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RrmA regulates the stability of specific transcripts in response to both nitrogen source and oxidative stress

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Summary

Differential regulation of transcript stability is an effective means by which an organism can modulate gene expression. A well-characterized example is glutamine signalled degradation of specific transcripts in *Aspergillus nidulans*. In the case of *areA*, which encodes a wide-domain transcription factor mediating nitrogen metabolite repression, the signal is mediated through a highly conserved region of the 3′ UTR. Utilizing this RNA sequence we isolated RrmA, an RNA recognition motif protein. Disruption of the respective gene led to loss of both glutamine signalled transcript degradation as well as nitrate signalled stabilization of *niaD* mRNA. However, nitrogen starvation was shown to act independently of RrmA in stabilizing certain transcripts. RrmA was also implicated in the regulation of arginine catabolism gene expression and the oxidative stress responses at the level of mRNA stability. Δ*rrmA* mutants are hypersensitive to oxidative stress. This phenotype correlates with destabilization of *eifE* and *dhsA* mRNA. *eifE* encodes eIF5A, a translation factor within which a conserved lysine is post-translationally modified to hypusine, a process requiring DhsA. Intriguingly, for specific transcripts RrmA mediates both stabilization and destabilization and the specificity of the signals transduced is transcript dependent, suggesting it acts in consort with other factors which differ between transcripts.

Introduction

Transcript stability is fundamental to gene expression, with transcript abundance being a product of both the rate of synthesis and degradation. In particular, the ability to respond rapidly to changes in environmental stimuli is limited by the rate of mRNA decay. Consequently decay rates differ significantly between transcripts with specific functional classes having either short or long half-lives, and the stability of transcripts encoding stoichiometric complexes are often closely matched (Wang *et al.*, 2002; Yang *et al.*, 2003). Importantly, the stability of individual transcripts can vary significantly in response to specific stimuli (Iwai *et al.*, 1993; Stoecklin *et al.*, 1994; Morozov *et al.*, 2001; Shim and Karin, 2002; Bevilacqua *et al.*, 2003; Puig *et al.*, 2005) and groups of transcripts can be co-ordinately regulated by specific RNA binding proteins (Parker and Song, 2004). In eukaryotes distinct pathways mediate transcript degradation, with the process being initiated by either de-capping, endonuclease cleavage or, most frequently, commencing with degradation of the 3′ poly(A) tail (Parker and Song, 2004). The rate of deadenylation differs widely between mRNAs and appears to be the rate-limiting step in many decay pathways (Tucker *et al.*, 2001). When the poly(A) tail is reduced to a critical length, which in fungi and plants is ~A_{15}, transcripts are rendered susceptible to degradation either through decapping and subsequent 5′ to 3′ exonuclease activity or exosome mediated 3′ to 5′ degradation (Morozov *et al.*, 2010a; 2012). Since the poly(A) tail is also involved in the initiation and particularly re-initiation of translation (Sachs *et al.*, 1997), the control of deadenylation is a critical point for regulating gene expression. In *Aspergillus nidulans*, two active deadenylases Ccr4 and Caf1, which form a complex with Not proteins, are primarily responsible for cytoplasmic deadenylation of transcripts (Morozov *et al.*, 2010b). Ccr4 mediates basal degradation, while Caf1 appears to be responsible for accelerated degradation of transcripts in response to specific signals. Similar processes occur in other eukaryotes.

Drosophila melanogaster CAF1 has been identified as the target for an RNA binding protein, NANOS, which stimulates deadenylation (Kadyrova *et al.*, 2007). In *Saccharomyces cerevisiae*, where, atypically, Caf1 appears to be catalytically inactive, it is also the target of some RNA binding proteins which stimulate RNA degradation (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007).

One well-studied example of regulation mediated at the level of transcript stability is nitrogen metabolism in *A. nidulans*. Modulation of the GATA transcription factor AreA, involves the rapid degradation of the *areA* transcript in response to intracellular Gln levels. This is mediated through a highly conserved region of 218 nucleotides within the 3′UTR of *areA* mRNA (Platt *et al.*, 1996; Morozov *et al.*, 2000). This sequence is one of the most highly conserved non-coding sequences within Aspergilli and is sufficient to determine regulated stability in a heterologous transcript (Morozov *et al.*, 2000). High levels of intracellular Gln trigger deadenylation of the *areA* transcript, which promotes de-capping and rapid 5′ to 3′-mediated decay (Morozov *et al.*, 2000). Additionally, a large subset of genes directly involved in nitrogen metabolism is also regulated at the level of transcript stability in response to nitrogen availability (Caddick *et al.*, 2006b). These comprise structural genes involved in nitrate utilization including a high affinity ammonium permease (MeaA) and a second GATA transcription factor, AreB (Conlon *et al.*, 2001). Like *areA*, these transcripts become significantly less stable in the presence of ammonium or Gln. The number of genes involved indicates that this represents a major regulatory mechanism. Furthermore, it opens the possibility that in ascomycetes other major biological processes are also regulated at the level of transcript stability.

Surprisingly, with respect to nitrate metabolism there is another level of complexity (Caddick *et al.*, 2006b). The transcripts encoding genes involved in nitrate assimilation, nitrate reductase (*niaD*) and nitrite reductase (*niiA*), but not the nitrate transporter structural genes (*crnA and nrtB*) are stabilized by the substrates for the respective enzymes, nitrate and nitrite. Nitrate stabilization overrides Gln destabilization and for *niaD* transcripts, both signals are mediated via the 3′UTR. Also, Gln signalled degradation and nitrate dependent stabilization are both mediated via the poly(A) tail since analysis of *niaD* transcript deadenylation revealed that Gln accelerates the rate of poly(A) shortening, as is the case for *areA*. However, intracellular nitrate inhibits the degradation of the poly(A) tail even in the presence of Gln. This divergent regulation makes good physiological sense, as it is important that nitrate and nitrite reductase are retained by the cell until the potentially toxic metabolites (nitrate and nitrite) are removed from the cell.

The regulated degradation of *niaD* and *areA* transcripts is consistent with deadenylation being the trigger for mRNA decay. In *A. nidulans*, when the poly(A) tail reaches a critical threshold length of A_{15}, a 3′ pyrimidine tag is added to transcripts which promotes decapping and transcript degradation (Morozov *et al.*, 2010a,b), mediated by the general eukaryotic mRNA degradation pathways (Parker and Song, 2004). Therefore, deadenylation, as the critical rate limiting step, is the likely target of regulation (Tucker *et al.*, 2001). We hypothesized that this regulation will involve *trans*-acting factors which modulate the activity of the deadenylase, Caf1 (Morozov *et al.*, 2010a). The aim of this study was to identify *trans*-acting factors involved in regulating mRNA deadenylation.

Utilizing the *areA* 3′UTR, which is known to mediate Gln signalled regulation of transcript stability, we conducted pull down analysis and identified an RNA binding protein, RrmA. Disruption of *rrmA* resulted in loss of both Gln signalled degradation of *niaD* and *areA* mRNAs and nitrate signalled stabilization of the *niaD* mRNA. However, a distinct response to nitrogen starvation was not affected. RrmA was already known to have a role in amino acid metabolism and we have shown that it regulates the stability of the arginine catabolism gene transcripts, *agaA* and *otaA*. The *rrmA* deletion mutant is highly pleiotropic. A key aspect of *rrmA*⁻ strains is hypersensitivity to oxidative stress. We have shown that this correlates with the destabilization of *dhsA* and *eifE* transcripts, which encode deoxyhypusine synthase and eIF5A respectively. RrmA appears to target a diverse array of transcripts and can mediate a variety of environmental signals at the level of both stabilization and destabilization in a transcript dependent manner.

