the recruitment of protein factors to facilitate RNAPII activity, no analysis of the direct effect of Def1 on RNA transcripts has been described. We carried out RNA-seq in wildtype and *def1*Δ mutant strains to investigate Def1's roles in gene expression. Using DESeq2, we detected 6486 RNAs, which include all types of RNA. Using a cutoff of >2-fold change and a $p_{adj}<0.01$, 612 and 475 transcripts were observed to be downregulated and upregulated, respectively, (Figure 1A). All differentially expressed transcripts were mRNAs. These differentially expressed genes were subjected to GO term analysis, and genes that are upregulated in the mutant are associated with ribosomes and translation. Transcripts downregulated in the mutant returned terms related to carbohydrate metabolism and metabolic pathways (Supplemental Figure 1A-D).

RNA-seq measures steady state RNA level, which is a function of synthesis and decay of transcripts. Studies have shown that cells alter one pathway to respond to changes in the other called transcript buffering (HAIMOVICH *et al.* 2013a; TIMMERS AND

TORA 2018). We therefore investigated transcript synthesis and decay rates in the *def1*Δ mutant. We employed RNA-approach-to equilibrium (RATE-seq), a pulse-only metabolic labeling approach whereby cells are treated with 4-thiouracil for increasing labeling times as incorporation of the nucleoside analog approaches equilibrium (NEYMOTIN *et al.* 2014). 4-ThioU-labeled *S. pombe* RNA was used as a spike-in control (see Methods section). We carried out RATE-seq in wild type and *def1Δ* cells and estimated synthesis and decay rates for 3938 and 5631 transcripts, respectively. We next analyzed the median synthesis and the decay rates of the 3596 transcripts that were common to both the wild type and mutant data sets. The synthesis rate of mRNAs was repressed globally in the mutant. The median synthesis rate was about 3.7-fold lower in the mutant than in wild type cells, but the change in synthesis for some transcripts was significantly higher (Figure 1B). Considering that global synthesis rates were repressed, yet a few transcripts showed differential expression, we analyzed the data to calculate the decay rates and RNA half-lives. The median decay rate in wild type cells under our condition was 0.259/s, which corresponds to a median half-life of 2.7min (Figure 1C and Supplemental Figure 2A). The half-life value we estimated is shorter than some reported measurements, but the values of median mRNA half-life from other studies varied between 2-18 minutes and is highly dependent on methodology and labeling conditions (Supplemental Figure 2B). Most importantly, the decay rate was 3.2-fold lower in the mutant, indicating a stabilization of mRNAs (Figure 1C). The global stabilization of transcripts suggests that Def1 is required for the decay of transcripts.

Transcript buffering is a phenomenon whereby decrease or increase to RNA synthesis rate is compensated for by a corresponding (in a feedback fashion) decrease or increase in decay rate, and vice-versa (SUN *et al.* 2012; HAIMOVICH *et al.* 2013a; TIMMERS AND TORA 2018). Buffering maintains the levels of transcripts when cells are exposed to a condition that results in the perturbation of kinetic rates of mRNA synthesis or decay. The changes in both synthesis and decay rates in the *def1*Δ mutant suggest that transcript buffering is occurring. To demonstrate this more clearly, we plotted the change in decay rates of individual mRNA versus their change in synthesis rates (Figure 1D). As expected, a strong correlation was observed (R=0.834), indicating

that transcript buffering is occurring to balance gene expression in the mutant. This suggests that *DEF1* is not required for transcript buffering.

RNA stability correlates with transcript-specific attributes such as abundance, length and codon optimality/usage (COLLER AND PARKER 2005; PRESNYAK *et al.* 2015; BAE AND COLLER 2022). However, we found no correlation with Def1 regulation and these features (Supplemental Figure 2C).

If Def1 regulates RNAs of genes involved in specific cellular processes or pathways, we would expect that its effects on half-lives would be most pronounced in such transcripts. To determine if Def1 targets mRNAs within specific pathways, we subjected transcripts in the top quartile of half-life fold changes to GO term analysis. Notably, the biological process of mitochondrial translation, protein metabolism and translation were highly represented as targets of Def1 (Figure 1E).

Def1 interacts with RNA regulation factors

The proximity-dependent labeling approach BioID is used to label and subsequently identify proteins within 10 nm of a protein of interest, allowing for the identification of transient and weak interactions. BioID also offers the advantages of the lesser tendency of losing interacting proteins to stringent experimental wash steps and eliminates the dependence on antibody properties (KIM *et al.* 2016; KIM AND ROUX 2016). Recently, we conducted BioID on subunits of the Ccr4-Not complex and found that multiple bait proteins strongly labeled Def1 (PFANNENSTEIN J 2024). Furthermore, the human homolog of Def1, UBAP2/2L, interacted with hCcr4-Not subunits and other RNA binding proteins (YOUN *et al.* 2018; KULKARNI *et al.* 2024). Ccr4-Not and Def1 regulate RNA polymerase II elongation and ubiquitylation, so it was unclear if this interaction is attributable to this transcription function. Therefore, we conducted the reciprocal experiment of performing BioID on Def1, fusing the TurboID (TID) version of the enzyme to the C-terminus. We expressed “free” TID as a control to levels close to that of Def1-TID and calculated the fold increase in the biotinylation of targets (Def1-TID versus free TID). We subjected the labelled proteins to mass spectrometry and then used a cutoff of 1.5-fold enrichment and $p<0.05$ to identify high confidence interacting or proximal partners of Def1 (**Figure 2A**). Def1 labelled a total of 93 proteins, of which 36

are involved in some aspect of the posttranscriptional regulation of RNA. We submitted the enriched proteins to GO term analysis, which returned terms associated with the regulation of translation, post-transcriptional control and mRNA decay and the component terms ribonucleoprotein granule, P-body, translation factor 4F, polysome and the Ccr4-Not core complex (Figures 2B and 2C). This data suggests that Def1 participates in three processes controlling the life of an mRNA, namely storage, translation and decay.

It was quite surprising that transcription factors or other nuclear proteins in mRNA synthesis were not labelled by Def1. This could however be explained by Def1's localization in the cytoplasm in the absence of transcription stress. We detected Def1 labeling Tfc1, a subunit of the RNA polymerase III transcription initiation factor complex (TFIIIC), however. This interaction of Def1 might translate into a direct or an indirect control of transcription of tRNA genes. Interestingly, we found that tRNA synthesis was decreased in the *def1*Δ mutant (not shown).

Directly tethering Def1 to mRNA suppresses expression, suggesting a role in posttranscriptional control

The extended half-lives of mRNA in the *def1*Δ mutant and the labeling of many mRNA regulators by Def1-TID suggests it regulates mRNA in the cytoplasm. However, the lower synthesis rate can result in suppressed turnover due to transcript buffering. The buffering mechanism confounds making the conclusion that Def1 directly regulates mRNA. Next, we used an MCP/MS2 tethered reporter assay to evaluate if Def1directly regulates mRNA. This assay has been used to demonstrate direct regulation of mRNA by decay factors (SWEET *et al.* 2012). The reporter construct expresses an mRNA with a green fluorescent protein (GFP) ORF and MS2 stem loops in the 3' UTR (Figure 3A). MCP was fused to either the N- or C-terminus of Def1 (MCP-DEF1 and DEF1-MCP respectively), and both were analyzed to rule out position effects. The *DEF1-MCP* fusion proteins fully complemented a *def1Δ* strain, indicating it was functional (Supplemental Figure 3A) and both Def1-MCP fusion proteins were expressed to equal levels as the free MCP protein (Supplemental Figure 3B). The translation repressor and

RNA decay factor Dhh1, was used as a control, as it has previously been shown to repress the reporter mRNA (SWEET *et al.* 2012; ZEIDAN *et al.* 2018).

To measure the activity of the reporter constructs, both protein and mRNA levels were measured. As expected, tethered Dhh1 significantly reduced mRNA and protein expression (Figure 3B and C). Tethering Def1 to the mRNA through either the N- or C-terminus of Def1 led to significant reduction in GFP mRNA- and protein levels relative to the free MCP control (Figure 3B and C). Both protein and mRNA were reduced approximately 40-50%, but not quite the magnitude of repression as the Dhh1-MCP control. The similar effects on both mRNA and protein implies that the reduction in protein level is the result of decreased mRNA. The destabilization of reporter mRNA validates the global reduction in RNA decay rates in a *def1Δ* mutant strain detected by RATE-seq (Figure 1C) and suggests that Def1 has a direct posttranscriptional function in the regulation of mRNAs.

Def1 acts like other factors implicated in both decay and synthesis of mRNAs. Previous studies have shown that tethering mRNA decay (HAIMOVICH *et al.* 2013b) or general transcription factors (CHATTERJEE AND STRUHL 1995) to promoters can activate transcription. We therefore, constructed fusion proteins of DNA binding domain of LexA and different fragments of Def1 (Full length and 1-380). A LexA-TBP, TATA-binding protein was analyzed in parallel. Whereas, the LexA-TBP fusion activated the reporter gene, neither LexA-Def1 proteins did (Supplemental Figure 3C). However, the tethering assay reports on effects on preinitiation complex formation, and a negative result does not definitely disprove Def1's effect on transcription, such as at elongation.

