Involvement of RNA granule proteins in meiotic silencing by unpaired DNA

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Abstract

In Neurospora crassa, expression from an unpaired gene is suppressed by a mechanism known as meiotic silencing by unpaired DNA (MSUD). MSUD utilizes common RNA interference (RNAi) factors to silence target mRNAs. Here, we report that Neurospora CAR-1 and CGH-1, homologs of two Caenorhabditis elegans RNA granule components, are involved in MSUD. These fungal proteins are found in the perinuclear region and P-bodies, much like their worm counterparts. They interact with components of the meiotic silencing complex (MSC), including the SMS-2 Argonaute. This is the first time MSUD has been linked to RNA granule proteins.

Keywords: meiotic silencing by unpaired DNA (MSUD); Neurospora crassa; P-bodies; RNA granules; RNA interference (RNAi)

Introduction

Neurospora crassa grows as an interconnected network of tubular cells (hyphae). Because the cross walls between individual cells are usually incomplete, viruses and transposons can potentially infiltrate the entire fungal colony. To defend against these invasive elements, Neurospora maintains several genome surveillance systems (Aramayo and Selker 2013; Gladyshev 2017). One example is known as meiotic silencing by unpaired DNA (MSUD), an RNA interference (RNAi) mechanism that targets unpaired genes for silencing during sexual development (Shiu et al. 2001; Hammond 2017). This process begins at meiotic prophase I, when a gene without a pairing partner is detected (presumably with the help of the SAD-6 homolog search protein; Samarajeewa et al. 2014). A single-stranded aberrant RNA (aRNA) is then produced from the unpaired region and exported out of the nucleus. In the perinuclear region, the aRNA is processed by the meiotic silencing complex (MSC), which contains several RNAi-related proteins (Decker et al. 2015). The SAD-1 RNA-directed RNA polymerase (RdRP), with the help of the SAD-3 helicase, converts the aRNA into a double-stranded RNA (dsRNA; Shiu and Metzenberg 2002; Hammond et al. 2011a). The DCL-1 Dicer cuts the dsRNA into small interfering RNAs (siRNAs), which are then made into single strands by the QIP exonuclease (Alexander et al. 2008; Xiao et al. 2010). The single-stranded siRNAs subsequently guide the SMS-2 Argonaute to target complementary mRNAs bound by nuclear cap-binding proteins NCBP1/2/3 (Lee et al. 2003; Decker et al. 2017; Boone et al. 2020). The SAD-2 scaffold protein functions to anchor many of the aforementioned factors to the perinuclear region (Shiu et al. 2006, Decker et al. 2015). SAD-4 and SAD-5 are required for siRNA production, although their precise roles in the pathway are currently unclear (Hammond et al. 2013a, 2013b). Finally, SAD-7 may coordinate nuclear and extranuclear silencing events (Samarajeewa et al. 2017).

Made up of RNA-protein aggregates, processing bodies (P-bodies) are cytoplasmic granules associated with the regulation of RNA translation, storage, and degradation (Jain and Parker 2013; Corbet and Parker 2019). Although P-bodies are enriched in proteins involved in translation repression and mRNA decay, the exact role of these structures remains to be determined (Luo et al. 2018; Tibble et al. 2021). In mammals, it has been suggested that mRNA decay may not take place inside P-bodies (Standart and Weil 2018), i.e., P-bodies may function primarily to store mRNAs for later translation or decay (Riggs et al. 2020; Borbolis and Syntichaki 2021). In addition to the above, P-bodies have also been associated with RNAi (Jakymiw et al. 2007; Standart and Weil 2018). In this investigation, we explored the possibility that P-bodies are present during sexual development in Neurospora and asked whether some of their components are involved in MSUD.