Results

(1) Identification of RrmA as an AreA mRNA binding protein

We have previously determined that the accelerated degradation of *areA* mRNA in response to increased intracellular Gln levels is dependent on the transcript's 3′ UTR (Platt *et al.*, 1996; Morozov *et al.*, 2000). One possibility is that specific proteins mediate this response. We therefore expressed the 3′ UTR *in vitro* and utilized this RNA element in electrophoretic mobility shift assays (EMSAs) (Fig. 1A). From this analysis it appeared that crude protein extracts led to gel retardation, indicative of protein binding to the RNA.

We therefore undertook to isolate these proteins utilizing RNA pull down. The 3′ end of *areA* mRNA, including a poly(A) tail, was synthesized *in vitro* and incubated with cell free extract. The RNA and associated proteins were then isolated utilizing oligo-dT immobilized on magnetic beads. After washing, the associated protein was eluted and analysed by gel electrophoresis. One strong band (approximately 50 kD) observed in the RNA pull down
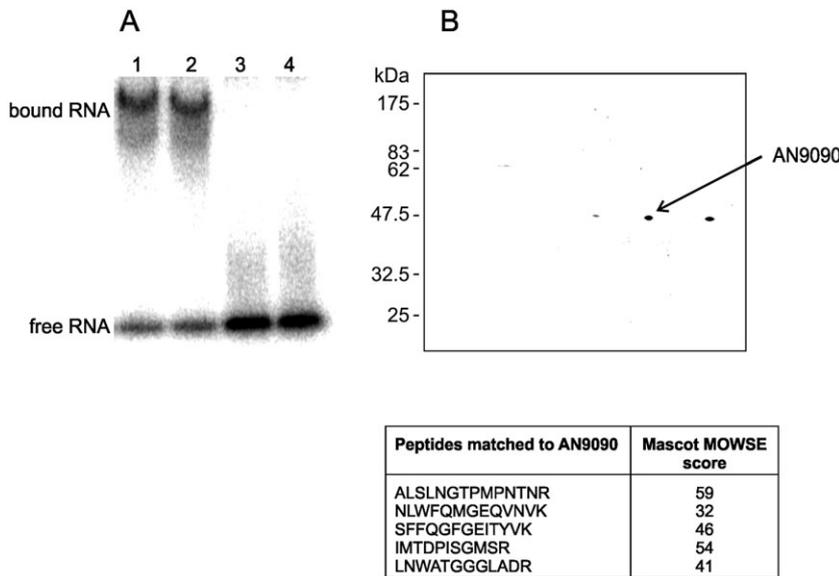
Peptides matched to AN9090	Mascot MOWSE score
ALSLNGTPMPNTNR	59
NLWFQMGEQVNVK	32
SFFQGFGEITYVK	46
IMTDPISGMSR	54
LNWATGGGLADR	41

was resolved to three spots using 2D gel electrophoresis (Fig. 1B). The same products were not observed using an unrelated RNA target (data not shown).

The three protein spots were extracted from the polyacrylamide gel, subjected to in-gel trypsin digestion and analysed using tandem mass-spectrometry. One of the three samples was identified using MASCOT, with five peptides mapping to AN0909 (Fig. 1). This had previously been identified as RrmA, a putative RNA binding protein, disruption of which influenced the expression of arginine catabolism genes and led to suppression of *proA1* with respect to proline auxotrophy (Olszewska *et al.*, 2007).

(2) RrmA is critical for the Gln signalled destabilization of specific transcripts

In order to determine the function of RrmA in Gln signalled mRNA degradation, we disrupted the gene utilizing homologous integration of a deletion cassette in which the *rrmA* coding region was replaced with *Af-pyrG*. The resulting transformants were confirmed by PCR and Southern analysis and out-crossed. Utilizing a Δ*rrmA* strain we undertook RNA degradation studies, monitoring transcript levels over a 30 min time-course after the inhibition of transcription. Quantitative northern analysis revealed that the rapid rate of *areA* and *niaD* transcript degradation observed in the wild type in response to the addition of Gln was suppressed in the Δ*rrmA* strains (Fig. 2, Tables S1 and S2). This implicates RrmA in mediation of the Gln response for these two transcripts. However, with regard to a third transcript, *meaA*, the significant increase in stability observed under nitrogen starvation was not disrupted in the Δ*rrmA* strain (Fig. 2, Table S3).

We have previously demonstrated that for *niaD* mRNA, in addition to being destabilized by Gln, nitrate leads to stabilization of the transcript (Caddick *et al.*, 2006b). These regulatory effects are lost in the Δ*rrmA* strain, although stabilization of the *niaD* transcript was observed in response to nitrogen starvation (Fig. 2, Table S2). These data indicate that RrmA mediates both the Gln signalled destabilization and nitrate signalled stabilization of *niaD* mRNA, while nitrogen starvation impacts on stability via a separate mechanism. The distinct nature of nitrogen starvation, as a signal which stabilizes transcripts in an RrmA independent manner, is consistent with data for *meaA* transcript degradation.

(3) RrmA regulates the rate of areA mRNA deadenylation

We have shown previously that the Gln signalled destabilization of *areA* mRNA correlates with accelerated deadenylation of the poly(A) tail (Morozov *et al.*, 2000). To determine whether RrmA functions upstream of this event we monitored poly(A) tail length utilizing RNase H analysis. As can be seen in Fig. 3, the accelerated poly(A) shortening in the presence of Gln observed in the wild type is lost in the Δ*rrmA* strain. This would be consistent with RrmA being involved in accelerating deadenylation via the Ccr4-Caf1-Not complex. Caf1 has previously been shown to be the key deadenylase responsible for the Gln signalled effect (Morozov *et al.*, 2010a,b).

(4) RrmA modulates the stability of both agaA and otaA mRNA

RrmA was originally identified via random transposon mutagenesis and selection to obtain suppressors of proline

A

B
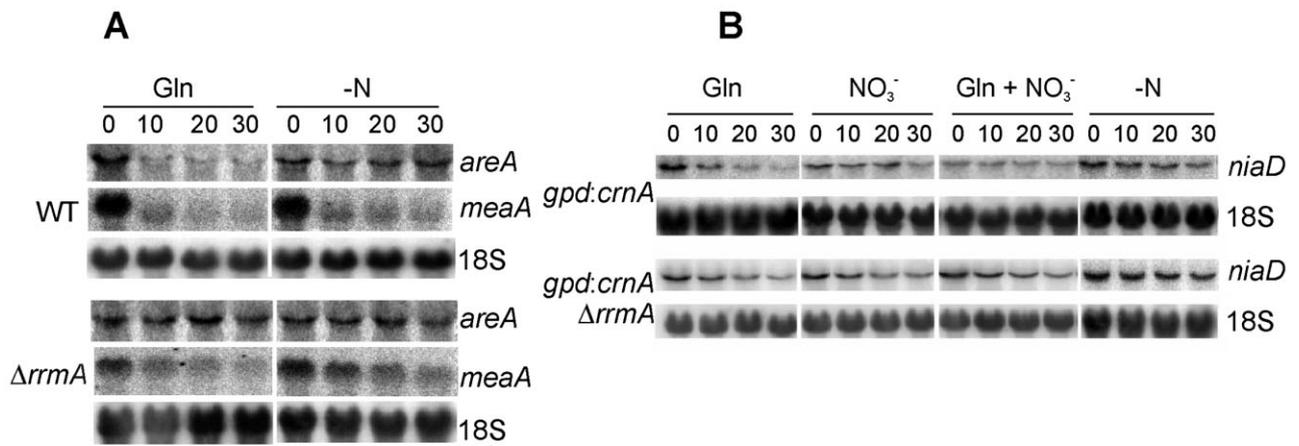
C

areA median half-life			Significance			
			WT		ΔrrmA	
Strain	N	Half-life (min)	Gln	- N	Gln	- N
WT	Gln	11				
	- N	36	*			
ΔrrmA	Gln	134	*	-		
	- N	317	-	*	NS	

