Comparative poly(A)+ RNA interactome capture reveals new proteins implicated in RNA regulation in fission yeast

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ABSTRACT (150 /175)

The nuclear RNA exosome plays a key role in mediating degradation, quality control and processing of multiple protein-coding and non-coding transcripts. Recognition of the specific RNA substrates by the exosome is mediated by the RNA-binding co-factors. Transient binding of co-factors either to substrate RNA or the exosome as well as rapid decay of the RNA substrates make identification of the co-factors challenging. Here we employ a comparative poly(A)+ RNA interactome capture approach in three different exosome mutant and unperturbed fission yeast cells to identify proteins that interact with poly(A)+ RNA in the exosome-dependent manner. Our analyses identified multiple proteins whose occupancy on RNA is altered in the exosome mutants. We demonstrate that zinc-finger protein Mub1 is required to maintain the levels of a subset of the exosome RNA substrates including mRNAs that encode for stress-responsive proteins. This finding highlight importance of exosome-dependent mRNA degradation in mediating cellular adaptation to stress.

INTRODUCTION

Regulation of RNA maturation and degradation is crucial to accurate gene expression (1). The nucleolytic RNA exosome complex has a central role in biogenesis of multiple types of transcripts produced by RNA polymerases I, II, and III (Pol I, II, and III) (2–9). The nuclear RNA exosome functions in RNA processing (3'end trimming) of stable non-coding (nc) RNA species such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), telomerase RNA, small nuclear and nucleolar RNAs (snRNAs and snoRNAs) and quality control where it degrades precursors of unprocessed or incorrectly processed mRNAs and ncRNAs (2–11). The exosome also degrades unstable transcripts produced by Pol II (Cryptic Unstable Transcripts, CUTs) in yeast and multiple unstable ncRNAs such as promoter upstream transcripts (PROMPTs), enhancer RNAs and products of wide-spread premature transcription termination in higher eukaryotes (12–18). Recent studies have also demonstrated that the exosome not only removes unprocessed mRNAs but is also required for proper mRNA processing since exosome mutants show splicing and mRNA 3'end processing defects (19–23). Finally, it has also been recently demonstrated that the exosome regulates the levels of specific mRNA transcripts in response to environmental changes and is an important player in executing specific gene expression programmes during development (9, 23–27). Unsurprisingly, mutations in the nuclear exosome lead to severe neurological diseases in humans, such as spinal muscular atrophy and pontocerebellar hypoplasia (27, 28).

The nuclear RNA exosome is a 3′–5′ exonuclease complex that consists of a 9-protein catalytically inactive core complex (EXO-9) and two catalytic subunits, Rrp6 (EXOSC10), and Dis3/Rrp44 (hDIS3). EXO-9 forms a double-layered barrel-like structure that comprises six ribonuclease (RNase) (PH)-like proteins (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, and Mtr3) and three S1/K homology (KH) "cap" proteins (Rrp4, Rrp40, and Csl4) (29). The two catalytic subunits occupy opposite ends of EXO-9 to constitute EXO-11 (30, 31). Rrp6 is located at the top of the S1/KH cap ring near the RNA entry into the channel formed by the exosome cap and core, and Dis3 is found at the bottom of EXO-9 channel near the RNA exit pore. Both Rrp6 and Dis3 are 3′–5′ exonucleases, but the latter also has endonucleolytic activity (2). In yeast, Rrp6 is restricted to the nucleus and Dis3 is found in both nuclear and cytoplasmic compartment (32, 33).

The conserved helicase Mtr4 is essential for RNA degradation by the exosome (34). In the fission yeast, *Schizosaccharomyces pombe* (*S. pombe*), Mtr4 shares its function with the highly homologous Mtr4-like helicase (Mtl1) (35). Mtr4/Mtl1 interacts with the RNA-binding proteins and the exosome and was proposed to play a role in exosome recruitment to substrate RNAs (1, 36–38). In addition to the exosome core, Mtr4/Mtl1 co-purify with the RNA-binding proteins involved in substrate recognition. In *Saccharomyces cerevisiae* (*S. cerevisiae*), Mtr4 is a part of the TRAMP complex (Trf4/5–Air1/2–Mtr4) which consists of two Zinc-finger proteins Air1 and Air2 (only Air1 in fission yeast), poly(A) polymerases Trf4 and Trf5 and Mtr4 (39–41). The TRAMP complex is recruited to RNA by the RNA and Pol II-binding protein Nrd1 during transcription (14, 42–45). However, in contrast to *S. cerevisia*e, the TRAMP complex seems more specialized in regulation of rRNA processing in fission yeast and human (34).

In fission yeast, Mtl1 interacts with the conserved YTH-domain containing protein Mmi1 and associated proteins, Red1, Iss10 and Erh1 (16, 35, 46–48). Mmi1 is needed for degradation of a selected group of mRNAs encoding for proteins involved in meiosis, cell cycle regulation, biosynthetic enzymes, RNA biology and ncRNAs by the exosome. Mmi1 is co-transcriptionally recruited to these transcripts by binding to an UUAAAC sequence motifs known as 'determinants of selective removal' (DSRs), and this is proposed to lead to their degradation (23, 24, 26, 49). It was recently demonstrated that Mmi1 interaction with Red1 and Mtl1 as a part of MTREC/NURS (Mtl1-Red1 Core / Nuclear RNA Silencing) complex is important to mediate recruitment of the exosome to RNAs (50). Mmi1 is also important for mRNA quality control and in particular for degradation of inefficiently spliced mRNAs and proper transcription termination of selected transcripts (19, 23, 51). In addition to Mmi1, Iss10, Erh1 and Red1, Mtl1 also co-purifies with other factors that have been functionally linked to the exosome regulation: the Zinc-finger protein Red5, the poly(A) binding protein Pab2, RRM (RNA-Recognition-Motif) and PWI (Pro-Trp-Ile signature) domain containing protein Rmn1 and the CAP-binding proteins Cbc1, Cbc2 and Ars2 (16, 35). Nevertheless, the mechanisms by which these factors regulate substrate recognition and exosome targeting to substrate RNAs are not entirely clear.

In addition to a group of RNAs recognised by Mmi1, levels of multiple other transcripts have been reported to increase in nuclear exosome mutants suggesting that additional Mmi1-independent mechanisms could contribute to their recognition (16, 35). We therefore employed quantitative proteomics approach to identify RNA-binding proteins that could potentially be involved in mediating exosome targeting to RNAs. We hypothesized that occupancy of the potential exosome co-factors on RNA should increase upon stabilisation of the substrate RNAs in the exosome mutants. We have compared the poly(A)+ transcriptomes and poly(A)+ RNA-bound proteomes from control and three different exosome mutants (*mtl1-1*, *rrp6Δ* and *dis3-54*). Our data suggest that nuclear exosome plays more prominent role in controlling fission yeast transcriptome than previously anticipated. Interestingly, analysis of the impact of the mutations in different exosome subunits on poly(A)+ bound proteome has identified proteins with increased RNA binding that could function as potential novel factors related to exosome regulation in fission yeast. We focus on the uncharacterised zf-MYND (MYeloid, Nervy, and DEAF-1) protein Mub1, which is highly enriched on poly(A)+ RNA in the exosome mutant. Mub1 physically interacts with the exosome and its deletion leads to increased levels of a specific subset of the exosome substrates supporting its role in exosome regulation.

RESULTS

Poly(A)+ RNA interactome capture in the exosome mutants.

To gain further insights into the exosome function in RNA regulation, we have applied an unbiased quantitative proteomic approach, RNA interactome capture (RIC), and identified proteins directly interacting with polyadenylated RNA (poly(A)+) in wild type (WT) cells and in three exosome mutants: *rrp6Δ*, lacking exonuclease Rrp6; *dis3-54*, a Dis3 mutant which contains an amino acid substitution (Pro509 to Leu509) located within RNB domain that was reported to show reduced catalytic activity (52, 53) and *mtl1-1,* a mutant of the helicase Mtl1, which has mutations in the region

surrounding the arch domain (35) (Figure 1A). The underlying hypothesis behind this approach was that inactivation of the exosome would not only stabilise RNAs targeted by the exosome but also affect RNA occupancy of the proteins that are functionally linked to the exosome (Figure 1B). The three mutant strains were cultured alongside a WT control in the presence of 4-thiouracil (4sU) to facilitate RNA-protein crosslinking with 365nm UV light. Following UV crosslinking, poly(A)+ RNA was enriched by oligo-d(T) selection and poly(A)+ RNA-associated proteins were identified by mass spectrometry (Figure 1B). The abundance of individual proteins in the whole cell extract (WCE) was also determined and used to normalise the RIC data (see Material and Methods) as in (54, 55). The RIC/WCE ratio was used to determine the enrichment of each individual protein on poly(A)+ RNA in the mutants relative to the WT (Supplementary Table 1). Additionally, RNA sequencing (RNA-seq) was carried out for oligo-d(T) enriched samples to assess levels of individual polyadenylated RNAs in the WT cells and exosome mutants (Figure 1B).

RNA sequencing analysis of poly(A)+ transcriptome from the exosome mutants.

