Role of the RNA-Binding Protein Nrd1 in Stress Granule Formation and Its Implication in the Stress Response in Fission Yeast

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Abstract

We have previously identified the RNA recognition motif (RRM)-type RNA-binding protein Nrd1 as an important regulator of the posttranscriptional expression of myosin in fission yeast. Pmk1 MAPK-dependent phosphorylation negatively regulates the RNA-binding activity of Nrd1. Here, we report the role of Nrd1 in stress-induced RNA granules. Nrd1 can localize to poly(A)-binding protein (Pabp)-positive RNA granules in response to various stress stimuli, including heat shock, arsenite treatment, and oxidative stress. Interestingly, compared with the unphosphorylatable Nrd1, Nrd1DD (phosphorylation-mimic version of Nrd1) translocates more quickly from the cytoplasm to the stress granules in response to various stimuli; this suggests that the phosphorylation of Nrd1 by MAPK enhances its localization to stress-induced cytoplasmic granules. Nrd1 binds to Cpc2 (fission yeast RACK) in a phosphorylation-dependent manner and deletion of Cpc2 affects the formation of Nrd1-positive granules upon arsenite treatment. Moreover, the depletion of Nrd1 leads to a delay in Pabp-positive RNA granule formation, and overexpression of Nrd1 results in an increased size and number of Pabp-positive granules. Interestingly, Nrd1 deletion induced resistance to sustained stresses and enhanced sensitivity to transient stresses. In conclusion, our results indicate that Nrd1 plays a role in stress-induced granule formation, which affects stress resistance in fission yeast.

Stress granules (SGs) are non-membranous cytoplasmic foci, composed of non-translating messenger ribonucleoproteins (mRNAs) that rapidly accumulate in cells exposed to a broad range of environmental stresses, including oxidative, genotoxic, hyperosmotic, or heat shock stresses [1,2,3]. Several components of SGs have been identified, including the related RNA-binding proteins TIA-1 and TIAR, poly(A)-binding protein (PABP), and translation factors such as eIF3, eIF4E, and eIF4G [4]. In mammalian cells, the key event leading to the formation of SGs is the stress-induced phosphorylation of the translation initiation factor eIF2α [5]. The assembly of SGs in response to the phosphorylation of eIF2α is dependent on TIA-1 and TIAR; thus, these proteins are key regulators of SG formation and assembly [5]. The structural domains of these proteins required for the assembly of SGs are the RNA recognition motifs (RRMs) at their N-termini and the prion-related domains at their C-termini. Identification of TIA-1 mRNA targets showed that this protein binds to a U-rich motif localizing preferentially to the 3′-untranslated regions of target genes [6].

Stress granules have been observed in yeast, such as fission yeast and budding yeast, protozoa and metazoa [1,2,3]. In budding yeast, the components and kinetics of SG assembly are extensively studied and although many components of SGs are highly conserved in this organism, stress-granule assembly and its composition can vary in a stress-specific manner in yeast [7]. Recently, some of the proteins that localize to SGs in fission yeast have been identified, including Vgl1, a multi-KH-type RNA-binding protein [8], and the role of PKA in the regulation of SGs has also been reported [9]. However, only a few players of the fission yeast SGs have been identified to date and the physiological significance of SGs in stress response has not been fully elucidated in this organism.

We previously identified Nrd1, an RRM-type RNA-binding protein, as a regulator of cytokinesis [10] by demonstrating that Nrd1 directly binds and stabilizes Cdc4 mRNA encoding a myosin light chain in fission yeast [10], in addition to its well-known role as a negative regulator of sexual differentiation [11,12,13]. We also demonstrated that the Pmk1 MAPK-dependent phosphorylation negatively regulates Nrd1 activity and cytokinesis through myosin

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Introduction

Stress granules (SGs) are non-membranous cytoplasmic foci, composed of non-translating messenger ribonucleoproteins (mRNAs) that rapidly accumulate in cells exposed to a broad range of environmental stresses, including oxidative, genotoxic, hyperosmotic, or heat shock stresses [1,2,3]. Several components of SGs have been identified, including the related RNA-binding proteins TIA-1 and TIAR, poly(A)-binding protein (PABP), and translation factors such as eIF3, eIF4E, and eIF4G [4]. In mammalian cells, the key event leading to the formation of SGs is the stress-induced phosphorylation of the translation initiation factor eIF2α [5]. The assembly of SGs in response to the phosphorylation of eIF2α is dependent on TIA-1 and TIAR; thus, these proteins are key regulators of SG formation and assembly [5]. The structural domains of these proteins required for the assembly of SGs are the RNA recognition motifs (RRMs) at their N-termini and the prion-related domains at their C-termini. Identification of TIA-1 mRNA targets showed that this protein binds to a U-rich motif localizing preferentially to the 3′-untranslated regions of target genes [6].
mRNA stability [10]. Intriguingly, Nrd1 shares significant sequence similarity and a common preferred RNA-binding sequence (UCUU) with TIA-1 or TIAR [10]. This prompted us to investigate whether Nrd1, like TIA-1/TIAR, plays a role in SG assembly in response to adverse environmental stimuli. In this study, we showed that Nrd1 forms RNA granules in response to various stresses. Notably, Nrd1 localization to stress granules is modulated by phosphorylation and Cdc2, which is a RACK homologue in fission yeast. In addition, deletion of Nrd1 affects the sensitivity to these stresses in fission yeast. We propose that Nrd1 is a key component of SGs coordinating stress responses and SG formation.