Materials and methods

Fungal methods and genotypic information

Standard fungal protocols were followed throughout this study (http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm). Genotypes of Neurospora strains used are provided in Table 1.
Quantification of sexual spore production

Fluffy (fl) strains were grown for six days in 24-well microplates (Corning #3524) at room temperature for use as designated females. Conidia (asexual spores) from each male strain were suspended in sterile water and adjusted to a concentration of 1000 counts/µl. For fertilization, 50 µl conidial suspension was inoculated on the female strain within a well. Ascospores (sexual spores) were collected from the lids 21 days post-fertilization and counted on a hemocytometer.

MSUD assays

Most assessments of MSUD proficiency were performed according to the method of Xiao et al. (2019), with crosses conducted in 24-well microplates and analyses based on shot ascospores. For cgh-1-null crosses, sexual development is too impaired for proficient spore shooting. Accordingly, spores from these crosses were extracted out of the fruiting bodies for progeny phenotyping.

Strain construction and confirmation

Green fluorescent protein (GFP) and mCherry tagging vectors were constructed using double-joint polymerase chain reaction (DJ-PCR; Hammond et al. 2011b; Samarajeewa et al. 2014). For strain confirmation, genomic DNA was isolated from conidia (Henderson et al. 2005) or vegetative hyphae (Qiagen DNeasy Plant Mini Kit). PCR-based validation of genotypes was conducted using the Promega GoTaq Green Master Mix or the Roche Expand Long Range dNTPack. When necessary, DNA sequencing was conducted by the University of Missouri (MU) DNA Core. Primers for strain construction and confirmation are listed in Supplementary Table S1.

Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) is an in vivo assay to detect protein–protein interaction. In BiFC, a functional fluorophore is restored when two halves of the yellow fluorescent protein (YFP) are brought together by the association of two interacting proteins (Hu et al. 2002; Bardiya et al. 2008). Tagging of YFP halves was performed as previously reported (Hammond et al. 2011b).

Photography and microscopy methods

Light microscopy images of protoperithecia (female structures), perithecia (fruiting bodies), and ascii (spore sacs) were captured according to the methods of Decker et al. (2017). For fluorescent microscopy, a Leica TCS SP8 system was used. Preparation and visualization of asci were essentially as described (Alexander et al. 2008; Xiao et al. 2010).

Image analysis of P-bodies

Images of asci in the single-nucleus (diploid) stage were analyzed with Fiji v2.0.0-rc-69-1.52p (Schindelin et al. 2012). The mCherry-DCAP-2 signal was used as a marker for P-bodies. After using the “Set Scale” function to input the pixel/µm ratio for an image, the number and average size (area) of P-bodies were obtained with the “Analyze Particles” function. For each cross, 8–24 ascis were analyzed. The P-values were calculated using the two-tailed Student’s t-test.

Results

Presence of P-bodies in Neurospora meiotic cells

We asked if P-bodies are present during the sexual phase of Neurospora. The DCAP-2 decapping enzyme is often used as a

