Translational Capacity of a Cell Is Determined during Transcription Elongation via the Ccr4-Not Complex

Ishaan Gupta,[2,6] Zoltan Villanyi,[1,6] Sari Kassem,[1] Christopher Hughes,[3] Olesya O. Panasenko,[1] Lars M. Steinmetz,[2,4,5] and Martine A. Collart[1,*]

[1]Department of Microbiology and Molecular Medicine, Faculty of Medicine, Institute of Genetics and Genomics, University of Geneva, 1211 Geneva 4, Switzerland
[2]Genome Biology Unit, European Molecular Biology Laboratory (EMBL), 69117 Heidelberg, Germany
[3]Genome Sciences Center, British Columbia Cancer Research Agency, Vancouver, BC V5Z 1L3, Canada
[4]Stanford Genome Technology Center, Stanford University, Palo Alto, CA 94304, USA
[5]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA
[6]Co-first author
*Correspondence: martine.collart@unige.ch
 http://dx.doi.org/10.1016/j.celrep.2016.04.055

SUMMARY

The current understanding of gene expression considers transcription and translation to be independent processes. Challenging this notion, we found that translation efficiency is determined during transcription elongation through the imprinting of mRNAs with Not1, the central scaffold of the Ccr4-Not complex. We determined that another subunit of the complex, Not5, defines Not1 binding to specific mRNAs, particularly those produced from ribosomal protein genes. This imprinting mechanism specifically regulates ribosomal protein gene expression, which in turn determines the translational capacity of cells. We validate our model by SILAC and polysome profiling experiments. As a proof of concept, we demonstrate that enhanced translation compensates for transcriptional elongation stress. Taken together, our data indicate that in addition to defining mRNA stability, components of the Ccr4-Not imprinting complex regulate RNA translatability, thus ensuring global gene expression homeostasis.

INTRODUCTION

There is growing evidence that different levels of gene expression are interconnected to form a network. Constant feedback in all directions is given by components of the different cellular machineries acting to finally produce functional proteins. This ensures homeostasis in gene expression, for example, by inducing compensatory changes in production and degradation of mRNAs to maintain a steady-state level. The circuitry buffering mRNA abundance was revealed first by the finding that different yeast species with different mRNA decay rates nevertheless had similar mRNA levels (Dori-Bachash et al., 2011). These findings were supported and extended by evidence that

mutations affecting machineries involved in mRNA synthesis and decay have co-evolved (Sun et al., 2012). It was also demonstrated that mutations in promoter elements induced coupled changes in synthesis and decay rates, suggesting that transcription factors (TFs) binding to promoters might ensure that the two processes are linked (Dori-Bachash et al., 2012; Trcek et al., 2011).

The first evidence that a factor involved in transcription can have functions in decay emerged from the discovery of the roles of the RNA polymerase II (Pol II) subunit Rpb4 in the decay of a specific class of mRNAs (Lotan et al., 2005). Our finding that Not5 is necessary for cytoplasmic functions of Rpb4 suggested that the multi-subunit Ccr4-Not complex might also play a key role in regulating the gene expression circuitry (Villanyi et al., 2014). Ccr4-Not is a conserved multi-functional eukaryotic regulator composed of nine subunits in the yeast *S. cerevisiae*. It has been proposed that Ccr4-Not is responsible for the integration of environmental signals that coordinate multiple nuclear and cytoplasmic steps in gene expression (reviewed in Chapat and Corbo, 2014; Collart and Panasenko, 2012; Collart et al., 2013; Collart and Timmers, 2004). The Ccr4-Not complex plays roles in both regulation of transcription in the nucleus and degradation of mRNA in the cytoplasm. Hence, it is tempting to hypothesize that components of the Ccr4-Not complex are also loaded onto mRNAs to play the role of a global orchestrator of gene expression that defines mRNA fate later in the cytoplasm (Haimovich et al., 2013).

Current models of gene expression circuitry ignore the possible cross-talk between the processes of transcription and translation. Evidence points toward the Ccr4-Not complex mediating this cross-talk. We have shown that Not5 plays a role in translatability and assembly of the Pol II complex (Villanyi et al., 2014). In addition, components of the Ccr4-Not complex, particularly Not4 and Not5, are important for transcription elongation (Kruk et al., 2011) and protein quality control (Dimitrova et al., 2009; Halter et al., 2014; Preissler et al., 2015). Hence, these components of the Ccr4-Not complex are prime candidates to coordinate transcription with translation.

Studies investigating the role played by the Ccr4-Not complex in regulating the fate of mRNA genome-wide have followed two lines of experimentation in yeast. First, analysis of deletion mutants of the subunits of the Ccr4-Not complex revealed that the complex controls expression of most of the genome (Azzouz et al., 2009b; Cui et al., 2008), with particular impacts on small nucleolar RNAs (Azzouz et al., 2009a) and SAGA-regulated genes (Cui et al., 2008). Second, genome-wide chromatin immunoprecipitation experiments revealed the presence of the Ccr4-Not complex on SAGA-regulated genes (Venters et al., 2011). The former studies indicated targets of the Ccr4-Not complex, both direct and indirect, without differentiating between transcriptional and post-transcriptional regulation of mRNA levels. The latter study shed some light on regulation by Ccr4-Not at the transcriptional level. To date, no genome-wide study has addressed which mRNAs are bound by the components of the Ccr4-Not complex, what determines the binding specificity of the complex subunits, and finally what the extent is to which the complex subunits might regulate the post-transcriptional fate of mRNAs genome-wide.

In this work, to find the mRNAs whose cytoplasmic fate might be directly affected by the Ccr4-Not complex, we first determined the core set of mRNAs that are bound by the Not1 scaffold of the Ccr4-Not complex using native RNA immunoprecipitation (RIP). Next, we determined what defines the binding of mRNAs by Not1. We extended previous single-gene studies genome-wide to show that the Not5 subunit of the complex regulates Not1 mRNA binding, specifically on ribosomal and nuclear-encoded mitochondrial protein mRNAs. Using polysome profiling and stable isotope labeling by amino acids in cell culture (SILAC) experiments, we found that Not5-dependent Not1-bound mRNAs are actively translated and that Not5 affects the translation of ribosomal genes. We determined that inhibition of transcriptional elongation enhanced Not5-dependent Not1 binding of mRNAs and their translatability. We show that tethering Not5 to the cytoplasm affects the polysomal presence of specific mRNAs, as predicted by our model. These findings indicate that Not5-dependent Not1 binding of mRNAs occurs during transcription and regulates translation. We therefore refer to this binding as mRNA imprinting, as proposed previously for proteins that associate with mRNA co-transcriptionally to regulate the cytoplasmic fate of the imprinted mRNA (Choder, 2011). Moreover, because ribosomal mRNAs are a major target of Not5, these findings establish a direct role for Not5 in regulating the abundance of the translation machinery and hence defining global translation levels in the cell.

RESULTS

Not1 Is Enriched over One-Fifth of the Yeast Transcriptome

We performed RIP experiments with a yeast strain expressing tagged Not1 (Figure 1A). RNA from total extracts and Not1-immunoprecipitated samples were sequenced using base-pair resolution mapping of polyadenylation isoforms to a read depth of more than 4 million (Figure S1A). Biological duplicates showed high reproducibility at the level of gene expression (Figure S1B). We calculated the differential enrichment of mRNAs in

the RIP sample over the total extract at a false discovery rate (FDR) < 10%, removing the bias of gene expression from the signal for RIP enrichment (Figure 1B), as described previously (Gupta et al., 2014). To account for non-specific binding of mRNAs, we performed a negative control RIP using a strain without tagged Not1, and the non-specific binders were removed from further analysis. We found that 1,030 (of 5,400) protein-coding mRNAs were significantly enriched in Not1 RIPs (Table S1), with enrichment for gene ontology (GO) terms belonging to the mitochondrion and ribosome categories (Figure 1C). Only 25 of the annotated non-coding RNAs, such as cryptic unannotated transcripts (CUTs) and stable unannotated transcripts SUTs, were significantly enriched in Not1 RIPs (Figure S1C).

Next, we compared the binding of Not1 to specific polyadenylation isoforms of a gene. In line with the known role played by the Ccr4-Not complex in mRNA degradation, we found that all polyadenylation isoforms of genes enriched in Not1 RIPs had higher degradation rates, as defined in a previous study (Gupta et al., 2014), when compared to other polyadenylation isoforms from the same genes that were not bound by Not1 (Figure 1D). Furthermore, we probed the intervening sequence between two differentially bound polyadenylated isoforms of the same gene for existing sequence elements like RNA binding protein (RBP) motifs (Figure S1D) (Riordan et al., 2011). Confirming previous evidence of a functional interaction between the RBPs and the Ccr4-Not complex, we found that the presence of an RBP motif in the 3′ UTR of a gene, such as Puf3 (Chatenay-Lapointe and Shadel, 2011), Khd1 (Ito et al., 2011), Vts1 (Rendl et al., 2008), and Pab1 (Hogan et al., 2008), was associated with increased Not1 binding. We also observed increased Not1 binding to mRNA isoforms that carried motifs recognized by RBPs, such as Pub1 or Nrd1, suggesting functional connections between these RBPs and the Ccr4-Not complex (Figure S1D).

Not5 Regulates Not1 Binding to Determine RNA Abundance

Previous studies have shown that Not5 is required for association of Not1 to specific mRNAs (Villanyi et al., 2014) and that in the absence of Not5, the expression of nuclear-encoded mitochondrial protein mRNAs was upregulated (Azzouz et al., 2009b; Cui et al., 2008). In *not5*Δ, Not1 interaction with most other Ccr4-Not subunits is reduced (Figure S2A). Many of these subunits have affinity for RNA. Therefore, we hypothesized that Not5 might globally affect the way Not1 is associated with mRNAs. To test this, we performed RIP with tagged Not1 in the *not5*Δ background.