D

meaA median half-life			Significance			
			WT		ΔrrmA	
Strain	N	Half-life (min)	Gln	- N	Gln	- N
WT	Gln	9.0				
	- N	26	***			
ΔrrmA	Gln	10.3	NS	-		
	- N	32	-	NS	*	

E

niaD median half-life			Significance							
			gpd:crnA				gpd:crnA ΔrrmA			
Strain	N	Half-life (min)	Gln	NO₃⁻	Gln + NO₃⁻	- N	Gln	NO₃⁻	Gln + NO₃⁻	- N
gpd:crnA	Gln	10								
	NO₃⁻	47	***							
	Gln+NO₃⁻	41	**	-						
	- N	26	**	-	-					
gpd:crnA ΔrrmA	Gln	18	*	-	-	-				
	NO₃⁻	23	-	**	-	-	NS			
	Gln+NO₃⁻	19	-	-	**	-	NS	-		
	- N	50	-	-	-	**	**	-	-	

Fig. 2. RrmA is required to destabilize specific transcripts in response to Gln and nitrate stabilization of *niaD* mRNA.
A. Northern analysis of *areA* (encoding a global transcription factor mediating nitrogen regulation) and *meaA* (encoding a high-affinity ammonium transporter) in both wild type and Δ*rrmA* strains is shown. In order to determine RNA degradation rates under conditions of nitrogen sufficiency (Gln) or nitrogen starvation (−N), transcript levels were monitored over a 30 min time-course after the inhibition of transcription. These two nitrogen regimes were chosen since both *areA* and *meaA* transcript stability is differentially regulated under these conditions.
B. Northern analysis of *niaD* (encoding nitrate reductase) transcript levels in *rrmA*[+] and Δ*rrmA* strains. Both strains include the *gpd::crnA* construct which constitutively expresses CrnA, a nitrate permease, thus facilitating nitrate uptake in the presence of repressing nitrogen sources such as Gln (Caddick *et al.*, 2006b). Transcript levels were monitored under four nitrogen regimes; glutamine sufficiency (Gln), nitrogen starvation (−N), nitrate (NO_3^-) or both glutamine and nitrate (Gln + NO_3^-).
Quantitative data from multiple (≥ 3) experiments was utilized to estimate the transcript median half-life (min) under each growth condition for *areA* (C), *meaA* (D) and *niaD* (E). Degradation rates were monitored over a 30 min time-course after transcription was inhibited. 18S rRNA was used as a loading control. Full statistical analysis of these data is presented in Tables S1, S2 and S3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS non-significant ($P > 0.05$).

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auxotrophy in a *proA1* strain (Olszewska *et al.*, 2007), *proA* being required for proline biosynthesis. We have confirmed that deletion of *rrmA*, as with the insertional mutation, results in a slower growth phenotype (Fig. 4) and suppression of *proA1* with respect to proline auxotrophy (data not shown). It was previously shown that the suppression mechanism is neither allele nor gene specific as the *rrmA* insertional mutation suppresses not only *proA1* but also *proA7*, *proB3* and *proB4* mutations. The suppression mechanism was also shown to require arginine catabolism enzymes arginase and ornithine aminotransferase (OAT) activities (Olszewska *et al.*, 2007). Exogenous arginine is catabolized by arginase to urea and ornithine. In a transamination reaction OAT converts ornithine to glutamate-5-semialdehyde which is a proline precursor. Previous analysis suggested that suppression of *proA*[−] and *proB*[−] alleles might be due to changes in the expression of arginine catabolism genes *agaA* and *otaA* encoding arginase and OAT respectively. Additionally a homologue of RrmA from *Schizosaccharomyces pombe*, Csx1, has been implicated in modulating the oxidative stress response, with gene disruption leading to destabilization of *atf1* mRNA, which encodes a bZIP transcription factor essential

for stress responses, and decreased resistance to H_2O_2 (Rodriguez-Gabriel *et al.*, 2003).

We therefore analysed the effect of Δ*rrmA* on *agaA* and *otaA* transcript stability under conditions including nitrogen repression and oxidative stress. In order to induce expression of both *agaA* and *otaA* in a wild type background it is necessary to grow the fungus in the presence of arginine. Northern analysis of transcript stability in Δ*rrmA* and wild type strains revealed some differences in transcript stability but these were generally subtle. The strongest effect was the stabilization of *otaA* mRNA in the Δ*rrmA* strain compared with the wild type grown in the presence of arginine or after transfer to arginine and glutamine (Arg→Arg+Gln) (Table S4A). For *agaA* mRNA the only significant effect was a slight destabilization of the transcript with glutamine (Arg→Arg+Gln) (Table S5A). It has been shown previously that the *agaA* transcript binds arginine *in vitro* through an aptamer-like element within the 5′UTR (Borsuk *et al.*, 2007). To test whether this direct binding of arginine influences the transcript's stability, masking the Δ*rrmA* phenotype, we utilized an *arcA*[d]*47* strain. This bears a mutation in *arcA*, the pathway-specific activator for arginine catabolism, resulting in induction of

areA WT   *areA* Δ*rrmA*

Gln   −N   Gln   −N

0 10 20 30 0 10 20 30   0 10 20 30 0 10 20 30

-A   -A
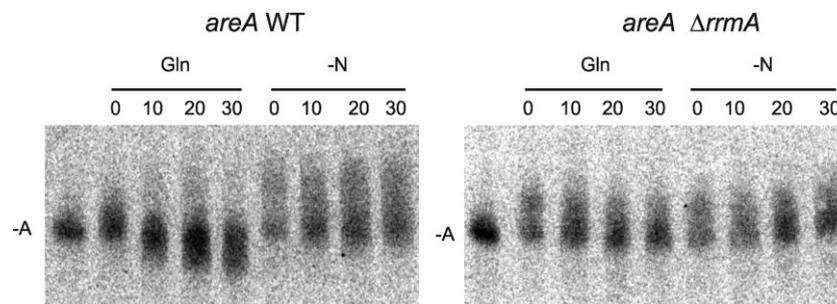
Fig. 3. Analysis of deadenylation of *areA* mRNA using RNase H assay. Total RNA was subjected to RNase H analysis using oligonucleotides specific to the *areA* 3′ UTR (Morozov *et al.*, 2001). Total RNA was extracted from both wild type and Δ*rrmA* strains over a 30 min. time-course after the inhibition of transcription. Cultures were incubated either in the absence of a nitrogen source (−N) or in the presence of Gln, as indicted. A deadenylated control was included [-A], providing a reference point (A_0). Cycloheximide was added to the culture before the beginning of the time-course to prevent decapping and 5′-3′ degradation of the mRNA. The deadenylation and 3′-5′ degradation observed for the wild type in the presence of Gln is lost in the Δ*rrmA* strain.