### Table 1 Neurospora strains used in this study

| Strain | Genotype |
|--------|----------|
| F2-01  | fl A (FGSC 4317) |
| F2-29  | rA::hph; fl A |
| F5-36  | fl; sad-S::hph A |
| F7-18  | cgh-1::hph A |
| F8-01  | car-1::hph fl A |
| F8-10  | rid; fl, mCherry-DCAP-2::hph; mus-51::bar A |
| F8-11  | fl, mCherry-DCAP-2::hph; mus-51::bar, sad-5::hph A |
| F8-01  | fl, mCherry-DCAP-2::hph; mus-51::bar, cgh-1::hph A |
| P3-25  | mep sad-1::hph A |
| P9-42  | Oak Ridge wild type (WT) A (FGSC 2490) |
| P11-67 | cgh-1::hph A |
| P17-70 | rA::hph; sad-5::hph A |
| P24-40 | rid; mCherry-DCAP-2::hph; mus-51::bar, gfp-cgh-1::hph A |
| P24-41 | rid his-3; mCherry-DCAP-2::hph, gfp-cgh-1::hph A |
| P24-42 | gfp-car-1::hph; mCherry-DCAP-2::hph, mus-51::bar A |
| P24-43 | rid his-3; gfp-car-1::hph; mCherry-DCAP-2::hph, mus-51::bar A |
| P24-64 | car-1::hph A |
| P25-19 | rid; gfp-car-1::hph; mCherry-cgh-1::nat A |
| P25-20 | rid his-3; gfp-car-1::hph; mCherry-cgh-1::nat A |
| P25-21 | rid; yfpc-car-1::hph; yfpc-1::hph; mus-51::bar A |
| P25-22 | rid; yfpc-car-1::hph; yfpc-nsm-2::hph A |
| P25-25 | rid; yfpc-car-1::hph; yfpc-nms-2::hph A |
| P25-28 | rid; yfpc-car-1::hph A |
| P25-29 | rid; yfpc-nsm-2::hph yfpc-nm-2::hph A |
| P25-30 | rid; yfpc-nms-2::hph yfpc-nm-2::hph A |
| P25-31 | rid; yfpc-car-1::hph A |
| P25-32 | rid; yfpc-car-1::hph; mus-51::bar, yfpc-sad-2::hph A |
| P25-33 | rid; yfpc-car-1::hph; mus-51::bar, yfpc-sad-2::hph A |
| P25-19 | rid his-3; mus-51::bar, yfpc-cgh-1::nat A |
| P25-20 | rid his-3; mus-51::bar, yfpc-cgh-1::nat A |
| P25-21 | rid; mus-51::bar, yfpc-dcl-1::hph; yfpc-cgh-1::nat A |
| P25-22 | rid; mus-51::bar, yfpc-dcl-1::hph; yfpc-cgh-1::nat A |
| P25-23 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-24 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-25 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-26 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-27 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-28 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-29 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-30 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-31 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-32 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-33 | rid; mus-51::bar, yfpc-cgh-1::nat A |
| P25-34 | rA::hph; yfpm-2::bar A |
| P25-35 | rA::hph; yfpm-2::bar A |
| P25-36 | mCherry-DCAP-2::hph A |
| P25-37 | rid; mCherry-DCAP-2::hph; mus-51::bar, sad-5::hph A |
| P25-38 | car-1::hph; mCherry-DCAP-2::hph A |
| P25-39 | car-1::hph; mCherry-DCAP-2::hph; mus-51::bar A |
| P25-40 | rid; mCherry-DCAP-2::hph; mus-51::bar, cgh-1::hph A |

Genetic loci are described in the Neurospora crassa e-Compendium (http://www.bioinformatics.leeds.ac.uk/~genefar/newgenelist/gene_list.htm).

Genetic markers and knockout mutants are originally from the Fungal Genetics Stock Center (FGSC; McCluskey et al. 2010) and the Neurospora Functional Genomics Group (Colot et al. 2006). Culturing and crossing media were prepared according to Vogel (1956) and Westergaard and Mitchell (1947), respectively.

Transcript analysis

For comparison of gene expression, Neurospora vegetative (SRR080688, SRR081479, SRR081546, and SRR081586) and sexual (SRR957218) RNA-seq datasets were obtained from the European Bioinformatics Institute (EBI)’s European Nucleotide Archive (ENA) (Ellison et al. 2011; Samarsjewa et al. 2014). Computational analysis of these datasets was performed as described (Decker et al. 2017).
cellular marker for P-bodies (Sheth and Parker 2003; Gallo et al. 2008). Using mCherry-DCAP-2 (NCU07889), we have demonstrated that P-bodies can be seen in the asci (Figure 1B). To our knowledge, this is the first published P-body marker for Neurospora.

CAR-1 and CGH-1 localize in the perinuclear region and P-bodies

CAR-1 (Sm-domain protein) and CGH-1 (RNA helicase) family proteins are conserved P-body components (Jain and Parker, 2013). In Caenorhabditis elegans, they are also parts of germline P granules (Updike and Strome 2010; Sundby et al. 2021). We have identified the corresponding homologs in Neurospora (NCU03366 and NCU06149) and tagged them with GFP. As seen in Figure 1, A–H, CAR-1 and CGH-1 localize in both the perinuclear region and P-bodies. This is reminiscent of the observations in worms, in which CAR-1 and CGH-1 are found in both perinuclear P granules and cytoplasmic P-bodies (Updike and Strome 2010; Jain and Parker 2013; Ko et al. 2013).