First, we assessed how mutations in different exosome subunits affect levels of poly(A)+ RNA globally by analysing poly(A)+ RNA-seq data. Consistent with the function of the exosome in degradation of ncRNAs (snRNAs, snoRNAs, CUTs), levels of multiple nc transcripts were increased in all three exosome mutants (Figure EV (Expanded View)1A, Supplementary Table 3). In agreement with previously reports, a high number of mRNAs is also increased in the exosome mutants (>1.5-fold, *p*-value<0.05) suggesting that the nuclear exosome regulates multiple mRNAs in addition to its known role in degradation of ncRNAs (Figure EV1B-D) (16, 35). Compared to *rrp6Δ* and *mtl1-1* mutants, lower number of RNAs are increased in *dis3-54* mutant (>1.5-fold, *p*-value<0.05), possibly reflecting an only partial loss of Dis3 function in this mutant under conditions tested (Figure EV1A). Furthermore, a large fraction of the transcripts increased in *rrp6Δ* was also increased in *mtl1-1* (corresponding to ~80% of RNAs increased in Mtl1 mutant) in agreement with the suggested functional connection between Mtl1 and Rrp6 (35, 50). Interestingly, approximately half of the transcripts that show increased levels in Dis3 mutant (54%) is also dependent on Mtl1. This corresponds to a smaller fraction of Mtl1-regulated transcripts (25%). One of the reasons for this could be that P509L mutation in *dis3-54* has a milder effect on Dis3 function compared to effect of the multiple mutations in *mtl1-1* (Figure 1A). Our data supports a model where Mtl1 regulates both exosomes-associated nucleases (Rrp6 and Dis3), with Rrp6 being perhaps slightly more dependent on Mtl1 compared to Dis3.

Effect of the mutations in the exosome subunits on poly(A)+ RNA interactome.

To assess how inactivation of the different subunits of the exosome impact RNA-protein binding profile, we next performed comparative analysis of proteins differentially enriched in interactomes from *mtl1-1*, *rrp6Δ* and *dis3-54* compared to WT. For each round of RIC experiment, proteins that are detected at least in two out of three biological repeats of this experiment are used in the analysis. A total number of 1146 of proteins were considered (see Material and Methods). From this analysis, 152, 180 and 83 proteins are 2-fold more abundant on poly(A)+ RNA in *mtl1-1*, *rrp6Δ*

and *dis3-54* compared to WT respectively (*p*-value<0.1, Figure EV1E and Supplementary Table 2). Consistently with the RNA-seq data, poly(A)+ RNA-protein interaction profile is more similar between *rrp6Δ* and *mtl1-1* than between *dis3-54* and *mtl1-1* further supporting a functional link between Mtl1 and Rrp6 (Figure EV1E). On the other hand, we detected 137, 95 and 86 proteins whose abundance on poly(A)+ RNA was reduced in *mtl1-1*, *rrp6Δ* and *dis3-54* (*p*-value<0.1, Figure EV1F and Supplementary Table 2).

We performed gene ontology (GO) analyses across three different mutants to assess whether proteins with altered RNA-binding in the exosome mutants are part of a specific biological process. The analysis has revealed that nuclear proteins are most noticeably affected in all three exosomes mutants including *dis3-54* mutant where both nuclear and cytoplasmic forms of the exosome are compromised (Figure EV1G, Supplementary Table 4). This further supports the prominent role of the exosome in nuclear RNA metabolism. Additionally, RNA-binding activity of proteins related to “mRNA metabolic process” (GO:0016071) and “ribosome biogenesis” (GO:0042254) is altered in all mutants (Supplementary Table 4) suggesting a profound reorganisation of RNPs in exosome mutant. We noticed the over-representation for the RNA-binding activity of depleted proteins in catalytic exosome mutants corresponding to GO “cytoplasmic translation” (GO:0002181) with 22 of the 95 proteins whose abundance on poly(A)+ RNA was reduced significantly for *rrp6Δ* (*p*-value = $2{,}02^{e-7}$) and 31 of the 86 proteins considered for *dis3-54* (*p*-value = $7{,}16^{e-17}$). This may reflect a change in subcellular localisation of poly(A)+ RNA in these strains. Indeed, it was previously reported that poly(A)+ RNA is retained in the nucleus in Rrp6 mutants (56–58). To further assess the subcellular localisation of the poly(A)+ RNA in exosome mutants we performed poly(A)+ RNA FISH. Consistently, *rrp6Δ* and *mtl1-1* mutants showed accumulation of poly(A)+ RNA inside the nucleus (Figure EV1H-I). In contrast, *dis3-54* shows modest poly(A)+ RNA accumulation in the nucleus (Figure EV1J). This might indicate that Dis3 plays less dominant role in regulation of RNA in the nucleus compared to Mtl1 and Rrp6. However, milder nuclear retention of poly(A)+ RNA observed in *dis3-54* compared to other exosome mutants might be due to only partial inactivation of Dis3 in this mutant as discussed above. Interestingly, even though only mild accumulation of poly(A)+ RNA is observed, increased RNA-binding of nuclear proteins is more pronounced compared to cytoplasmic proteins in *dis3-54* (Figure EV1G). This might reflect that exosome has more prominent impact on nuclear rather than cytoplasmic RNPs.

Altogether, this analysis reveals that proteins affected in exosome mutants tend to be linked to similar biological processes. Additionally, these data demonstrate significant changes in RNA-protein interactions in exosome deficient cells, highlighting the important role of the exosome in RNA metabolism.

Comparative RIC identifies known RNA binding proteins linked to the nuclear RNA exosome

To assess whether comparative interactome capture approach leads to enrichment of known RNA-binding proteins linked to the exosome, we first analysed how RNA occupancy of Mmi1 is affected in the exosome mutants as a proof of principle. As predicted, Mmi1 association with poly(A)+

RNA is 1.5, 2 and 4 times increased in *dis3-54*, *mtl1-1* and *rrp6Δ* RIC respectively, compared to WT cells (*p*-value=0.768, *p*-value=0.039 and *p*-value=0.001) (Figure 1C). The increased RNA association of Mmi1 correlates with the increased abundance of Mmi1 RNA substrates in the exosome mutants (Figure 1D). Indeed, well characterised Mmi1 mRNAs targets such as transcripts encoding for meiotic proteins are noticeably enriched in the *rrp6Δ* and *mtl1-1* mutants and also to some extend in *dis3-54* mutant. Moreover, occupancy of other known exosome co-factors on poly(A)+ RNA is also increased in the exosome mutants. For example, RNA occupancy of Iss10 protein that is proposed to interact with Mmi1 is increased from 2 to more than 10 fold in *dis3-54* and *mtl1-1*, *rrp6Δ* RIC respectively, compared to WT cells (p-value=0.15, p-value=3.26x10-5 and p-value=1.73x10-5). Similarly, RNA occupancy of the components of the CAP-binding complex Cbc1, Cbc2 and Ars2 is also increased (>4 fold) in the exosome mutants (Supplementary table 1). Altogether, these results demonstrate that comparative poly(A)+ RNA interactome can identify known RNA-binding proteins involved in exosome targeting to substrates RNA and can potentially be applied for the identification of RNA-bound factors related to the exosome.

Identification of the RNA-binding proteins potentially linked to the exosome through comparative RIC approach

To narrow down the list of potential factors for subsequent analyses, we selected proteins containing classical RNA-binding domains (59) and annotated as “uncharacterised” out of the list of proteins whose occupancy on poly(A)+ RNA is increased (>2 fold) in the exosome mutants. This resulted in a list of 10 proteins (Figure 2A).

Next, the candidate genes were deleted to test whether they recapitulate phenotypes associated with compromised exosome function. First, we have assessed whether nuclear retention of poly(A)+ RNA characteristic for the exosome mutants, can be recapitulated in the deletion strains using poly(A)+ FISH (Figure 2B). In WT cells, poly(A)+ RNA is homogenously distributed between the cytoplasm and the nucleus (ratio of poly(A)+ signal in the Nucleus vs Cytoplasm is 1). However, nuclear retention of poly(A)+ RNA observed in *rrp6Δ* strain results in a ratio around 2 (Figure 2C). Based on these quantifications, we organised 10 candidates into 3 different groups affecting distribution of poly(A)+ RNA between the nucleus and the cytoplasm according to the ratio of poly(A)+ signal that ranges from moderately increased to no change (Figure 2C and Figure EV2A). The deletion of *SPCC126.11c* lead to noticeable nuclear retention of poly(A)+ RNA (ratio of poly(A)+ signal ~1.5). A retention of poly(A)+ RNA in the nucleus is also observed in strains lacking Srp40, Pof8, SPAC222.08, SPBC530.08, SPBC16G5.16, Swt1 and Mlo1 with a ratio around 1.2 (Figure 2C and Figure EV2A). In contrast, no retention is observed in *SPAC31F10.10c* and *SPABC18H10.09* mutants.

Next, we also assessed the steady-state levels of RNAs that are up-regulated in the exosome mutants but are independent of known exosome factor Mmi1 (such as the RNA produced from the Tf2 retro-transposable element (*SPAC9.04*)). Increase in *tf2-1* RNA levels is only observed in

SPBC31F10.10cΔ with a ratio of 1.24 compared to WT suggesting that SPBC31F10.10c protein could be implicated in controlling levels of this mRNA, similar to Rrp6 (Figure 2D).

