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Functional characterization of Upf1 targets in *Schizosaccharomyces pombe*

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Nonsense-mediated mRNA decay (NMD) is a highly conserved mechanism of mRNA degradation. NMD eliminates mRNAs containing premature termination codons (PTCs), preventing the production of truncated proteins with possible deleterious effects. However, there is mounting evidence that NMD factors, like Upf1, Upf2 and Upf3, participate in general regulation of gene expression, affecting the expression of genes lacking PTCs. We have used the fission yeast *Schizosaccharomyces pombe* to identify mRNAs directly regulated by NMD. Using a combination of genetic and biochemical approaches, we have defined a population of fission yeast mRNAs specifically regulated by Upf1. We show that other components of the Upf complex, Upf2 and Upf3, are required for binding of Upf1 to its RNA targets and for the proper response of fission yeast to oxidative stress. Finally, we investigated the physiological importance of this phenomenon, and demonstrate that the Upf1-dependent downregulation of some of its direct targets is necessary for normal resistance to oxidative stress.

Introduction

Nonsense-mediated mRNA decay (NMD) eliminates mRNAs carrying nonsense translation codons.[1-4] The source of those premature termination codons is varied: mutations, errors in splicing processes, DNA rearrangements during lymphocyte development, etc. NMD has been found in all eukaryotes studied so far, from yeast to human, although the specific mechanisms show some differences,[4-7] and modulates the expression of an important fraction of the genome in yeast, plants, fruit fly and humans.[6,8-15] There is little overlap in the functional groups regulated by NMD in different organisms,[6,13] although the strong conservation of NMD suggests an important role in eukaryotic cellular physiology. Consistently, NMD is essential for vertebrate embryonic viability.[16,17] In mammals, for example, failure to eliminate certain PTC-containing mRNAs can lead to the emergence of dominant-negative phenotypes due to the production of truncated proteins. In contrast, when those mRNAs are eliminated through NMD, the phenotypes are usually recessive.[18] Inhibition of NMD also leads to changes in general protein expression with potential uses in therapy, like tumor immunity.[19]

The mechanisms underlying NMD substrate recognition and targeting, however, appear to vary in different organisms and remain an interesting research field. Thus, identification of legitimate targets of the NMD pathway should help to shed light on this issue.

Some of the proteins involved in NMD are extremely well conserved through phylogeny. This is the case for Upf1 (RENT1), Upf2 and Upf3,[2] that work as a complex. However, how this machinery recognizes its substrates and targets them to accelerated degradation remains to be clearly defined.

There is also evidence that Upf1 and other components of NMD can play important functions in the cell independently of their role in the NMD process.[20-24] In fact, many targets of NMD factors do not have PTCs.[8,25]

The fission yeast *Schizosaccharomyces pombe* contains Upf1 and Upf2 proteins, although the NMD mechanism is somehow different from mammals.[7,26,27] Interestingly, a deficiency in Upf1 or Upf2 leads to oxidative stress sensitivity, possibly indicating a role for NMD in this process.[27] However, the biochemical mechanism by which NMD is involved in oxidative stress protection remains elusive.

In this report, we have described for the first time the direct targets of Upf1 in *Schizosaccharomyces pombe*. We have also provided evidence of the link among the three Upf proteins (Upf1, Upf2 and Upf3) in the response to oxidative stress. We finally propose a mechanism of gene regulation by which Upf1 may regulate oxidative stress resistance in fission yeast.

Results

Determination of Upf1 role in post-transcriptional regulation. In *Schizosaccharomyces pombe*, the role of Upf1 in post-transcriptional regulation is still unclear, although Upf1 is a bona fide NMD factor with an essential role in the oxidative stress response.[27] We have previously shown that deletion of *upf1*$^+$ leads to changes in gene expression even in the absence of oxidative stress, suggesting that Upf1 may have a constitutive role

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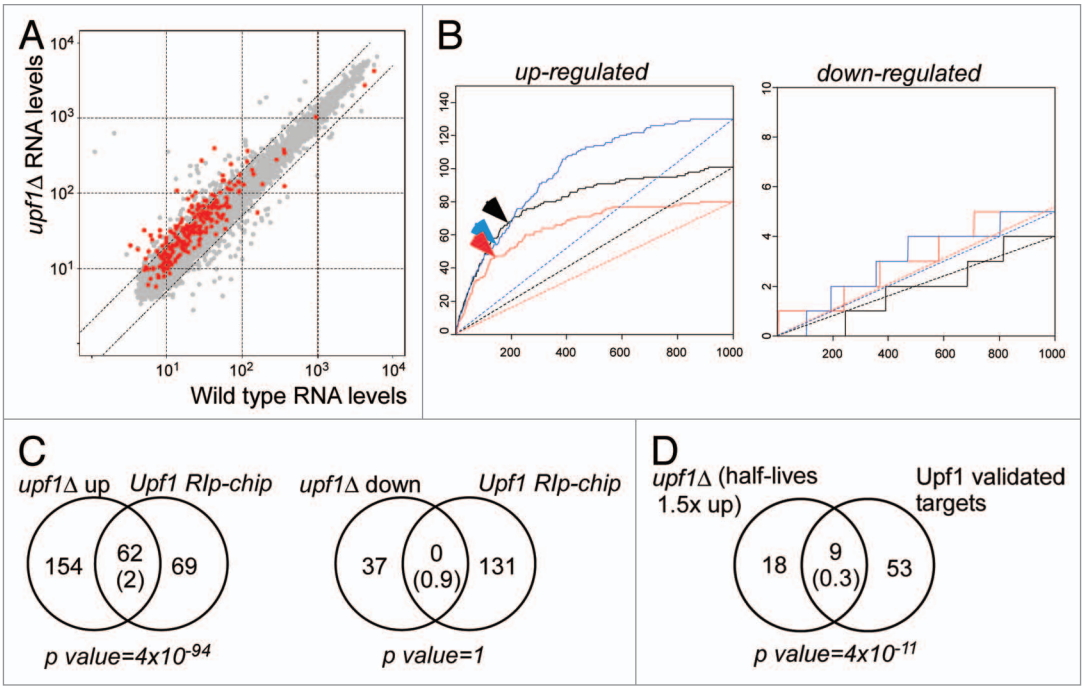