Def1 associates with polyribosomes

The BioID studies showed that Def1 labelled proteins involved in translation and the component term polysome was significantly enriched (Figure 2B and C). Translation and RNA decay are coupled processes(HU *et al.* 2009; BAE AND COLLER 2022). We therefore hypothesized that Def1 has a function in translation and/or interacts with polyribosomes. We performed polysome profiling to determine if Def1 associates with polyribosomes and to evaluate if Def1 is required for global translation by measuring the polysome to monosome ratio (henceforth referred to as P/M ratio).

Polysome-to-monosome ratio (P/M) is a reliable measure of translation in cells. Lysates from cycloheximide (CHX)-treated wild type cells were fractionated on 10-50% sucrose gradients and the fractions were probed with anti-Def1 antibodies. We found that the majority of Def1 migrates with the mono- and polyribosome fractions (Figure 4A). Some Def1 was also detected in the fractions containing and 40S and 60S subunits. Comparatively little was in the mRNP fraction. To verify Def1 is associated with polysomes, we prepared lysates and gradients in buffers containing EDTA, a polysome disassembling agent. EDTA treatment disrupted the migration of Def1 in the heavier fractions polysome fractions (Figure 4B), confirming that Def1 migrates with polyribosomes. Interestingly, Def1 still migrated with the 40- and 60S peaks, suggesting it interacts with the small and large subunits of the ribosome.

Association of a protein with ribosomes could imply that the factor exerts either a positive or negative influence on translation. We next examined the effects of deleting *DEF1* on bulk polysome density. We found that the polysome-to-monosome ratio (P/M) was not significantly affected by the loss of *DEF1* (Figure 4C, 4D). When the profiles of wildtype and *def1Δ* were overlaid, there was also no striking differences in the peaks of the 40S, 60S and 80S (Figure 4C). Although there was no difference in polysome profiles, polyribosome-associated proteins can affect co-translational processes, such as decay.

Mutants of some translation regulators, such as the repressor Dhh1, only displayed altered polysome profiles under stress conditions, especially nutrient stress (SWEET *et al.* 2012). We induced carbon source starvation stress in cells by transferring them from YP+dextrose to YP media for 10 min, followed by extract preparation and polysome analysis. We also analyzed a *dhh1*Δ mutant as a positive control. Transferring wild type cells to starvation conditions led to a sharp decrease in P/M ratio (Supplemental Figure 4), and as reported by others, translational repression was impaired in the *dhh1*Δ mutant, (SWEET *et al.* 2012; ZEIDAN *et al.* 2018). However, translation repression occurred in *def1Δ* mutant, and there was a slight, but reproducible enhanced repression of translation in the mutant. These findings together suggest that the loss of Def1 has no measurable effect on bulk translation under the conditions tested here and Def1 may have transcript or condition-specific effects on translation.

Def1 binding to polysomes requires its ubiquitin binding domain

Def1 contains two identifiable features, a C-terminal polyglutamine-rich domain (polyQ) that is required for its accumulation in the cytoplasm, and a N-terminal CUE domain that interacts with the ubiquitin-homology domain of Ela1 and ubiquitin (WILSON *et al.* 2013b) (Figure 5A). We next examined which region of Def1 is required for ribosome binding. We generated a strain that expresses a truncation mutant of Def1 ($Def1_{1\text{-}380}$) lacking the polyQ region by incorporating GFP into the C-terminus. Full length Def1-GFP was also generated and compared directly. The 1-380 mutant retained ribosome binding, and moreover, we reproducibly observed enhanced polysome binding, especially in the heavier (larger) polysome fraction (Figure 5B). An explanation for increased polysome interaction in the polyQ truncation mutant could be an intrinsic property of the region. Bioinformatic tools predict a large intrinsically disorder segment within the polyQ region (data not shown), and Def1 forms polyQ-dependent amyloid-like filaments *in vitro* (DAMODAREN *et al.* 2017). A portion of full length Def1 may be trapped in an insoluble fraction in cells and liberating the N-terminus from the polyQ domain may increase the amount available to bind ribosomes.

The N-terminus contains the ubiquitin-binding CUE domain. We used CRISPR-Cas9 gene editing to introduce two clusters of mutations in the CUE domain, Cue1mut and Cue2mut, that disrupt Def1 binding to ubiquitin and ubiquitin-homology domains (Wilson *et al*., 2013). Using strains that express these mutant versions of Def1 ($Def1_{CUE1}$ and $Def1_{CUE1,2}$), we found that the CUE domain, and likely ubiquitin binding, is required for Def1 association with ribosomes (Figure 5C). Furthermore, unlike what was observed in wild type extracts, the majority of mutant Def1 appears in the mRNP fractions. Interestingly, when the polysome profiles of the mutants were overlayed with the wild type, two differences were noted. First, the P/M ratio increased modestly in both CUE mutants, ~2.9 in mutants versus ~2.3 in wild type extracts (Figure 5C). And the second, the amount of monosomes and polysomes were decreased (Figure 5C, yellow and red lines). It should be noted that the amount of 40S and 60S subunits were not different between the wild type and the mutants, suggests that translation initiation may be impaired in the CUE mutants. These results were unexpected, considering no

difference was observed in the *def1*Δ mutant (Figure 4C). This can be explained by the CUE mutants acting in a dominant negative manner. The mechanism will be proposed and explained in the discussion section.

Not4-mediated ubiquitylation of eS7a (Rps7A) is required for the association of Def1 with polyribosomes

Ubiquitylation of ribosomal proteins (RPs) plays several roles in translation, such as rescuing stalled ribosomes and destroying aberrant nascent polypeptides by the ribosome quality control (RQC) pathway(INADA AND BECKMANN 2024). Based on our findings that the CUE domain of Def1 is necessary for its association with polysomes, we hypothesized that ubiquitylation of a RP plays a role in its recruitment. There are multiple ubiquitin ligases that carry out modification of RPs, and one is the Not4 subunit of the Ccr4-Not complex(PANASENKO AND COLLART 2012; PANASENKO 2014; IKEUCHI *et al.* 2019; BUSCHAUER *et al.* 2020; ALLEN *et al.* 2021; INADA AND BECKMANN 2024). As described above, BioID indicates that Def1 and Ccr4-Not are near each other (Figure 2) and Def1 and Not4 have genetic, physical and molecular interactions (JIANG *et al.* 2019; AKINNIYI AND REESE 2021). Not4 ubiquitylates eS7A (Rps7A) to facilitate the disassembly of the arrested ribosome (ALLEN *et al.* 2021; INADA AND BECKMANN 2024).

We determined if Ccr4-Not, Not4 specially, is required for Def1 binding to ribosomes. Not1 is the scaffold subunit of the complex, and is encoded by an essential gene. Thus, we used the auxin-inducible degron system to deplete it from cells (SHETTY *et al.* 2019). Depleting Not1 caused a remarkable reduction in the association of Def1 with polysomes (Figures 6A, 6B). Next, we analyzed Def1 recruitment to ribosomes in a Not4 RING domain deletion mutant that disrupts ubiquitin ligase activity, but leaves the Ccr4-Not complex otherwise intact (JIANG *et al.* 2019). Here too we found that Def1 association was greatly reduced (Figure 6C). Furthermore, deleting the entire *NOT4* coding sequence or depleting the protein by auxin-dependent degradation also disrupted Def1's association with polyribosomes (Supplemental Figure 5).
These findings indicate that Ccr4-Not complex recruits Def1 to polyribosomes, and requires the ubiquitin ligase activity of Not4.

Although these results indicate that Ccr4-Not, the RING domain of Not4 specifically, recruits Def1 to ribosomes, they only suggest it is mediated by eS7A (Rps7A) ubiquitylation. These results could be ascribed to a secondary effect resulting from disruption of other Not4 RING-dependent pathways. To directly link Not4's ubiquitylation of eS7A to the recruitment of Def1 to polyribosomes, we utilized a yeast strain that contains the Not4-dependent ubiquitylation sites mutated to arginine (eS7A-4KR) (BUSCHAUER *et al.* 2020). The wildtype and mutant strain were subjected to polysome analysis and the sedimentation of Def1 was analyzed by western blotting. We observed a striking loss of Def1 association with polysomes in the mutant, which mimics the *not4ΔRING* condition (Figure 6C and D). This further supports our hypothesis that Not4-mediated ubiquitylation of eS7a precedes the recruitment of Def1 to stalled ribosomes and demonstrates that ubiquitin signaling is important in the recruitment of Def1 to polyribosomes.