Exosome mutants have been demonstrated to show defects in heterochromatic silencing (35, 60). This results in accumulation of heterogeneous RNAs produced from telomeric and centromeric regions of the *S. pombe* genome, although the underlying mechanism remains unclear. To test whether the candidate proteins might contribute to expression of heterochromatic transcripts, each individual deletion mutant was crossed with the strain bearing a *ura4+* reporter gene inserted within transcriptionally silent telomeric region of chromosome I. We then monitored the ability of these reporter strains to grow on -URA plates or plates containing 5-Fluoroorotic acid (5-FOA) at 25°C and 30°C. Interestingly, the deletion of either *SPCC126.11c*, *srp40* or *SPAC222.18* led to moderate growth on –URA plates and attenuated growth on 5-FOA compared to WT (Figure 2E and Figure EV2B), suggesting that each of these candidates might play a role in heterochromatin formation/maintenance.

Taken together, this preliminary screen has identified several candidate proteins, including SPBC31F10.10c, SPCC126.11c, Srp40 or SPAC222.18, whose deletions phenocopy exosome mutants suggesting a potential relationship between these factors and the nuclear RNA exosome. It is interesting to note that SPCC126.11c and Srp40 were also previously reported to co-purify with the exosome further supporting a possible direct link between these proteins and the exosome (46, 61). Moreover, a recent study reports that SRSF3, the human orthologous of SPAC222.18 is involved in the exosome recruitment via the Nuclear Exosome Targeting (NEXT) complex suggesting a potential conserved role of the serine rich protein SPAC222.18 in *S.pombe (62)*. However, further studies are needed to test whether this is indeed the case.

Mub1 regulates levels of the stress-induced mRNAs targeted by the exosome.

One of the RNA-binding proteins that we selected for analysis, *SPBC31F10.10c* or Mub1 protein, which contains an Armadillo-type domain, and a potential nucleic acid binding region represented by a zf-MYND domain. Mub1 shows a >6-fold increase in *mtl1-1* RIC (*p*-value= 5,41E-08) (Figure 2A). To further understand how Mub1 contributes to RNA regulation, we investigated the functional consequences of Mub1 deletion. Interestingly, *mub1* deleted cells showed a rounded morphology, suggesting an alteration in processes related to cell morphology (Figure EV3A). To assess the contribution of Mub1 in regulation of RNA levels we carried out transcriptome analyses by RNA-seq in WT and *mub1Δ*, *mtl1-1* and double mutant (*mtl1-1 mub1Δ*). RNA-seq data was normalised to *S. cerevisiae* spike-in for calibration (see Material and Methods). This analysis has revealed that 248 transcripts (162 mRNAs and 86 ncRNAs) are upregulated more than 1.5-fold (*p*-value<0.05) in *mub1Δ* cells (Figure 3A, Supplementary Tables 5-6). The GO analysis of mRNAs increased in *mub1Δ* cells have shown a significant enrichment for GO “Core Environmental Stress Response induced (CESR)” (46.25% (74/160), *p*-value = 1.55246^{e-28}, AnGeLi tool (63)) implying that Mub1 may be required for cellular response to stress. Interestingly, 144 transcripts (92 mRNAs and 52 ncRNAs) that are affected by Mub1 deletion were also up-regulated in *mtl1-1* (Figure 3A-B) suggesting that Mtl1 and

Mub1 act in the same pathway. The GO analysis of the share up-regulated mRNAs revealed that more than half of them are enriched in GO "Core Environmental Stress Response induced" (64.44% (58/90), *p*-value = 3.32315^{e-32} , AnGeLi tool (63)) strongly supported a role of Mub1 and Mtl1 in stress response. In agreement with RNA-seq data, increased the steady-state levels of *gst2* and *SPCC663.08c* mRNAs in *mub1Δ* and *mtl1-1* mutants are also detected by northern blot analyses (Figure 3B). No additive effect was observed in the double mutant *mtl1-1 mub1Δ* compared to single mutants, which is consistent with Mtl1 and Mub1 acting together (Figure 3B). Then, to assess whether Mub1 and the exosome can interact, we carried out co-immunoprecipitation experiments. These experiments revealed that Mub1 co-purifies with the Rrp6 subunit of the exosome supporting a physical link between Mub1 and the nuclear exosome (Figure 3C).

Presence of Mub1 in the poly(A)+ RNA interactome suggests that Mub1 is an RNA-binding protein. Mub1 contains a predicted zf-MYND domain (471-528), which is likely to mediate its interaction with RNA. Similar to Mub1, multiple factors associated with the exosome contain Zinc-finger domains. In fission yeast these are: component of the TRAMP complex, Air1, Red1 and Red5 proteins (47, 64, 65). Zinc-finger domains of Red1 and Red5 were shown to be important for RNA degradation by the exosome as mutations in this region leads to RNA stabilisation (47, 65). We therefore hypothesised that this domain may be critical for Mub1 function. To test this, we deleted the zf-MYND domain of Mub1 (Figure 3D). Deletion of the zf-MYND domain led to expected change in size of the protein, which was assessed by visualising FLAG tagged protein by Western blotting (Figure 3E). Addition of triple FLAG tag to Mub1 did not have any effect on cell morphology, cell growth and cellular protein levels (Figure EV3A-C). The protein level of Mub1Δ471-528-3xFLAG (Mub1-ΔZ-3xFLAG) was comparable to Mub1-3xFLAG, suggesting that the deletion did not affect the stability of the truncated protein (Figure 3E). Cells expressing the truncated Mub1Δ471-528-3xFLAG displayed the characteristic rounded shape, alike *mub1Δ* cells suggesting that function of Mub1 is compromised in this mutant (data not shown). To test the importance of the zinc-finger domain for Mub1 function, we assessed levels of *gst2* and *SPCC663.08c* mRNAs in *mub1Δ471-528-3xFLAG* mutants by northern blot. This experiment revealed that similar to *mub1Δ,* levels of both mRNAs were increased in the *mub1Δ471-528-3xFLAG* mutant (Figure 3F). Taken together, these results suggest that Zinc-finger domain of Mub1 is essential for Mub1 function.

Mub1 plays a role in the heat shock response.

We then investigated the physiological role of Mub1. We noticed that *mub1Δ* growth at 37°C is impaired (Figure 4A). To further assess a role of Mub1 in the regulation of genes related to heat shock, we analysed expression of *hsp16*, encoding a heat shock protein implicated in cellular response to heat (66). Indeed, *hsp16* levels are increased in *mub1Δ* and *mtl1-1* (Figure 4B, compare lanes 2-4 to 1). Interestingly, *hsp16* mRNA levels are also altered during heat shock (4 hours at 37°C) in the *mub1Δ* and *mtl1-1* (compare lanes 6-7 to lane 5), suggesting that Mub1 is required for exosome dependent expression of the heat shock genes. Interestingly, similar to complete Mub1 ablation, deletion of the zf-MYND domain also impaired growth at 37°C (Figure 4C) further supporting the

importance of this domain in mediating Mub1 function to ensure proper expression of stress response genes at 37°C. Our data highlight a role of Mub1 in heat shock response potentially through the nuclear RNA exosome. This observation is in adequacy with a recent study proposing that exosome is required for cell survival during heat shock through the proper expression of cell wall integrity (CWI) genes in budding yeast (67). Furthermore, exosome was shown to be implicated in heat shock response in flies suggesting that function of the exosome in environmental adaptation is conserved (68). Further works will assess how Mub1 contributes to the proper expression of RNA through the nuclear RNA exosome.

MATERIAL AND METHODS

Yeast strains and manipulations

General fission yeast protocols and media are described in (69). All strains are listed in Supplementary Table 7. Experiments were carried out using YES medium at 30°C unless stated otherwise. Gene deletions and epitope tagging were performed by homologous recombination using polymerase chain reaction (PCR) products (70). All oligos are listed in Supplementary Table 8. Protein extracts and western blotting were done as described in (71).

Co-Immunoprecipitation

Total-protein extracts were prepared from cycling log-phase cells. Cells were collected, rinsed in ice-cold phosphate-buffered saline (PBS)-1 mM phenylmethylsulfonyl fluoride (PMSF), and frozen on dry ice. Lysis was performed in 125 µl ice-cold lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Triton X-100, 1 mM dithiothreitol (DTT), 10% glycerol) with inhibitors (complete EDTA free protease inhibitor, 40694200, Roche, and 1 mM PMSF) using a glass bead beater (Cryoprep). The volume was increased by the addition of 275 µl of ice-cold lysis buffer with protease inhibitors, and the extracts were clarified by two successive rounds of centrifugation. Samples were incubated 1h at 4°C with anti-MYC magnetic beads (TA150044, Origene). Beads were washed 5 times with lysis buffer without inhibitors. Hot reducing agent was added during 5min to eluate proteins.

Northern blotting

Northern blot experiments were essentially performed as described in (43). RNA was prepared as described in (23). 8 µg of RNA was resolved on a 1.2% agarose gel containing 6.7% formaldehyde in MOPS buffer. After capillary transfer in 10× SSC onto a Hybond N+ membrane (GE Healthcare), RNA was UV-crosslinked and stained with methylene blue to visualise ribosomal RNA bands. For snoRNAs analysis, 16µg of RNA was resolved in 8% Urea-PAGE. Electro-transfer was performed in TBE overnight onto a Hybond N+ membrane (GE Healthcare, RPN303B). Gene-specific probes were generated by random priming in the presence of ATP [$\alpha^{32}P$] using the Prime-It II Random Primer Labeling Kit (Agilent, 300385) using PCR generated DNA template produced from gDNA isolated from a wild type *S.pombe* strain (YP71) using oligonucleotides listed in Supplementary Table 8.