Materials and Methods

Strains, Media, and Genetic and Molecular Biology Methods

_Schizosaccharomyces pombe_ strains used in this study are listed in Table 1. The complete medium (yeast extract-peptone-dextrose [YPD], yeast extract with supplements [YES]) and the minimal medium (Edinburgh minimal medium [EMM]) have been described previously [14,15]. Standard genetic and recombinant DNA methods [14] were used except where otherwise noted. PCR-based genomic epitope tagging was performed using standard methods [16]. In all cases, proteins were C-terminally tagged with GFP, YFP, or tdTomato and expressed from the respective endogenous loci.

Protein Expression, Site-Directed Mutagenesis, and Phosphorylation Assays

For protein expression in yeast, the thiamine-repressible _nmt1_ promoter was used [17]. Expression was repressed by the addition of 4.0 μg/ml thiamine to EMM and was induced by washing and incubating the cells in EMM lacking thiamine. The GST-, the mCherry-, or the GFP-fused gene was subcloned into the pREP1, or pREP2 vectors.

**Table 1.** _Schizosaccharomyces pombe_ strains used in this study.

| Strain | Genotype | Reference |
|--------|----------|-----------|
| 1243   | his2-leu1-32 ura4-D18 ade6-M210 | Tvegård et al., 2007 [32] |
| AN102  | leu1-32 ured4-18 cpc2::KanMX6 | Núñez et al., 2009 [27] |
| HM123  | leu1-32 | Our stock |
| HT201  | ade6-M210 leu1-32 ura4-D18 cpc2::ura4 | Jeong et al., 2004 [13] |
| KAY296 | leu1-32 ade6-M216 leu1-32 ura4-D18 htr1::ura4 | Udagawa et al., 2008 [33] |
| KP456  | leu1-32 ade6-M216 | Our stock |
| KP616  | leu1-32 ade6-M216 nrd1::ura4 | Our stock |
| KP928  | his2-leu1-32 ura4-D18 | Our stock |
| SP755  | ade6-M216 ura4-D18 cpc2::ura4 | This study |
| SP1074 | ade6-M216 cpc2-GFP::KanMX6 | This study |
| SP1263 | ade6-M216 nmt1-pabp-GFP::KanMX6 | This study |
| SP1265 | ade6-M216 nmt1-pabp-GFP::KanMX6 | This study |
| SP1341 | ade6-M216 nmt1-pabp-GFP::KanMX6 | This study |
| SP1558 | ade6-M216 nrd1-GFP::KanMX6 | This study |
| SP1576 | ade6-M216 ade6-M210 pmk1::ura4 | This study |
| SP1651 | ade6-M216 ade6-M210 pmk1::ura4 | This study |
| SP1652 | ade6-M216 ade6-M210 pmk1::ura4 | This study |
| SP1660 | ade6-M216 ade6-M210 pmk1::ura4 | This study |
| YO15   | ade6-M210 ade6-M210 pmk1::ura4 | Jeong et al., 2004[13] |

**Fluorescence In Situ Hybridization**

The cells were grown to the mid-log phase in synthetic minimal medium at 26°C and exposed to heat shock at 42°C for 20 min or treated with 2.0 μM sodium arsenite for 40 min. Fluorescence in situ hybridization was then performed according to a previously published procedure with slight modifications [18]. Hybridization with a biotin-labeled oligo dT probe (50-mer) was performed at 37°C. After hybridization, the cells were washed and treated with fluorescein isothiocyanate (FITC)-conjugated avidin for 30 min at room temperature [19]. Photos of hybridized cells were acquired using Keyence BIORÈVO BZ-9000.

Microscopy and Miscellaneous Methods

Light microscopy methods, such as differential interference contrast (DIC) and fluorescence microscopy, were performed as described [20]. Cell extract preparation and immunoblot analysis were performed as previously described [21].
Image Quantification

The quantification of stress granule foci was done for 3 individual datasets which summed up to 150 counted cells.