Mitochondrial protein and ribosomal protein (RP) mRNAs were 2-fold less enriched in the Not1 RIP from *not5*Δ when compared to the RIP from the wild-type, indicating that Not5 plays an important role in Not1 binding to these transcripts (Figure 2A; Figure S2B). We observed that the change in Not1 binding of mRNAs genome-wide was negatively correlated with the change in gene expression between wild-type and *not5*Δ (Figure 2B). The enhanced expression of a gene upon loss of Not1 binding could be due to enhanced in vivo stability of transcripts that lose Not1 binding. To verify this, we tested the decay rate
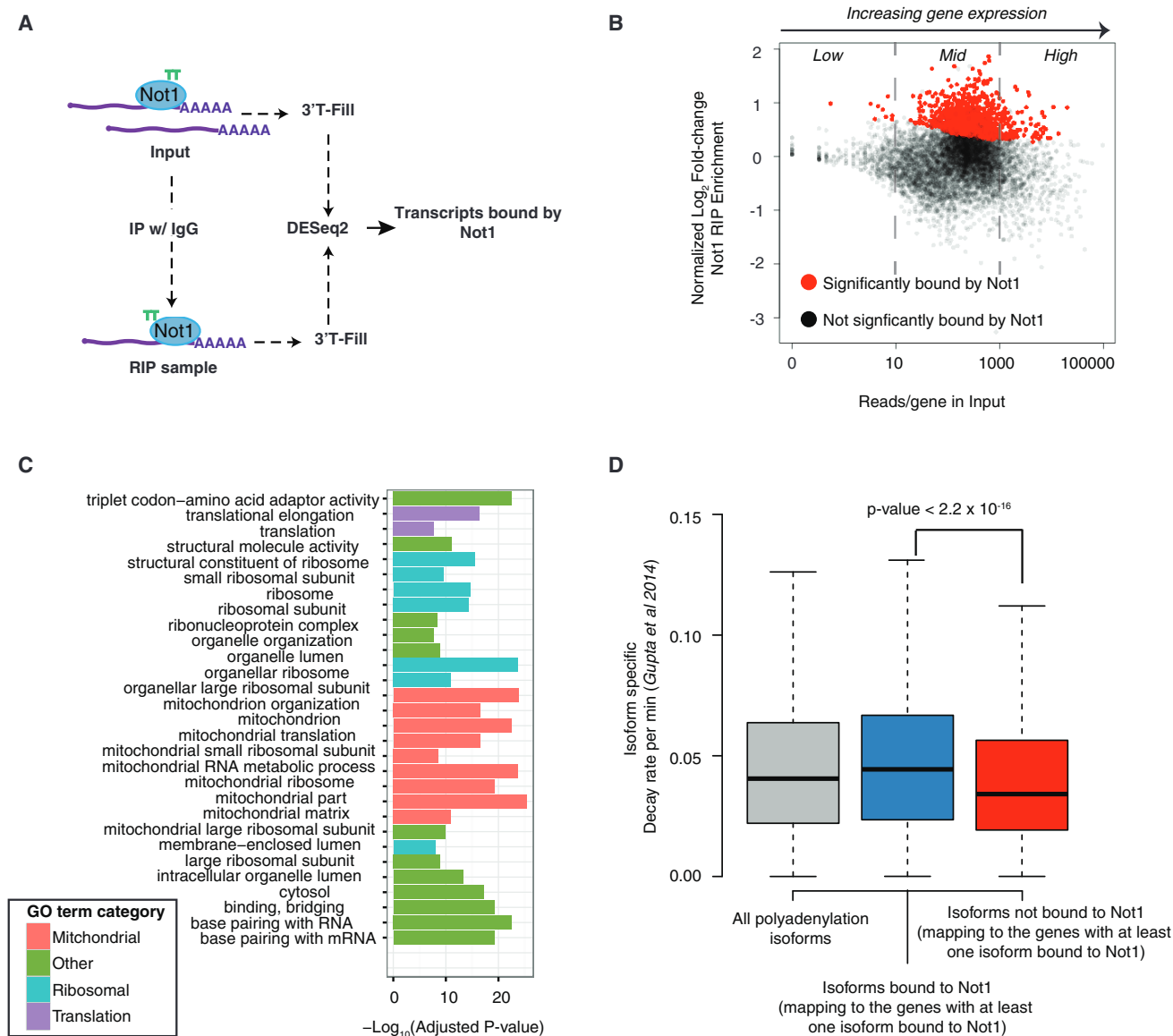
A

B

C

D

Figure 1. Native RIP Reveals that Not1 Binds One-Fifth of the Transcriptome
Native RIP was performed in biological duplicates.
(A) Schematic view of the workflow used to identify Not1-bound mRNAs.
(B) Plot of the applied cutoff to identify significantly bound or unbound mRNAs by Not1.
(C) Chart of GO categories of Not1-enriched mRNAs.
(D) Boxplots show decay rates of polyadenylation isoforms in wild-type yeast obtained in a previous study (Gupta et al., 2014) for all genes (gray), for poly-adenylation isoforms that are enriched in Not1 RIP (blue), and for polyadenylation isoforms that are not enriched in Not1 RIP but belong to genes that have at least one polyadenylation isoform enriched in Not1 RIPs (red).
See also Figure S1 and Table S1.

of two such Not1 target mRNAs (*NHP2* and *RPS8*) and one non-target (*SED1*), by a 1,10-phenanthroline pulse chase (Figure S2C). *NHP2* and *RPS8* mRNAs were more stable in the mutant, but in contrast, the *SED1* decay curve was not different between *not5Δ* and wild-type cells. Anti-correlation between the levels of the mRNAs and their detection by RIP of Not1 from extracts indicates that they are in vivo targets of Not5-dependent Not1 binding.

Although most slow-growth phenotypes related to a deficient growth medium or to stress conditions correlate with a global reduction in the RNA abundance of RP genes (Gasch et al., 2000), in the case of *not5Δ* cells, the expression of most RP genes is upregulated or unchanged (Figure S2D). Moreover, the global change in gene expression in *not5Δ* is not correlated with the slow-growth gene expression signature reported by O'Duibhir et al. (2014) (Figure S2E).
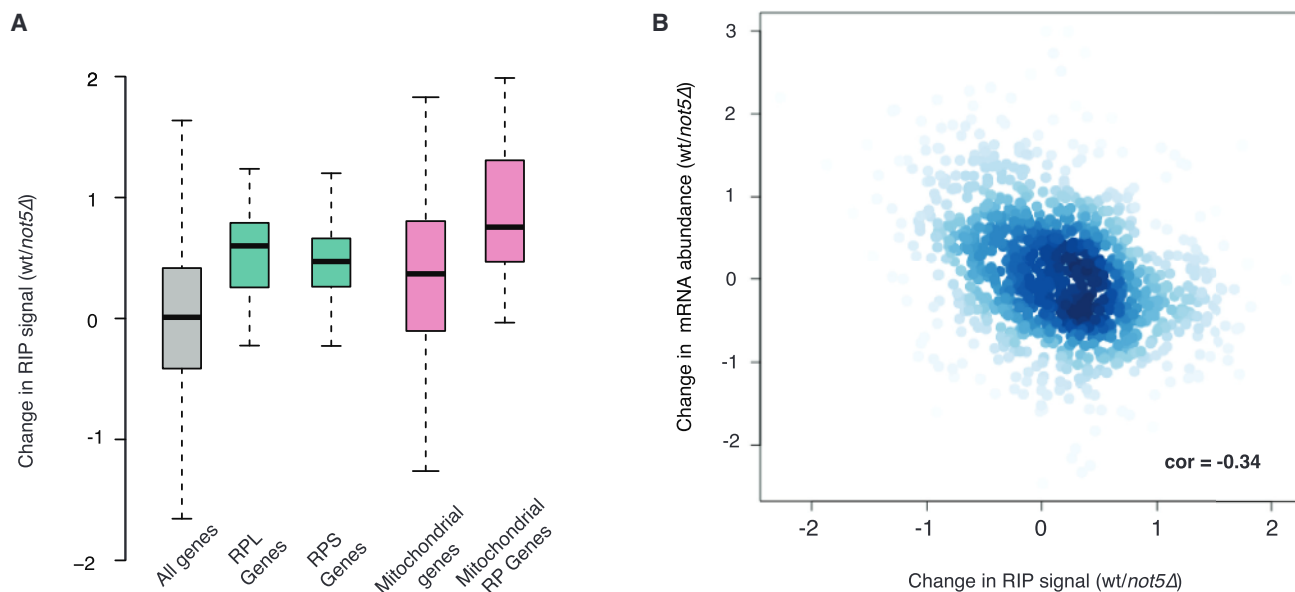
Figure 2. Not5 Regulates Not1 Binding to Determine RNA Abundance
(A) Boxplot representing the distribution of loss in log2 fold of Not1 enrichment in *not5Δ* (calculated as the difference in the RIP signal between wild-type [WT] and *not5Δ*) for all genes (gray), ribosomal large subunit (*RPL*) and ribosomal small subunit (*RPS*) genes (green), and mitochondrial genes and mitochondrial ribosomal genes (pink). All specific categories of genes lost Not1 binding significantly in *not5Δ*, as denoted by the p values from a Student's t test comparing the distribution of all genes in gray with the distribution of each category of genes.
(B) Scatterplot between change in per gene RNA abundance and change in log2 fold Not1 enrichment from WT to *not5Δ* shows a negative Spearman correlation.
See also Figure S2 and Table S1.

mRNAs Bound by Not1 in a Not5-Dependent Manner Are Translated

Previous work has indicated that Not5 is important for the presence of certain mRNAs in polysomes (Villanyi et al., 2014). Moreover, Not5 is needed for association of a newly produced protein with its chaperone (Villanyi et al., 2014). These findings have revealed that Not5 is needed for translation of specific mRNAs.