CAR-1 and CGH-1 interact in P-bodies

CAR-1 and CGH-1 family proteins are known to have direct or indirect interaction with each other (Decker and Parker 2006; Roy and Rajyaguru 2018). Because Neurospora CAR-1 and CGH-1 colocalize (Figure 1L), we tested whether they actually have physical association. Using BiFC, we have detected interaction between CAR-1 and CGH-1 in cytoplasmic P-bodies and not in the perinuclear region (Figure 2). It is possible that they do not interact in the perinuclear region at all or that their interaction there is indirect (e.g., through another protein).

Expression of car-1 and cgh-1 during both vegetative and sexual stages

To determine the expression profiles of car-1 and cgh-1, we examined their transcript levels through RNA-seq datasets. While presumed MSUD-exclusive genes have relatively low vegetative expression (as compared to their sexual expression), car-1 and cgh-1 are abundantly expressed in both developmental stages (Table 2). These results hint that the two RNA granule proteins are probably active in both sexual and asexual cycles.

car-1^D and cgh-1^D mutants are slow growers

Deletion of the car-1 or cgh-1 homolog in yeast (SCD6/DHH1) is not lethal to the fungus (Hata et al. 1998; Kolesnikova et al. 2013). While Neurospora car-1 and cgh-1 are also nonessential, their losses are associated with slower linear growth (Figure 3A).
Interestingly, deletion of cgh-1 leads to an abnormal conidiation pattern (Figure 3B), suggesting its possible involvement in asexual development.

**Loss of CAR-1 or CGH-1 impairs sexual development**

Because car-1 and cgh-1 are well expressed during sexual development, we asked if they are required for ascospore formation. Although crosses homozygous for car-1Δ or cgh-1Δ are not completely barren, they typically produce only 9 and 0.01% of the normal number of spores, respectively (Figure 4A). Examination of the mutant crosses showed that they are deficient in perithecial and ascus development (Figure 4B). These results demonstrate that normal sexual reproduction in *Neurospora* requires CAR-1 and CGH-1, similar to the case in worms (Audhya et al. 2005). In yeast, deletion of the car-1 homolog (DH1) also leads to severe mating defects (Ka et al. 2008).

**CAR-1 and CGH-1 are important for MSUD**

Because RNA granules are linked to post-transcriptional regulation (Anderson and Kedersha 2009; Tian et al. 2020), we asked whether CAR-1 and CGH-1 are required for MSUD in *Neurospora*. *Neurospora* normally produces American football-shaped spores. In an rþ/C2r− cross, the round spore gene is unpaired and silenced (Shiu et al. 2001), leading to the production of mostly round spores (or 2–4% football; Figure 5, A and B). This “silenced” phenotype could be reversed (or partially reversed) by having an MSUD gene deletion in one or both parents (Hammond et al. 2013b). When one parent of an r-unpaired cross contains a car-1Δ or cgh-1Δ allele, 20 and 9% of the progeny are of football-shaped, respectively, suggesting that the two deletion mutations act as semidominant suppressors of MSUD (Figure 5A). In crosses homozygous for car-1Δ or cgh-1Δ, the figures go up to 63 and 81%, respectively (Figure 5B). These results suggest that the two RNA granule proteins are involved in the MSUD pathway.

**CAR-1 and CGH-1 interact with components of the MSUD machinery**

The perinuclear localization of CAR-1 and CGH-1 suggests that they could be linked to MSC, the silencing complex surrounding the nucleus. Indeed, as shown by BiFC, CAR-1 and CGH-1 interact with known MSC factors (SAD-1, SAD-2, DCL-1, QIP, and SMS-2; Figure 6). These interactions are consistent with the notion that CAR-1 and CGH-1 are involved (directly or indirectly) in the meiotic silencing of target mRNAs.