Results

Nrd1 Localizes to Stress-Induced Granules

To examine the possible functions of Nrd1 in SG formation, we determined the subcellular localization of Nrd1 in response to environmental stimuli. In unstressed cells, chromosomally integrated GFP-tagged Nrd1 was localized diffusely in the cytosol (Figure 1A; untreated). Upon exposure to thermal stress for 20 min, small patches of granule-like structures were clearly observed throughout the cytoplasm (Figure 1A; 42°C; 20 min). Intriguingly, exposure of cells to arsenite, a chemical well-known to induce SGs in mammalian cells also induced the Nrd1-positive granules (Figure 1A; 2.0 mM arsenite; 120 min). In addition, as shown in Figure 1A, Nrd1-positive granules are also formed in cells treated with H2O2 (5.0 mM H2O2; 30 min), CdCl2 (10.0 mM CdCl2; 120 min), and hyperosmotic stress (1.0 M KCl; 10 min).

To precisely define the conditions of Nrd1 granules formation at each condition, we have performed a quantitative analysis of Nrd1-positive granule number/cell for each stress. The results showed that heat shock induced the strongest stimulation of Nrd1-positive granules (Figure 1A; right panel). Arsenite also induced the second strongest effect on Nrd1—granule formation. In contrast, H2O2 and CdCl2 exerted a relatively weak effect on granule formation as compared with heat and arsenite stress (Figure 1A; right panel). Regarding the kinetics of Nrd1 granule formation, heat shock caused a rapid emergence of granules, whereas oxidative stress induces gradual appearance of granules (Figure 1A; right panel). Notably, regarding the intensity of granule formation, KCl treatment exerted a very weak effect (0.5±0.1% at 10 min) as compared with the other stresses. In addition, the kinetics of Nrd1 granule formation in response to KCl treatment was different in comparison to other stimuli in that it induced granule formation very quickly, with its peak at 20 min (Figure 1A; right panel). The number of Nrd1 granules per cell decreased and reached zero after a 60-min exposure of the cells to KCl, whereas the cells still continued to induce Nrd1 granule formation at this time point when exposed to other stimuli (Figure 1A). We also examined the effect of Nrd1 overproduction on granule formation. For this, we expressed GFP-Nrd1 under the control of the nrd1 promoter, which is repressed in the presence of thiamine. Overproduction of Nrd1 stimulated Nrd1-positive granule formation in the absence of stress (Figure 1A; overexpressed for 18 h).

In mammalian cells and in both budding and fission yeasts, poly(A)-binding protein (Pabp) is an SG marker [5,9,22]. Therefore, we used GFP-tagged Pabp, the major poly(A)-binding protein of S. pombe, to examine its co-localization with Nrd1. For this experiment, we constructed a strain expressing Nrd1 protein tagged with tdTomato and Pabp protein tagged with GFP expressed from their respective endogenous loci. In unstressed cells, both Nrd1 and Pabp were localized diffusely in the cytosol (Figure 1B; untreated). The fluorescence of Nrd1 and Pabp largely co-localizes in cytoplasmic foci on heat stress (Figure 1B, 42°C 20 min).

To determine whether the Nrd1-positive granules actually contain mRNA, we visualized cellular poly(A)+ RNA by in situ hybridization in vivo in cells expressing mCherry-tagged Nrd1 and compared the subcellular localization of poly(A)+ RNA and Nrd1. Notably, arsenite treatment induced near-complete co-localization of poly(A)+ RNA and Nrd1 in the cytoplasm (Figure 1C). Thus, Nrd1-positive granules correspond to SGs comprising of ribonucleoprotein complexes in S. pombe.

Effects of Cycloheximide on Nrd1-positive RNA Granules

We investigated whether cycloheximide (CHX) could prevent Nrd1-positive RNA granule formation in S. pombe, since RNA granule formation by external stress can be blocked through inhibition of protein synthesis by CHX in mammalian cells and in fission yeast [9,23]. The localization of Nrd1 was not affected upon CHX treatment (Figure 1D; CHX). Pre-treatment with CHX largely prevented the formation of Nrd1-positive granules induced by heat shock or arsenite treatment (Figure 1D; CHX then 42°C for 20 min, CHX then arsenite for 120 min). It should be noted that the addition of CHX after 20-min exposure to heat shock or 120-min exposure to arsenite failed to block granule formation (42°C for 20 min then CHX, arsenite for 120 min then CHX). We also counted the number of SG per cell to quantitatively assess the effect of CHX on Nrd1 granule assembly, and the results confirmed the above findings (Figure 1D; lower panel). The fact that the known inhibitor of SG formation was effective in preventing Nrd1 granule formation suggests that the Nrd1 granules correspond to SGs in mammals and that this is a biologically regulated process.