Probes were added to the membrane and hybridized at 42°C overnight. After repeated washes in 2× SSC, 0.1% SDS, blots were exposed with Amersham Hyperfilm MP (GE Healthcare, 28-9068-44).

Poly(A)+ RNA interactome capture

Poly(A)+ *S. pombe* RNA interactome capture (RIC) was obtained from (54, 72). Briefly, two sets of triplicate experiments (Wild-type 1 (WT1) + *mtl1-1* = First eXperiment (FX); WT2 + *rrp6Δ* + *dis3-54* = Second eXperiment (SX)) were performed. *S. pombe* cells were grown at 30°C, in Edinburgh minimal media supplemented with glutamic acid (EMMG) with limited amounts of uracil (10 mg/l), and labelled with a final concentration of 1 mg/l 4-thiouridine for 4.5 h. Cells were harvested by filtration, snap-frozen in liquid nitrogen after UV-crosslinking at 3J/cm^2 in 50 ml PBS and lysed by grinding in liquid nitrogen. Clear lysates were resuspended in oligo-d(T) lysis buffer (20 mM Tris–HCl pH 7.5, 500 mM LiCl, 0.5% Lithium Dodecyl Sulfate (LiDS), 1 mM EDTA; 5 mM DTT, protease inhibitor cocktail IV (fungal) 1:10,000; 5 mM DTT). 1/48th of total volume of whole cell extract (WCE) was used for proteomics analysis, the rest was subjected to pull-down with oligo-d(T)x25 magnetics beads (NEB-S1419S), 1ml of slurry per 1L of cell culture during 1 hour at 4°C. Immobilized oligo-d(T)x25 magnetics beads were washed two times with oligo-d(T) wash buffer 1 (20 mM Tris–HCl pH 7.5, 500 mM LiCl, 0.1% LiDS, 1 mM EDTA, 5 mM DTT) at 4°C, two times with oligo-d(T) wash buffer 2 (20 mM Tris–HCl pH 7.5, 500 mM LiCl, 1 mM EDTA) at room temperature and two times with oligo-d(T) low salt buffer (20 mM Tris–HCl pH 7.5, 200 mM LiCl, 1 mM EDTA) at room temperature. RNA-proteins complexes were eluted from beads with oligo-d(T) elution buffer (20 mM Tris–HCl pH 7.5,1 mM EDTA), 330µl/L of culture during 10min at 55°C. 1/33 of the oligo-d(T) pull-down total volume was used for RNA sequencing analysis. The rest was subjected to RNase-A and RNase T1 treatment and subjected to mass spectrometry analyses as described in (54).

Statistical data analysis

Statistical analysis was performed essentially as described in (40) with the following modifications. To be considered for the analysis, protein was required to be present in at least one of the interactomes with two non-zero values. Raw intensities were log2 transformed, normalised to the same median and analysis was followed by the imputation of missing values using a minimal value approach (MinDet – where each sample is considered independently). Data manipulations, principal component analysis (PCA) and Pearson correlation plots were performed with the DEP package implemented in R (1). Median-normalised data values were used to estimate the log-fold changes between exosome mutants and WT cells, which were further normalised by the whole-cell extract values (WCE-normalisation). To minimise the batch effects control experiments (WT cells) were performed twice in triplicates alongside each of the sets of exosome mutants (First triplicate = FX = WT1 + *mtl1-1*; Second triplicate = SX = WT2 + *rrp6Δ* + *dis3-54*). To test the changes between whole-cell extract normalised (WCE-normalised) proteomes of mutants and WT cells, we used modified scripts from the

DEP package. Briefly, this software takes advantage of the Limma package that calculates moderated t-statistics on a linear model fit to the expression data (73). It allows defining custom contrasts (like comparing the difference of differences – as in the case of the WCE-normalised intensities). Proteins with a log2 (WCE-normalised RIC of exosome mutant/ WCE-normalised RIC of WT) > 1 were considered specifically enriched in exosomes mutants. Remaining figures and analyses were performed with custom scripts or ones modified from the DEP package. *S. pombe* GO term annotations and information on individual proteins were retrieved using PomBase (74).

Poly(A)+ RNA Fluorescence *In Situ* Hybridization (FISH)

Poly(A)+ RNA FISH was done as described in (75, 76), using oligo-d(T)(x20)-alexa488 (Invitrogen 7206906) DNA probe. Briefly, 5×10–1×10^8 cells were used for one hybridization reaction. Cells from an asynchronously growing culture were fixed by the addition of paraformaldehyde into the culture to a final concentration of 4%. Cell pellet was washed with 1ml of buffer B (1.2 M sorbitol, 100 mM KH_2PO_4 at pH 7.5, 4 °C) and immediately after, cells were resuspended in 1ml of spheroplast buffer (1.2 M sorbitol, 100 mM KH_2PO_4 at pH 7.5, 20 mM vanadyl ribonuclease complex and 20 µM β-mercaptoethanol) with 1% 100T zymolyase (MP Biomedicals, 083209-CF) and cell wall was digested for 60min. The reaction was stopped by washing with 1 ml of cold buffer B. Cells were incubated for 20 min in 0.01% Triton-X100/1X PBS and washed with 10% formamide/2× SSC at room temperature. Before hybridization, 50 ng of the oligo-d(T) probe was mixed with 2µl of a 1:1 mixture between yeast transfer RNA (10 mg/ml, Life Technologies, AM7119) and salmon-sperm DNA (10 mg/ml, Life Technologies, 15632-011) and the mixture was dried in a vacuum concentrator. Hybridization buffer F (20% formamide, 10 mM $NaHPO_4$ at pH 7.0; 50 µl per hybridization) was added, and the probe/buffer F solution was incubated for 3 min at 95 °C. Buffer H (4× SSC, 4 mg/ml BSA (acetylated) and 20 mM vanadyl ribonuclease complex; 50 µl per hybridization) was added in a 1:1 ratio to the probe/buffer F solution. Cells were resuspended in the mixture and incubated overnight at 37 °C. After three washing steps (10% formamide/2× SSC; 0.1% Triton-X100/2x SSC and 1x PBS), cells were resuspended in 1× PBS/DAPI and mounted into glass slides for imaging. *Z*-planes spaced by 0.2 µm were acquired on a Ultraview spinning-disc confocal. Acquisition was done with DAPI filter (405nm) and a FITC filter (488nm for alexa488 acquisition). Images were analysed using ImageJ software (77). Signal intensity was measured in the same square surface containing the nucleus (i.e. DAPI) or the cytoplasm. Average intensity of each square was calculated and the ration of Nucleus Signal/ Cytoplasm Signal was calculated for each cell. The mean and the confidence interval of the mean were calculated with α=0.05. Statistical analysis (un-paired *t-test*) was performed using GraphPad Software.

RNA sequencing

For spike-in normalisation, the *S. cerevisiae* cells were added to *S. pombe* at 1:10 ratio prior to RNA isolation. Total RNA was extracted from cultures in mid-log phase using a standard hot phenol method and treated with RNase-free DNase RQ1 (Promega, M6101) to remove any DNA

contamination. For total RNA sequencing, experiments were done in duplicates, and ribodepletion was performed using ribo-minus transcriptome isolation kit (Invitrogen, K155003). Poly(A)+ RNA sequencing was performed by using 1/33 of the oligo-d(T) pull-down total volume, subjected to proteinase K treatment for 1h at 50°C. Poly(A)+ RNA was recovered by a standard hot phenol method. Experiments were done in triplicate. cDNA libraries were prepared using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (NEB#E7760S) for 50ng of total RNA and using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420) for 100ng of WT1, *mtl1-1*, *rrp6Δ* and *dis3-54* purified oligo-d(T) RNA. Paired-end sequencing was carried out on the Illumina HiSeq 500 platform. RNA sequencing data are available with the GEO number GSE148799 and GSE149187.

RNA-sequencing data analyses

Quality trimming of sequenced reads was performed using Trimmomatic (Galaxy Version 0.32.3, RRID:SCR_011848). Reads were aligned to the concatenated *S. pombe* (ASM294v2.19) using Bowtie 2 (TopHat) (78). For spike-in normalisation, reads derived from different *S. pombe* and *S. cerevisiae* chromosomes were separated. Reads mapped only once were obtained by SAMTools (79) and reads were mapped to the genome using genome annotation from (80). Differential expression analyses were performed using DESeq2 (81) in R and using the spike-in normalisation. For poly(A)+ RNA sequencing total read count normalisation using DEseq2 (81) in R was used. The significance of RNAs list overlaps was calculated using a standard Fisher's exact test. For gene ontology analysis, up or down-regulated protein-coding gene lists were submitted to AnGeLi (http://bahlerweb.cs.ucl.ac.uk/cgi-bin/GLA/GLA_input), a web based tool (63).

ACCESSION NUMBERS

Raw (fastq) and processed sequencing data (bedgraph) can be downloaded from the NCBI Gene Expression with the GEO number GSE148799 and GSE149187. Mass spectrometry data are available via ProteomeXchange with identifier PXD016741.