Figure 1. Upf1 RNA immunoprecipitation - DNA chips (RIp-chip) experiments. (**A**) Comparison of expression levels between wild type and *upf1*Δ cells. RNAs identified in RIp-chip experiments are shown in red, other RNAs are displayed in gray. RNAs outside the dashed lines differ more than 1.75-fold in levels between both strains. (**B**) Selection of functionally relevant Upf1-associated transcripts (see text for details). The number of genes selected from a ranked list of RIp-chip enrichments is plotted against the number of genes in that list whose expression levels are altered in *upf1*Δ mutant cells. Each line represents an independent RIp-chip experiment. The dashed lines show the rate at which genes miss-expressed in *upf1*Δ cells would be expected to be found if chosen randomly for each of the corresponding experiments. The analysis was performed separately for RNAs overexpressed (left) and underexpressed (right) in *upf1*Δ mutants. The arrows show the threshold used to choose functionally relevant Upf1 targets. (**C**) Venn diagrams showing the overlap between Upf1 targets identified by RIp-chip and genes overexpressed in *upf1*Δ mutants (left) or underexpressed (right). The number in brackets shows the expected overlap if randomly generated gene lists of the corresponding sizes were compared, and the number below is the probability of obtaining the observed overlap with randomly generated lists. (**D**) Venn diagrams showing the overlap between Upf1 validated target mRNAs (overexpressed in *upf1*Δ and bound in RIp-chip experiments) and those mRNAs with half-lives increased 50% or more in *upf1*Δ mutants. See (**C**) for the description of p values and expected overlaps.

in regulating RNA levels. To characterize this effect further, we used DNA microarrays to directly compare the transcriptomes of wild-type and *upf1*Δ vegetatively growing cells. To gain statistical power, we pooled our new experiments (two independent biological replicates) with two previously published experiments. The results of the analysis showed that 216 RNAs were significantly upregulated in *upf1*Δ cells, while 37 were downregulated.

To identify direct physiological targets of NMD, we performed RIp-chip experiments using TAP-tagged versions of Upf1. We purified *Upf1:TAP* and identified associated RNAs using DNA microarrays, and the results were compared with those of wild type and *upf1*Δ expression arrays (**Fig. 1A**). RIp-chip experiments result in a continuum of enrichments, and it is difficult to determine the limit between real enrichment and background. To define a physiologically relevant threshold in the RIp-chip experiments, we ranked all RNAs present in each of the immunoprecipitates according to their enrichment. We then made lists of increasing size, and quantified the number of genes in each list whose expression was affected in *upf1*Δ mutants ("functional targets"). The number of functional targets in each of the lists of Upf1-bound mRNAs was plotted against the total number of genes in the list (**Fig. 1B**) and compared with the rate at which functional targets would be expected to be randomly found. For genes upregulated in the mutant, the initial slope in the ranked RIp-chip is higher than expected by random sampling, indicating that the targets identified by RIp-chip are enriched in functional targets. The point at which the slope changes (arrows in **Fig. 1B**) indicates when the RIp-chip experiment ceases to identify functional targets at a rate higher than random, and was chosen as a threshold for the definition of Upf1p targets (between 150–200, depending on the experiment). To require consistency between replicates, we defined Upf1 direct targets as those RNAs identified by RIp-chip in at least two out of three experiments. This resulted in a list of 131 RNAs, of which 62 were significantly upregulated in the mutant (**Fig. 1C**, **Table 3**; **Table S1**). By contrast, genes downregulated in the mutant were not identified at a rate substantially higher than randomly selecting genes. Consistently, the overlap between RIp-chip targets and RNAs expressed at higher levels in *upf1*Δ is highly significant (*P* value ~0), while RIp-chip targets are not enriched in RNAs downregulated in *upf1*Δ (**Fig. 1C**). These results demonstrate that the major effect of Upf1 is to downregulate the expression of its targets, and suggests that effects in the opposite direction (downregulation in the mutant) are likely to be indirect. We investigated

whether Upf1 targets were enriched in genes with common features, and only found a small but significant over-representation of RNAs induced upon nitrogen starvation[28] (12 out of 62, p value = 5×10^{-4}). By contrast, there was no enrichment in genes regulated after exposure to oxidative stress. We also investigated if *S. pombe* and *S. cerevisiae* use NMD to regulate similar genes. Of 765 Upf1-associated mRNAs in *S. cerevisiae*,[9,25] 265 had orthologs in fission yeast. A comparison of this list with that of the validated 62 *S. pombe* targets (the 62 mRNAs bound by Upf1 and expressed at higher levels in the *upf1*Δ mutant background) showed no significant overlaps (p value = 0.24).