Nrd1 Localization to Stress Granules is Modulated by Phosphorylation Under Certain Stresses

Our previous results showed that heat-shock induced Pmk1 MAPK-dependent phosphorylation of Nrd1 [10]. This prompted us to investigate whether various stimuli that induced Nrd1-positive SG formation also affect the phosphorylation levels of Nrd1. We utilized anti-phospho Nrd1 T40 antibodies and anti-phospho Nrd1 T126 antibodies that recognize the phosphorylated Thr40 or Thr126, respectively [10]. As shown in Figure 2A, the levels of Nrd1 phosphorylation were markedly increased in response to heat shock (42°C for 20 min) and arsenite treatment (2.0 mM arsenite for 120 min). In addition, H2O2, and CdCl2 treatment moderately induced the phosphorylation of Nrd1 at T40, whereas KCl treatment, did not significantly affect the phosphorylation levels of Nrd1. It should be noted that the strength of Nrd1 phosphorylation by each stress roughly parallels that of SG formation as judged by the number of SG/cell treated by each stress as shown in Fig. 1A. Thus, the distinct kinetics of SG assembly on exposure to KCl and the lack of Pmk1 action on the Nrd1 substrate on KCl may be because of negative feedback regulation, such as induction of phosphatases that dephosphorylate Pmk1 MAPK. Our previous reports showed that KCl treatment activates a broad range of signaling pathways including the p38/Spc1 MAPK pathway, which induces Ptc1/Ptc3 phosphatase to inactivate both Spc1 and Pmk1 MAPK [24]. In addition, it should be noted that H2O2 and CdCl2 treatment promotes Nrd1 phosphorylation predominantly at T40 (Figure 2A). It can be interpreted that the selectivity of MAPK and/or phosphatases that inactivate Nrd1 may be different in these two threonine residues.

We then investigated whether phosphorylation enhanced Nrd1 translocation to SGs in response to stimuli. For this, we expressed the GFP-fused phosphorylation-mimic Nrd1DD, wherein T40 and T126 had been replaced with alanine [10], and compared their localization with that of the wild-type GFP-Nrd1. In untreated cells (27°C), wild-type Nrd1 and Nrd1DD were localized diffusely in the cytosol, whereas a small portion of Nrd1DD accumulated as patch-like structures (Figure 2B;
Figure 1. Nrd1 localizes to stress granules under various environmental stresses. (A) Analysis of Nrd1-GFP localization under stress. Localization of Nrd1-GFP in living cells grown at 27°C (untreated) after a shift to 42°C for 20 min (42°C 20 min) and after exposure to 2.0 mM arsenite (2.0 mM arsenite 120 min), 5.0 mM H2O2 (5.0 mM H2O2 30 min), 10.0 mM CdCl2 (10.0 mM CdCl2 120 min), or 1.0 M KCl (1.0 M KCl 10 min) for the times indicated. Wild-type (wt) cells transformed with pREP1-GFP-Nrd1 were grown in EMM (thiamine-free medium) for 18 h to induce overproduction of GFP-Nrd1 (overproduction 18 hr). Bar, 10 μm. The number in the picture indicates the SG number/cell in each experiment. Right panel: Quantitative analysis of the number of SGs/cell on each stress. Graph depicting the number of stress granules per cell formed before...
Stress Granule Formation by Nrd1

Phosphorylation-Dependent Binding of Nrd1 and Cpc2, a RACK Homologue in Fission Yeast

The above findings that Nrd1DD translocated to the granule structures faster than the unphosphorylatable form of Nrd1 suggested the possibility that compared with the Nrd1AA, Nrd1DD might have higher affinity to the component(s) of cytoplasmic granule structures. We then examined whether the phosphorylation of Nrd1 affects its ability to bind to Cpc2, the RACK1 orthologue in fission yeast, since Cpc2 was identified as a binding partner for Nrd1 [13] and Cpc2 has roles in translation, G2/M transition, and stress responses in fission yeast [26,27]. We co-expressed various versions of GST-fused Nrd1 proteins in cells expressing the chromosomally tagged Cpc2-GFP from its endogenous promoter. As shown in Figure 4A, Cpc2-GFP bound more strongly to GST-Nrd1DD as compared with the wild-type Nrd1 and Nrd1AA, both before (untreated) and after heat shock or arsenite treatment.

We next examined the functional relationship between Cpc2 and Nrd1 by assessing the effect of Cpc2 deletion on the localization of Nrd1 in SGs. In untreated conditions, the localization of GFP-fused Nrd1 and Nrd1DD proteins in wild-type cells and *qpc2* deletion cells (Δqpc2) was indistinguishable (Figure 4B; untreated). Notably, in *qpc2* deletion cells, the assembly of Nrd1 and Nrd1DD granules was markedly inhibited upon arsenite stress (Figure 4B; 2.0 mM arsenite). In contrast, Cpc2 did not affect Nrd1 and Nrd1DD granule formation after heat shock, as *qpc2* deletion cells formed granules with normal kinetics and intensity under these circumstances (Figure 4B; 42°C). Thus, arsenite-induced Nrd1 localization to SGs is Cpc2-dependent. Quantitative