**The absence of MSUD does not hinder visible P-body formation**

In mammals, siRNA-mediated silencing induces P-body assembly (Lian et al. 2007). We asked if the absence of MSUD would diminish the production of visible P-bodies in *Neurospora* ascis. SAD-5 is crucial for the production of siRNAs, and MSUD becomes nonfunctional in its absence (Hammond et al. 2013b). As seen in Figure 7, the absence of SAD-5 (and MSUD) does not reduce visible P-body formation (in terms of number and average size).

Because CAR-1 and CGH-1 are P-body factors (Jain and Parker 2013), we examined whether their absence would affect P-body formation in *Neurospora*. While the number of visible P-bodies is not obviously reduced in a cross devoid of car-1 or cgh-1, their average size is (Figure 7).

**Discussion**

In this study, we have demonstrated the presence of P-bodies during sexual development in *Neurospora*. P-body formation could be a consequence of RNA silencing (Eulalio et al. 2007b). The silencing machinery could promote mRNA degradation and trigger

### Table 2 Expression of RNA silencing genes

| RNA granules         | Gene no. | Vegetative expression (FPKM) | Sexual expression (FPKM) |
|----------------------|----------|-----------------------------|--------------------------|
| car-1                | ncu03366 | 98.7509                     | 65.0277                  |
| cgh-1                | ncu06149 | 34.2955                     | 72.2677                  |
| Housekeeping         |          |                             |                          |
| actin                | ncu04173 | 2638.3425                   | 905.4051                 |
| MSUD                 |          |                             |                          |
| sad-1                | ncu02178 | 0.3684                      | 14.4495                  |
| sad-2                | ncu04294 | 0.0000                      | 38.5137                  |
| sad-5                | ncu06147 | 0.0000                      | 13.2599                  |
| sms-2                | ncu09434 | 0.0496                      | 673.0190                 |
| MSUD/Quelling        |          |                             |                          |
| dcl-1                | ncu08270 | 4.4300                      | 31.0978                  |
| qip                  | ncu00076 | 18.6841                     | 107.2514                 |

Quelling and MSUD refer to the vegetative and sexual silencing systems in *Neurospora*, respectively (Gladyshev 2017). FPKM, fragments per kilobase of exon per million mapped reads.
the assembly of decay mRNA-protein complexes that aggregate into P-bodies (Anderson and Kedersha 2009). We did not observe a reduced P-body production in SAD-5’s absence, which affects siRNA biogenesis during MSUD. If MSUD indeed involves P-bodies, one possibility is that visible P-body formation can be saturated by other pathways in MSUD’s absence. Alternatively, MSUD may mainly involve submicroscopic ribonucleoprotein complexes (Leung and Sharp 2013). Aggregation into a visible entity may not confer additional advantages and is often not essential for RNA granule functions (Anderson and Kedersha 2009; Thomas et al. 2011; Leung and Sharp 2013).

CAR-1 and CGH-1 are involved in MSUD. The absence of either protein markedly reduces the severity of meiotic silencing. CAR-1 and CGH-1 are found in cytoplasmic P-bodies as well as the perinuclear region (the center of MSUD activity). The latter localization brings to mind the case in C. elegans, in which the two proteins are components of perinuclear P granules (germline structures important for post-transcriptional regulation; Sundby et al. 2021). The fact that RNA surveillance often happens in the perinuclear region is hardly a coincidence (also see examples in mammals and flies; Meikar et al. 2011; Kloc et al. 2014). It could provide an environment in which exported RNAs can meet up with their developmental regulators and have their fates effectively controlled (Voronina 2013; Decker et al. 2015).

Using BiFC, we have shown that CGH-1 has interaction with the SMS-2 Argonaute. This is similar to the situation in human cells, in which a CGH-1 homolog (RCK) interacts with Argonaute proteins (to mediate translation repression; Chu and Rana 2006). It is possible that certain complex formations are conserved among different post-transcriptional networks. Interestingly, CAR-1 has also been shown to interact with the SMS-2 Argonaute in this study.