To determine whether Not5 may have a global function in translation, we compared the Not5-dependent Not1-bound RIP signal on mRNAs (measured as the difference between Not1 binding in wild-type and *not5Δ*) with the published RIP enrichment signal for the Btt1 protein (Figure 3A). Btt1 is a ribosome-associated chaperone that binds to nascent peptides (for review, see Rospert et al., 2002). It has been shown to interact with the Ccr4-Not complex (Liu et al., 2001) and to be associated with mRNAs being translated, in particular with mRNAs encoding mitochondrial proteins and RPs (del Alamo et al., 2011), the same category of mRNAs bound by Not1 in a Not5-dependent manner. We found that the Not5-dependent Not1 RIP signal correlated (Spearman correlation of 0.49) with the Btt1 RIP signal for genes that were significantly enriched in the wild-type Not1 RIP. In contrast, less of a correlation was obtained if we looked at genes enriched in Not1 RIPs in *not5Δ* but not in the wild-type (Spearman correlation of 0.3) (Figure S3).

Btt1 was reported to have a pattern of RIP enrichment over specific mRNAs different from the pool of mRNAs being translated globally, as reflected by the RIP signal with the two RPs Rpl16 and Rpl17 (del Alamo et al., 2011). Nevertheless, we found that the Not5-dependent Not1 RIP signal correlated (Spearman

correlation of 0.41) with the RIP enrichment of RP subunits Rpl16 and Rpl17 (Figure 3B). Again, less of a correlation was obtained if we looked at genes enriched in Not1 RIPs in *not5Δ* only (Figure S3). Taken together, these results suggest that Not5-dependent Not1-bound mRNAs are being translated.

Not5 Affects the Translation of RP Genes

To study the role of Not5 in regulating translation genome-wide, we profiled mRNA from the polysome fraction in both wild-type and *not5Δ* (Table S2). The abundance of mRNAs in the polysomes should directly reflect the translatability of a particular mRNA.

We saw drastically reduced polysomes in *not5Δ* (Figure 4A), as previously observed (Panasenko and Collart, 2012). 273 mRNAs had greater than 40% loss in polysome occupancy (calculated as enrichment in polysomes over total RNA abundance) in *not5Δ* compared to wild-type cells (Figure 4B). Of these, 125 mRNAs had similar or higher mRNA abundance in total extracts of *not5Δ*, clearly demonstrating that reduction from *not5Δ* polysomes was due to reduced translatability and not due to reduced mRNA abundance. Among the 273 mRNAs, we found that almost all RP mRNAs and most other mRNAs encode ribosome biogenesis factors (Table S2). Unlike most stress responses, in which the mRNA abundance of RP genes globally decreases (Weiner et al., 2012), in *not5Δ*, we found that the level of most RP mRNAs is either unchanged or upregulated, yet most of these mRNAs are less abundant in polysomes (98 of 139) (Figure 4B). Although the number of polysomes in the cell is diminished in *not5Δ*, only the class of RP mRNAs is significantly
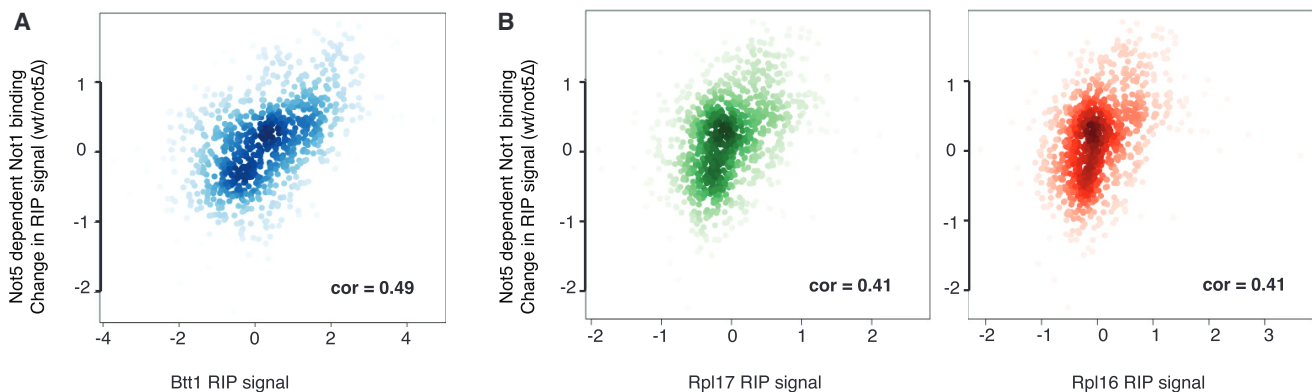
Figure 3. Not5-Dependent Not1 mRNA Binding Correlates with Rpl16, Rpl17, and Btt1 mRNA Binding

(A and B) Scatterplot of the log2 fold change in Not1 RIP signal strength (wild-type or *not5Δ*) with previously published Btt1 (A) and Rpl17 and Rpl16 (B) RIP signals (del Alamo et al., 2011) for genes significantly enriched in Not1 RIP in the wild-type background only shows a positive Spearman correlation.

See also Figure S3.

depleted from the polysomes. Therefore, RP genes lost both Not1 binding and polysome occupancy in *not5Δ* (Figure 4C).

To get a global picture of the translation phenotype in *not5Δ*, we conducted SILAC for 3 hr by substituting growth medium with a heavier amino acid (Experimental Procedures). We were able to quantify protein turnover of 4,350 proteins (Table S3). We found that the amount of heavy-label incorporation, and subsequently the total protein abundance, was lower in the mutant than in the wild-type globally (Figure 4D), consistent with reduced polysomes in *not5Δ* cells. We found that heavy-label incorporation for RPs was significantly lower (p value < 2.2 × 10^{-16}) than for the bulk of proteins in *not5Δ* (Figure 4E). This was also true for the total abundance of RPs (p value < 2.2 × 10^{-16}). Consistently, the protein turnover rates, measured as the ratio of heavy amino acid incorporation to total amino acid incorporation, remained the same for RPs in *not5Δ* compared to the wild-type (Figure 4G). Thus, the deletion of Not5 led to loss of Not1 binding for RP mRNAs and to their reduced translation.

### Not1 Binding Is a Co-transcriptional Event and Depends on Not5

Not5-dependent Not1 binding to RP mRNAs correlates with reduced expression of these mRNAs, indicative of a role for Not1 in decay of these mRNAs, but it also correlates with presence of these mRNAs in polysomes and production of RPs. Therefore, we questioned where Not5 might be important for Not1 binding to mRNAs: in the nucleus or in the cytoplasm.

Gene length-dependent accumulation of mRNA assays have previously shown that Ccr4-Not contributes to transcription elongation (Kruk et al., 2011), and a more recent study has indicated that Not1 is associated with elongating polymerase and contributes to TFIIS's function in transcription elongation (Dutta et al., 2015). Hence, Not1 mRNA binding could be coupled to transcription elongation. In support of this hypothesis, we found that mRNAs bound by Not1 in a Not5-dependent manner (enriched in Not1 RIPs in wild-type cells with at least 40% reduction in binding in *not5Δ* cells) have significantly higher occupancy of Pol II subunits such as Rpb3 (Figure 5A, left) (Mayer et al., 2010)

and Rpb7 (Figure 5A, center) (Jasiak et al., 2008) on the encoding genes. We found a similar pattern of significant accumulation of the NET-seq (native elongating transcript sequencing) signal (Figure 5A, right) (Churchman and Weissman, 2011).

These findings suggested that Not5 could be defining Not1 binding to mRNAs during transcription. To determine whether Not1 was binding mRNAs before their export to the cytoplasm, we determined whether intronic sequences were present in the Not1 immunoprecipitates. Few genes in yeast have introns, but fortunately for this study, they are mostly RP genes, a major target of Not5-dependent Not1 binding. Introns are removed from newly produced RNAs before their export to the cytoplasm. Therefore, we analyzed total RNA, polysomal RNA, and Not1-immunoprecipitated RNA for the levels of exonic and intronic sequences belonging to *RPL30*, *RPS7A*, and *ACT1*. We detected intronic sequences in Not1 RIPs. The relative levels of intron to exon sequences were a magnitude lower in polysomal RNA than in total extracts and in Not1 RIPs (Figure S4). These results support that Not1 is binding mRNAs before their association with ribosomes.