(untreated) and after each condition as indicated in Figure 1(A) plotted against time after exposure to each stress and the inset is a magnification of the results obtained on KCl treatment. (B) Co-localization of Nrd1 with poly(A)-binding protein (Pabp). Merged image of fluorescence micrographs showing Pabp-GFP (green) and Nrd1-TdTomato (red) in untreated cells and after a 20-min incubation at 42°C. Bar, 10 μm. (C) Fluorescence micrographs of the wild-type cells expressing mCherry-tagged Nrd1 grown at 26°C (untreated); and these were subjected to in situ hybridization with a digoxigenin-labeled oligo (dT)20 probe after a 40-min exposure to 2.0 mM arsenite (2.0 mM arsenite 40 min). The hybridized probe was detected by treatment with mouse anti-digoxigenin antibody, followed by a fluorescein-conjugated goat anti-mouse IgG antibody (FITC). Nrd1 was detected using mCherry fluorescence (mCherry-Nrd1). Nuclei were counterstained using DAPI dye (DAPI). Bar, 10 μm. (D) Cycloheximide (CHX) prevents the formation of heat-shock- and arsenite-induced Nrd1 granules. Fluorescent images of cells expressing Nrd1-GFP incubated (from left to right) at 27°C with 100 μg/ml CHX for 30 min (CHX); at 42°C for 20 min; pre-incubated with CHX for 30 min at 27°C followed by 20-min incubation at 42°C (CHX then 42°C 20 min); 20-min incubation at 42°C followed by CHX incubation (42°C 20 min then CHX); with 2.0 mM arsenite for 120 min at 27°C pre-incubated with CHX for 30 min followed by 120-min incubation with 2.0 mM arsenite; and 120-min pre-incubation with arsenite followed by 30-min incubation at with CHX. Bar, 10 μm. Lower panel: Graph depicting the number of stress granules per cell in each condition plotted against time after exposure to each stress.

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Phosphorylation-dependent binding of Nrd1 and Cpc2.
analysis obtained after scoring the SG number/cell indicated that Cpc2 deletion did not significantly affect the localization of wild-type Nrd1 to SGs on heat shock. However, it affected the localization of Nrd1 and Nrd1DD to SGs under arsenite treatment, although the effect is more prominent with Nrd1DD (Figure 4B). It should be noted that the effect of Cpc2 deletion was greater in Nrd1DD as compared with Nrd1, presumably because of the higher affinity between Cpc2 and Nrd1DD as compared with Nrd1.

Nrd1 Plays a Role in SG Formation
As described in Figure 1A, overproduction of Nrd1 stimulated SG formation in the absence of stress. Notably, granule formation was faster in the phosphorylated mutant version of Nrd1DD (Figure 4C; GFP-Nrd1DD, overexpressed for 16 h) than in the wild-type Nrd1 (Figure 4C; GFP-Nrd1, overexpressed for 18 h). This finding also demonstrated that Nrd1-positive granule formation induced by its overproduction was modulated by phosphorylation.

It should be mentioned that significantly larger granules were induced by Nrd1DD overproduction for more than 18 h as compared with those induced by various stimuli (Figure 4C; GFP-Nrd1, 22 h, GFP-Nrd1DD, 18 h and 22 h). These structures may represent a consequence of the fusion of Nrd1 and/or SGs. Therefore, in this situation, the SG number/cell in Nrd1DD-overexpressing cells for 18 h and 22 h was lower than that for 16 h. In addition, the number of SG/cell in Cpc2-deletion cells was higher than that in the wild-type cells when Nrd1 or Nrd1DD was overproduced for 22 h. This unexpected result may reflect the aggregation of Nrd1 induced by its overproduction, which apparently lowered the SG number/cell. Thus, the effect of Nrd1DD overproduction, similar to that of Pek1DD may be appropriate to assess by monitoring the SG size, but not SG number/cell.

Interestingly, Cpc2 deletion also affected the formation of overproduction-induced Nrd1 granules, because in Δcpc2 cells, the formation of large granules of both wild-type GFP-Nrd1 and GFP-Nrd1DD was clearly inhibited (Figure 4C; Δcpc2). We then measured Nrd1 protein levels in both wild-type and Cpc2 deletion cells at the same time points shown in the above experiments. The results showed that protein levels of Nrd1 and Nrd1DD were markedly lower in Cpc2 deletion cells (Figure 4D). The effect of Cpc2 deletion was more prominent in Nrd1DD, presumably because of its stronger physical interaction as shown in Figure 4A.