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CONFLICT OF INTEREST

None declared

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FIGURE LEGENDS

Figure 1: Comparative poly(A)+ RNA interactome capture from fission yeast exosome mutants. **A.** Schematic diagram describing domain organisation of *S. pombe* (Sp) Dis3 and Mtl1 and position of the mutations in *dis3-54* (P509L) and *mtl1-1* (I522M, L543P, Y551H, L557P, D793G, A998V) shown in yellow. **B.** Schematic diagram describing the poly(A)+ interactome capture approach. Cells were grown in the presence of 4-thiouracil (4sU) and exposed to UV (3J/cm^2) to allow protein-RNA crosslinking. Poly(A)+ RNA and associated proteins were pulled down by oligo-d(T) beads and subjected to RNA sequencing and mass spectrometry analyses (RBP= RNA-Binding Proteins, EF = Exosome Factors, involved in recognition of the exosome substrates such as Mmi1). **C**. Volcano plots showing distribution of Mmi1 enrichment on poly(A)+ RNA in the three exosome mutants. In the volcano plot, *p*-values (-log10, moderated Student's *t-test*) are plotted against the ratio of log2-fold changes in mass spectrometry (MS) intensities for the whole-cell extract normalised proteomes of mutants versus WT cells recovered from the oligo-d(T) pull-downs of UV-crosslinked samples (3 J/cm^2). In all panels, individual proteins are depicted as a single dot. **D**. Heat map analysis of poly(A)+ RNA-seq showing differential expression of the Mmi1 regulon in exosome mutants compared to WT.

Figure EV1: Effect of the exosome mutations on poly(A)+ RNA transcriptome and protein interactome. A. mRNAs and ncRNAs are up-regulated (>1.5-fold, *p*-value<0.05) in *mtl1-1*, *rrp6Δ* and *dis3-54* mutants compared to WT. **B.** Venn diagram showing overlap between RNAs that show increased levels (>1.5-fold, *p*-value<0.05) in *mtl1-1*, *rrp6Δ* and *dis3-54*. The *p*-values indicate the probabilities that the observed overlaps occurred by chance (6949 genes analysed). **C.** Venn diagram showing overlap between mRNAs increased levels in *mtl1-1*, *rrp6Δ* and *dis3-54* mutants compared to WT (>1.5-fold, *p*-value<0.05). The *p*-values indicate the probabilities that the observed overlaps

occurred by chance (5153 mRNAs analysed). **D**. Venn diagram showing overlap between ncRNAs that show increased levels in *mtl1-1*, *rrp6Δ* and *dis3-54* mutants compared to WT (>1.5-fold, *p*-value<0.05). The *p*-values indicate the probabilities that the observed overlaps occurred by chance (1796 ncRNAs analysed). **E.** Venn diagram showing proteins commonly enriched in poly(A)+ RNA pull-down of *mtl1-1* and either *rrp6Δ* or *dis3-54* relative to WT. The *p*-values indicate the probabilities that the observed overlaps occurred by chance. (1146 proteins analysed). **F**. Venn diagram showing proteins depleted in poly(A)+ pull-down of *mtl1-1* and either *rrp6Δ* or *dis3-54* relative to WT. The *p*-values indicate the probabilities that the observed overlaps occurred by chance (1146 proteins analysed). **G**. RNA association of nuclear proteins (GO:0005575) is primarily affected in the exosome mutants. **H-J**. Poly(A)+ RNA FISH illustrating poly(A)+ RNA accumulation in the nucleus in *rrp6Δ* (**H**) *mtl1-1* (**I**) and *dis3-54* (**J**). DAPI is shown in blue. Poly(A)+ RNA is represented in green. Scale bar = 5µm.

Figure 2: Comparative RIC uncovers putative novel exosome factors. **A.** List of the candidate proteins enriched on poly(A)+ RNA in the exosome mutants, their predicted function and volcano plot showing their distribution in *mtl1-1*, *rrp6Δ* and *dis3-54* mutants. **B.** Poly(A)+ RNA FISH illustrating cellular localisation of poly(A)+ RNA in the indicated strains. DAPI is shown in blue. Poly(A)+ RNA is visualised in green. Scale bar = 10µm. **C.** Quantification of Poly(A)+ RNA FISH analysis, relative to figure 2B and EV2A (***: *p*-value<0.0001, un-paired *t-test*). **D.** Analyses of *tf2-1* mRNA levels by northern blot in the indicated strains. Band corresponding to *tf2-1* is indicated with an arrow. Schematics shows location of the probe with a black bar. The *adh1* mRNA is used as a loading control. Numbers indicated the relative abundance of *tf2-1* transcript normalise to *adh1* transcript. **E.** Serial dilutions of the indicated strains plated on complete medium supplemented with adenine (YES+A), medium lacking uracil (-URA) and containing 5-FOA (5-FOA) and grown at 25°C. A schematic representation of the Chromosome I (Chr I) depicting centromeric and telomeric regions (grey circle and grey rectangles correspondently). Position of where *ura4*$^{+}$ reporter integrated into the left arm of the telomere is shown.

Figure EV2: A. Poly(A)+ RNA FISH illustrating poly(A)+ RNA location in the indicated strains. DAPI is shown in blue. Poly(A)+ RNA is visualised in green. Scale bar = 10µm. **B.** Serial dilutions of the indicated strains plated on complete medium (YES+A), lacking uracil (-URA) and containing 5-FOA (5-FOA) at 30°C. Related to Figure 2E.

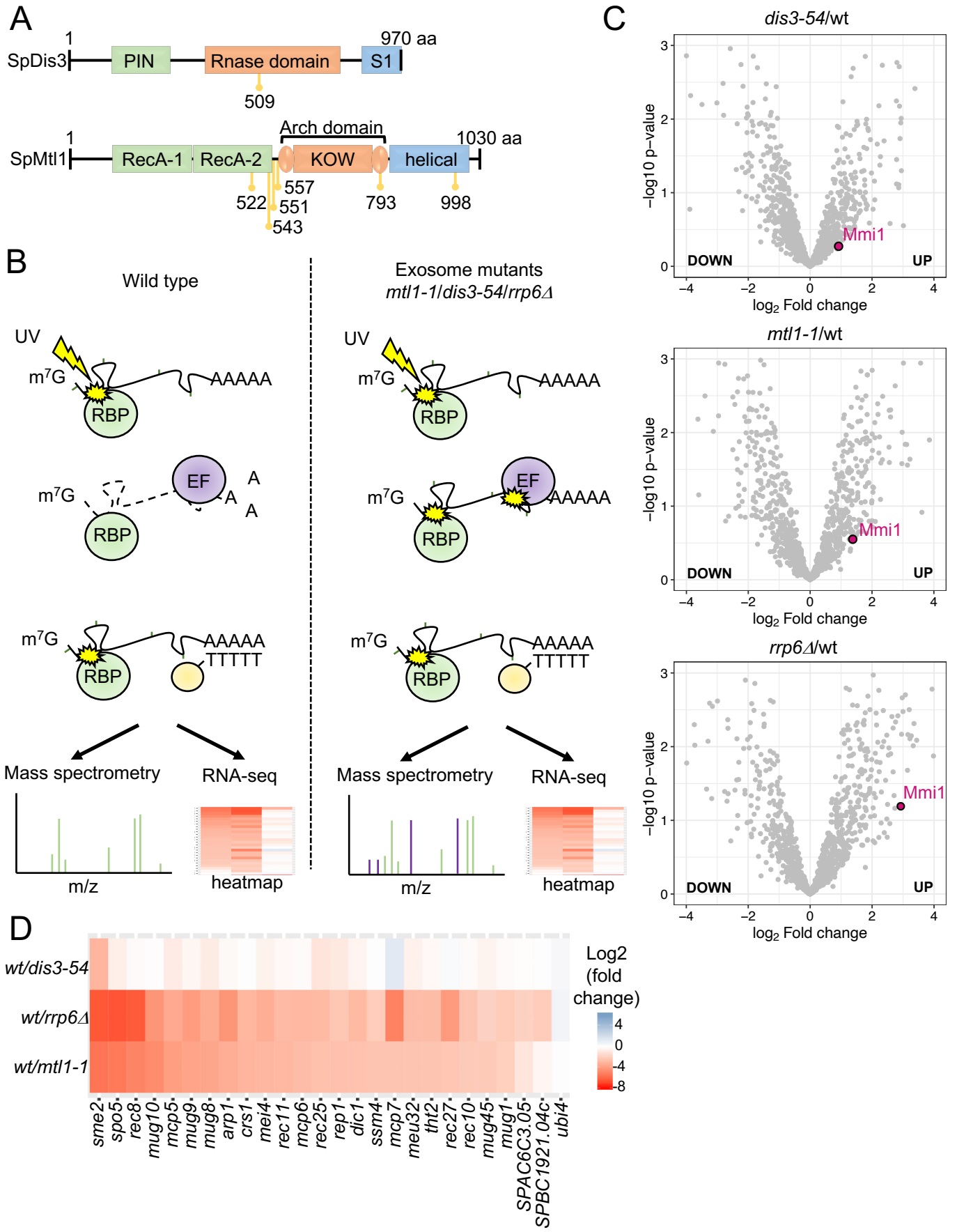
Figure 3: Mub1 protein functions in maintaining levels of the exosome RNA targets. A. Venn diagram showing overlap between RNAs that show an increase in levels in *mtl1-1* and *mub1Δ* (>1.5-fold, *p*-value<0.05) **B.** Genome browser snapshots showing RNA-seq data and northern blot analyses for two representative transcripts (*gst2* and *spcc663.08c*) upregulated in *mtl1-1* and *mub1Δ* compared to WT. Bands corresponding to *gst2* and *spcc663.08c* are indicated with arrows. Positions of the probes are indicated with black bars. 28S ribosomal RNAs (rRNA) visualised with methylene blue served as the loading control **C.** Co-immunoprecipitation of Rrp6-Myc with Mub1-3xFLAG**. D.** Graphical representation of WT Mub1 and Mub1-Δ-Zinc-Finger. **E.** Western blot showing levels of

endogenously expressed Mub1-3xFLAG and Mub1-ΔZ-3xFLAG proteins. **F.** Analyses of *SPCC663.08c* and *gst2* mRNAs from *mub1-3xFLAG* and *mub1-ΔZ-3xFLAG* strains by northern blot as in **B**.