To confirm that the changes in RNA levels caused by Upf1 inactivation were caused by alterations in RNA turnover, we used a genome-wide in vivo labeling method to directly compare decay rates between *upf1*Δ mutants and wild-type cells (see Material and Methods). There was a highly significant overlap between mRNAs with increased stabilities in *upf1*Δ and Upf1 validated targets (**Fig. 1D**). Moreover, stabilized mRNAs were enriched in genes expressed at high levels in *upf1*Δ mutants, but not in those underexpressed (**Fig. S1A and B**). These results show that Upf1 destabilizes at least some of the mRNAs it binds to, and indicate a strong biological link between binding to Upf1, mRNA levels and mRNA half-lives. The fact that some mRNAs show changes in levels that are not mirrored by changes in RNA stability (**Fig. S1A**) may be a technical issue due to the difficulty of measuring decay rates genome-wide, or may indicate that some of these mRNA are not regulated at the stability level.

NMD components are necessary for an efficient Upf1-mRNA binding and oxidative stress response. The proper function of NMD pathway requires the binding of Upf1 to Upf2 and Upf3.[29] A full NMD complex is required for this response and, thus, in cells lacking Upf2 or Upf3 the NMD complex is not active. In order to determine if the presence of these two proteins was required for efficient Upf1-mRNA binding, we performed Upf1 immunoprecipitation assays followed by RNA extraction in strains lacking *upf2*$^+$and *upf3*$^+$. For this assay, we selected a total of seven mRNAs that were quantified using qPCR. Of these, six were chosen from the list of 62 validated targets, while the remaining one (*SPAC3A12.06c*) did not show any expression changes in *upf1*Δ in order to monitor whether differential expression in *upf1*Δ strain was related to the binding efficiency in the presence/absence of NMD components. The genes used for this assay are described in **Table 4**.

We observed that in *upf2*Δ and in *upf3*Δ genetic backgrounds, the mRNA binding efficiency to *Upf1:TAP* was about 20% of that of the wild-type strain (**Fig. 2A**). These changes were not due to alterations in the level of Upf1 in the mutant backgrounds (**Fig. 2B**). These results demonstrate that in fission yeast Upf2 and Upf3 are required for efficient association of Upf1 to its mRNA targets.

In order to understand the functional relationship between Upf1, Upf2 and Upf3, we compared the sensitivity of strains lacking each of their encoding genes to oxidative stress. We hypothesized that if all NMD components cooperate in the oxidative stress response, they should have similar sensitivity to hydrogen peroxide. As shown in **Figure 2C**, *upf1*Δ, *upf2*Δ and *upf3*Δ strains were more sensitive to oxidative stress than a wild-type control and double mutants *upf1*Δ *upf2*Δ and *upf1*Δ *upf3*Δ did not show any additive sensitivity. This experiment reinforces the idea of an NMD complex working together in oxidative stress response.

To further investigate the relationship among the three proteins we used DNA microarrays to monitor gene expression in *upf2*Δ and *upf3*Δ strains (**Fig. 2D**). Consistent with the idea that the three proteins function as a complex, we found statistically significant overlap among genes regulated by the three components of NMD, (**Fig. 2D**; **Table S1**). Most of the genes enriched in *upf2*Δ and *upf3*Δ strains, were those previously classified as Upf1 targets, that is, genes upregulated in *upf1*Δ strains and whose mRNA is bound by Upf1.

Deletion of Upf1 targets can rescue *upf1*Δ sensitive phenotype under oxidative stress conditions. Cells lacking Upf1, Upf2 or Upf3 activity are sensitive to oxidative stress, although it is unclear how Upf1 targets are involved. We reasoned that the *upf1*Δ strain might be sensitive to oxidative stress because some Upf1 targets have a deleterious effect when expressed above their normal levels. If this hypothesis was true, the double deletion mutants lacking *upf1*$^+$ and some of those targets should have a lower sensitivity to hydrogen peroxide than *upf1*Δ strains.

We therefore performed survival assays to characterize Upf1 targets phenotypes under oxidative stimuli. We constructed double mutants with deletions in *upf1*$^+$ and the previously mentioned Upf1 targets: *rex2*$^+$, *SPAC11D3.09c*, *rad8*$^+$, *SPCC63.13* and *SPAC25H1.04/mug105*$^+$. We plated serial dilutions of the single and double mutants on hydrogen peroxide-containing plates (**Fig. 3A**). After 48 h incubations, we observed that the sensitivity of three of the double *upf1*Δ mutants tested, *rex2*Δ *upf1*Δ, *SPAC11D3.09c*Δ *upf1*Δ and *SPCC63.13*Δ *upf1*Δ, was lower than the sensitivity shown by the *upf1*Δ strain, while all single mutants displayed a wild-type phenotype under hydrogen peroxide treatment.