A.

B.

Fig. 4. Loss-of-function mutations in *rrmA* result in hypersensitivity to oxidative stress.
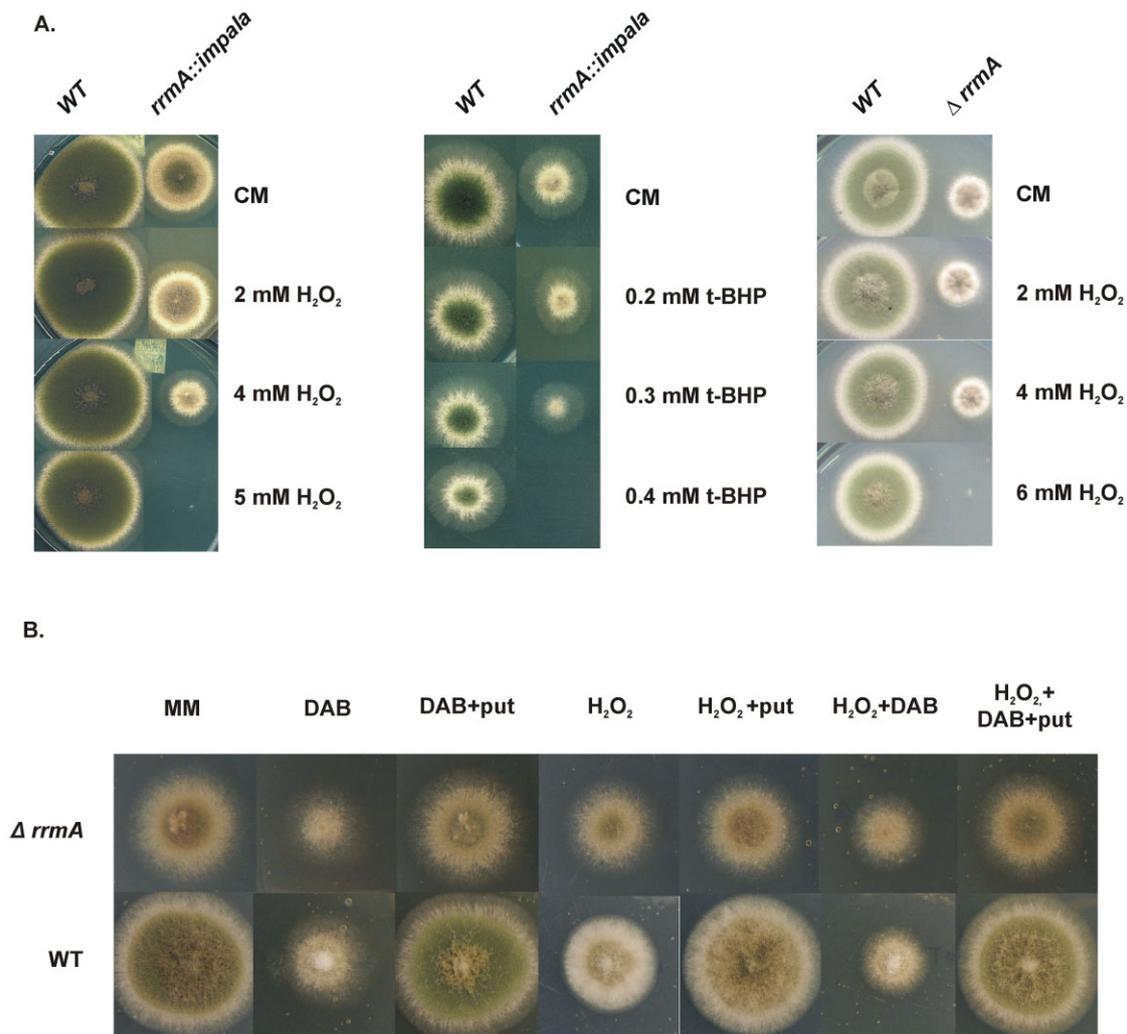A. The *rrmA* insertional mutant (*rrmA::impala*), the deletion strain (*ΔrrmA*) and the wild type (WT) were grown on complete medium with H_2O_2 or *tert*-butyl hydroperoxide (*t*-BHP). This demonstrates increased sensitivity of *rrmA*− mutants to oxidative stress.
B. The involvement of polyamines in the oxidative stress response was shown by comparing wild type and *ΔrrmA* on minimal medium with H_2O_2, putrescine (put) and/or the ornithine decarboxylase inhibitor 1,4-diamino-2-butanone (DAB). In order to compare the response of the wild type and *ΔrrmA* strains to DAB and putrescine, H_2O_2 was used at the maximum concentrations which allowed growth; 12 mM for the wild type and 4 mM for the *ΔrrmA* strain.

the pathway in the absence of arginine (Empel *et al.*, 2001). From these assays, clear differences were observed between *rrmA*+ and *ΔrrmA* strains for *agaA* and *otaA* transcript stability. In particular, *otaA* mRNA was stabilized by deletion of *rrmA* under all conditions that we tested, including nitrogen repression and oxidative stress (point estimates for half-life medians being 2.7 and 3.3 times higher in the mutant respectively) (Table 1A and Table S4B). Potentially this could lead to higher OAT activity which may explain the suppression of both *proA*− and *proB*− in *rrmA*− mutants. For *agaA* mRNA, more than sevenfold increase in stability was observed in the *ΔrrmA* strain under oxidative stress (Table 1A and Table S5B). To

determine if the role of RrmA in mediating altered transcript stability in response to oxidative stress extended to other transcripts whose stability is regulated by RrmA, we monitored *areA* mRNA in the presence of H_2O_2. From this analysis there was no evidence that RrmA affected the *areA* transcript stability under oxidative stress (Table S7).

Finally, the effects of nitrogen and carbon starvation on both *agaA* and *otaA* mRNAs were assayed in the wild type and *ΔrrmA* backgrounds. From this it was shown that disruption of *rrmA* leads to a slight but significant stabilization of the *otaA* transcript under nitrogen starvation conditions (Table 1B and Table S4C), while *agaA* mRNA is

Table 1. *otaA* and *agaA* transcript stability in Δ*rrmA* and *rrmA*⁺ strains.

Conditions	Strain	otaA		agaA	
		Half-life (min)	Significance	Half-life (min)	Significance
A.					
NO₃→NO₃	*arcA^d47*	17	**	25	*
	Δ*rrmA, arcA^d47*	26		16	
NO₃→Gln	*arcA^d47*	33	**	30	NS
	Δ*rrmA, arcA^d47*	88		33	
NO₃→NO₃+H₂O₂	*arcA^d47*	23	**	14	***
	Δ*rrmA, arcA^d47*	75		102	
B.					
Arg→–C	wt	44	NS	92	***
	Δ*rrmA*	44		29	
Arg→–N	wt	22	*	21	NS
	Δ*rrmA*	37		19	

Both *otaA* and *agaA* transcript stability were determined in *arcA^d47* (A) or wild type (B) backgrounds under conditions of nitrogen repression (Gln), oxidative stress (H₂O₂), nitrogen starvation (–N) or carbon starvation (–C). Either arginine (Arg) or nitrate (NO₃⁻) were used as nitrogen sources, as indicated. The median half-life was estimated based on quantitative northern analysis of a minimum of three replicate experiments (data not shown). Full statistical analysis of these data is presented in Tables S4B, C and S5B, C. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS non-significant ($P > 0.05$).

destabilized in carbon starvation conditions (Table 1B and Table S5C).