Although many components of the meiotic silencing machinery have been revealed, the final fates of MSUD-targeted mRNAs remain unclear. Our current model proposes that the SMS-2 Argonaute uses siRNAs to guide the slicing of complementary mRNAs (presumably in the perinuclear region). It is conceivable that targeted mRNA slicing is not 100% efficient and that unsliced (and possibly sliced) mRNAs are associated with certain RNA granule proteins for degradation, repression, and/or storage. CAR-1 and CGH-1 family proteins are presumed to be translation repressors and/or decapping activators (Eulalio et al. 2007a; Jain and Parker 2013; Zeidan et al. 2018). In the absence of CAR-1 or CGH-1, unsliced mRNAs could be inadvertently released for translation, allowing some expression of unpaired genes.
Figure 5 car-1 and cgh-1 are involved in MSUD. In Neurospora, a normal cross yields American football-shaped ascospores. Here, crosses heterozygous for r+ were tested. In an MSUD-proficient background, an unpaired r+ is silenced and nearly all of the progeny are round (with 2–4% football; crosses 1 and 5). (A) However, if one parent of an r-unpaired cross is car-1D or cgh-1D, the percentage of normal progeny goes up noticeably (with 20 and 9% football; crosses 2 and 3, respectively), suggesting that MSUD is impaired. (B) If both parents are car-1D or cgh-1D, progeny are predominantly normal (with 63 and 81% football; crosses 6 and 7, respectively). Also shown here are results for sad-1D and sad-5D (with 100 and 97% football; crosses 4 and 8, respectively), two standard MSUD suppressors used as positive controls (Hammond et al. 2013b). (1) F2-29/C2 P9-42. (2) F2-29/C2 P24-64. (3) F2-29 × P11-67. (4) F2-29 × P3-25. (5) F2-29 × P9-42. (6) F8-01 × P26-34. (7) F7-18 × P26-32. (8) F5-36 × P17-70.

Figure 6 CAR-1 and CGH-1 interact with SAD-1 (RdRP), SAD-2 (scaffold protein), DCL-1 (Dicer), QIP (exonuclease), and SMS-2 (Argonaute). Micrographs illustrate prophase asci expressing mCherry-dcap-2 in an MSUD-proficient cross (1, F8-10 × P26-36), a car-1-null cross (2, P27-18 × P27-19), a cgh-1-null cross (3, F9-01 × P27-20), and a sad-5-null cross (4, F8-11 × P26-37). Bar, 5 μm. (B) When compared to the control (+), none of the mutant crosses have a substantially lower P-body count. (C) The average size of P-bodies is markedly lower in a car-1D or cgh-1D background. For + versus mutant, * indicates P < 0.05 and *** indicates P < 0.001.

Figure 7 Visible P-body formation in various asci. (A) Micrographs illustrate prophase asci expressing mCherry-dcap-2 in an MSUD-proficient cross (1, F8-10 × P26-36), a car-1-null cross (2, P27-18 × P27-19), a cgh-1-null cross (3, F9-01 × P27-20), and a sad-5-null cross (4, F8-11 × P26-37). (B) When compared to the control (+), none of the mutant crosses have a substantially lower P-body count. (C) The average size of P-bodies is markedly lower in a car-1D or cgh-1D background. For + versus mutant, * indicates P < 0.05 and *** indicates P < 0.001.
Although the above hypothesis could explain our observations thus far, other possibilities abound. For example, CAR-1 and CGH-1 could enhance the activity of the SMS-2 Argonaute and/or other MSUD factors (i.e., their usual P-body functions are not involved in MSUD). Future work on these and other RNA granule components will shed light on how they regulate gene expression and diverse processes.

Data availability

Strains are available upon request. Supplementary material is available at figshare: https://doi.org/10.25387/g3.14575689.

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Conflicts of interest

None declared.

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