We have previously described that cells deficient in Upf1 have a lower abundance of *atf1*$^+$ mRNA, encoding a key transcription factor in the response to oxidative stress in fission yeast.[27] One possible explanation for the phenotypic rescue could be that the double mutants can promote an efficient induction of *atf1*$^+$ mRNA. This possibility was tested by using qPCR to quantify *atf1*$^+$ mRNA abundance in different backgrounds in the absence or presence of hydrogen peroxide. We selected a wild-type strain as positive control of *atf1*$^+$ induction and an *upf1*Δ strain as negative control. This experiment was performed in *rex2*Δ *upf1*Δ strain, where we observed a rescue of *upf1*Δ strain phenotype, and in *rad8*Δ *upf1*Δ strain, that did not show this phenotypic rescue. Single mutants *rex2*Δ and *rad8*Δ were also included in the assay (**Fig. 3B**). As expected, *atf1*$^+$ mRNA levels in cells lacking *upf1*$^+$ were reduced compared with those of wild-type cells. The double mutant *rex2*Δ *upf1*Δ showed similar induction of the transcription factor to that observed in wild-type cells, while *rad8*Δ *upf1*Δ strain only showed a slight enhancement in the induction compared with *upf1*Δ strain. These results indicate that some Upf1 targets, like Rex2, may exert a negative effect on oxidative stress survival mediated by their regulation of *atf1*$^+$ mRNA abundance.