To determine the role of Nrd1 in SGs assembly in more detail, we examined the effect of Nrd1 deletion on the assembly of SGs. For this, we compared the accumulation of the Pabp-positive granules after heat stress in wild-type cells and Nrd1 deletion cells. We found that the accumulation of the Pabp-positive granules (Pabp-YFP) in response to thermal stress was markedly inhibited in Δnrd1 cells compared to wild-type.
Phosphorylation of eIF2 involved in stress-induced granule assembly independent of the Nrd1 mutants was not distinguishable from that of the wild-type cells (Figure S1). Taken together, these results suggest that Nrd1 is involved in SG formation usually requires the stress-induced phosphorylation of the translation initiation factor eIF2α [28]. An allele expressing a phosphomimetic version of eIF2α is sufficient to induce SGs, whereas the expression of a mutant, unphosphorylatable version of eIF2α blocks SG formation upon stress [5]. In fission yeast, the kinetics of the appearance of RNA granules after hyperosmotic stress was slower in eIF2α-S52A cells than in wild-type cells [9]. To characterize the role of Nrd1 in SG formation and its relationship with eIF2α, we examined the effect of the eIF2α-S52A mutation on Nrd1 localization after stress. We observed dot-like structures of the endogenous Nrd1-GFP protein in eIF2α-S52A cells after heat shock and arsenite treatment with kinetics similar to that observed in the wild-type cells (Figure S1). In addition, we examined the localization of Nrd1-GFP in triple mutants of eIF2α kinases (Δga2ΔBnl1Δknl2), and found that the Nrd1 localization in the triple eIF2α kinase mutants was not distinguishable from that of the wild-type cells (Figure S1). Taken together, these results suggest that Nrd1 is involved in stress-induced granule assembly independent of the phosphorylation of eIF2α.

Nrd1 Deletion Confer Resistance to Various Stresses

To test whether the disassembly of SGs (as shown above) caused by Nrd1 deletion would alter stress sensitivity, we exposed the Nrd1 deletion cells to heat, arsenite, CdCl₂, H₂O₂, and KCl. Surprisingly, Nrd1 deletion cells were highly resistant to the high temperatures of 38°C, a temperature at which the wild-type cells barely grew (Figure 5C). While it is clear that Nrd1 deletion enhances cell tolerance against thermal stress, cell viability under arsenite or CdCl₂ is only slightly enhanced, by less than an order of magnitude. No difference in growth can be observed in the presence of H₂O₂ (Figure 5C). Nrd1 deletion cells did not show resistance to KCl, and instead, the growth of Δnrd1 cells was slightly slower than that of the wild-type cells (Figure 5C; 1.0 M KCl). These results may be related to the stress-dependent nature of Nrd1 phosphorylation as shown in Figure 2A, wherein heat shock induced the strongest phosphorylation, followed by arsenite; CdCl₂ and H₂O₂ give only a modest induction of Nrd1 phosphorylation, and KCl barely induced phosphorylation of Nrd1. The strength of Nrd1 phosphorylation by each stress roughly parallels that of SG formation as judged by number of SGs/cell treated by each stress (Figure 2B). Therefore, Nrd1 deletion cells were more tolerant of thermal stress by delaying SG formation. The degree of tolerance to each stress associated with Nrd1 deletion may be the reflection of the avoidance of SG formation by Nrd1 deletion. Alternatively, Nrd1 may possess a variety of functions other than SG assembly, and thus, the deletion phenotypes may not be explained only by defects in SG assembly.

Nrd1 is Involved in Recovery from Various Environmental Stresses

We determined the time required for the disassembly of SGs that were formed when wild-type cells were treated with various stimuli for specific periods of time (that varies depending on the stimuli). We found that after 60 min of recovery from thermal stress (Figure 6A; recovery from 42°C for 60 min), GFP-Nrd1 and GFP-Nrd1DD granules had resolved, whereas Nrd1DD granules persisted longer, with visible fluorescence still observed 60 min after heat shock. In addition, after arsenite treatment for 15 min, cells were washed and allowed to recover from the stress. In cells exposed to sodium arsenite (2.0 mM) for 15 min, translocation to the cytoplasmic granules was more rapid in the case of Nrd1DD than in the wild-type Nrd1 and Nrd1ΔA (Figure 6A; 2.0 mM arsenite for 15 min), because Nrd1 and Nrd1ΔA granules were formed 45 min after the exposure of the cells to arsenite (Figure 6A; 2.0 mM arsenite for 45 min). After 240 min of recovery from arsenite stress, GFP-Nrd1DD still formed granules, whereas GFP-Nrd1 and GFP-Nrd1ΔA had already resolved (recovery from arsenite 240 min). Similarly, in cells exposed to H₂O₂, CdCl₂, and KCl, after 30 min of recovery from H₂O₂, 60 min recovery from CdCl₂, and 30 min recovery from KCl, GFP-Nrd1DD remained within the granules, whereas GFP-Nrd1 and GFP-Nrd1ΔA granules had almost disappeared. It should be noted that 60 min after the continuous exposure to KCl resolved the Nrd1-positive granules (Figure 6A, 1.0 M KCl 60 min), suggesting that this phenomenon may be functionally related to the sensitivity to KCl associated with Nrd1 deletion cells (Figure 5C).