(5) Disruption of rrmA results in hypersensitivity to oxidative stress

We used growth tests to determine whether the disruption of *rrmA* impacts on the phenotype during oxidative stress induced with H_2O_2 or *tert*-butyl hydroperoxide. As can be seen from Fig. 4A, disruption of *rrmA* leads to hypersensitivity to oxidative stress in both deletion and insertional mutants.

Based on these data, we hypothesized that RrmA may play a key role in modulating the stability of transcripts directly involved in the oxidative stress response, so we assayed the stability of three transcripts required in this process, SrrA (AN3688), the response regulator of a two component signal transduction system (Hagiwara *et al.*, 2007) and two bZIP transcription factors, AtfA (AN2911) and NapA (AN7513) (Asano *et al.*, 2007; Hagiwara *et al.*, 2008). We did not observe any significant differences in mRNA stability between the *rrmA* deletion strain and the wild type under stress conditions (data not shown). Similarly, no difference was observed in stability of *catB* mRNA, encoding catalase B (AN9339). An alternative possibility was that the effect was indirect, with enzyme levels and not mRNA stability being altered. We therefore assayed the activity of catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX) under oxidative stress conditions. Analysis of these enzyme activities did not reveal any consistent significant differences between the Δ*rrmA* and wild type strains (data not shown).

(6) RrmA modulates stability of eIF5A and deoxyhypusine synthase mRNAs

In addition to proline biosynthesis, ornithine is utilized for the production of polyamines, including putrescine and spermidine, which are important during oxidative stress as free radical scavengers (Ha *et al.*, 1998; Rider *et al.*, 2007). They have also been implicated in a variety of distinct cellular processes including translation, RNA binding and chromatin modification (Igarashi and Kashiwagi, 2000). Specifically, spermidine is necessary for a post-translational modification of eIF5A, a translation factor implicated in polysome disassembly, translational repression and stress granule assembly (Li *et al.*, 2010; Ohn and Anderson, 2010). eIF5A is the only protein known to contain hypusine, a post-translationally modified lysine. In the first step of modification, a specific lysine residue in eIF5A is converted to deoxyhypusine by deoxyhypusine synthase using spermidine as donor of a 4-amino-butyl moiety. Then in the second step, deoxyhypusine is converted to hypusine. This modification is essential for the activity of eIF5A (Park, 2006).

As *rrmA* is implicated in both ornithine metabolism and the oxidative stress response we undertook growth tests to determine whether polyamines are implicated in the *A. nidulans* oxidative stress response and if *rrmA* is involved. As can be seen from Fig. 4B, the ornithine decarboxylase inhibitor DAB, which prevents the conversion of ornithine to putrescine, inhibits the growth of both wild type and Δ*rrmA* strains and extracellular putrescine reverses this effect. In the presence of DAB and H_2O_2, addition of putrescine reverses the growth inhibition in both the wild type and the mutant. This confirms that in

A

	ΔrrmA			WT		
	0	10	30	0	10	30
eifE						
18S						

B

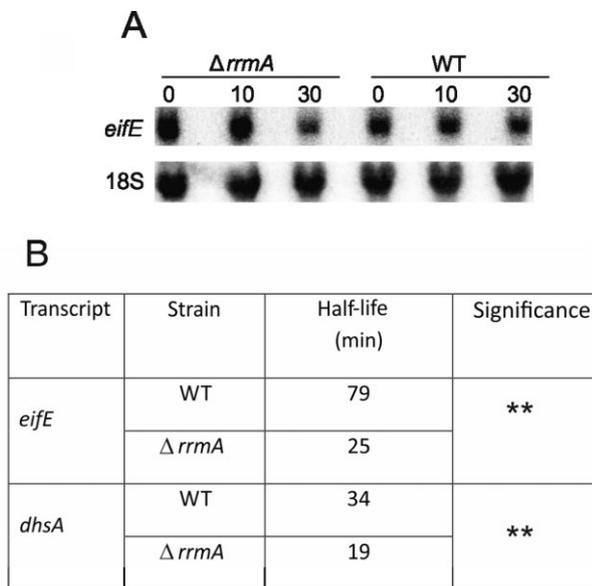| Transcript	Strain	Half-life (min)	Significance
eifE	WT	79	**
	Δ rrmA	25	
dhsA	WT	34	**
	Δ rrmA	19	

Fig. 5. RrmA influences the stability of *eifE* and *dhsA* mRNAs under oxidative stress conditions. Stability of the *eifE* transcript was determined by quantitative northern analysis with 18S rRNA as a control (A). For *dhsA*, qPCR was utilized due to the low level of expression (data not shown). In this case *tubC*, which encodes β-tubulin (May and Morris, 1988), was used as the reference. Stability was monitored in both *wild type* (WT) and Δ*rrmA* strains over a 30 min time-course after transcription was inhibited. The estimated median half-life for each transcript is given (B). In both cases there is a significant difference between the two strains ($P < 0.01$; **). Full statistical analysis of these data is presented in Table S6.

A. nidulans polyamines play an important role in the oxidative stress response, which is consistent with other organisms. However, putrescine did not reverse the sensitivity of the Δ*rrmA* mutant to oxidative stress, showing only a marginal effect, unlike the wild type (Fig. 4B). This suggests that the sensitivity of *rrmA*⁻ strains to oxidative stress is not primarily due to reduced intracellular levels of polyamines.

To determine if the Δ*rrmA* mutation directly alters eIF5A or deoxyhypusine synthase expression we assayed the stability of their respective transcripts. The orthologue for eIF5A was identified as AN4015, and designated *eifE*. Its transcript stability under oxidative stress conditions was analysed by quantitative northern analysis (Fig. 5A and Table S6) and from these data it is clear that the *eifE* mRNA is significantly less stable in the Δ*rrmA* strain (point estimates for half-life medians three times lower in the mutant). In the case of the deoxyhypusine synthase mRNA (*dhsA*, AN8075), qRT-PCR was used since transcript levels were too low for northern analysis (Fig. 5B and Table S6) and stability was again significantly reduced in the Δ*rrmA* background (point estimates for half-life medians 1.8 times lower in the mutant). These results show that deletion of *rrmA* results in destabilization of both *eifE* and *dhsA*

transcripts and might potentially lead to lower eIF5A activity. This may in part explain the sensitivity of the *rrmA* mutants to the oxidative stress.

(7) RrmA subcellular localization and role in P-body function

In order to determine the subcellular localization of RrmA we constructed a C-terminal *rrmA*::GFP fusion construct and transformed this into *A. nidulans* replacing the wild-type allele. The fusion is functional *in vivo* as demonstrated by the resistance of the tagged strain to oxidative stress being similar to wild type (Fig. 6A). The resulting strain was analysed using confocal fluorescence microscopy and the protein appeared to be located in the cytoplasm (Fig. 6B). Considering the apparent role of RrmA in mRNA degradation, and in particular the alteration of specific differential responses, we investigated the effect of Δ*rrmA* on processing (P) body formation. P-bodies are implicated in mRNA degradation, being highly dynamic RNA-protein complexes, which include translationally repressed transcripts and key factors involved in RNA decapping and degradation. To undertake these experiments we introduced the Δ*rrmA* allele into a strain expressing Dcp1::GFP which we have previously shown to localize to P-bodies in *A. nidulans* (Morozov *et al.*, 2010b). Confocal microscopy revealed that P-bodies are still formed in the Δ*rrmA* strain (Fig. 6C and D). However, unlike the wild type, perturbations, including oxidative stress or altered nitrogen regime lead to relatively minor shifts in P-body number (Fig. 6E–I). Additionally, treatment with cycloheximide, which leads to rapid loss of P-bodies in the wild type showed a significantly delayed response. A similar phenotype has previously been observed in *caf1*⁻ mutants, in which differential deadenylation and subsequent transcript degradation appears to be lost (Morozov *et al.*, 2010b).The general effects on P-bodies would be consistent with RrmA playing a key role in the regulated degradation of a wide array of transcripts.