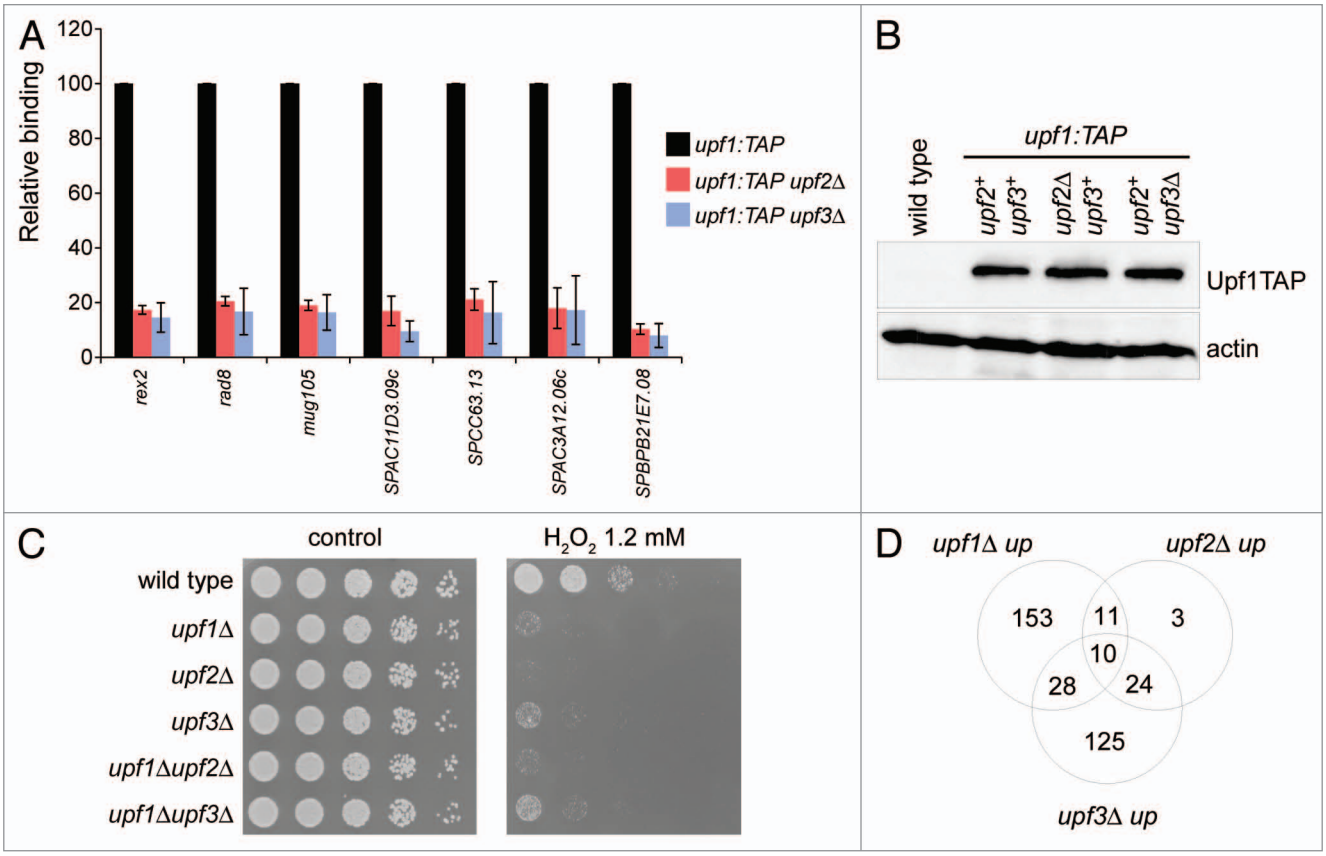

Figure 2. Upf1 relationship with Upf2 and Upf3 in mRNA binding and oxidative stress response. (**A**) Upf1-mRNA targets binding efficiency in different genetic backgrounds. Total and IP data of *Upf1:TAP* strain was used as reference for data normalization. Binding efficiency was calculated comparing each IP RNA to the corresponding total RNA. Black columns represent the binding efficiency of Upf1 to *rex2*$^{+}$, *rad8*$^{+}$, *mug105*$^{+}$, *SPAC11D3.09c*, *SPCC63.13*, *SPAC3A12.06c* and *SPBPB21E7.08* in basal conditions. Grey columns represent Upf1 binding efficiency in cells lacking Upf2 and white columns represent Upf1 binding efficiency in *Upf1:TAP upf3*Δ strain. Data was normalized using actin as control for background binding. Bars indicate standard error. (**B**) Upf1 abundance in cells lacking Upf2 or Upf3. *Upf1:TAP* protein was monitored in total cell extracts using anti-peroxidase antibodies conjugated to peroxidase in wild type, *upf2*Δ and *upf3*Δ strains. Actin was used as a loading control. (**C**) Oxidative stress sensitivity in NMD mutants. Wild type cells or lacking Upf1, Upf2, Upf3 or double mutants lacking Upf1Upf2 or Upf1Upf3, were grown in plates with rich media and in the presence of hydrogen peroxide. Pictures were taken after 48 h of growth at 30°C. (**D**) Genes regulated by Upf1, Upf2 and Upf3. Venn diagram displaying the number of up-regulated genes in mutants lacking Upf1, Upf2 and Upf3, respectively.

Discussion

In this report, we systematically identified mRNAs associated with Upf1 in *S. pombe* and used genome-wide methods to characterize the effect of Upf1 on their abundance and stability. Although similar experiments have been performed in other organisms such as *Saccharomyces cerevisiae*,[9,25] this is the first time that such targets have been investigated in *S. pombe*. The identification of biochemical targets of Upf1 proteins may shed light on the mechanisms that select mRNAs for degradation in different eukaryotes. Interestingly, the genes regulated by NMD in *S. pombe* are not conserved in *S. cerevisiae*, consistent with the lack of conservation of NMD targets in *S. cerevisiae* and various metazoans.[1,13]

Our results show that Upf1 in *S. pombe* has an important role in regulating mRNA levels. Many of the mRNAs bound by Upf1 are directed to degradation by Upf1, as shown by their increased level and decreased half-lives in Upf1-deficient strains. Another group of mRNAs displayed increased levels in *upf1*Δ mutants without clear binding to Upf1. This observation could be explained if these mRNAs are targeted for degradation in a very fast manner (so they would not remain associated with Upf1 long enough to be detected) or by indirect biochemical effects of the bona fide targets of Upf1.

A group of Upf1-bound mRNAs did not appear to be affected by the presence of Upf1. This is consistent with results reported in other organisms where Upf1 preferentially associates, but not exclusively, with NMD targets.[30,31] This is an intriguing result, because this binding may indicate additional functionality of Upf1 independently of the degradation of mRNAs.

We observed that many of the targets of Upf1 (bound and targeted for degradation) have features commonly found in NMD targets, like pseudogenes (*SPBPB21E7.08*), long UTRs (*SPAC11D3.09, rad8*$^{+}$) or the presence of introns (*rex2*$^{+}$). However, other targets like *SPCC63.13*, *SPAC3A12.06* and *mug105*$^{+}$ lack these features (**Table S2**). Therefore, Upf1 might be able to target for degradation mRNAs without PTCs, as described in other systems.[6,9,12,13] This observation also demonstrates that fission yeast is a good model organism to dissect the