Def1 recruits the translation control factor, Dhh1 to ribosomes

The C-terminus of Def1 retains it in the cytoplasm and progressively deleting the polyQ domain phenocopies the deletion mutant (Wilson paper and see Supplemental Figure 6A). We hypothesized the polyQ region may recruit proteins to ribosomes to impact translation or co-translational decay. To test this, we generated strains expressing truncated versions of Def1-TID; $Def1_{1-738}$, $Def1_{1-380}$, $Def1_{1-500}$ and $Def1_{1-530}$. Growth spot assays showed that fusing TID to full length Def1 did not result in a growth defect, and the expression levels of all Def1 TID fusions were equivalent (Supplementary Figure 6B). As an initial qualitative check of the biotin-transfer activities of the different mutants, we examined the levels of biotinylated proteins enriched on streptavidin beads by probing these fractions with streptavidin-horseradish peroxidase (HRP). There were some differences in the number and intensity of biotinylated proteins, but strong biotinylation was observed except for the $Def1_{1-500}$ derivative (Supplemental Figure 6C). Mass spec analysis identified 59 enriched proteins labeled by the $Def1_{1-500}$ mutants, even though this crude analysis of labeled protein suggested a "fail". Both $Def1_{1-530}$ and $Def1_{1-380}$ TID fusion proteins labeled a few more interacting partners than the full-length protein, labeling 105 and 120 versus 93, respectively

(Figure 7A). Interestingly, when we compared the percentage of proteins labeled among the derivates that are posttranscriptional RNA regulators or RNA binding proteins, we found that derivatives lacking the polyQ region labeled less RNA regulators than the full length Def1-TID protein. (Figure 7A). Compared to the 34 (37% of total) labelled regulators of RNA by the full length protein, the $Def1_{1-380}$, $Def1_{1-500}$ and $Def1_{1-530}$ proteins labeled, 17 (14%), 10 (17%) and 14 (13%), respectively (Figure 7A).

The loss of RNA regulators suggests that the polyQ domain binds RNA regulators and that Def1 acts as a ubiquitin-dependent scaffold to recruit factors to the ribosome. We therefore scanned the BioID dataset to identify a polyQ region-dependent interacting partner that might play a role in ribosome dynamics or mRNA degradation. Notably, we found the DEAD box RNA helicase Dhh1, which is a translation repressor and mRNA degradation factor(COLLER AND PARKER 2005) (SWEET *et al.* 2012; RADHAKRISHNAN *et al.* 2016), was labeled by the full-length protein, but not the truncation proteins. We performed polysome profiling to determine if the association of Dhh1 with polysomes is Def1-dependent. Dhh1 was found to associate with polyribosomes, as described by others (SWEET *et al.* 2012). Dhh1 was more enriched in the early polysome fractions (Figure 7B). This might be explained by Dhh1's role in clearing ribosomes from mRNA during repression as ribosomes stalling increases throughout the body. We note that others have reported that crosslinking is needed to see significant Dhh1 in the heavier polysome fractions (SWEET *et al.* 2012). Importantly, Dhh1 migration with polyribosomes was lost in the *def1*Δ mutant (Figure 7B). This suggests that Def1 recruits Dhh1 to ribosomes.

It was recently reported that Dhh1 association with ribosomes required Not4 (see supplement of (BUSCHAUER *et al.* 2020)), and considering our data indicating that Not4-dependent ubiquitylation is required for Def1 association. We verified this result under our conditions and indeed found that that Dhh1 lost its interaction with polyribosomes in the *not4*Δ mutant (Figure 7C). Likewise, Not4 depletion by AID-dependent proteolysis diminished the association of Dhh1 with polyribosomes (data not shown).

Finally, to establish an order of events, we examined if Def1 requires Dhh1 to bind to polysomes. We carried out polysome profiling on *dhh1Δ* mutant lysates and observed that Def1 migrates with polysomes in the absence of Dhh1, confirming that

Def1 recruitment precedes that of Dhh1 (Figure 7D). Finally, we determined if directly tethering $Def1_{1-380}$, $Def1_{1-500}$ to the GFP reporter mRNA caused repression in the MS2/MCP assay. We found that the truncation mutants were not as effective as the wild type at suppressing expression of the reporter construct (Supplemental Figure 6D and E). We have therefore established a pathway for recruitment of factors to polysomes led by Not4-dependent ubiquitylation of eS7a, followed by Def1 binding the ubiquitylated eS7a, and the recruitment of RNA regulators to the ribosome such as Dhh1 (Figure 8). These results strongly suggests that Def1 acts as a ubiquitin-dependent scaffold or adaptor protein to link translation status and downstream events such as co-translational mRNA decay.

Discussion

An ever-growing number of genes are found to have unknown or underappreciated activities. For example, Ccr4-Not once thought only to control TBP and transcription initiation, is now recognized for its important functions in posttranscriptional control of mRNAs. Degradation factor 1, Def1, was first identified as a DNA damage mitigating factor, and a substantial amount of work has established it as an elongation and a degradation factor that clears arrested RNA polymerase II from genes. Other nuclear functions have been attributed to protein (AKINNIYI AND REESE 2021). Despite its localization to the cytoplasm, no known cytoplasmic functions had been identified.

We have shown that *DEF1* maintains RNA synthesis and decay rates. Def1 is an elongation factor that co-purifies with TFIIH and affects start site selection in reconstituted transcription reactions (DAMODAREN *et al.* 2017). Thus, its role in maintaining synthesis rates was expected, but not so for its requirement for normal mRNA decay. Even though transcript buffering could have accounted for the slowed mRNA decay in a *def1*Δ mutant due to perturbed transcription, we provide strong evidence that Def1 directly controls posttranscriptional gene regulation, probably by associating with polyribosomes. Def1 is associated with many cytoplasmic posttranscriptional regulators that bind to polyribosomes and is required for normal

mRNA degradation. The MS2/MCP reporter assay suggests it directly regulates mRNA, versus the change in decay resulting from buffering of reduced synthesis.

Our work reconciles the perplexing cytoplasmic localization and the very high abundance of Def1. Estimates of median abundance for Def1 cited in the Saccharomyces cerevisiae database (SGD) is ~21,000, but that value can be as high as 80,000. As noted above, DNA damage causes the proteasomal processing of a small fraction of Def1, but a larger fraction of unprocessed protein remains in the cytoplasm (WILSON *et al.* 2013b). Def1 has a function in the cytoplasm and its accumulation there serves a purpose greater than preventing it from prematurely entering the nucleus and interfering with transcription. Def1 certainly has transcription functions, but it also has important cytoplasmic functions in mRNA decay. As such, Def1 adds to a list of factors involved in transcription in the nucleus and translation/decay in the cytoplasm. Ccr4-Not, which we describe as an initiator of Def1 recruitment to ribosomes, connects transcription elongation to translation (GUPTA *et al.* 2016; BUSCHAUER *et al.* 2020). Def1, too, may be important for nuclear-cytoplasmic communication by coordinating transcription elongation and translation.

We propose a model where Not4-dependent ubiquitylation recruits Def1 to the ribosome through Def1's CUE domain, where it, in turn, recruits regulators of translation and/or decay (Figure 8). Interestingly, two other CUE domain-containing proteins, Cue2 and Cue3, play roles in recruiting factors to ribosomes. Cue2 is recruited to ubiquitylated ribosomes during ribosome collision to cleave mRNA in non-ribosome quality control (RQC)-coupled no-go decay (D'ORAZIO *et al.* 2019; TOMOMATSU *et al.* 2023). Likewise, the resolution of colliding ribosomes in the RQC pathway is facilitated by the RQC-Trigger complex (RQT), which contains Cue3 (MATSUO *et al.* 2017; MATSUO *et al.* 2023). Def1 is another example of a CUE domain-containing protein that monitors translation and establishes this domain as necessary in assessing cell translation status.

Although Def1 is associated with polyribosomes, deleting the gene has no detectable effect on the polysome profiles (Figure 4). On the other hand, we found reduced monosome and polysome abundance in cells expressing the ubiquitin-binding mutants (CUEmut1,2). The reason for this is unclear, but we speculate that the CUE

domain mutant may act as a dominant negative by sequestering or titrating away a translation factor from ribosomes. The CUE mutant retains the polyQ-rich segment that our BioID studies indicate is required for Def1 derivatives to label multiple mRNA regulators (Figure 7A). As proof of principle, we showed that one such factor recruited by Def1 is the translation and decay factor Dhh1, but there are likely others that remain to be tested (Figure 7B). So, it is feasible that the CUE mutant interferes with translation through this mechanism. Another explanation for not observing changes in the polysome profile of the deletion mutant is that Def1 plays a message-specific role in translation, or it does so under a condition not examined here. *DHH1* is essential for nutrient stress- dependent repression of translation (SWEET *et al.* 2012; RADHAKRISHNAN *et al.* 2016; ZEIDAN *et al.* 2018). We were surprised to find that the *def1*Δ mutant was dispensable for the repression of translation under nutrient stress (Supplemental Figure 4) because Dhh1 recruitment to ribosomes was compromised in the *def1*Δ mutant. This can be explained by a Def1-independent Dhh1 recruitment mechanism in cells, which is insufficient to maintain Dhh1's binding to ribosomes in polysome fractionation conditions. Interestingly, RNA-seq studies on steady-state mRNA levels revealed that ribosome biogenesis and translation factors are significantly upregulated in the *def1*Δ mutant (Supplemental Figures 1). These results can be interpreted as the mutant cells sensing subtle changes in translation and then adjusting by increasing these factors. The mutant may have compensation mechanisms that lead to the underestimation of Def1's impact on translation.