We observed that expression of longer genes was reduced in *not5Δ* (Figure 5B) and that the increase in Not1 binding in *not5Δ* was significantly correlated with gene length (Figure 5C). These findings confirmed that binding of Not1 to mRNAs correlated with reduced expression and indicated that in the absence of Not5, association of Not1 with mRNAs is mainly defined by length. In contrast, binding of Not1 in the presence of Not5 seems to favor RP mRNAs, some of which are very short, so this led us to question what might define RP mRNAs as a target for Not5-dependent Not1 binding. Transcription of RP genes has been well characterized and depends upon two prevalent ribosomal protein-encoding genes (RPG) promoter types, with respect to the localized binding of four TFs (Rap1, Fhl1, Ifh1, and Hmo1). Nine RP genes do not seem bound by any of these four TFs (Knight et al., 2014). Not1 RIP and the polysome presence of RP mRNAs in wild-type versus *not5Δ* were not correlated with any specific promoter type, suggesting that promoter elements are unlikely to determine the specificity of Not1 targeting to RP mRNAs. Transcription of RP genes has been reported
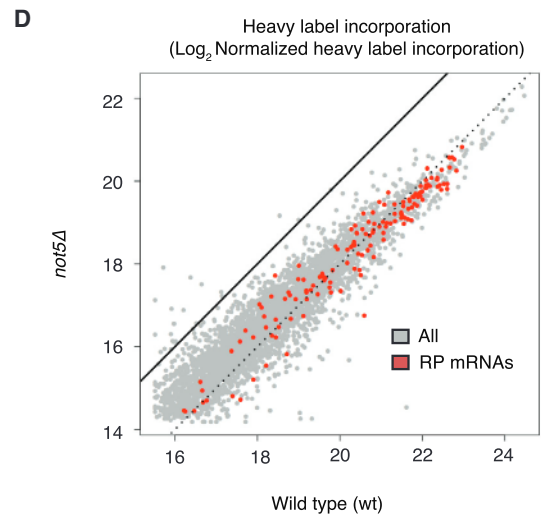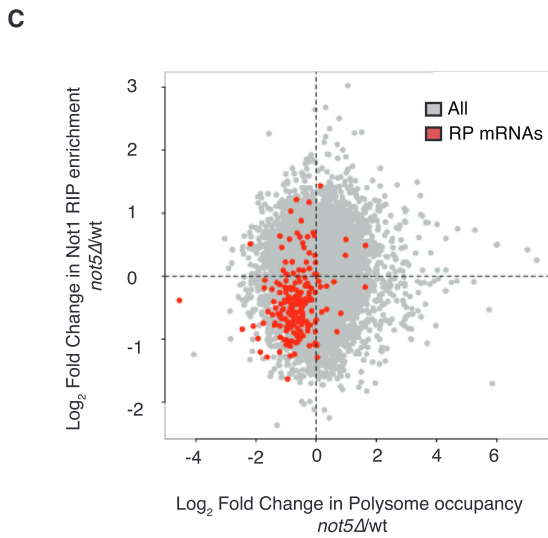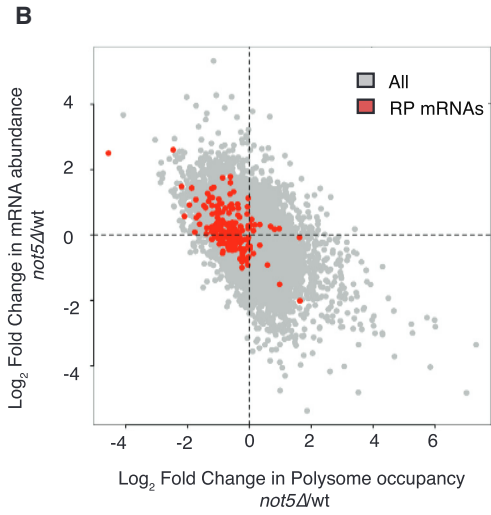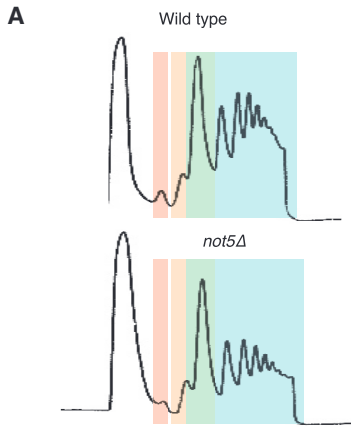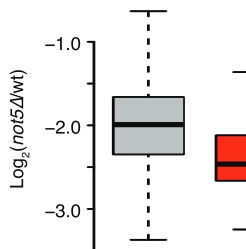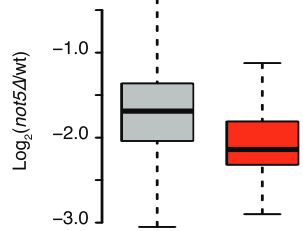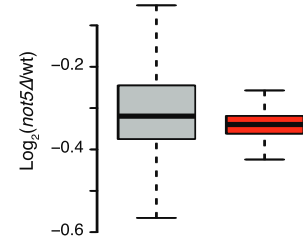
to occur with intense backtracking of Pol II (Gómez-Herreros et al., 2012). The Ccr4-Not complex can associate with the elongating polymerase and promote elongation from the backtracked polymerase (Kruk et al., 2011), so we considered the possibility that Not5 might promote Not1 association to newly produced mRNAs under these conditions. If this model is correct, then by impairing transcription elongation, we might promote association of Not1 with mRNAs.

To test this model, we treated wild-type and not5Δ cells with 6-azauracil (6AU), which impairs transcription elongation by limiting available guanosine triphosphates, and then performed the Not1 RIP. In cells treated for 90 min with 6AU, Not1 association with mRNAs was globally improved in wild-type cells (Figure 5D). This was not the case in the absence of Not5 (Figure 5D).

To orthogonally test whether Not1 was associating co-transcriptionally with mRNAs, we analyzed cells lacking TFIIS (dst1Δ). Efficient transcription elongation of RP genes is highly dependent on TFIIS (Gómez-Herreros et al., 2012). We performed Not1 RIP from wild-type and dst1Δ and compared the presence of six mRNAs in the RIPs: three RP-encoding mRNAs, namely, RPS8, RPS22A, and RPL30, and three mRNAs as controls, namely, NIP1, MNN4, and SED1. The RP mRNAs were associated with Not1 to a greater extent in dst1Δ compared to the wild-type (Figure 5E), whereas none of the control mRNAs had significant enrichment in the Not1 RIP from dst1Δ. This effect was not due to changes in Not1 protein abundance, because Not1 was similarly immunoprecipitated from wild-type, not5Δ, and dst1Δ strains (Figure S5).

Taken together, these results indicate that binding of Not1 to mRNAs is coupled to transcription elongation. They also indicate that transcription elongation stress permits improved Not1 binding but that this requires Not5.

Transcriptional Stress Leads to a Not5-Dependent Increase in Translation

Not1 binding to RP mRNAs under transcription elongation stress conditions requires Not5, which is also needed for the optimal presence of RP mRNAs in polysomes in normal conditions. To determine whether better Not1 binding under elongation stress is connected to better polysome presence of the bound mRNA, we tested for the presence of mRNAs in polysomes in cells treated with 6AU. Inhibition of transcription elongation led to increased presence of all tested mRNAs in polysomes for wild-type cells, but this was less the case in the absence of Not5 (Figure 6). We then tested the presence of mRNAs in wild-type and dst1Δ polysomes and similarly observed that the increased binding of Not1 to RPS8, RPS22A, and RPL30 shown

earlier correlated with increased presence in polysomes. In contrast, in the case of the SED1 and MNN4 controls, neither a significant change in Not1 binding nor any increased polysomal presence was observed in dst1Δ (Figures 5E and 6B).
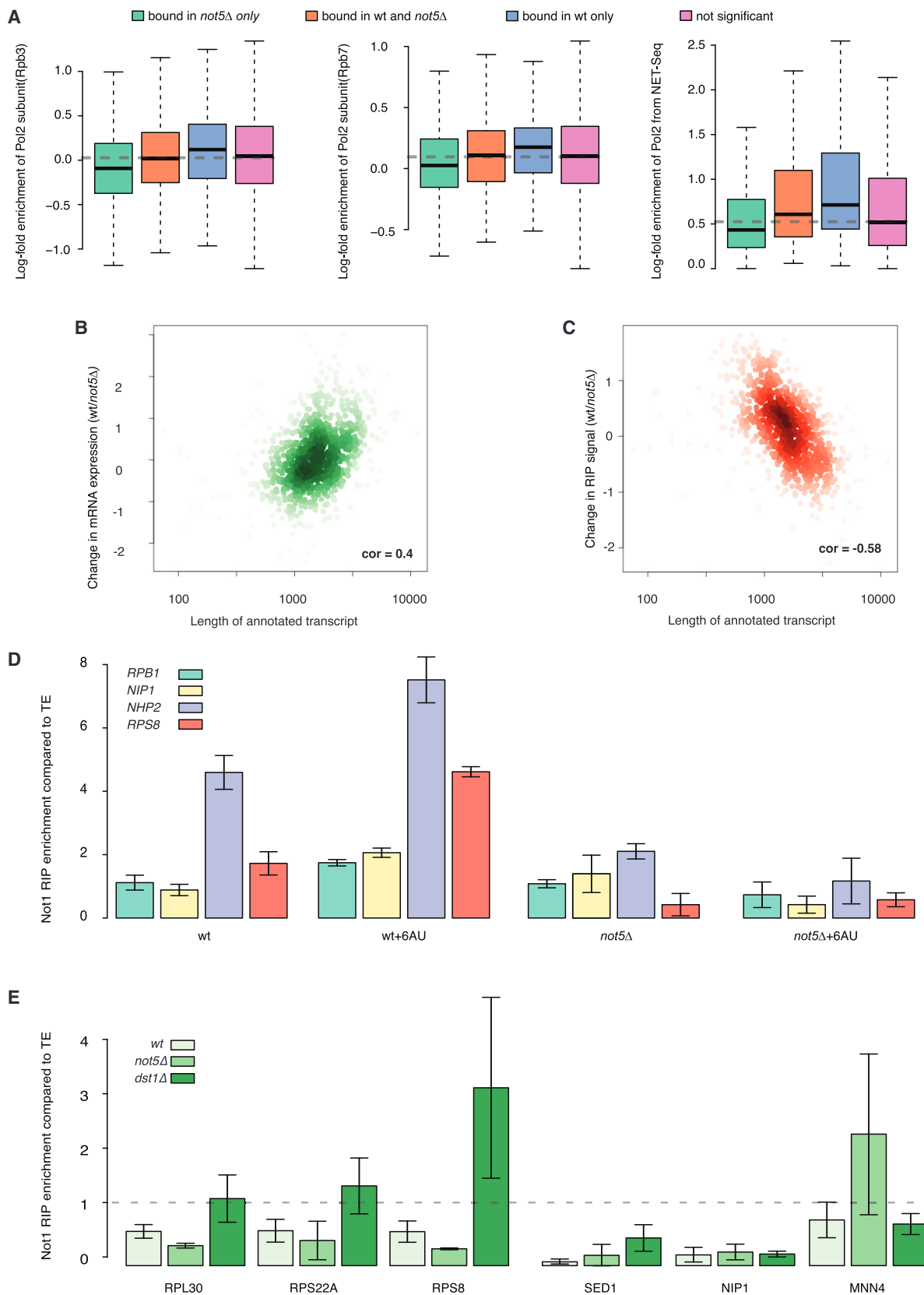
Because transcription elongation stress leads to a Not5-dependent increase in Not1 association with mRNAs that enhances their abundance in polysomes, particularly for RP mRNAs, we wanted to confirm that nuclear Not5 has an impact on RP mRNA translatability. We used the tether-away system, in which fusing a protein of interest to the FKBP12-rapamycin binding (FRB) domain of human mechanistic target of rapamycin (mTOR) in a rapamycin-resistant strain in which the human FKBP12 is fused to Rpl13A leads to tethering of the protein of interest to the cytoplasm upon treatment of cells with rapamycin (Haruki et al., 2008). We fused Not5 to FRB in the parental strain. Cells expressing the fusion protein did not display any detectable growth phenotype and, when treated with rapamycin, started growing slower than the parental strain only after 2 hr. We first looked at localization of fused Not5 before and after treatment with rapamycin for 5 min (Figure 7A). Not5 was localized in both the cytoplasm and the nucleus before treatment, but it lost nuclear localization after treatment. Consistently, the distribution of fused Not5 across a sucrose gradient was changed 5 min after rapamycin treatment: the Not5 that was initially detected in the free fraction had been removed and accumulated in polysomes (Figure 7B). This was not the case in the control strain that did not express tagged Not5 (Figure S6A). The tethering of Not5 to ribosomes did not change the distribution of Not1 across the sucrose gradient (Figure 7B) or Not1's nuclear staining (Figure S6B).