Discussion

The regulatory response to the level and quality of available nitrogen has been studied extensively in filamentous fungi (Platt *et al.*, 1996; Morozov *et al.*, 2000; Caddick *et al.*, 2006a). The intracellular glutamine levels represent a key regulatory signal, and this is known to modulate transcript stability for a number of genes involved in nitrogen metabolism, including structural genes such as *niaD* and *meaA* as well as *areA*, which encodes the key transcription factor (Caddick *et al.*, 2006b). In the case of *areA* mRNA, a 218 nucleotide region of the 3′ UTR, which is highly conserved between the Aspergilli orthologues, is required and sufficient for regulated transcript stability
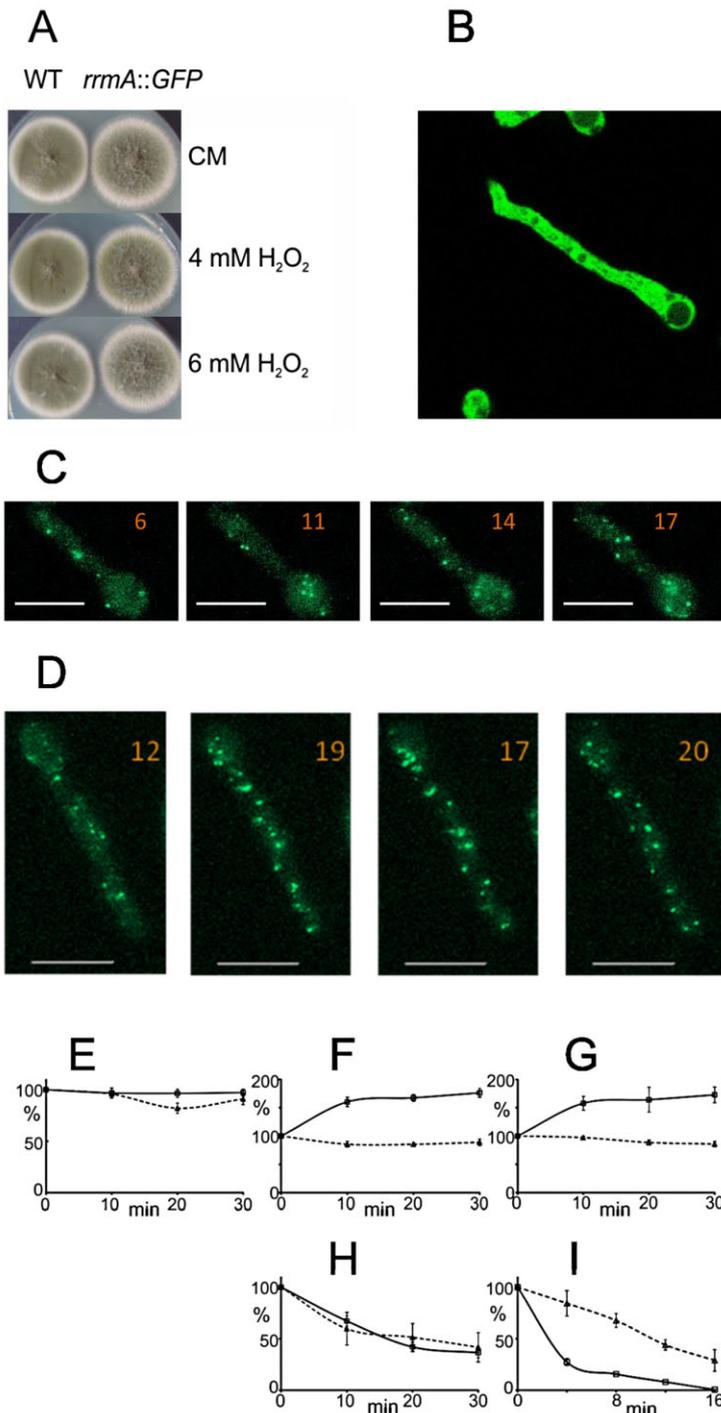
Fig. 6. RrmA localization and role in P-body formation.

A. Functionality of an RrmA::GFP fusion was assessed utilizing growth tests, with a near wild type growth phenotype and sensitivity to oxidative stress.

B. Fluorescence microscopy revealed that RrmA::GFP is evenly distributed throughout the cytoplasm.

C and D. P-body formation, as observed using Dpc1::GFP (Morozov *et al.*, 2010b) in *rrmA*⁺ (C) or Δ*rrmA* (D) strains. In the Δ*rrmA* strain the P-bodies appeared normal, being evenly distributed and highly dynamic, as can be seen by comparing the four images taken at 10 min intervals. The number of P-bodies in each image is indicated and a 10 μm scale included.

E–I. Quantitative analysis of the number of the P-bodies, normalized by cell volume, based on a minimum of four independent experiments with five to seven cells in each. The numbers given (± SE) were those observed in the *rrmA*⁺ (solid line) and Δ*rrmA* mutant (broken line) after different growth shifts. As a control, P-body number was monitored without shifting conditions (NH₄⁺) for 30 min (E). Compared with wild type no shift in numbers was observed on transfer to carbon free media (F) or after the addition of H₂O₂ to the media (G) suggesting the loss of a regulatory response. On transfer from ammonium to nitrate (NH₄⁺ to NO₃⁻), the number of P-bodies did change but this was less dramatic than in the wild type background (H). Cycloheximide treatment which leads to loss of P-bodies, probably due to disruption of RNA decapping and degradation (Beelman and Parker, 1994), was observed and they were lost at a greatly reduced rate in the Δ*rrmA* strain compared with wild type (I). This would be consistent with delayed degradation of mRNA associated with P-bodies.

(Platt *et al.*, 1996; Morozov *et al.*, 2000). In order to identify proteins with the potential to regulate the stability of *areA* mRNA we undertook RNA pull down analysis to identify proteins which bind to the 3′ UTR of the transcript. This analysis led to the identification of RrmA, which had previously been implicated in the regulation of arginine/proline metabolism (Olszewska *et al.*, 2007). Consistent with its role in modulating RNA stability, RrmA has three RRM domains (RNA Recognition Motif; Pfam Accession No. PF00076; SMART Accession No. SM00360) that are likely to mediate RNA binding. Disruption of RrmA leads to significant stabilization of the *areA* transcript, both in the presence of Gln and in nitrogen starvation conditions. However, in the case of *meaA*, which encodes an ammonium transporter, transcript stability was not significantly different in the Δ*rrmA* background compared with wild type,

although differential stability was observed between nitrogen starvation and Gln sufficiency, as in the wild type. This suggests that more than one mechanism is involved in regulating transcript stability in response to nitrogen availability.