Def1 interacts with ribonucleoprotein granules (RNPs) markers such as Ccr4-Not subunits, Edc3, Dhh1, Lsm7, and others. Ribonucleoprotein granules, such as stress granules and processing bodies, are cytosolic compartments for translational repression, whereby mRNAs are stored together with initiation factors and decay factors during stress (VAN TREECK AND PARKER 2018). However, UV stress (WILSON *et al.* 2013b) or nutrient stress (data not shown) did not result in Def1-containing foci , such as P-bodies. Recently, the human homolog of Def1 was identified, UBAP2/2L (HERLIHY *et al.* 2022). UBAP2/2L has a ubiquitin-binding domain, but lacks a polyQ-rich domain found in Def1. UBAP2/2L also does not reside in P-bodies or stress granules; however, it is required for stress granule formation (YOUN *et al.* 2018; SANDERS *et al.* 2020).

Interestingly, UBAP2/2L co-sedimented with mammalian ribosomes, but its function there or how it was recruited was unknown (LUO *et al.* 2020). Def1 could regulate the partitioning of mRNAs between the repressed versus the actively translated pool. Future studies evaluating the roles of Def1 in regulating P-bodies may provide evidence of this function.

Def1 is recruited to ribosomes in a ubiquitin-dependent manner. Ubiquitylation of the small subunit of the ribosome occurs when arrested ribosomes recruit the Ccr4-Not complex when the ribosome encounters messages rich in non-optimal codons or rendered untranslatable due to, for example, nucleotide damage (MATSUDA *et al.* 2014; MATSUO *et al.* 2017; YAN AND ZAHER 2019b; YAN AND ZAHER 2019a; TAKEHARA *et al.* 2021; INADA AND BECKMANN 2024). Agents that damage DNA and arrest RNA polymerase II and initiates Def1 processing in the cytoplasm also damage RNA and arrests ribosomes. RNA polymerase II degradation and Def1 processing is also triggered by transcriptional stress initiated by depleting GTP pools using 6-azauracil (6-AU) and mycophenolic acid(WILSON *et al.* 2013a). GTP is essential for ribosome movement, and 6-AU treatment causes ribosomes to accumulate on mRNAs(GUPTA *et al.* 2016). Just like how RNAPII detects DNA damage as it moves along the gene, ribosomes can sense untranslatable mRNAs and trigger ribosome quality control. It would be advantageous for cells to sense untranslatable mRNAs in the cytoplasm and shut down transcription, or the new transcripts would meet the same fate. It is feasible, if not likely, that Def1 senses and plays a role in shutting down gene expression at multiple levels in response to nucleic acid damage.

In addition to its well-characterized role in resolving DNA-damage arrested RNA polymerase II, Def1 has other nuclear functions associated with genomic integrity mechanisms (for review see). One such function is the destruction of Pol3, the catalytic subunit of replicative DNA polymerase delta (DARABA *et al.* 2014). The ubiquitylation and removal of Pol3 leads to the exchange of replicative for translesion DNA polymerase, allowing replication to continue. Here, Def1 functions with DNA polymerase delta to detect DNA damage and directs its destruction to allow for continued DNA replication by translesion DNA polymerase. Another example is the requirement for Def1 in maintaining telomere structure and function. Deleting *DEF1* caused telomere shortening, by ~200 bp (CHEN *et al.* 2005). This function likely involves its interaction

with Rrm3, a DNA helicase that promotes replication at telomeres and suppresses DNA damage at G4-quadruplex structures (MAKOVETS *et al.* 2004; CHEN *et al.* 2005).

We propose the exciting idea that Def1 acts as a ubiquitin-dependent scaffold protein that plays a role in surveying the activity of molecular motors that scan nucleic acids for damage. Def1 is well connected to molecular motors that scan DNA, such as DNA and RNA polymerase II and now RNA, the ribosome. In this capacity, Def1 plays an essential role in ubiquitin-mediated signaling to resolve damage to cells and guard the genome from errors.

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Figure legends:

Figure 1: **DEF1 is required for mRNA synthesis and decay.** (A). Total RNA-seq was carried out in both wild type (WT) and *def1Δ* strains. DEseq2 was used to determine changes in expression. *S. pombe* spike-in was used at 1:14 OD ratio of cells to normalize the data. Upregulated transcripts (>2-fold change, p_{adj}<0.01) appear in red and downregulated transcripts (<2-fold change, p_{adj}<0.01) appear in blue. (B). RNA synthesis rates are reduced in the *def1*Δ mutant. RATE-seq was used to determine global RNA synthesis rates. Rates obtained for the 3596 transcripts that overlap in the wild type and *def1*Δ samples are displayed in the figure. *p* value was determined using an unpaired *t*-test. (C). *DEF1* globally controls mRNA decay rates. (D). Evidence for transcript buffering in the *def1*Δ mutant. Scatterplot of the fold change (FC) in RNA decay and synthesis rates were plotted on the y- and x-axis, respectively. A Spearman correlation coefficient is shown in the scatterplot. (E). GO term analysis of the processes associated with the top quartile (Q4) transcripts whose decay rates change the most in the mutant.

Figure 2: BioID analysis reveals that Def1 interacts with posttranscriptional regulatory factors. (A). BioID labeling was performed in cells expressing Def1-Turbo ID (TID) and cells expressing free TID enzyme. Biotinylated proteins were pulled down using streptavidin beads and identified by mass spectrometry. Data is presented as a Volcano plot of fold change of protein abundance ratio (Def1-TID versus free TID) versus p-value. The enriched proteins (>1.5fold, p<0.05) appear in red and underrepresented proteins (<1.5fold, p<0.05) appear in blue. Proteins enriched in the Def1-TID sample were analyzed using ShinyGO 0.80 and the enriched process (B) and component (C) are shown.

Figure 3: **MS2-MCP Reporter assays indicate that Def1 functions in posttranscriptional control of mRNAs.** (A) Schematic of the MS2 coat protein (MCP)-MS2 tethering assay. (B). Graph of steady-state GFP mRNA. RT-qPCR measured the abundance of both GFP and ACT1 (control) mRNA. GFP mRNA amounts were

normalized to *ACT1*. The relative abundance of GFP mRNA in the fusion protein-expressing cells, compared to that expressing free MCP protein, was set at 1.0 (N=3). Def1-MCP has MCP fused to the C-terminus; MCP-Def1 has MCP fused to the N-terminus; Dhh11-MCP was used as a positive control. Expression of the free MCP and the Def1-MCP fusion proteins were driven by the DEF1 promoter. Dhh1-MCP was driven by the *DHH1*-promoter. (C). Analysis of GFP protein abundance. GFP protein expression level was determined by Western blotting using anti-GFP antibody. Taf14 was used as a loading control and GFP protein signals were normalized to the control (N=3).

Figure 4: **Def1 associates with polyribosomes.** (A). Ribosome profiling. Cell lysate was fractionated on a 10-50% sucrose gradient as described in the Methods section. A_{254} recording of the gradient fractions are displayed in the chart. Def1 and Rpl3-s (large subunit protein) were detected by Western blotting. Input from a *def1*Δ was loaded on the blot to validate the antibody. The locations of the mRNP, 40S, 60S, 80S monosomes and polysomes are indicated. (B). As in "A" except comparing the fractionation of Def1 in the presence (red) or absence (black) of the polysome disrupting agent EDTA. (C). Representative polysome profiles of wild type and *def1Δ* cells overlayed (wild type cells in black and *def1Δ* in red). (D). The average polysome-to-monosome ratios (P/M) were determined and represented with a column graph. The area under the curve (AUC) for the monosome (80S) and polysome fractions were calculated using PeakChart 3 software (Brandel). The average and standard deviations (N=3) are shown.

Figure 5: **The N-terminus of Def1, the CUE domain specifically, binds ribosomes.** (A). Schematic of the Def1 protein with the poly-Q and CUE domains illustrated. (B). Polysome profile of the Def1 (1-380) mutant. Western blot was performed using anti-GFP antibodies. (C). Polysome fractions of wild type, CUEmut1 and CUEmut1,2. The amino acid substitutions were described in Supplemental Table 1 and (WILSON *et al.* 2013b). Western blotting was performed using Def1 antiserum.

Figure 6. Ubiquitylation of eS7a by Not4 recruits Def1 to ribosomes. (A). Western blot showing Not1 depletion using the auxin-induced degron system. Antibodies to Not1 and Dhh1 (loading control) were used. Two isolates of the Not1-AID strain were compared. Cells were treated with auxin for 2 hr where indicated. (B). Lysates of untreated and auxin-treated Not1-AID cells were subjected to polysome analysis. (C). Analysis of Def1 binding to polysomes in a *not4Δring* mutant. Blots were probed with anti-Def1 and Rpl3-s antibodies. (D). Lysates from strains expressing wild type eS7a or a version with lysine to arginine substitution in the Not4-dependent ubiquitylation sites (eS7$_{KR}$ mut) (BUSCHAUER *et al.* 2020) were fractionated on sucrose gradients.

Figure 7. **The poly-glutamine-rich (polyQ) domain of Def1 mediates its interaction with mRNA regulators and recruits the decapping factor Dhh1 to ribosomes.** (A). BioID was conducted with Def1 truncation mutants fused to TID. Labeled proteins that were enriched for each Def1 derivative versus free TID (>1.5-fold, $p<0.05$) were tabulated. The number and percent of RNA regulators were calculated and displayed in the table. Dhh1 binding to ribosomes in a *def1*Δ (B) and *not4*Δ (C) mutants. (D). Def1 binding to ribosomes in a *dhh1*Δ mutant.