We then measured the presence of different mRNAs in polysomes before and after treatment. In parallel, we determined the association of these mRNAs with Not1 by RIP. RPS8A, RPS22A, and RPS7A mRNAs were already decreased in polysomes 5 min after rapamycin addition (Figure 7C). This correlated with a reduced association with Not1 after treatment (Figure 7D). Inversely, the polysomal presence of IMH1, an mRNA that gains Not1 binding in not5Δ and that gained binding after rapamycin addition (Figure 7D), was enhanced after tethering of Not5 to the cytoplasm (Figure 7C). These correlated changes in polysome presence and Not1 RIP were not observed in the control strain. Moreover, NIP1 mRNA presence in polysomes or Not1 RIP did not change after rapamycin treatment.

Taken together, these results confirm that Not1 mRNA targets are imprinted in the nucleus, that nuclear Not5 defines the mRNA targets for Not1 imprinting, and that Not1 imprinting is facilitating translation.

Figure 4. Not5 Affects the Translation of RP Genes

(A) Polysome traces in wild-type (WT) cells (top) and not5Δ cells (bottom), with different ribosomal fractions highlighted.

(B) Scatterplot showing the log2 fold change in mRNA abundance from not5Δ to WT against the log2 fold change in polysomal occupancy from not5Δ to WT.

(C) Scatterplot of the log2 fold change of Not1 RIP enrichment from not5Δ to WT is plotted against the polysomal occupancy change from not5Δ to WT.

(D) Scatterplot of log2-transformed heavy-label (lysine) incorporation in WT and not5Δ strains from the SILAC experiment.

(E) Boxplots for the log2 fold change in heavy-label incorporation from not5Δ to WT.

(F) Boxplots for the log2 fold change in total protein abundance (sum of both heavy and light lysine) from not5Δ to wild-type.

(G) Boxplots for the log2 fold change in protein turnover (measured by the log2 ratio of heavy-label incorporation over total protein abundance) from not5Δ to WT.

(B–G) All mRNAs are in gray, and RP mRNAs are in red.

See also Tables S2 and S3.

A

bound in *not5Δ only* bound in wt and *not5Δ* bound in wt only not significant

B

C

D

RPB1
NIP1
NHP2
RPS8

E

wt
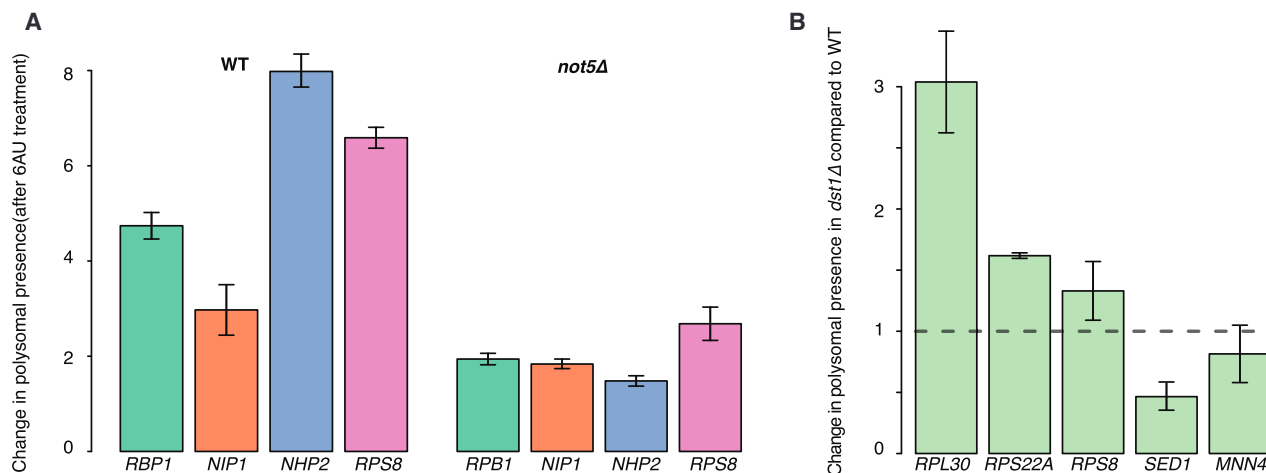not5Δ
dst1Δ

(legend on next page)

Figure 6. Inhibition of Transcription Elongation Is Compensated by Upregulation of Translatability

(A) Barplots of mRNA abundance in polysomes upon treatment or not with 6AU for four mRNAs tested previously for RIP enrichment (Figure 5D) in wild-type and not5Δ.

(B) Barplots showing the abundance of various mRNAs normalized to *NIP1* mRNA levels in not5Δ and dst1Δ polysomes relative to wild-type polysomes.

Error bars represent SD.

See also Table S4.

DISCUSSION

Specific mRNAs Are Imprinted by Not1 Co-transcriptionally

In this work, we have investigated at a genome-wide scale, using RIP experiments, which mRNAs can be detected in association with Not1, the core subunit of the Ccr4-Not complex. We found that Not1-bound mRNAs define one-fifth of the coding transcriptome, with GO enrichment for mitochondrial protein and RP genes. Association of Not1 with these specific targets in particular greatly depended on the Not5 subunit of the Ccr4-Not complex.

A caveat with RIP experiments is that some level of binding could be occurring in vitro. We observed that Not1 was less bound to mRNAs in not5Δ even when they were more abundant in the extracts, but Not1 also lost association with several subunits of the Ccr4-Not complex that have RNA binding affinity in not5Δ. However, we found a genome-wide correlation between loss of Not1 binding to RP mRNAs and reduced translation of these mRNAs, measured by polysome profiling and SILAC (Figure 4), suggesting that the RIP values reported in our study describe an in vivo phenomenon. Our single-gene experiments

reinforced that Not1 binding measured by RIP correlates with translatability of mRNAs (Figures 5D and 6A).

Our findings strongly indicate that besides the RBP- or microRNA-associated tethering of Not1 to mRNAs in the cytoplasm (for review, see Collart and Panasenko, 2012), Not1 binds to mRNA co-transcriptionally. We provide experimental evidence for the existence of this nuclear Not1 mRNA imprinting. First, Not1 was associated with intronic RNA sequences that are hardly detectable in polysomes (Figure S4). Second, generating transcription elongation stress by treating cells with 6AU leads to better Not1 binding to mRNAs (Figure 5D). Third, impairing transcription elongation by deletion of TFIIS also leads to better Not1 mRNA imprinting (Figure 5E). Fourth, Not1 binding, as measured by the amount of mRNA that can be co-immunoprecipitated with Not1, correlates with polymerase occupancy on genes globally. This correlation is the strongest for mRNAs that are bound by Not1 in a Not5-dependent manner (Figure 5A). Finally, the tethering of Not5 away from the nucleus leads within 5 min to a correlated change in the presence of mRNAs in polysomes and binding of these mRNAs to Not1, which additionally correlate with how the binding of these mRNAs by Not1 changes in not5Δ (Figure 7). These results confirm that Not5 is needed in

Figure 5. Not5 Dependency for Not1 mRNA Binding Correlates with Elongating Polymerase

(A) Boxplots show how four categories of genes categorized by their Not1 binding properties (bound in not5Δ only, bound in wild-type [WT] and not5Δ, bound in WT only, and not significantly bound) correlate with occupancy of RNA Polymerase II subunit Rpb3 (Mayer et al., 2010) and subunit Rpb7 (Jasiak et al., 2008), as well as with the NET-seq signal identifying nascent mRNAs (Churchman and Weissman, 2011).

(B) Scatterplot between change in RNA abundance from WT to not5Δ (WT/not5Δ) and transcript length is positively correlated.