In the case of *niaD*, which encodes nitrate reductase, it has previously been shown that the transcript is rapidly degraded in the presence of Gln but that nitrate stabilizes the transcript and that this stabilization overrides Gln signalled destabilization (Caddick *et al.*, 2006b). From the data presented it is apparent that both Gln signalled destabilization and nitrate signalled stabilization are lost in ∆*rrmA* strains. However, the stabilization in response to nitrogen starvation is still observed in the mutant and appears to be accentuated – the half-life being equivalent to the wild type in the presence of nitrate. Combining these data the simplest model is that RrmA mediates Gln signalled destabilization of specific transcripts. However, there is a separate mechanism mediating stabilization in response to nitrogen starvation which is independent of RrmA. Additionally, RrmA mediates a nitrate signal which stabilizes specific transcripts (e.g. *niaD* and *niiA*). The specificity of the nitrate signal may involve other RNA binding proteins which could interact with a limited subset of RrmA regulated transcripts. Importantly this also demonstrates that RrmA can have both a role in stabilization and destabilization, mediating distinct signals to specific transcripts.

Gln signalled transcript degradation has previously been associated with accelerated deadenylation and this is mediated primarily by Caf1, an active deadenylase which appears to effect regulated deadenylation as part of the Ccr4-Caf1-Not complex (Morozov *et al.*, 2010a,b). Consistent with this we found that deadenylation of the *areA* transcript in the response to Gln signalling was lost in the *rrmA* deletion strain. It is therefore possible that RrmA interacts directly with Caf1, as has been shown to be the case for other RNA binding proteins that modulate transcript stability (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007; Kadyrova *et al.*, 2007).

RrmA was originally identified via transposon mutagenesis, being selected on the basis of suppression of proline auxotrophy in a *proA1* strain and shown to suppress other *proA* and *proB* alleles (Olszewska *et al.*, 2007). It was proposed that the basis for *rrmA⁻* suppression of these mutations may relate to overexpression of the arginine catabolism genes *agaA* and *otaA*. One possibility is that RrmA modulates the stability of certain transcripts involved in amino acid metabolism, with *agaA* and *otaA* being prime candidates. Our data confirms that under various growth regimes disruption of *rrmA* leads to increased stability of *otaA* mRNA. This could explain the mechanism of proline suppression as the elevated level of OAT should result in a higher concentration of glutamic-

5-semialdehyde which is a proline precursor. However, with regards to *agaA*, both stabilization and destabilization were observed suggesting that RrmA mediates distinct signals at this transcript, as for *niaD*.

The specific regimes that displayed differential transcript stability between the wild type and the *rrmA* deletion strains include oxidative stress. This was of particular interest as an RrmA homologue in *S. pombe*, Csx1, had previously been implicated in the oxidative stress response (Rodriguez-Gabriel *et al.*, 2003). In the presence of hydrogen peroxide both the *agaA* and *otaA* transcripts are significantly more stable in the ∆*rrmA* strain, although in both cases the presence of arginine masked the effect. Interestingly this role in mediating the oxidative stress response was not extended to *areA* mRNA, again emphasizing the transcript specificity of particular responses. As with the effect of nitrogen regime on *areA*, *meaA* and *niaD*, the implication is that further factors are involved and the specificity is potentially defined by the specific combination of proteins able to bind particular transcripts. Consistent with this, the oxidative stress response mediated by Csx1 in *S. pombe* is complex and involves at least two other RRM proteins, Cip1 and Cip2, which potentially bind the same transcripts and counter the effect of Csx1 (Martin *et al.*, 2006). However, although structurally related RNA binding proteins from *A. nidulans* and *S. pombe* are implicated in oxidative stress response, disruption of Csx1 has a major effect on the expression of a key transcription factor, Atf1, by destabilizing its transcript. This results in *csx1* mutants having a major defect in their response to oxidative stress at the transcriptional level. Csx1 is also implicated in modulation of the sexual cycle by modulating the stability of *ste11* mRNA, which encodes a key transcription factor that regulates sexual development (Matia-Gonzalez *et al.*, 2012). Intriguingly, a key regulatory signal for sexual development in *S. pombe* is nitrogen availability, which suggests conservation of a second function between Csx1 and RrmA. We have observed no effects on sexual development in *rrmA⁻* strains (data not shown).

The role of RrmA in the oxidative stress response was explored further and we have shown that *rrmA⁻* strains are hypersensitive to both H_2O_2 and *tert*-butyl hydroperoxide. In order to determine the primary basis for this phenotype we assayed several key transcripts and activities known to be involved in the oxidative stress response, including superoxide dismutase and catalase activity but these appeared unaltered by disruption of RrmA. The role of RrmA in the catabolism of ornithine, which is a precursor for putrescine and subsequently spermidine biosynthesis, suggested a possible link with polyamine biosynthesis, polyamines having diverse biological roles including the binding of oxygen radicals (Ha *et al.*, 1998; Rider *et al.*, 2007). It has been shown that modulation of expression of

arginase coding genes results in change in tolerance to multiple abiotic stresses in *Arabidopsis* and this correlates with changes in concentration of polyamines and ROS in the cell (Shi *et al.*, 2013). In yeast arginine synthesis from proline confers resistance to oxidative stress (Nishimura *et al.*, 2010).

This postulated link between polyamine biosynthesis and resistance to oxidative stress in *A. nidulans* was supported by plate tests which show that disruption of putrescine biosynthesis with an ornithine decarboxylase inhibitor (DAB) exacerbates sensitivity to oxidative stress in wild type *A. nidulans*. Furthermore, this was reversible on addition of extracellular putrescine.

One specific role for spermidine is the post-translational modification of a specific Lys residue to hypusine in eIF5A. There is strong evidence that both eIF5A expression and its post-translational modification are altered in Δ*rrmA* strains, with both the *eifE* and *dhsA* transcripts being significantly less stable in the Δ*rrmA* background. eIF5A is a highly conserved translation factor involved in initiation and/or elongation (Saini *et al.*, 2009; Henderson and Hershey, 2011). It has also been implicated as having a role in eukaryotic stress responses (Ohn and Anderson, 2010; Wang *et al.*, 2012) and nonsense mediated decay (Kang and Hershey, 1994; Schrader *et al.*, 2006) and as a key determinant of cell proliferation making it a key factor in pathogenicity of various diseases including cancer (Kaiser, 2012). However, from our data we cannot exclude the possibility that the effect of the Δ*rrmA* mutation on *eifE* and *dhsA* transcript stability is indirect, for example as a consequence of an altered concentration of a specific polyamine within the cells.

The function of RrmA in modulating specific transcripts at the level of stability would be consistent with it acting in the cytoplasm. The subcellular location of its GFP fusion shows this appears to be the case, with abundant RrmA::GFP evenly distributed. There is no obvious association with P-bodies suggesting that it does not specifically associated with translationally repressed or degrading mRNAs. This is consistent with RrmA being associated with functional transcripts and having a role in co-ordinating different signals by modulating their deadenylation. Interestingly, there appears to be a marked effect of Δ*rrmA* on P-bodies themselves, these being generally less responsive to specific environmental shifts, consistently with partial loss of regulation at the level of RNA stability. Additionally, the P-bodies show a delayed dissociation in response to cycloheximide treatment – both of which would be consistent with a general delay to mRNA degradation.