Figure EV3. A. Mub1 deletion leads to altered cellular morphology. Phase contrast images of the cells from the indicated strains. Scale bar = 10µm. **B.** Serial dilutions of the indicated strains plated on complete medium (YES+A) at the indicated temperatures. **C.** Western blot showing levels of endogenously expressed Mub1-3xFLAG in WT and exosome mutants. Cellular proteins visualised with ponceau S are used to control for loading.

Figure 4: Mub1 and nuclear exosome are involved in regulation of heat shock response. A. Serial dilutions of the indicated strains plated on complete medium (YES+A) at the indicated temperatures. **B.** Analyses of *hsp16* mRNAs levels by northern blot in the indicated strains at the indicated temperatures. Band corresponding to *hsp16* mRNA is indicated with arrow. Schematic diagram shows location of the probe with a black bar. 28S ribosomal RNAs (rRNA) visualised with methylene blue served as the loading control. **C.** Serial dilutions of the indicated strains plated on complete medium (YES+A) at the indicated temperatures.

Figure 1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PIN
Rnase domain
S1
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509
SpMtl1
1
RecA-1
RecA-2
Arch domain
KOW
helical
1030 aa
522
557
551
543
793
998
B
Wild type
Exosome mutants
mtl1-1/dis3-54/rrp6Δ
UV
m7G
AAAAA
RBP
EF
A
TTTTT
Mass spectrometry
RNA-seq
m/z
heatmap
C
dis3-54/wt
mtl1-1/wt
rrp6Δ/wt
−log10 p−value
log2 Fold change
Mmi1
DOWN
UP
D
wt/dis3-54
wt/rrp6Δ
wt/mtl1-1
Log2 (fold change)
sme2
spo5
rec8
mug10
mcp5
mug9
mug8
arp1
crs1
mei4
rec11
mcp6
rec25
rep1
dic1
ssm4
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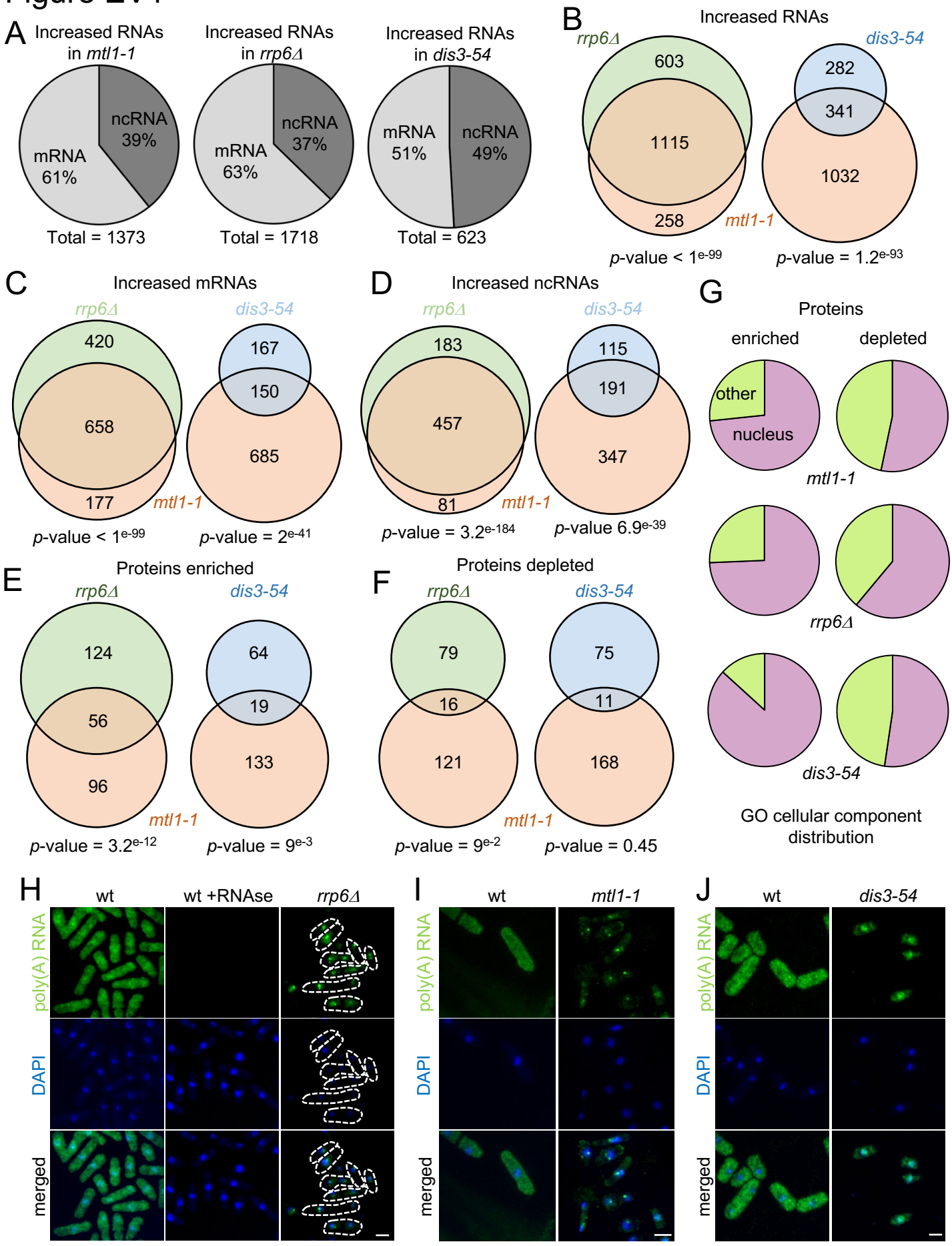
ubi4

Figure EV1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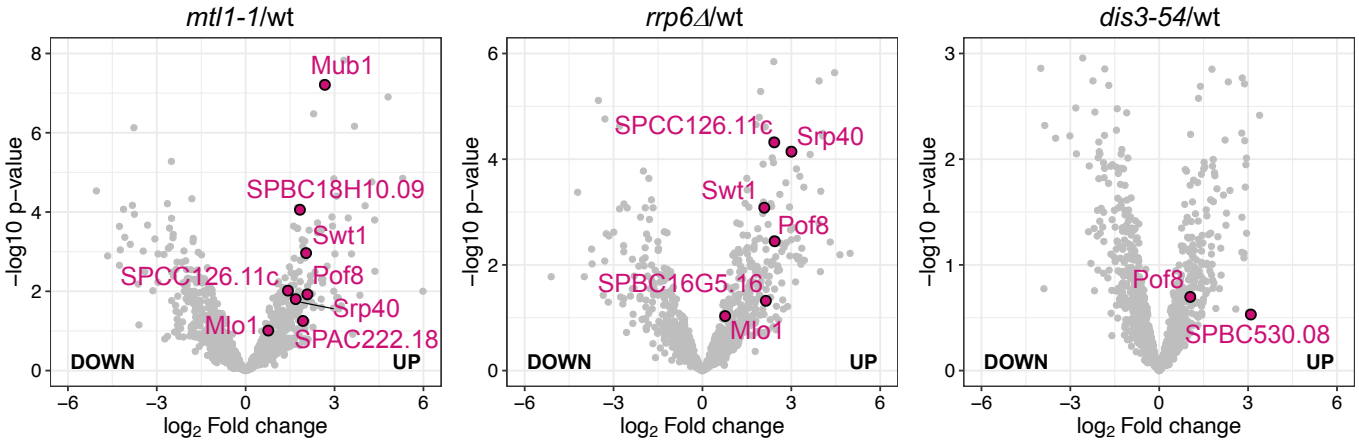
Increased RNAs in mtl1-1
ncRNA 39%
mRNA 61%
Total = 1373
Increased RNAs in rrp6Δ
ncRNA 37%
mRNA 63%
Total = 1718
Increased RNAs in dis3-54
mRNA 51%
ncRNA 49%
Total = 623
B
Increased RNAs
rrp6Δ
603
1115
258
mtl1-1
dis3-54
282
341
1032
p-value < 1e-99
p-value = 1.2e-93
C
Increased mRNAs
rrp6Δ
420
658
177
mtl1-1
dis3-54
167
150
685
p-value < 1e-99
p-value = 2e-41
D
Increased ncRNAs
rrp6Δ
183
457
81
mtl1-1
dis3-54
115
191
347
p-value = 3.2e-184
p-value 6.9e-39
G
Proteins
enriched
depleted
other
nucleus
mtl1-1
rrp6Δ
dis3-54
GO cellular component distribution
E
Proteins enriched
rrp6Δ
124
56
96
mtl1-1
dis3-54
64
19
133
p-value = 3.2e-12
p-value = 9e-3
F
Proteins depleted
rrp6Δ
79
16
121
mtl1-1
dis3-54
75
11
168
p-value = 9e-2
p-value = 0.45
H
wt
wt +RNAse
rrp6Δ
poly(A) RNA
DAPI
merged
I
wt
mtl1-1
poly(A) RNA
DAPI
merged
J
wt
dis3-54
poly(A) RNA
DAPI
merged