(C) Scatterplot between change in Not1 RIP signal strength (WT/not5Δ) and transcript length is negatively correlated.

(D) Barplot for log2 fold change in Not1 enrichment over four mRNAs (*RPS8*, *NHP2*, *RPB1*, and *NIP1*) in WT and not5Δ after transcriptional inhibition upon 6AU treatment.

(E) Barplot for fold enrichment in Not1 RIP compared to total extract for several mRNAs in WT, not5Δ, and dst1Δ.

(D and E) Error bars represent SD.

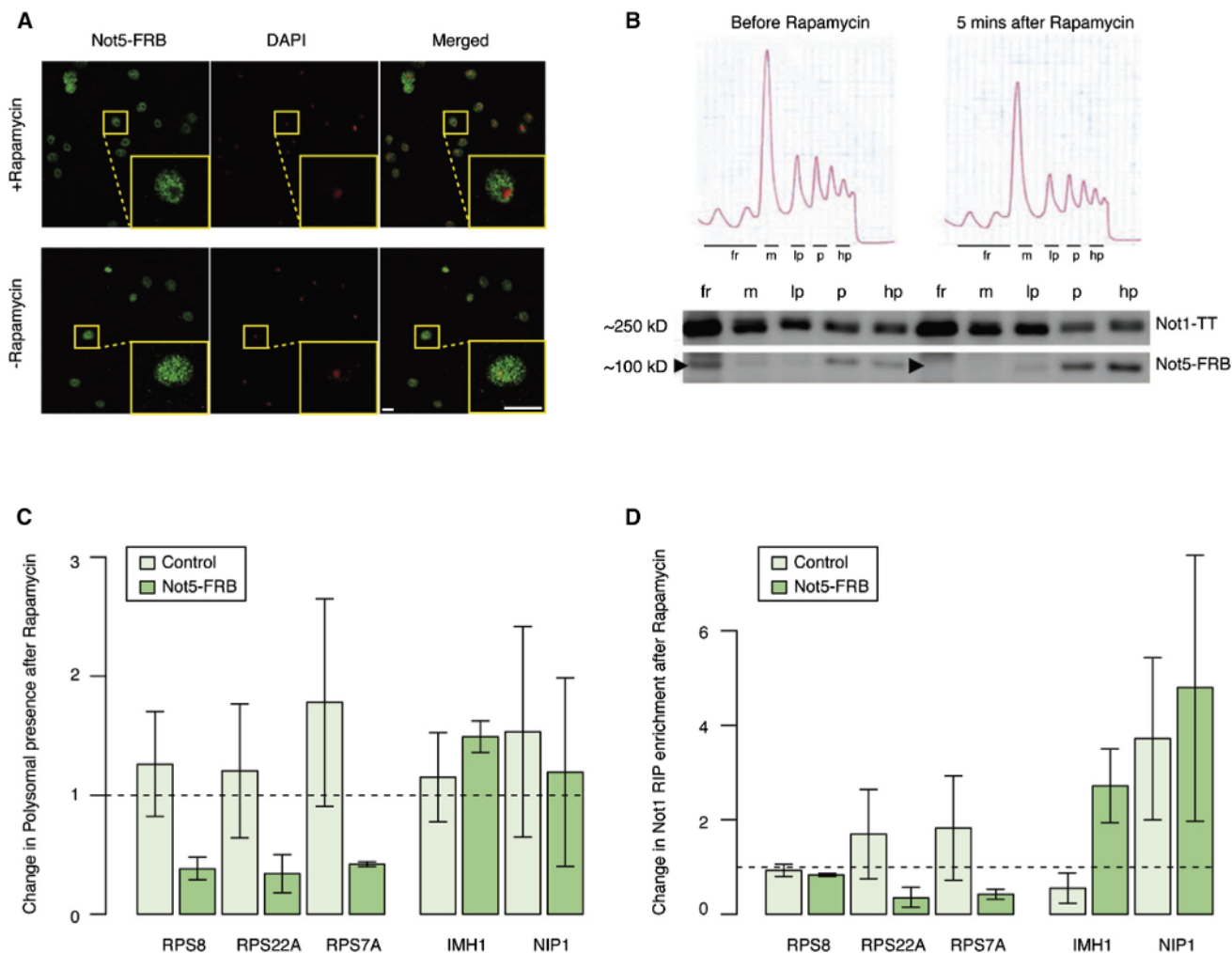See also Figures S4 and S5 and Table S4.

Figure 7. Translatability of Specific mRNAs Is Regulated by Nuclear Not5

Tethering of Not5 to the cytoplasm leads to a reduced presence of RP-encoding mRNAs and an increased presence of *IMH1* mRNA in polysomes.

(A) Localization of FRB-fused Not5 in cells before and after treatment for 5 min with rapamycin. The nucleus is shown in red (DAPI), and Not5-FRB is shown in green (Alexa Fluor). In response to rapamycin, nuclear localization of Not5-FRB was lost, and the nuclei are detected as dark holes, which co-localize with DAPI staining. Scale bars represent 5 μm.

(B) Presence of Not5-FRB and Not1 before and after 5 min of rapamycin treatment in free fractions (fr), monsomes (m), and in three polysome fractions: light (lp), medium (p), and heavy (hp). Arrows indicate Not5-FRB. Polysome traces before and after rapamycin treatment for 5 min are displayed above the corresponding blots.

(C) Barplots of the change in abundance of several mRNAs in polysomes after treatment for 5 min with rapamycin in control cells or cells expressing Not5-FRB.

(D) Barplot for the fold change in Not1 RIP enrichment over total extract after rapamycin compared to before for several mRNAs in control cells and in cells expressing Not5-FRB.

(C and D) Error bars represent SD.

See also Figure S6 and Table S4.

the nucleus to promote Not1 binding, which in turn promotes mRNA translatability.

Our study suggests that Not1 associates better with mRNAs under transcription elongation stress. The Ccr4-Not complex binds to the elongating polymerase and promotes elongation, but it does not affect elongation of un-arrested polymerase (Babbarwal et al., 2014; Dutta et al., 2015). The window of opportunity for Not1 to associate with the nascent transcripts is likely to be greater when the elongation process is slowed by backtracking

or limiting nucleotides. Under normal conditions, this concerns specifically RP mRNAs (Dutta et al., 2015; Gómez-Herreros et al., 2012; Pelechano et al., 2009). Under global transcription elongation stress such as 6AU treatment, it concerns all genes.

How might Not5 be important for the association of Not1 with specific mRNAs during transcription? Association of Ccr4-Not with the elongating polymerase complex was shown to depend upon transcript length, suggesting that Ccr4-Not is interacting with the nascent transcript (Babbarwal et al., 2014). Not5 might

connect Not1 to the elongating polymerase or to the mRNAs as a direct tether or because it is important for other Ccr4-Not subunits to associate with Not1. Not5 has RNA binding activity (Bhaskar et al., 2013), as do other subunits of the Ccr4-Not complex, such as the deadenylase subunits or Not4, which has an RRM (RNA recognition motif) motif (Albert et al., 2000). As mentioned earlier, association of Not1 with different isoforms of the same gene was more likely for the isoforms with binding sites for RBPs. This could indicate that RBPs facilitate the retention of Not1 on mRNAs after Not1 is imprinted during the transcription elongation phase. Finally, the poly(A) binding protein might contribute to anchoring Not1 to the mRNA.

Co-transcriptional Imprinting by Not1 Regulates Translation and mRNA Degradation

Polyadenylation isoforms of a gene that showed better association with Not1 were those previously determined to have higher degradation rates (Figure 1D) (Gupta et al., 2014). This is in line with the role of the Ccr4-Not complex as the major yeast deadenylase. Moreover, RP mRNAs that are a major target of Not5-mediated Not1 binding have been reported to be a preferred target of Ccr4 (Grigull et al., 2004). This raised the question of whether Not1 was co-transcriptionally associated with mRNAs solely to define their subsequent decay rate. We were able to globally correlate the mRNAs dependent on Not5 for Not1 binding with translated mRNAs, defined as those associated with one of two RPs, Rpl16 or Rpl17, or those associated with a ribosome-associated chaperone, Btt1 (Figure 3). Previous work has suggested that mRNA decay and translation are coupled processes (Hu et al., 2009; Pelechano et al., 2015). We observed that fewer RPs were produced in not5Δ. So we reflected on how an impact on mRNA degradation could indirectly affect translation. The Ccr4-Not complex is important for mRNA deadenylation, and interaction of Not1 with the Ccr4 deadenylase is reduced in not5Δ. However, in this study, we measured polyadenylated mRNAs and found that polyadenylated RP mRNAs are reduced in polysomes in not5Δ. Inefficient deadenylation of RP mRNAs in the mutant cannot explain this observation.

Not1 Imprinting Determines Translational Capacity

Our findings reveal that Not1 imprinting plays an important role in coupling different steps of gene expression, facilitating both translation of the imprinted mRNA and its subsequent decay. Because Not1 imprinting of RP mRNA happens co-transcriptionally, it also links the decay and translation machinery to the status of global transcription. An exciting finding in this work is that we were able to correlate the necessity of Not5 for Not1 imprinting during transcription with translation of RP mRNAs and the level of total polysomes. Without Not5, RP mRNAs are less imprinted by Not1 (Figure 4C), fewer RPs are produced, and polysome levels are reduced (Figures 4A and 4E). During transcriptional elongation stress that renders transcription generally less efficient (Gómez-Herreros et al., 2012), Not1 associates with mRNAs better via Not5 (Figure 5D); consequently, these mRNAs are better translated (Figure 6A). Because RP mRNAs are particularly affected by this mechanism, this allows the cell to produce more ribosomes and increase global translation levels to overcome transcriptional stress even for mRNAs not imprinted by

Not1. Our data indicate that loss of Not5-mediated Not1 imprinting is visible within 5 min of removing Not5 from the nucleus, and it affects the presence of RP mRNAs in polysomes within 5 min. There is still substantial binding of Not1 to *RPS8*, and newly produced *RPS8* mRNAs are unlikely to constitute an essential part of the steady-state *RPS8* mRNA; nevertheless, these observations suggest that newly exported and imprinted RP mRNAs contribute to the pool of RP mRNAs being translated.