Figure 8. Model for Def1 recruitment to ribosomes. The model is modified from (BUSCHAUER *et al.* 2020)**.** Ribosome arrest or stalling causes Not5 binding to the empty E-site, recruiting Ccr4-Not to the ribosome and causing eS7a ubiquitylation. Def1 binds ubiquitylated eS7a through its CUE domain and recruits RNA decay and translation factors to the ribosome through the C-terminal polyQ-rich domain. The CDC48 complex and the proteosome assist in ribosome quality control and ribosome removal.

Figure 1

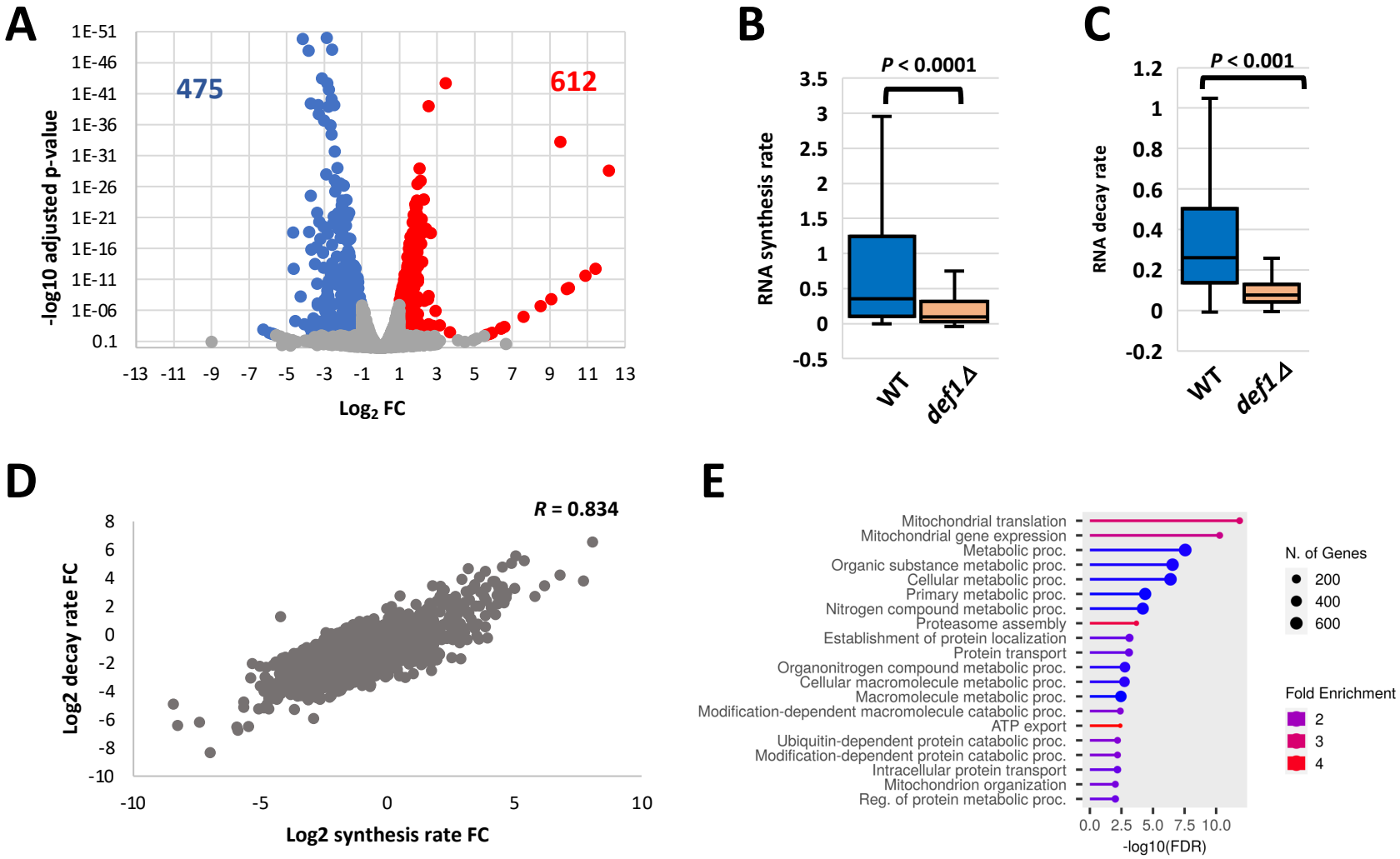
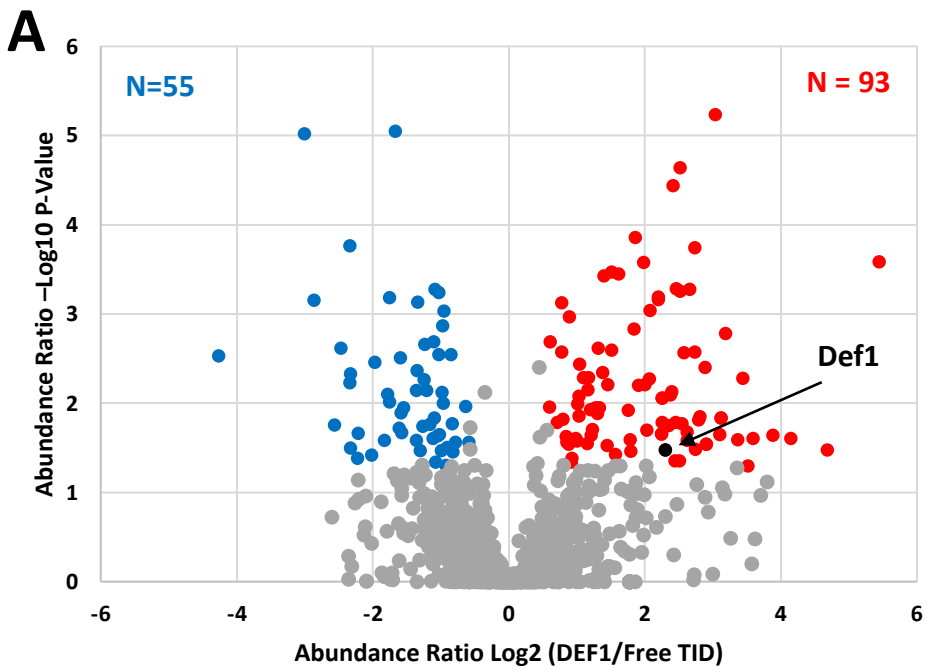
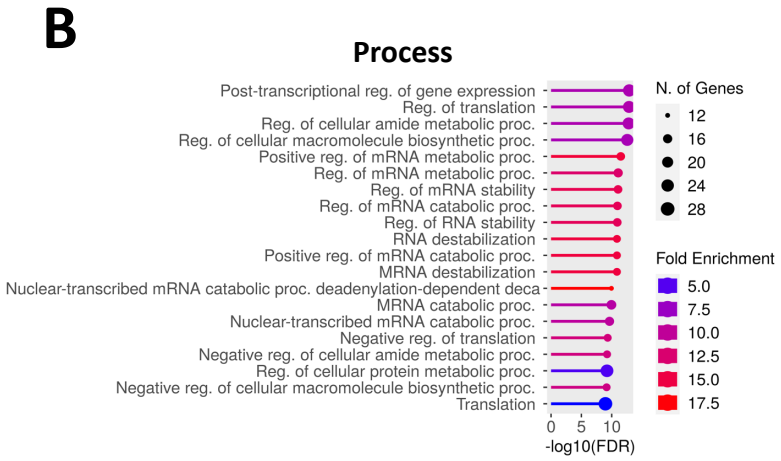

A
475
612
-log10 adjusted p-value
Log2 FC
B
P < 0.0001
RNA synthesis rate
WT
def1Δ
C
P < 0.001
RNA decay rate
WT
def1Δ
D
R = 0.834
Log2 decay rate FC
Log2 synthesis rate FC
E
Mitochondrial translation
Mitochondrial gene expression
Metabolic proc.
Organic substance metabolic proc.
Cellular metabolic proc.
Primary metabolic proc.
Nitrogen compound metabolic proc.
Proteasome assembly
Establishment of protein localization
Protein transport
Organonitrogen compound metabolic proc.
Cellular macromolecule metabolic proc.
Macromolecule metabolic proc.
Modification-dependent macromolecule catabolic proc.
ATP export
Ubiquitin-dependent protein catabolic proc.
Modification-dependent protein catabolic proc.
Intracellular protein transport
Mitochondrion organization
Reg. of protein metabolic proc.
-log10(FDR)
N. of Genes
200
400
600
Fold Enrichment
2
3
4

Figure 2


A
N=55
N = 93
Def1
Abundance Ratio –Log10 P-Value
Abundance Ratio Log2 (DEF1/Free TID)
6
5
4
3
2
1
0
-6
-4
-2
0
2
4
6