Figure 2

A

Name	Function
SPBC31F10.10c	Mub1-zf-MYND type zinc finger protein
SPBC18H10.09	zf-CHY type zinc finger protein
Swt1	RNA endoribonuclease involved in mRNP quality control Swt1 (predicted)
Pof8	F-box protein Pof8
SPAC222.18	Srp1 family splicing factor (predicted)
Srp40	SPBC1711.05 nucleocytoplasmic transport chaperone Srp40 (predicted)
SPCC126.11c	RNA-binding protein, RRM type
SPBC16G5.16	transcription factor. zf-fungal binuclear cluster type (predicted)
SPBC530.08	membrane-tethered transcription factor (predicted)
Mlo1	SPCC31H12.03c RNA-binding protein (predicted)



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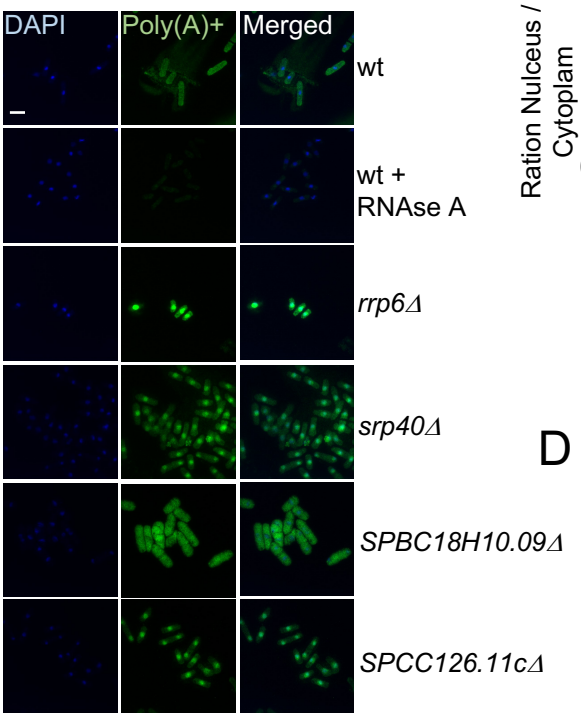

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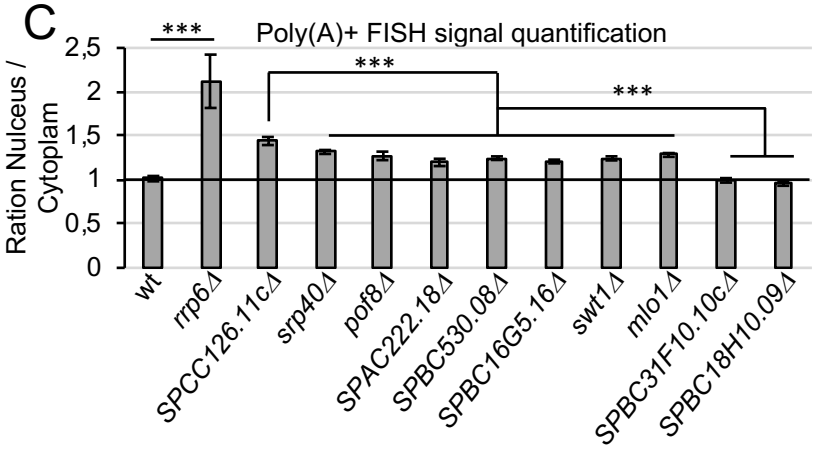

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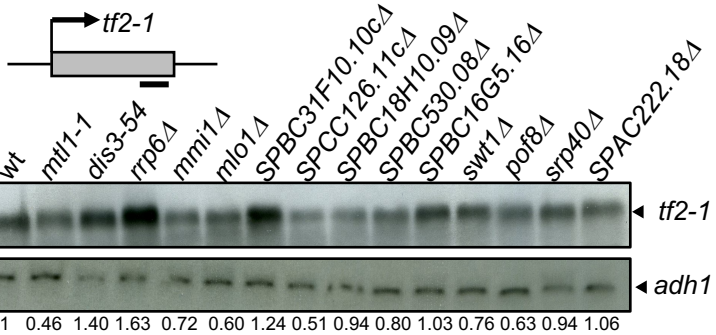


E

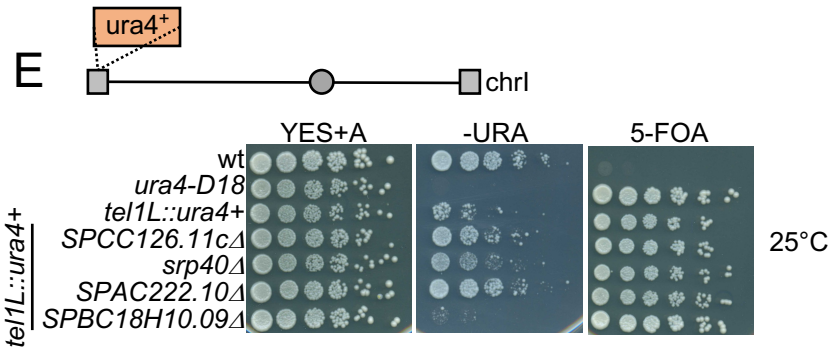
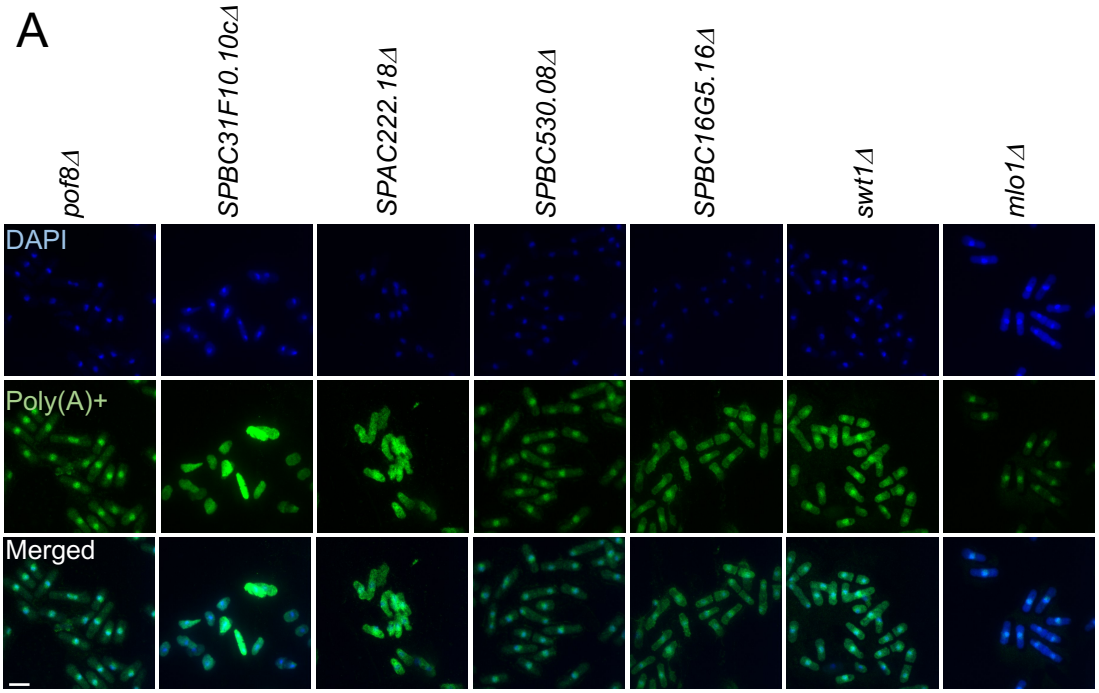


Figure EV2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pof8Δ
SPBC31F10.10cΔ
SPAC222.18Δ
SPBC530.08Δ
SPBC16G5.16Δ
swt1Δ
mlo1Δ
DAPI
Poly(A)+
Merged