We observed that tethering of Not5 to ribosomes did not lead to co-tethering of Not1, suggesting either that the tethering artificially pulled Not5 away from the Not1 scaffold or that, in vivo, the Ccr4-Not complex is not a single entity at all times. This is an exciting observation because the question of whether the Ccr4-Not complex exists only in one form in cells to perform its multiple and sometimes conflicting functions is an open and important one.

We recently showed that Not5 controls assembly of Pol II during translation (Villanyi et al., 2014). While we have not demonstrated that the level of transcription is directly affected by the co-translational assembly of the polymerase, these results are highly suggestive that Not5 could in turn determine transcription levels during translation.

Our findings demonstrate that inhibition of global transcription can be compensated by a direct physical link to an increase in translation globally. They demonstrate that the different gene expression levels, transcription, translation, and mRNA degradation, are linked in gene expression circuitry by the Ccr4-Not complex, which clearly plays a major role in gene expression that extends beyond its function as the major deadenylase of eukaryotic cells.

EXPERIMENTAL PROCEDURES

Strains and Growth Media
Strains used in the present study are summarized in Table S4. Growth media were standard. In some experiments, 6AU was added at 25 μg/ml for 90 min. For others, to obtain complete transcription inhibition to follow mRNA decay rates, we used 1,10-phenanthroline as described previously (Grigull et al., 2004).

Native RIP
Exponentially growing cells were harvested at an optical density (OD) of 0.6–0.9 and broken with glass beads. An aliquot of lysate was taken as input control; the remaining was immunoprecipitated with Dyna M280 sheep anti-rabbit immunoglobulin G (IgG) for 2 hr at 4°C and analyzed as described previously (Gupta et al., 2014). To follow single mRNAs with RIP, we performed real-time qPCR on input and immunoprecipitated samples using specific primers as described (Villanyi et al., 2014). The input and immunoprecipitated RNA samples obtained were subjected to polyadenylation profiling using sequencing as previously described (Gupta et al., 2014), and the reads were aligned to the R64 *S. cerevisiae* genome.

qPCR Analysis
The 1 μg of total RNA obtained from polysomes or from total extracts was reverse transcribed with oligo(dT) primers in a total volume of 25 μl. After synthesis, cDNAs were diluted to a final volume of 250 μl, and 5 μl were used for qPCR using gene-specific primers as described in Villanyi et al. (2014). Gene-specific primers are summarized in Table S4.

Polysome Profiling
The polysome profiling was done as described previously (Panasenko and Collart, 2012). The input and polysomal RNA samples obtained were subjected

to polyadenylation profiling using sequencing as previously described (Wilkening et al., 2013), and the reads were aligned to the R64 *S. cerevisiae* genome.

SILAC Experiment

Wild-type and *not5Δ* cells were grown until saturation overnight in synthetic complete (SC)-complete medium (2% glucose) containing light lysine (12C6, 14N2). The next day, the samples were diluted to an OD of 0.1–0.2 and grown for 3 hr in SC-complete medium with light lysine. The cells were then shifted to medium containing heavy lysine (13C6, 15N2), grown for another 3 hr, harvested, and sent for liquid chromatography-mass spectrometry as described previously (Hughes et al., 2014).

Not5 Tether Away and Immunolocalization of Not5-FRB

Yeast cells expressing *NOT5-FRB* and *RPL13A-FKBP12* alleles were cultured in YPD medium at 30°C. Exponentially growing cultures were treated with 1 μg/ml rapamycin (Enzo Laboratories), and 100 ml of cells were collected for polysome profiling. Then, 5 ml of cells were collected for immunofluorescence microscopy. For microscopy, cells were fixed with 1 ml of 37% formaldehyde at room temperature for 2 hr. Cell pellets were washed with PBS containing 0.1% Tween 20 and resuspended in 1 ml of spheroblasting buffer (1.2 M sorbitol, 20 mM potassium phosphate [pH 7.4]). Then, 0.1 ml of cell suspension was treated with 3.2 μl of 1.42 M β-mercaptoethanol and 5 μl of 5 mg/ml zymolyase 100T for 30 min at 30°C. Next, 20 μl of spheroblasts were immobilized on polylysine-coated microscope slides. Staining was done using rabbit polyclonal anti-Not5 antibody, followed by Alexa Fluor 488 anti-rabbit IgG (Life Technologies) and DAPI. Images were taken before and 15 min after addition of rapamycin using an Olympus DeltaVision microscope equipped with a GFP/monomeric red fluorescent protein (mRFP) filter set (Chroma) and a CoolSNAP HQ camera. Images were obtained by optical sectioning (taking several z stacks) with a step size of 0.2 μm and further processed with ImageJ-win64.

Statistical Methods

To calculate fold change enrichments between input and Not1 RIPs, we used the DESeq2 R Bioconductor package as described previously (Gupta et al., 2014), and these values are given in Table S1. The polysomal occupancy was calculated as the fold change enrichment in the polysomal fraction over the total input RNA, also using DESeq2 (Love et al., 2014), and these values are provided in Table S2. The raw values from the mass spectrometry run were normalized and log transformed by variance stabilizing using the vsn package in R Bioconductor and are provided in the Table S3. For calculating changes in light- or heavy-label incorporation, we variance stabilized either only light-labeled or only heavy-labeled wild-type and mutant samples together. To calculate changes in total protein levels, the heavy-labeled and light-labeled raw values were added and then variance stabilized for wild-type and mutant samples together. To calculate the protein turnover for each sample, the heavy-labeled raw values by total protein, as calculated previously, were variance stabilized for wild-type and mutant samples together.

ACCESSION NUMBERS

The accession number for the datasets reported in this paper is EBI ArrayExpress: E-MTAB-3195. The accession number for the mass spectrometry proteomics data reported in this paper is PRIDE: PXD003871.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and four tables can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.055.

AUTHOR CONTRIBUTIONS

Conceptualization, I.G., Z.V., and M.A.C.; Investigation – Methodology, I.G., Z.V., S.K., C.H., and O.O.P.; Software, I.G.; Formal Analysis, I.G., Z.V., L.M.S., and M.A.C.; Resources, I.G., Z.V., S.K., C.H., O.O.P., L.M.S., and M.A.C.; Writing – Original Draft, I.G., Z.V., L.M.S., and M.A.C.; Writing – Review

& Editing, I.G., Z.V., O.O.P., L.M.S., and M.A.C.; Visualization, I.G. and Z.V.; Supervision, L.M.S. and M.A.C.; Funding Acquisition, L.M.S. and M.A.C.

REFERENCES

Albert, T.K., Lemaire, M., van Berkum, N.L., Gentz, R., Collart, M.A., and Timmers, H.T. (2000). Isolation and characterization of human orthologs of yeast CCR4-NOT complex subunits. Nucleic Acids Res. *28*, 809–817.

Azzouz, N., Panasenko, O.O., Colau, G., and Collart, M.A. (2009a). The CCR4-NOT complex physically and functionally interacts with TRAMP and the nuclear exosome. PLoS ONE *4*, e6760.

Azzouz, N., Panasenko, O.O., Deluen, C., Hsieh, J., Theiler, G., and Collart, M.A. (2009b). Specific roles for the Ccr4-Not complex subunits in expression of the genome. RNA *15*, 377–383.

Babbarwal, V., Fu, J., and Reese, J.C. (2014). The Rpb4/7 module of RNA polymerase II is required for carbon catabolite repressor protein 4-negative on TATA (Ccr4-not) complex to promote elongation. J. Biol. Chem. *289*, 33125–33130.

Bhaskar, V., Roudko, V., Basquin, J., Sharma, K., Urlaub, H., Séraphin, B., and Conti, E. (2013). Structure and RNA-binding properties of the Not1-Not2-Not5 module of the yeast Ccr4-Not complex. Nat. Struct. Mol. Biol. *20*, 1281–1288.

Chapat, C., and Corbo, L. (2014). Novel roles of the CCR4-NOT complex. Wiley Interdiscip. Rev. RNA *5*, 883–901.

Chatenay-Lapointe, M., and Shadel, G.S. (2011). Repression of mitochondrial translation, respiration and a metabolic cycle-regulated gene, SLF1, by the yeast Pumilio-family protein Puf3p. PLoS ONE *6*, e20441.

Choder, M. (2011). mRNA imprinting: additional level in the regulation of gene expression. Cell. Logist. *1*, 37–40.

Churchman, L.S., and Weissman, J.S. (2011). Nascent transcript sequencing visualizes transcription at nucleotide resolution. Nature *469*, 368–373.

Collart, M.A., and Panasenko, O.O. (2012). The Ccr4-not complex. Gene *492*, 42–53.

Collart, M.A., and Timmers, H.T. (2004). The eukaryotic Ccr4-not complex: a regulatory platform integrating mRNA metabolism with cellular signaling pathways? Prog. Nucleic Acid Res. Mol. Biol. *77*, 289–322.

Collart, M.A., Panasenko, O.O., and Nikolaev, S.I. (2013). The Not3/5 subunit of the Ccr4-Not complex: a central regulator of gene expression that integrates signals between the cytoplasm and the nucleus in eukaryotic cells. Cell. Signal. *25*, 743–751.