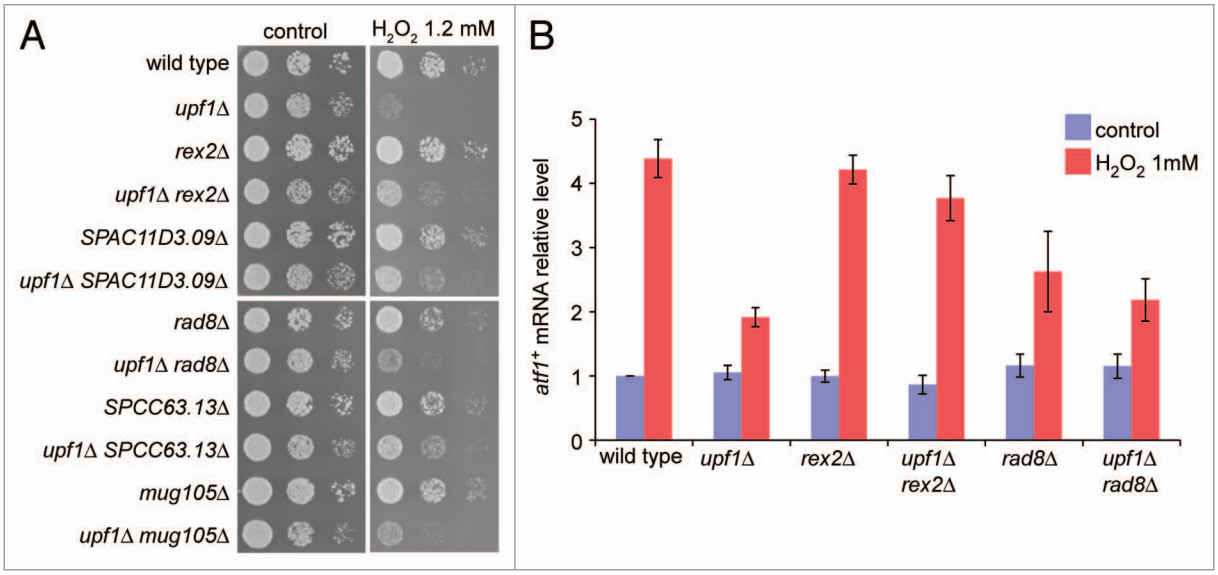

Figure 3. Survival assays in oxidative stress conditions. (**A**) Serial dilutions (1/5) of wt, *upf1Δ*, *rex2Δ*, *upf1Δ rex2Δ*, *SPAC11D3.09Δ*, *SPAC11D3.09Δ upf1Δ*, *rad8Δ*, *rad8Δ upf1Δ*, *SPCC63.13Δ*, *SPCC63.13Δ upf1Δ*, *mug105Δ*, *mug105Δ upf1Δ* strains were plated in rich media and in the presence of 1.2 mM hydrogen peroxide. Plates were incubated at 30°C for 2 d. (**B**) Quantitative real-time PCR analysis of *atf1*$^{+}$ mRNA in wt, *upf1Δ*, *rex2Δ*, *upf1Δ rex2Δ*, *rad8Δ*, *rad8Δ upf1Δ* strains in the presence and absence of 1 mM hydrogen peroxide. Bars indicate standard error.

different roles of Upf1 in NMD pathways and in non-NMD mechanisms. What defines a substrate for degradation and how some mRNAs bound by Upf1 escape from degradation are still open questions in the field.[32]

As demonstrated in other eukaryotes like *S. cerevisiae*,[25] we show that Upf2 and Upf3 are essential for the correct performance of NMD in *S. pombe*. First, Upf1, Upf2 and Upf3 are all required for survival under oxidative stress condicions. Second, microarray analyses show that the three proteins regulate a common set of genes. Our data also shows that Upf2 regulates a smaller set of genes than Upf1 or Upf3 and, on the other hand, many genes are regulated by Upf1 or Upf3 independently of the other two proteins. This result is interesting and points to a mechanism where the three proteins share many of their biological targets, but could also have independent roles in gene expression regulation. However, it is unclear whether some of the functions of Upf1 in fission yeast can be performed in the absence of Upf2 or Upf3, as described in other systems.[20,21,23] Addressing this question will require additional experiments.

We have also uncovered a mechanism of oxidative stress resistance mediated by some Upf1 targets. This result is reminiscent of that described in *S. cerevisiae*, where inactivation of Upf1 leads to increased expression of the magnesium transporter Alr1 and, as a consequence, translation termination fidelity is affected.[33] We propose a somewhat similar mechanism in fission yeast, where inactivation of NMD by deletion of Upf1 would lead to increased levels of Rex2 and, as a consequence, to increased sensitivity to oxidative stress. It is still unclear by which mechanism Rex2 overexpression reduces oxidative stress resistance. Rex2 sequence homology indicates that it may have a 3'-5' exonuclease activity, but it has not been characterized in fission yeast.

The data presented in this manuscript give insight into how the physiological functions of NMD factors are mediated by target mRNAs that lack nonsense codons, and into the importance of Upf1 in different physiological situations.

Materials and Methods

Yeast strains and general techniques. *Schizosaccharomyces pombe* strains with different gene modifications have been used. Details of these strains appear in **Table 1**.

Culture media were prepared using de-ionized water based on Millipore system.

For solid media, Bacto™ Agar (BD) was added at 20 g/l.

The media used in this study was purchased from Formedium (UK), and sterilized at 1 atm/121°C for 20 min.

Viability assays. To determine sensitivity or resistance of the strains to hydrogen peroxide, cells were cultivated in YES rich media (Formedium) in the presence/absence of hydrogen peroxide ranging from 0.25–4 mM.

Western blotting. Total cell extracts from fission yeast cells were obtained as previously described.[27] *Upf1:TAP* protein was detected using PAP antibodies (Sigma). Antibodies against actin were obtained from MP Biomedical Immuno.

Quantitative PCR. RNA was extracted with phenol:chloroform acid as is described by Bähler.[34] Once obtained RNA is purified by Qiagen RNeasy Mini Kit. For reverse transcription, 1 μg RNA was used for the reaction, using Reverse Transcription System kit (Promega®), following manufacturer's guidelines. Finally, quantification was performed with the equipment ABI 7700 (Applied Biosystems).

Oligonucleotides used as PCR primers are listed in **Table 2**.