In conclusion, RrmA is an intriguing RNA binding protein implicated in the regulation of specific transcripts in response to distinct signals. Its role varies depending on both the signal and the transcript, implicating other factors, possibly additional RNA binding proteins, which combine to exert appropriate regulation. In this respect RrmA appears to be the equivalent of a DNA binding protein which can act at different enhancers and promoters through its interaction with co-activators and co-repressors. It will be interesting to determine the full spectrum of proteins that RrmA interacts with and the transcripts it regulates. Although our study has focused on mRNA stability, closely coupled with this is translational efficiency (Hoshino, 2012) and it is possible that RrmA may also influence this. Recent global analysis of the full spectrum of proteins associated with mammalian mRNA has revealed a diverse array of proteins (Castello *et al.*, 2012) suggesting a level of complexity not dissimilar to that observed for transcriptional regulation and emphasizing the biological importance of the differential regulation of both mRNA stability and translational efficiency.

Experimental procedures

A. nidulans *strains, genetic techniques and imaging*

Aspergillus nidulans strains carried markers in standard use (Clutterbuck, 1974; 1993). Standard genetic techniques were used (Clutterbuck, 1974). Growth media were as described by Cove (1966). The Δ*rrmA* and *rrmA:GFP* strains were constructed by direct transformation of recombinant PCR constructs utilizing *Aspergillus fumigatus pyrG* as the selectable marker, as described by Szewczyk *et al.* (2006). The recipient strain was *pyrG89 sE15 wA3 fwA1 chaA1 pyroA4 nirA⁻ ΔnkuA::argB*.

The *A. nidulans* strains employed for growth tests, transcript analysis and protein production were: (1) *biA1* (wild type), (2) Δ*rrmA*, (3) *rrmA::(impala::yA) proA6 pabaA1 ΔyA::pyr4* (4) *arc^{d}47proA6 pabaA9 yA* (5) Δ*rrmA arc^{d}47 proA6 pyroA4* (6) *gpd::crnA fwA1 biA1* (7) Δ*rrmA gpd::crnA pabaA1 pyroA4 sE15*.

Growth tests of *rrmAΔ* and *WT* (*biA1*) were performed on complete medium (CM) with different concentrations of H_2O_2, *tert*-butyl hydroperoxide (*t*-BHP), 1 mM putrescine and/or 1.5 mM 1,4-diamino-2-butanone (DAB), at 37°C for three days. Confocal fluorescence imaging of P-bodies utilized strains expressing Dcp1::GFP as described previously (Morozov *et al.*, 2010b).

Preparation of protein extracts

Cells were grown overnight in fully supplemented minimal medium at 30°C in the presence of 10 mM NH_4^+ and transferred into fresh fully supplemented minimal medium lacking any nitrogen source (−N). After incubation for 1 h the cells were either harvested for the −N samples or 10 mM of Gln was added for 5 min to prior harvesting. Cells were washed with cold wash buffer, containing 20 mM HEPES pH 7.6, 100 mM KCl, 0.1 mM EDTA and 10 % glycerol. The mycelium was blotted dry, ground in liquid nitrogen and immediately transferred into cold wash buffer supplemented with 2 mM DTT, 0.5 mM PMSF and a protease inhibitor cocktail (Sigma,

P8215), in the ratio 5 ml buffer per 1 g frozen cells. The extracts were agitated in ice for 30 min followed by two high speed centrifugation stages at 30 000 *g* for 1 h and 100 000 *g* (Ti-1284 rotor) for 90 min. The resulting S100 cell-free extracts were frozen in liquid nitrogen and stored at −80°C.

Electrophoretic mobility shift assay (EMSA)

The EMSA was performed as described previously (Baker *et al.*, 2002). Briefly, 1 nM of gel-purified 5′ end-labelled RNA [200 nucleotide region of the conserved 3′ UTR of the *areA* mRNA (Morozov *et al.*, 2000)] was incubated with 10 μg protein (according the Bradford concentration assay) from S100 extracts of mycelia grown with Gln, −N or without any protein extract. The volume of the reaction mixture was adjusted to 20 μl with the extraction buffer, and then incubated for 30 min at 37°C. The competition assay contained 100× excess (0.1 μM) of the same but unlabelled RNA. Samples were then fractionated on native 10% polyacrylamide gels and visualized using a Storm phosphorimager.

RNA pull down assay

Ten micrograms polyadenylated 3′ end *areA* RNA (see above) was incubated with 1 mg S100 cell free extract (Gln or nitrate) for 30 min at 37°C, as for the gel shift assay. The RNA and associated proteins were then isolated utilizing oligo-dT immobilized on magnetic beads (Dynabeads, no. 610.05) following the supplier's protocol. After washing, the associated proteins were eluted and analysed by 2D gel electrophoresis, following Bio-Rad protocols, utilizing 11 cm ReadyStrip IPG strips with linear 3–10 pH gradient followed by Laemmli 10% PAGE. Spots, after detection according to Heukeshoven and Dernick method (Heukeshoven and Dernick, 1988) were extracted from the gel and in-gel trypsin digests were performed (Shevchenko *et al.*, 1996). The extracted peptides were used directly for MALDI-TOF mass spectrometry using a Waters Q-Tof micro coupled to an LC-Packings nano LC-MS. This was operated in the positive ion electrospray mode, and data acquisition was done using the data directed analysis mode. The data files are Waters MassLynx raw files. Local MASCOT searches were conducted using the resulting peptide mass fingerprints.

RNA isolation and transcript analysis

Growth of mycelia, transcriptional inhibition, RNA extraction, Northern blot and RNase H analysis was conducted as described previously (Platt *et al.*, 1996; Morozov *et al.*, 2000; Macios *et al.*, 2012). Cultures were grown in fully supplemented minimal media for 14 h at 30°C. Appropriate nitrogen sources were added at a final concentration of 10 mM nitrate or arginine. Mycelia were harvested, washed and transferred to the same medium or to nitrogen/carbon free media and incubated for a further 2 h. To inhibit transcription, proflavin (15 μg ml^{-1}) was added 10 min prior the time-course commencing. As indicated, glutamine (5 mM), nitrate (5 mM) or H_2O_2 (1 mM) were added at t_0. For quantitative northern analysis, probes for specific transcripts were produced by

PCR and signals were quantified using a phosphorimager (Storm). The full list of primers used is given in the supplementary data (Table S8). For *dhsA* transcript analysis, qRT-PCR was performed. cDNA was synthesized using High-Capacity cDNA Reverse transcription Kit (Applied Biosystems) and *dhsA* specific primer deox3end. qPCR was performed using SYBRGreen JumpStart Taq Ready Mix for Quantitative PCR (Sigma-Aldrich) and a LightCycler 480 (Roche). For qPCR primers deox3P and deox3L were used for *dhsA*, and tub1L/tub1P for a reference *tubC* transcript. For both northern and qRT-PCR at least three independent experiments were conducted and the data subjected to statistical analysis. RNase H analysis was performed as described previously (Caddick *et al.*, 2006b).

Statistical analysis

For each biological experiment, point estimate of transcript half-life time (HLT) was obtained using linear regression fitted to log-transformed data by the ordinary least squares method (SAS/REG procedure; SAS Institute Inc. 2010). Then, assuming approximate log-normality of HLTs, comparisons were made by computing Satterthwaite confidence intervals for the mean difference of log-transformed HLTs (SAS/TTEST procedure; SAS Institute 2010. SAS/STAT® 9.22 User's Guide. Cary, NC: SAS Institute). This yielded also a *P*-value for the test of no difference. Confidence intervals for the ratio of median HLTs, to avoid over-interpretation of median HLTs, were found by applying inverse transformation to confidence limits for the mean difference of log-transformed values. Conventionally, confidence coefficient was set at $1 − \alpha = 0.95$.

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Supporting information

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