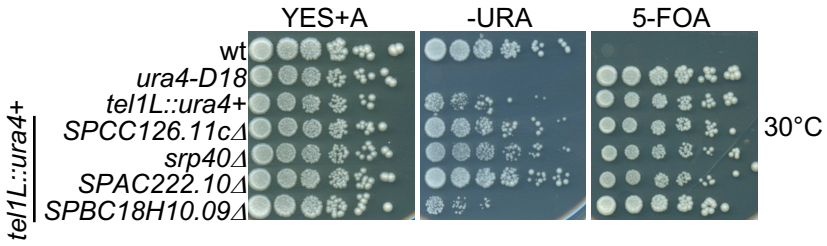
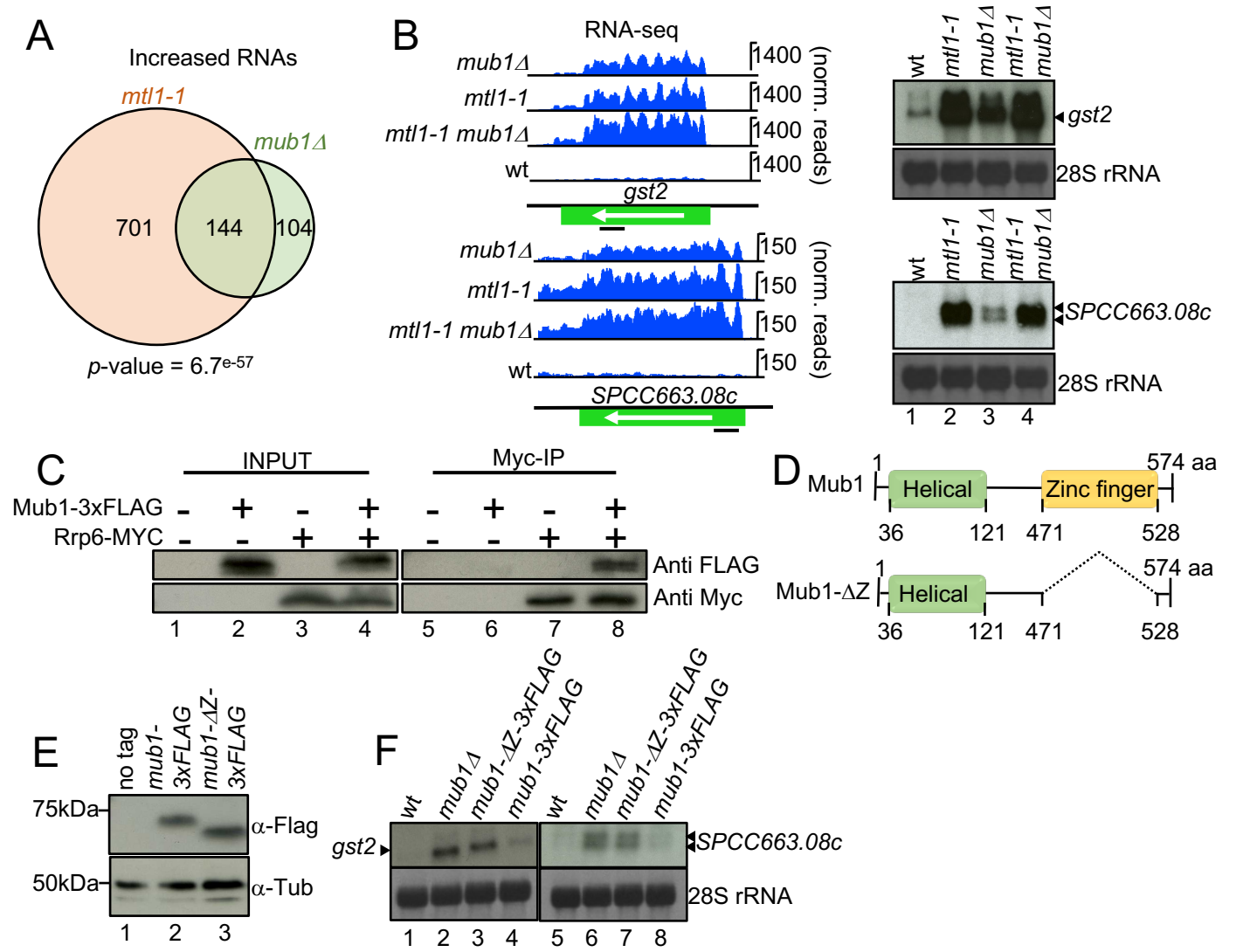

B
YES+A
-URA
5-FOA
wt
ura4-D18
tel1L::ura4+
SPCC126.11cΔ
srp40Δ
SPAC222.10Δ
SPBC18H10.09Δ
tel1L::ura4+
30°C

Figure 3


A
Increased RNAs
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mub1Δ
701
144
104
p-value = 6.7e-57
B
RNA-seq
mub1Δ
mtl1-1
mtl1-1 mub1Δ
wt
1400
(norm. reads)
gst2
150
SPCC663.08c
wt
mtl1-1
mub1Δ
mtl1-1
mub1Δ
gst2
28S rRNA
SPCC663.08c
1 2 3 4
C
INPUT
Myc-IP
Mub1-3xFLAG
Rrp6-MYC
Anti FLAG
Anti Myc
1 2 3 4 5 6 7 8
D
Mub1
1
574 aa
Helical
Zinc finger
36 121 471 528
Mub1-ΔZ
E
no tag
mub1-3xFLAG
mub1-ΔZ-3xFLAG
75kDa
50kDa
α-Flag
α-Tub
1 2 3
F
wt
mub1Δ
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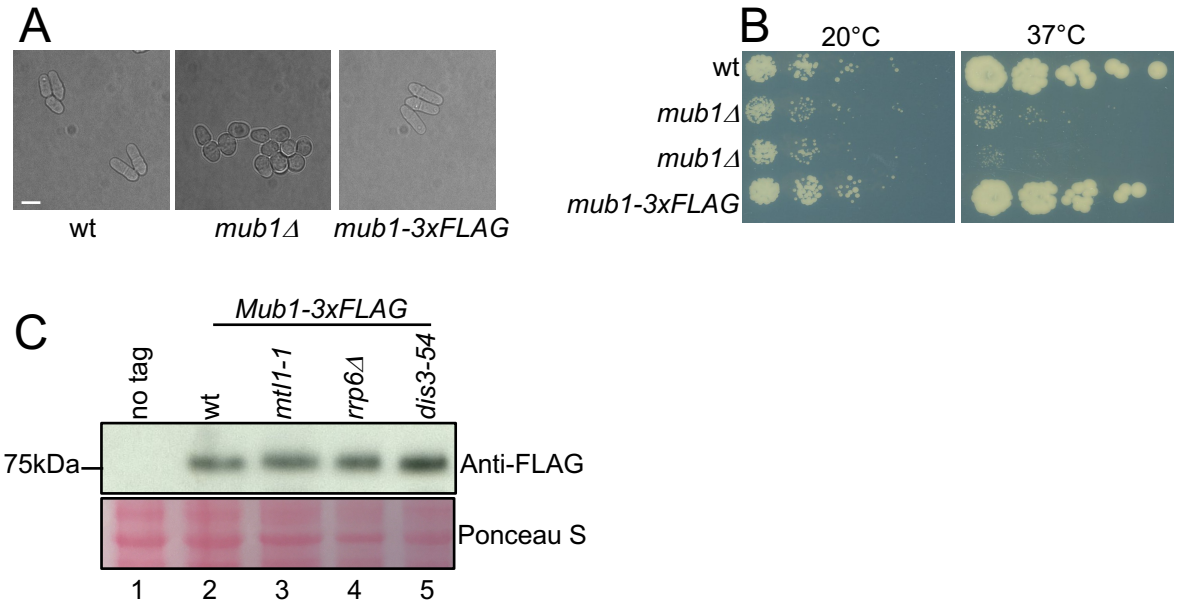
mub1-3xFLAG
gst2
SPCC663.08c
28S rRNA
1 2 3 4 5 6 7 8

Figure EV3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mub1Δ
mub1-3xFLAG
B
20°C
37°C
wt
mub1Δ
mub1Δ
mub1-3xFLAG
C
Mub1-3xFLAG
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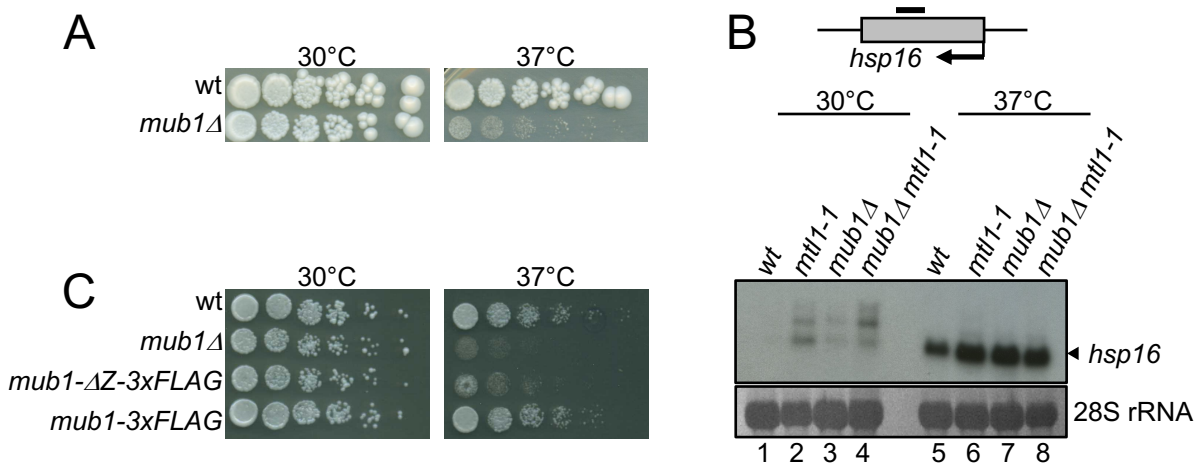
rrp6Δ
dis3-54
75kDa
Anti-FLAG
Ponceau S
1
2
3
4
5

Figure 4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37°C
wt
mub1Δ
B
hsp16
30°C
37°C
wt
mtl1-1
mub1Δ
mub1Δ mtl1-1
wt
mtl1-1
mub1Δ
mub1Δ mtl1-1
hsp16
28S rRNA
1 2 3 4 5 6 7 8
C
30°C
37°C
wt
mub1Δ
mub1-ΔZ-3xFLAG
mub1-3xFLAG