B
Process
Post-transcriptional reg. of gene expression
Reg. of translation
Reg. of cellular amide metabolic proc.
Reg. of cellular macromolecule biosynthetic proc.
Positive reg. of mRNA metabolic proc.
Reg. of mRNA metabolic proc.
Reg. of mRNA stability
Reg. of mRNA catabolic proc.
Reg. of RNA stability
RNA destabilization
Positive reg. of mRNA catabolic proc.
MRNA destabilization
Nuclear-transcribed mRNA catabolic proc. deadenylation-dependent deca
MRNA catabolic proc.
Nuclear-transcribed mRNA catabolic proc.
Negative reg. of translation
Negative reg. of cellular amide metabolic proc.
Reg. of cellular protein metabolic proc.
Negative reg. of cellular macromolecule biosynthetic proc.
Translation
0
5
10
-log10(FDR)
N. of Genes
12
16
20
24
28
Fold Enrichment
5.0
7.5
10.0
12.5
15.0
17.5

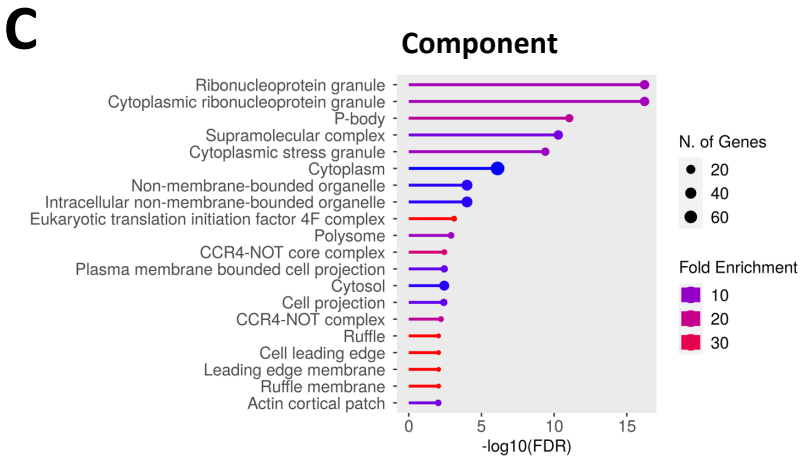

C
Component
Ribonucleoprotein granule
Cytoplasmic ribonucleoprotein granule
P-body
Supramolecular complex
Cytoplasmic stress granule
Cytoplasm
Non-membrane-bounded organelle
Intracellular non-membrane-bounded organelle
Eukaryotic translation initiation factor 4F complex
Polysome
CCR4-NOT core complex
Plasma membrane bounded cell projection
Cytosol
Cell projection
CCR4-NOT complex
Ruffle
Cell leading edge
Leading edge membrane
Ruffle membrane
Actin cortical patch
0
5
10
15
-log10(FDR)
N. of Genes
20
40
60
Fold Enrichment
10
20
30

Figure 3

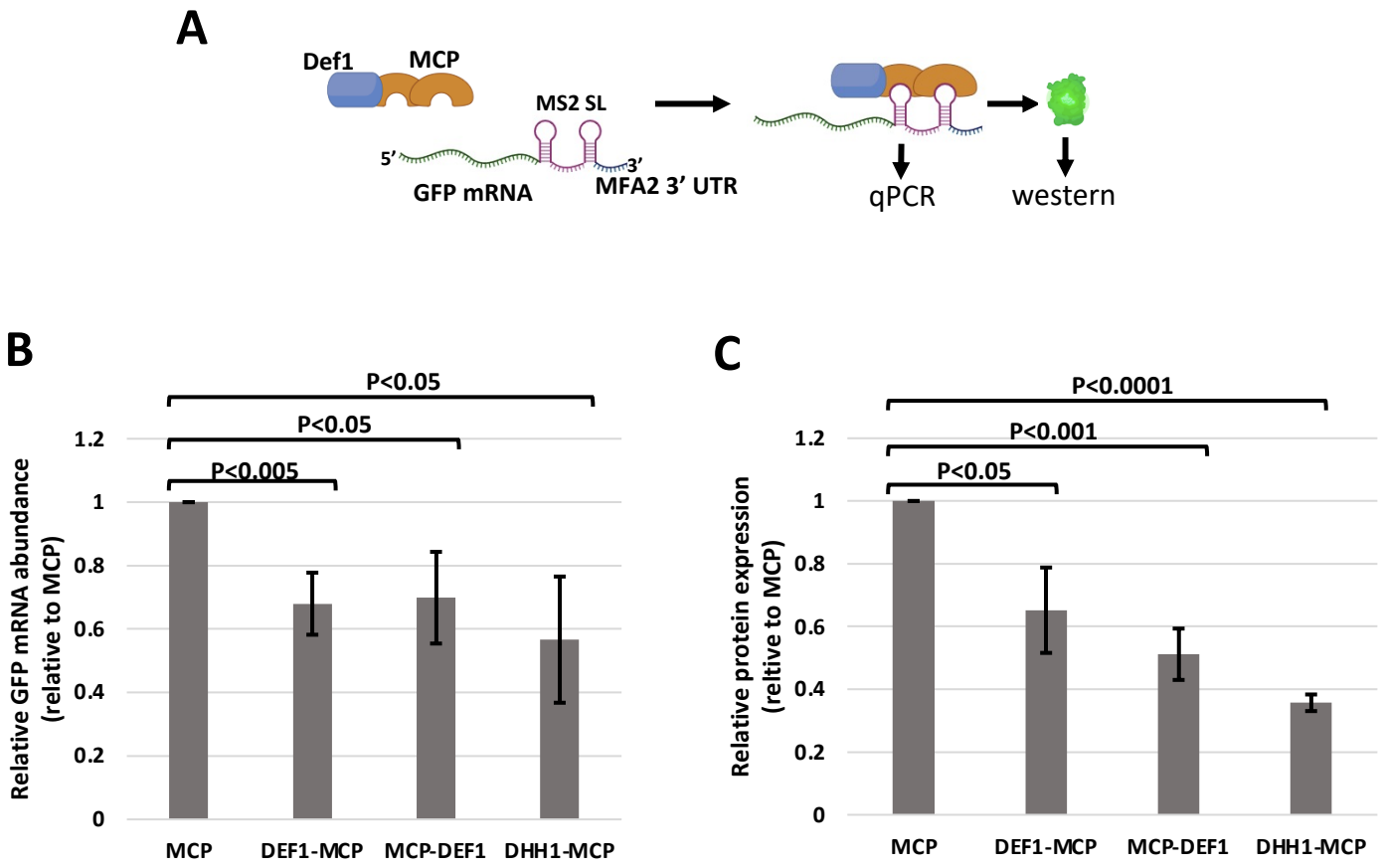

A
Def1
MCP
MS2 SL
5'
3'
GFP mRNA
MFA2 3' UTR
qPCR
western
B
P<0.05
P<0.05
P<0.005
Relative GFP mRNA abundance (relative to MCP)
1.2
1
0.8
0.6
0.4
0.2
0
MCP
DEF1-MCP
MCP-DEF1
DHH1-MCP
C
P<0.0001
P<0.001
P<0.05
Relative protein expression (reltive to MCP)
1.2
1
0.8
0.6
0.4
0.2
0
MCP
DEF1-MCP
MCP-DEF1
DHH1-MCP