Cui, Y., Ramnarain, D.B., Chiang, Y.C., Ding, L.H., McMahon, J.S., and Denis, C.L. (2008). Genome wide expression analysis of the CCR4-NOT complex indicates that it consists of three modules with the NOT module controlling SAGA-responsive genes. Mol. Genet. Genomics *279*, 323–337.

del Alamo, M., Hogan, D.J., Pechmann, S., Albanese, V., Brown, P.O., and Frydman, J. (2011). Defining the specificity of cotranslationally acting chaperones by systematic analysis of mRNAs associated with ribosome-nascent chain complexes. PLoS Biol. *9*, e1001100.

Dimitrova, L.N., Kuroha, K., Tatematsu, T., and Inada, T. (2009). Nascent peptide-dependent translation arrest leads to Not4p-mediated protein degradation by the proteasome. J. Biol. Chem. *284*, 10343–10352.

Dori-Bachash, M., Shema, E., and Tirosh, I. (2011). Coupled evolution of transcription and mRNA degradation. PLoS Biol. *9*, e1001106.

Dori-Bachash, M., Shalem, O., Manor, Y.S., Pilpel, Y., and Tirosh, I. (2012). Widespread promoter-mediated coordination of transcription and mRNA degradation. Genome Biol. *13*, R114.

Dutta, A., Babbarwal, V., Fu, J., Brunke-Reese, D., Libert, D.M., Willis, J., and Reese, J.C. (2015). Ccr4-Not and TFIIS function cooperatively to rescue arrested RNA polymerase II. Mol Cell Biol. *35*, 1915–1925.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell *11*, 4241–4257.

Gómez-Herreros, F., de Miguel-Jiménez, L., Morillo-Huesca, M., Delgado-Ramos, L., Muñoz-Centeno, M.C., and Chávez, S. (2012). TFIIS is required for the balanced expression of the genes encoding ribosomal components under transcriptional stress. Nucleic Acids Res. *40*, 6508–6519.

Grigull, J., Mnaimneh, S., Pootoolal, J., Robinson, M.D., and Hughes, T.R. (2004). Genome-wide analysis of mRNA stability using transcription inhibitors and microarrays reveals posttranscriptional control of ribosome biogenesis factors. Mol. Cell. Biol. *24*, 5534–5547.

Gupta, I., Clauder-Münster, S., Klaus, B., Järvelin, A.I., Aiyar, R.S., Benes, V., Wilkening, S., Huber, W., Pelechano, V., and Steinmetz, L.M. (2014). Alternative polyadenylation diversifies post-transcriptional regulation by selective RNA-protein interactions. Mol. Syst. Biol. *10*, 719.

Haimovich, G., Medina, D.A., Causse, S.Z., Garber, M., Millán-Zambrano, G., Barkai, O., Chávez, S., Pérez-Ortín, J.E., Darzacq, X., and Choder, M. (2013). Gene expression is circular: factors for mRNA degradation also foster mRNA synthesis. Cell *153*, 1000–1011.

Halter, D., Collart, M.A., and Panasenko, O.O. (2014). The Not4 E3 ligase and CCR4 deadenylase play distinct roles in protein quality control. PLoS ONE *9*, e86218.

Haruki, H., Nishikawa, J., and Laemmli, U.K. (2008). The anchor-away technique: rapid, conditional establishment of yeast mutant phenotypes. Mol. Cell *31*, 925–932.

Hogan, D.J., Riordan, D.P., Gerber, A.P., Herschlag, D., and Brown, P.O. (2008). Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. PLoS Biol. *6*, e255.

Hu, W., Sweet, T.J., Chamnongpol, S., Baker, K.E., and Coller, J. (2009). Co-translational mRNA decay in *Saccharomyces cerevisiae*. Nature *461*, 225–229.

Hughes, C.S., Foehr, S., Garfield, D.A., Furlong, E.E., Steinmetz, L.M., and Krijgsveld, J. (2014). Ultrasensitive proteome analysis using paramagnetic bead technology. Mol. Syst. Biol. *10*, 757.

Ito, W., Li, X., Irie, K., Mizuno, T., and Irie, K. (2011). RNA-binding protein Khd1 and Ccr4 deadenylase play overlapping roles in the cell wall integrity pathway in *Saccharomyces cerevisiae*. Eukaryot. Cell *10*, 1340–1347.

Jasiak, A.J., Hartmann, H., Karakasili, E., Kalocsay, M., Flatley, A., Kremmer, E., Strässer, K., Martin, D.E., Söding, J., and Cramer, P. (2008). Genome-associated RNA polymerase II includes the dissociable Rpb4/7 subcomplex. J. Biol. Chem. *283*, 26423–26427.

Knight, B., Kubik, S., Ghosh, B., Bruzzone, M.J., Geertz, M., Martin, V., Dénervaud, N., Jacquet, P., Ozkan, B., Rougemont, J., et al. (2014). Two distinct promoter architectures centered on dynamic nucleosomes control ribosomal protein gene transcription. Genes Dev. *28*, 1695–1709.

Kruk, J.A., Dutta, A., Fu, J., Gilmour, D.S., and Reese, J.C. (2011). The multifunctional Ccr4-Not complex directly promotes transcription elongation. Genes Dev. *25*, 581–593.

Liu, H.Y., Chiang, Y.C., Pan, J., Chen, J., Salvadore, C., Audino, D.C., Badarinarayana, V., Palaniswamy, V., Anderson, B., and Denis, C.L. (2001). Characterization of CAF4 and CAF16 reveals a functional connection between the CCR4-NOT complex and a subset of SRB proteins of the RNA polymerase II holoenzyme. J. Biol. Chem. *276*, 7541–7548.

Lotan, R., Bar-On, V.G., Harel-Sharvit, L., Duek, L., Melamed, D., and Choder, M. (2005). The RNA polymerase II subunit Rpb4p mediates decay of a specific class of mRNAs. Genes Dev. *19*, 3004–3016.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Mayer, A., Lidschreiber, M., Siebert, M., Leike, K., Söding, J., and Cramer, P. (2010). Uniform transitions of the general RNA polymerase II transcription complex. Nat. Struct. Mol. Biol. *17*, 1272–1278.

O'Duibhir, E., Lijnzaad, P., Benschop, J.J., Lenstra, T.L., van Leenen, D., Groot Koerkamp, M.J., Margaritis, T., Brok, M.O., Kemmeren, P., and Holstege, F.C. (2014). Cell cycle population effects in perturbation studies. Mol. Syst. Biol. *10*, 732.

Panasenko, O.O., and Collart, M.A. (2012). Presence of Not5 and ubiquitinated Rps7A in polysome fractions depends upon the Not4 E3 ligase. Mol. Microbiol. *83*, 640–653.

Pelechano, V., Jimeno-González, S., Rodríguez-Gil, A., García-Martínez, J., Pérez-Ortín, J.E., and Chávez, S. (2009). Regulon-specific control of transcription elongation across the yeast genome. PLoS Genet. *5*, e1000614.

Pelechano, V., Wei, W., and Steinmetz, L.M. (2015). Widespread co-translational RNA decay reveals ribosome dynamics. Cell *161*, 1400–1412.

Preissler, S., Reuther, J., Koch, M., Scior, A., Bruderek, M., Frickey, T., and Deuerling, E. (2015). Not4-dependent translational repression is important for cellular protein homeostasis in yeast. EMBO J. *34*, 1905–1924.

Rendl, L.M., Bieman, M.A., and Smibert, C.A. (2008). *S. cerevisiae* Vts1p induces deadenylation-dependent transcript degradation and interacts with the Ccr4p-Pop2p-Not deadenylase complex. RNA *14*, 1328–1336.

Riordan, D.P., Herschlag, D., and Brown, P.O. (2011). Identification of RNA recognition elements in the *Saccharomyces cerevisiae* transcriptome. Nucleic Acids Res. *39*, 1501–1509.

Rospert, S., Dubaquié, Y., and Gautschi, M. (2002). Nascent-polypeptide-associated complex. Cell. Mol. Life Sci. *59*, 1632–1639.

Sun, M., Schwalb, B., Schulz, D., Pirkl, N., Etzold, S., Larivière, L., Maier, K.C., Seizl, M., Tresch, A., and Cramer, P. (2012). Comparative dynamic transcriptome analysis (cDTA) reveals mutual feedback between mRNA synthesis and degradation. Genome Res. *22*, 1350–1359.

Trcek, T., Larson, D.R., Moldón, A., Query, C.C., and Singer, R.H. (2011). Single-molecule mRNA decay measurements reveal promoter- regulated mRNA stability in yeast. Cell *147*, 1484–1497.

Venters, B.J., Wachi, S., Mavrich, T.N., Andersen, B.E., Jena, P., Sinnamon, A.J., Jain, P., Rolleri, N.S., Jiang, C., Hemeryck-Walsh, C., and Pugh, B.F. (2011). A comprehensive genomic binding map of gene and chromatin regulatory proteins in *Saccharomyces*. Mol. Cell *41*, 480–492.

Villanyi, Z., Ribaud, V., Kassem, S., Panasenko, O.O., Pahi, Z., Gupta, I., Steinmetz, L., Boros, I., and Collart, M.A. (2014). The Not5 subunit of the ccr4-not complex connects transcription and translation. PLoS Genet. *10*, e1004569.

Weiner, A., Chen, H.V., Liu, C.L., Rahat, A., Klien, A., Soares, L., Gudipati, M., Pfeffner, J., Regev, A., Buratowski, S., et al. (2012). Systematic dissection of roles for chromatin regulators in a yeast stress response. PLoS Biol. *10*, e1001369.

Wilkening, S., Pelechano, V., Järvelin, A.I., Tekkedil, M.M., Anders, S., Benes, V., and Steinmetz, L.M. (2013). An efficient method for genome-wide polyadenylation site mapping and RNA quantification. Nucleic Acids Res. *41*, e65.