Table 1. List of strains used in this work

Strain	Genotype	Reference
PR109	*h⁻ ura4-D18 leu1-32*	Paul Russell Lab
MR3567	*h⁻ upf1::kanMX6 ura4-D18 leu1-32*	Laboratory collection
MR158	*h⁻ upf1:TAP:kanMX6 ura4-D18 leu1-32*	Laboratory collection
MR163	*h⁻ upf2::kanMX6 ura4-D18 leu1-32*	Laboratory collection
V2–01-G07	*h⁻ upf3::kanMX4 ura4-D18 leu1-32*	Bioneer
MR349	*h⁻*	Jürg Bähler Lab
MR350	*h⁻ upf1::kanMX6*	Jürg Bähler Lab
MR164	*h⁻ upf1::kanMX6 upf2::kanMX6 ura4-D18 leu1-32*	Reference 27
MR979	*h⁻ upf1::kanMX6 upf3::kanMX6 ura4-D18 leu1-32*	This work
MR402	*h⁺ upf1:TAP:kanMX6 upf2::kanMX6 ura4-D18 leu1-32*	This work
MR433	*h⁻ upf1:TAP:kanMX6 upf3::kanMX4 ura4-D18 leu1-32*	This work
MR591	*h⁻ upf1::kanMX6 mug105::kanMX4 ura4-D18 leu1-32*	This work
MR596	*h⁻ upf1::kanMX6 SPCC63.13::kanMX4 ura4-D18 leu1-32*	This work
MR595	*h⁻ upf1::kanMX6 SPAC11D3.09::kanMX4 ura4-D18 leu1-32*	This work
MR589	*h⁻ upf1::kanMX6 rad8::kanMX4 ura4-D18 leu1-32*	This work
V2–17-G02	*h⁺ mug105::kanMX4 ura4-D18 leu1-32 ade-*	Bioneer
V2–11-F11	*h⁺ rad8::kanMX4 ura4-D18 leu1-32 ade-*	Bioneer
V2–14-D03	*h⁺ SPAC11D3.09::kanMX4 ura4-D18 leu1-32 ade-*	Bioneer
V2–27-C04	*h⁺ SPCC63.13::kanMX4 ura4-D18 leu1-32 ade-*	Bioneer
MR252	*h⁻ rex2::kanMX6 ura4-D18 leu1-32*	This work
MR256	*h⁻ upf1::kanMX6 rex2::kanMX4 ura4-D18 leu1-32*	This work

Table 2. RT-PCR primer list

Primer	Sequence
185.Atf1_5	AACCCCTACT GGAGCTGGAT
186.Atf1_3	GGGAACCTGG GAGAGTAAGC
189.Act1_5_probe	AGCACCCTTG CTTGTTGACT
190.Act1_3_probe	CTCATGAATA CCGGCGTTTT
415.SPAC11D3.09c_FWD	tacccctgag agtgcaggtt
416.SPAC11D3.09c_REV	gagtttgatc ccatccaagc
417.REX2_FWD	GCGTTAATCG CTGGAAACAGT
418.REX2_REV	GATTATCTTT GGCATCTCGA CAGA
419.MUG105_FWD	cggtattgat cgtggatgg
420.MUG105_REV	aaccaatttg gattggtgta ttg
421.RAD8_FWD	acttgtgcca accatgtttt ta
422. RAD8_REV	gaatacgatc gatggcctgt
423.SPCC63.13_FWD	tgcagtagaa aaagctcgca ta
424.SPCC63.13_REV	aggaggtgaa ctgcttggaa
425.SPAC3A12.06c_FWD	GTTTAAGTGA CCTCATTGCG GATA
426.SPAC3A12.06c_REV	CCACCCATTG CCATTTCG
427.ACTIN_FWD	tcctagctcc atgaaggtca a
428.ACTIN_REV	gaatggatcc accaatccag
429.SPBPB21E7.08_FWD	ACCTCACACC CACTCTCATT ACC
430.SPBPB21E7.08_REV	CAGGAAGGAA TTTGGAAAAT GG

RNA immunoprecipitation. RNA immunoprecipitation was performed as described by Amorim and Mata.[35]

Microarray data analysis. Microarray data processing and normalization were performed as previously described.[34] Selection of differentially expressed genes between wild-type and *upf1*Δ mutants was performed using SAM (Significance Analysis of Microarrays).[36] For *upf2* and *upf3* mutants, we selected as differentially expressed those genes that showed differences of at least 1.5-fold in two independent biological replicates. RIp-chip targets were identified as described[37] using custom-made scripts written in R. Upf1 targets identified by RIp-chip were not enriched in RNAs present in RIp-chip experiments using untagged strains or other RNA-binding proteins,[37] demonstrating the specificity of the protocol.

Gene annotations were retrieved from Pombase-GeneDB.[38]

All microarray data have been deposited in ArrayExpress with accession numbers E-MTAB-931 (*upf1*Δ expression arrays), E-MTAB-909 (RIp-chip experiments), E-MTAB-1325 (effects of *upf2*Δ and *upf3*Δ on gene expression) and E-MTAB-1324 (effects of *upf1*Δ on mRNA stability).

After that, query for the accession number of the corresponding experiment.

mRNA stability experiments. mRNA decay rates were determined using an in vivo labeling method with 4-thiouridine (4sU).[37] Cells were grown in EMM at 32°C, and mRNAs were labeled by the addition of thiouridine to the medium at a final concentration of 75 μg/ml. Cells were collected after 7 min,