Figure 4

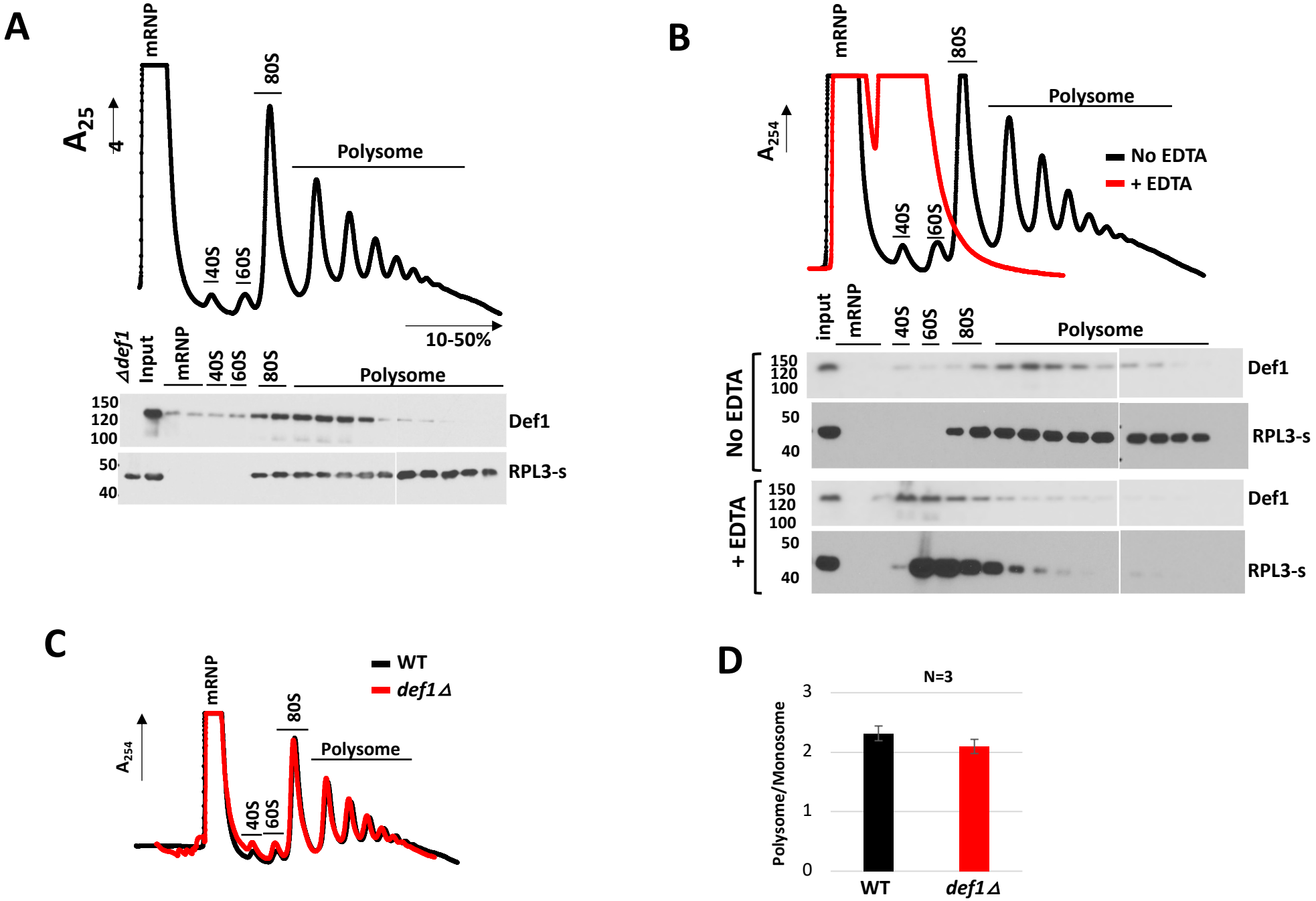

A
mRNP
80S
A_{25}
4
Polysome
40S
60S
10-50%
Δdef1
Input
mRNP
40S
60S
80S
Polysome
150
120
100
Def1
50
40
RPL3-s
B
mRNP
80S
Polysome
A_{254}
No EDTA
+ EDTA
40S
60S
input
mRNP
40S
60S
80S
Polysome
No EDTA
150
120
100
Def1
50
40
RPL3-s
+ EDTA
150
120
100
Def1
50
40
RPL3-s
C
WT
def1Δ
mRNP
80S
A_{254}
Polysome
40S
60S
D
N=3
Polysome/Monosome
3
2
1
0
WT
def1Δ

Figure 5

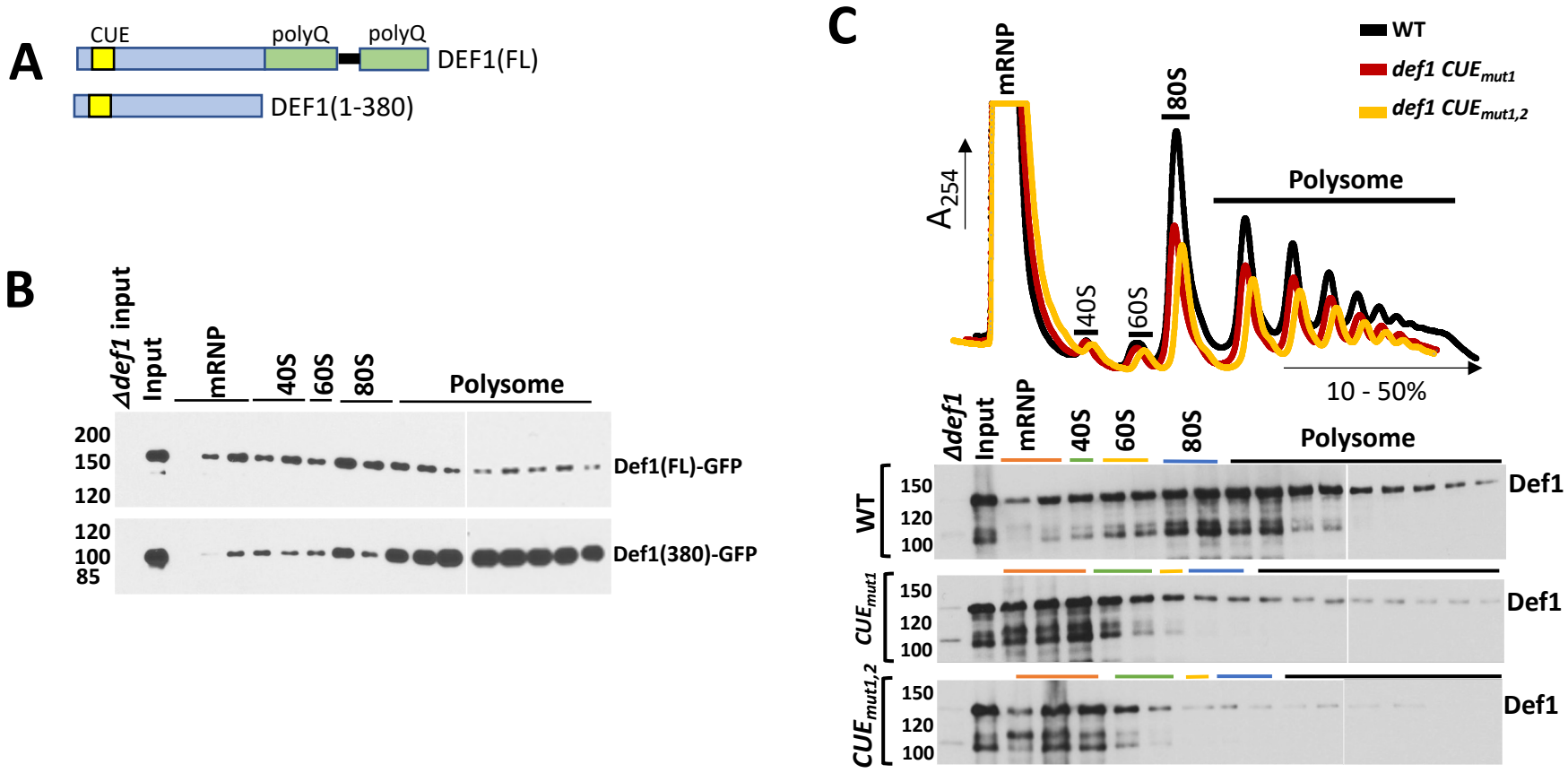

A
CUE
polyQ
polyQ
DEF1(FL)
DEF1(1-380)
B
Δdef1 input
Input
mRNP
40S
60S
80S
Polysome
200
150
120
Def1(FL)-GFP
120
100
85
Def1(380)-GFP
C
WT
def1 CUEmut1
def1 CUEmut1,2
mRNP
80S
Polysome
A254
40S
60S
10 - 50%
Δdef1
Input
mRNP
40S
60S
80S
Polysome
WT
CUEmut1
CUEmut1,2
150
120
100
Def1