We then examined whether the deletion and overexpression of Nrd1 would alter the tolerance of the cells to the transient exposure to the thermal stress. The wild-type cells harboring the control vector or the nrd1Δ strain expressed the Δnrd1 cells transformed with the control vector were exposed to thermal stress (48°C for 90 min), arsenite stress (2.0 mM for 120 min), oxidative stress (5.0 mM H₂O₂ for 30 min; or 10.0 mM CdCl₂ for 180 min), or...
osmotic stress (1.0 M KCl for 180 min), then incubated at 27°C. As shown in Figure 6B, in contrast to the sustained thermal stress shown in Figure 5C, Nrd1 deletion cells were more sensitive than the wild-type cells to the transient stresses. In contrast, the growth of the wild-type cells overexpressing the Nrd1 protein was almost similar to that harboring the control vector alone (Figure 6B).

In addition, we performed time-course experiments wherein liquid cultures were subjected to transient stresses and number of...
Figure 6. Nrd1 is necessary for recovery from under certain stresses. (A) Disassembly of stress-induced Nrd1 granules. Wild-type cells expressing GFP-tagged Nrd1 were grown in EMM+thiamine at 27°C. After a 20-min incubation at 42°C (recovery from 42°C 60 min), they were incubated at 27°C (recovery from 27°C 60 min). After a 15-min exposure to 2.0 mM arsenite at 27°C (2.0 mM arsenite 15 min), the cells were washed and allowed to recover for 30-2.0 mM arsenite 45 min) or 240-min (recovery from arsenite 240 min). After a 30-min exposure to 5.0 mM H₂O₂ at 27°C, the cells were washed and allowed to recover for 30 min (recovery from H₂O₂ 30 min). After a 120-min exposure to 10.0 mM CdCl₂ at 27°C, the cells were washed and allowed to recover for 60 min (recovery from CdCl₂ 60 min). A 10-min exposure to 1.0 M KCl at 27°C, the cells were washed and allowed to recover for 30 min (recovery from KCl 30 min). After a 60-min exposure to 1.0 M KCl, Nrd1-positive granules resolved (1.0 M KCl 60 min). Bar, 10 μm. Lower panel: Graphs showing the number of stress granules per cell from each strain after each condition as indicated. (B) Δnrd1 cells displayed transient stress sensitivity. Wild-type cells transformed with vector or the nrd1+ genes, or the Δnrd1 cells transformed with control vector were grown to mid-log-phase in EMM+thiamine at 27°C. The indicated cells were then exposed to thermal stress (48°C 90 min), 2.0 mM arsenite for 120 min, 5.0 mM H₂O₂ for 30 min, 10.0 mM CdCl₂ for 180 min, and 1.0 M KCl for 180 min and were then spotted onto YES plates and incubated at 27°C. (C) CPU assay of the cells as indicated in Figure 6(B). Cells were treated as indicated in Figure 6(B), and the colony forming ability of each strain after each condition as indicated was determined by counting the number of viable colonies and normalized to the number of colonies in unstressed condition for each strain. This experiment is representative of two independently performed experiments. doi:10.1371/journal.pone.0029683.g006

Identification of Nrd1 as a component of fission yeast SGs

Here, we have presented several lines of evidence demonstrating that Nrd1 plays a role in SG assembly. Subcellular localization studies have shown that in response to various stresses, Nrd1 localized to the cytoplasmic granules, which partly co-localized with Pabp and contained mRNA (Figure 1). Like mammalian SGs, the assembly of the Nrd1-positive granules was blocked with cycloheximide, which traps mRNAs in polysomes (Figure 1D). Importantly, in Nrd1 deletion cells, the assembly of Pabp-positive granule formation was delayed, and when overproduced, Nrd1 formed granules per se without stimuli. Furthermore, Nrd1 overproduction leads to aggregation of Pabp-positive granules, thus raising the possibility that Nrd1 plays a central role in SG formation. This latter observation was reminiscent of the spontaneous formation of granules with TIA-1/TIAR overproduction [5]. Overexpression of recombinant TIA-1 represses the production of co-expressed reporter genes in the absence of exogenous stress, and endogenous TIA-1/TIAR represses the translation of the TNP2 transcripts in the absence of stress [23]. Several protein components of yeast or mammalian SGs cause growth inhibition and/or granule formation when overexpressed [29]. Interestingly, overexpression of Nrd1 induced cell growth arrest (data not shown), suggesting that Nrd1 may play a role in translation repression in addition to a role in mRNA stability [10].

Nrd1 was identified as a negative regulator of differentiation [30], as well as a regulator of cytokinesis [10]; this indicated that Nrd1 is involved in 2 physiological processes. Nrd1 localizes to the dot-like structures in response to glucose deprivation, a situation that induces sexual differentiation [25], in addition to the responses to various environmental stimuli as we have shown in this study. These results suggest that the localization of Nrd1 to SGs is intimately connected to its regulation.