Table 3. List of putative Upf1 targets

Gene name	Gene db	Gene description
SPAC22F3.05c	*alp41*	ADP-ribosylation factor Alp41
SPAC3A12.06c		Member of the sodium or calcium exchanger protein family of membrane transporters
SPAC11D3.13		ThiJ domain protein
SPCC1281.07c		Glutathione S-transferase (predicted)
SPAC1039.04 (*)		Nicotinic acid plasma membrane transporter
SPAC823.06	*taf3*	Transcription factor TFIID complex subunit Taf3 (predicted)
SPCC1450.01c (*)		Pseudogene
SPAC4F10.12	*fta1*	CENP-L homolog Fta1
SPBC1773.14	*arg7*	Argininosuccinate lyase
SPAC25H1.04	*mug105*	Ubiquitin-fold modifier-specific protease (predicted)
SPBC1683.11c (*)		Isocitrate lyase (predicted)
SPBC21C3.16c	*spt4*	Transcription elongation factor complex subunit Spt4
SPBC16E9.07	*mug100*	Sequence orphan
SPBC1773.13		Aromatic aminotransferase (predicted)
SPBC1778.05c		Sequence orphan
SPBC21C3.15c		Aldehyde dehydrogenase (predicted)
SPAC17A5.05c		Conserved fungal protein
SPAC17A2.07c		Sequence orphan
SPBC18E5.09c		Sequence orphan
SPBC577.09	*ckn1*	ERCC-8 DNA repair homolog
SPAC17A5.04c	*mde10*	Spore wall assembly ADAM family peptidase Mde10
SPAC11D3.09 (*)		Agmatinase (predicted)
SPAC139.04c	*fap2*	L-saccharopine oxidase
SPAC20G4.05c (*)		UPF0061 family protein
SPBC1683.07	*mal1*	Maltase α-glucosidase
SPCC63.13 (*)		DnaJ domain containing protein
SPBC21C3.06		Sequence orphan
SPAC24C9.07c	*bgs2*	1,3-β-glucan synthase subunit Bgs2
SPAC25H1.03	*mug66*	Meiotically upregulated gene Mug66
SPBC1198.14c	*fbp1*	Fructose-1,6-bisphosphatase Fbp1
SPBC27B12.12c		CorA family magnesium ion transporter (predicted)
SPBP8B7.27	*mug30*	Ubiquitin-protein ligase E3 (predicted)
SPAC32A11.02c		Conserved fungal protein
SPBC354.03	*swd3*	WD repeat protein Swd3
SPAC13G6.01c	*rad8*	Ubiquitin-protein ligase E3
SPAPB24D3.03 (*)		Agmatinase (predicted)
SPAC3C7.13c (*)		Glucose-6-phosphate 1-dehydrogenase (predicted)
SPCC970.02		Mannan endo-1,6-α-mannosidase (predicted)
SPCC24B10.20		Protein containing a short chain dehydrogenase domain
SPBPB21E7.08		Pseudogene
SPBC530.06c		Translation initiation factor eIF3 α subunit (p135) (predicted)
SPBC11C11.11c		Mitochondrial ATP-dependent DNA helicase Irc3 (predicted)
SPCC550.08		N-acetyltransferase (predicted)
SPCC736.05	*wtf7*	Wtf element Wtf7
SPBC8D2.07c	*sfc9*	Transcription factor TFIIIC complex subunit Sfc9 (predicted)

Genes induced upon nitrogen starvation (ref. 28) are indicated with an asterisk (*).

Table 3. List of putative Upf1 targets

SPBC8E4.02c (*)		Sequence orphan
SPBC1347.07	*rex2*	RNA exonuclease (predicted)
SPAC922.03 (*)		1-aminocyclopropane-1-carboxylate deaminase (predicted)
SPAC11D3.06		Member of the MatE family transporter
SPBC1347.08c		Ribonuclease H2 complex subunit (predicted)
SPAC922.06 (*)		3-oxoacyl-[acyl-carrier-protein]reductase(predicted)
SPNCRNA.28	*prl28*	Non-coding RNA, poly(A) bearing (predicted)
SPAP7G5.03	*prm1*	Conjugation protein Prm1 (predicted)
SPCC1494.01 (*)		Iron/ascorbate oxidoreductase family
SPBC28E12.01c	*apc13*	Anaphase-promoting complex subunit Apc13
SPCC550.10	*meu8*	Aldehyde dehydrogenase Meu8 (predicted)
SPBC32H8.13c	*mok12*	Alpha-1,3-glucan synthase Mok12
SPBP23A10.03c		Mitochondrial ACN9 family protein (predicted)
SPAC2F7.05c		Translation initiation factor eIF5 (predicted)
SPNCRNA.131	*tos2*	Antisense RNA (predicted)
SPNCRNA.132	*tos3*	Antisense RNA (predicted)
SPBC1289.02c	*uap2*	U2 snRNP-associated protein Uap2

Genes induced upon nitrogen starvation (ref. 28) are indicated with an asterisk (*).

Table 4. Description of targets used to analyze the requirement of the presence of Upf2 and Upf3 for an efficient Upf1-mRNA binding

Gene name	Function
***rex2*⁺**	3'-to-5' exoribonuclease specific for small oligoribonucleotides
***rad8*⁺**	Ubiquitin-protein ligase E3 Rad8
***mug105*⁺**	Ubiquitin-fold modifier-specific protease
SPAC11D3.09c	Agmatinase
SPCC63.13	DNAJ domain protein
SPBPB21E7.08	Pseudogene
SPAC3A12.06c	Sodium/calcium exchanger

total RNA extracted and 4sU-labeled RNA was biotinylated as described.[37] Finally, 4sU-labeled fractions and total RNA were compared using DNA microarrays. The fraction of 4sU labeled RNA was then used to estimate the mRNA half-lives in wild type and mutant cells.[37] mRNAs that showed increases in half-lives of at least 1.5-fold in two independent biological replicates were considered stabilized in the *upf1*Δ mutant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/rnabiology/article/24569/

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