Figure 6

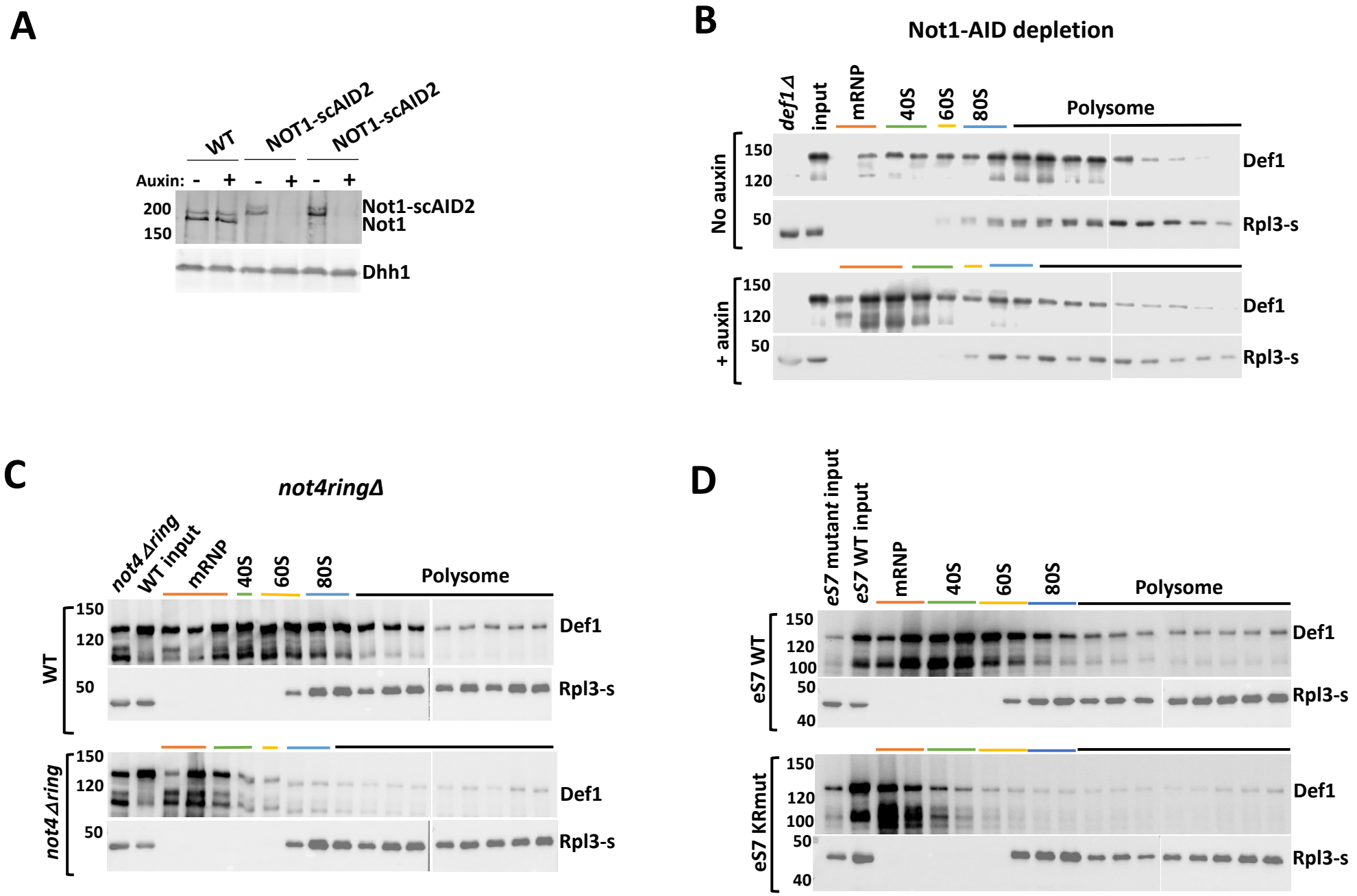
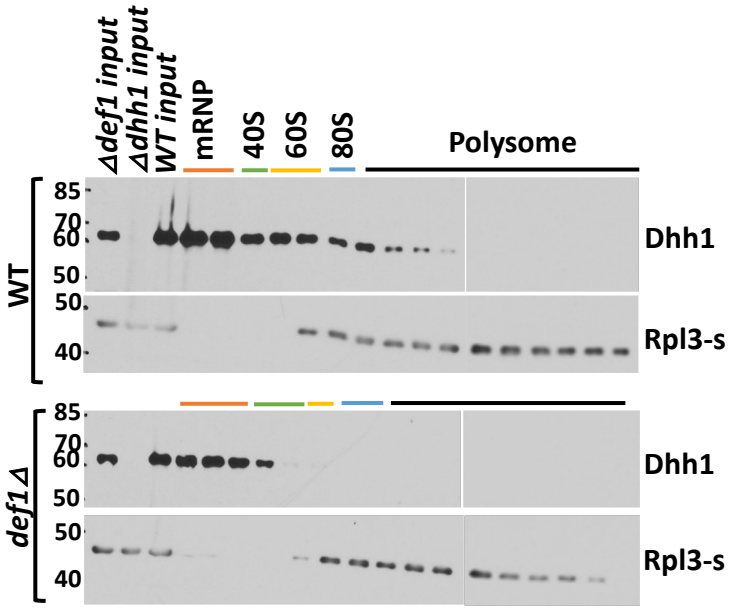

A
WT
NOT1-scAID2
NOT1-scAID2
Auxin: - + - + - +
200
150
Not1-scAID2
Not1
Dhh1
B
Not1-AID depletion
def1Δ
input
mRNP
40S
60S
80S
Polysome
No auxin
+ auxin
150
120
50
Def1
Rpl3-s
C
not4ringΔ
not4Δring
WT input
mRNP
40S
60S
80S
Polysome
WT
not4Δring
150
120
50
Def1
Rpl3-s
D
eS7 mutant input
eS7 WT input
mRNP
40S
60S
80S
Polysome
eS7 WT
eS7 KRmut
150
120
100
50
40
Def1
Rpl3-s

Figure 7

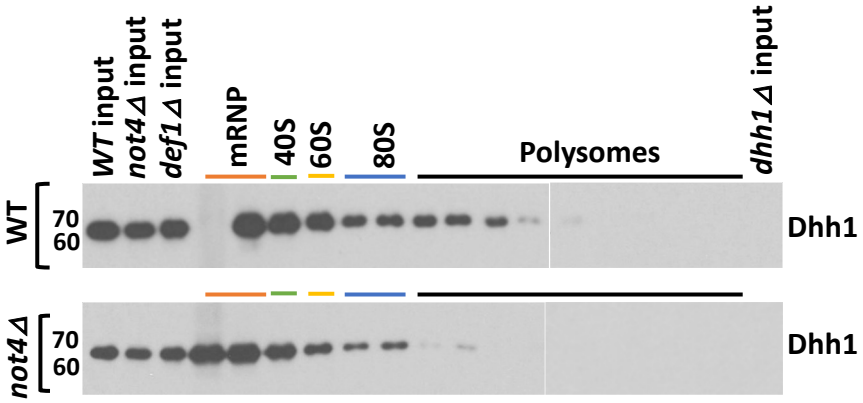
A.

TID fusion	labelled proteins	labelled RNA regulators (% of total labelled)
Def1(1-738)	93	34 (37%)
Def1(1-530)	105	14 (13%)
Def1(1-500)	59	10 (16.9%)
Def1(1-380)	120	17 (14%)

B.



C.



D.

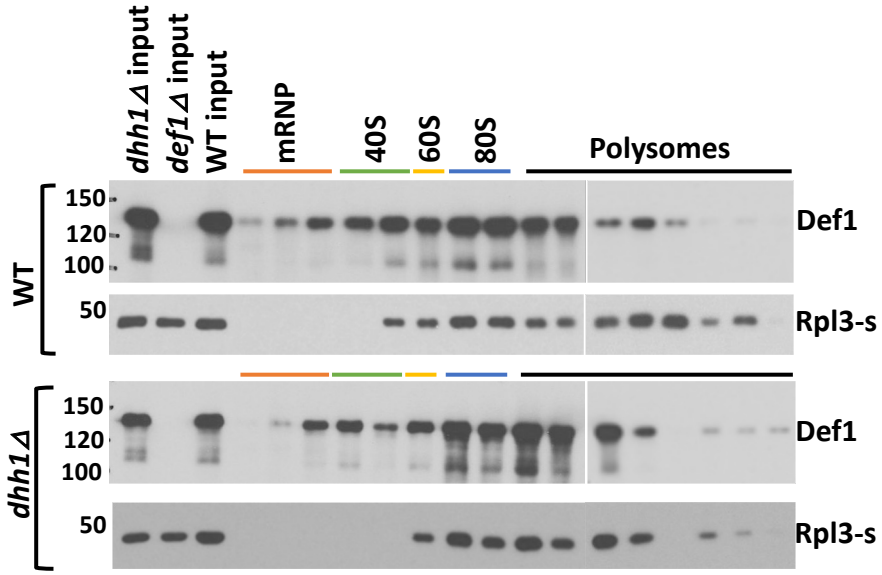
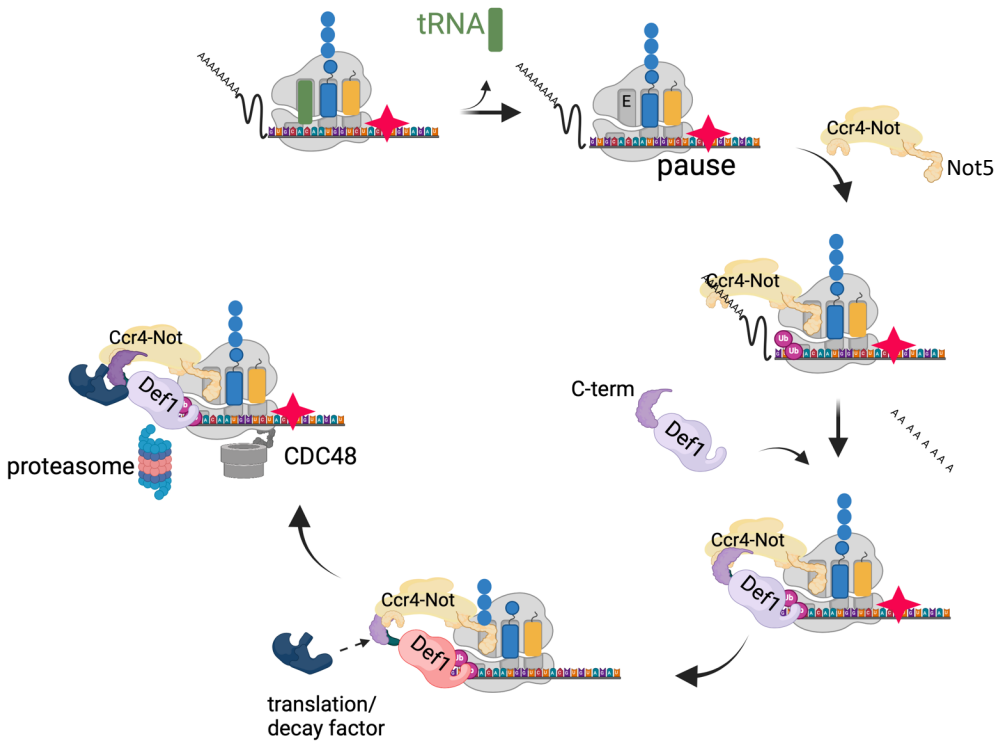


Figure 8


tRNA
E
pause
Ccr4-Not
Not5
Ccr4-Not
C-term
Def1
Ccr4-Not
Def1
proteasome
CDC48
Ccr4-Not
Def1
translation/
decay factor