Role of MAPK-mediated phosphorylation and Cpc2 in Nrd1-mediated SG formation

What is the physiological significance of Nrd1 localization to SGs? In our previous study, we found that Nrd1 binds to its target mRNAs in its unphosphorylated form and that Nrd1DD, the phosphorylation mimic version of Nrd1 failed to bind and stabilize Cdc4; one of the target mRNAs of Nrd1; this suggested that Pmk1 MAPK phosphorylation negatively regulates Nrd1 activity [10]. In this study, we showed that, compared to unphosphorylatable Nrd1, Nrd1DD translocates to the cytoplasmic granules more rapidly and is more prone to aggregate. It should be noted, however, that even unphosphorylatable Nrd1DD translocated to the RNA granules, although much later than Nrd1DD. Thus, the phosphorylation of Nrd1 by MAPK is not itself essential for Nrd1 localization to granules; instead, it may enhance the characteristics of Nrd1 to self-aggregate or translocate into the granules in response to stress. Recently, Arimoto et al. identified RACK1 as a binding partner for MEKK4 (MTK1) and showed the role of RACK in MTK1 activation [31]. Interestingly, stress treatments that caused the formation of SGs resulted in the association of RACK1, but not MTK1, with the granules; this indicated that stress treatment causes sequestration of RACK1 into granules, thereby preventing the activation of MTK1. In this study, we showed that the various stresses that triggered SG formation also stimulated Nrd1 phosphorylation. When Nrd1 was phosphorylated, its RNA-binding activity was reduced and Nrd1 was strongly bound to Cpc2. Environmental stress promotes sequestration of the Nrd1/Cpc2 complex into SGs, which may serve as a platform for the nucleation of Pabp-positive RNA granules. After the stress was resolved, Nrd1 restarted binding and stabilizing target mRNAs required for cytokinesis, and the absence of Nrd1 will affect recovery from stresses. Thus, it would be intriguing to speculate that the phosphorylation-dependent sequestration of Nrd1 to SGs might represent a mechanism to suppress the ability of Nrd1 to bind and stabilize target mRNAs such as Cdc4.

In conclusion, this is the first study to demonstrate the role of Nrd1 in SG assembly and stress tolerance and its control by MAPK and the RACK homologue in fission yeast. Given the remarkable conservation of MAPK and RACK, a similar mechanism may regulate SG formation and stress tolerance in other eukaryotes. Further functional and molecular characterization of Nrd1 function may help in gaining an understanding of how eukaryotic cells integrate signaling information to regulate the mitotic cycle and differentiation.

Supporting Information

Figure S1 Nrd1 is involved in stress-induced granule assembly independent of the phosphorylation of eIF2α.

Wild-type, eIF2α-s432A, or Agn28kn1/Ahn2 cells expressing GFP-tagged Nrd1 were grown in YES medium at 27°C (untreated) and were subjected to a 5- or 10-min incubation at 42°C (42°C 5 min or 10 min) or 120-min incubation to 2.0 mM arsenite at 27°C (2.0 mM arsenite 120 min). Bar, 10 μm. (TIF)
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References

1. Gull S, Long JC, Caceres JF (2006) mRNAs: mRNAs relocalization to the stress granules reflects a role in the stress response. Mol Cell Biol 26: 5754–5760.
2. Anderson P, Kedersha N (2009) Stress granules.Curr Cell Biol 19: R397–R398. S0960-9822(09)00315-3 [pii];10.1016/j.cub.2009.03.013 [doi].
3. Anderson P, Kedersha N (2006) RNA granules. J Cell Biol 172: 893–903. jcb.200512082 [pii];10.1083/jcb.200512082 [doi].
4. Kedersha N, Anderson P (2002) Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. Biochem Soc Trans 30: 963–969. 10.1042/ bst030963 [doi].
5. Kedersha NL, Gupta M, Li W, Miller I, Anderson P (1999) RNA-binding protein Nrd1 and Pmk1 mitogen-activated protein kinase in the regulation of mRNA stability and translatability. Mol Biol Cell 10: 1324–1332. mbc.9811035 [pii];10.1091/mbc.10.10.1324 [doi].
6. Toda T, Dhut S, Superti FG, Gotoh Y, Nishida E, et al. (1996) The fission yeast gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. Mol Cell Biol 16: 6752–6764.
7. P-values and fold changes were calculated using the Student’s t-test. A significant p-level of 0.05 was considered. 8. Kedersha NL, Anderson P (2009) Stress granules. Curr Biol 19: R397–R398. S0960-9822(09)00315-3 [pii];10.1016/j.cub.2009.03.013 [doi].
9. Toda T, Dhut S, Superti FG, Gotoh Y, Nishida E, et al. (1996) The fission yeast gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. Mol Cell Biol 16: 6752–6764.
10. Bahlé J, Wu JQ, Longtime MS, Shah NG, McKenzie A, III, et al. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast 14: 943–951.
11. MaudeRR K (1999) mntf of fission yeast. A highly transcribed gene completely repressed by thiamine. J Biol Chem 263: 10857–10860.