Complex Formation of RNA Silencing Proteins in the Perinuclear Region of *Neurospora crassa*

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ABSTRACT In *Neurospora*, genes not paired during meiosis are targeted by meiotic silencing by unpaired DNA (MSUD). Here, our bimolecular fluorescence complementation (BiFC) study suggests that RNA-directed RNA polymerase, Dicer, Argonaute, and others form a silencing complex in the perinuclear region, with intimate interactions among the majority of them. We have also shown that SAD-2 is likely the anchor for this assembly.

KEYWORDS RNA interference; *Neurospora crassa*; meiotic silencing by unpaired DNA (MSUD); bimolecular fluorescence complementation (BiFC); fluorescent protein tagging

THE filamentous fungus *Neurospora crassa* consists of a hyphal network where nuclei and other cellular components share a common cytoplasm. This trait, while beneficial for distributing resources, may promote the spread of detrimental elements such as transposons and viruses. Perhaps for this reason, *Neurospora* possesses several surveillance mechanisms that operate during different phases of its life cycle (Aramayo and Selker 2013; Billmyre *et al.* 2013; Nicolás and Ruiz-Vázquez 2013). For example, quelling silences tandem transgenes during vegetative growth (Romano and Macino 1992). Repeat-induced point mutation (RIP), a premeiotic system functioning before nuclear fusion, introduces C → T mutations to duplicated DNA sequences (Cambareri *et al.* 1989). Finally, meiotic silencing by unpaired DNA (MSUD) targets genes not paired with a homologous partner during meiosis (Shiu *et al.* 2001). MSUD begins inside the nucleus when an unpaired gene is detected (Samarajeewa *et al.* 2014) and acts as a template for the production of an aberrant RNA (aRNA; abnormal RNA that would enter the silencing pathway). A working model for MSUD holds that the aRNA is exported to the perinuclear region, where it is met by a host of RNAi-related proteins, including SAD-1, an RNA-directed RNA polymerase (RdRP) that converts aRNAs into double-strand RNAs (dsRNAs) (Shiu and Metzenberg 2002); SAD-3, a helicase thought to increase SAD-1's processivity on RNA templates (Hammond *et al.* 2011a); DCL-1, an RNAse III Dicer that cuts dsRNAs into small interfering RNAs (siRNAs) (Alexander *et al.* 2008); QIP, an exonuclease that converts siRNAs into single strands (Xiao *et al.* 2010); and SMS-2, an Argonaute protein that uses siRNAs to guide selective destruction of homologous mRNAs (Lee *et al.* 2003). SAD-2, unlike the others, is not found in other RNA silencing systems but is essential for the perinuclear localization of SAD-1 (Shiu *et al.* 2006).

Understanding how proteins interact is a key aspect to revealing how complex cellular processes, like MSUD, function at the molecular level. Numerous methods exist to study protein–protein interactions, *e.g.*, the yeast two-hybrid system (Y2H), co-immunoprecipitation (Co-IP), fluorescence resonance energy transfer (FRET), and bimolecular fluorescence complementation (BiFC) (Syafrizayanti *et al.* 2014). BiFC is capable of revealing the location of direct protein interactions *in vivo* (Hu *et al.* 2002). It is based on the reconstitution of a fluorescent protein when its nonfluorescing halves are brought within close proximity to one another by the association of two interacting proteins. Previously, we adopted the BiFC technology for use in *Neurospora* (Bardiya *et al.* 2008; Hammond *et al.* 2011a,b). In this study, we set out to define the precise

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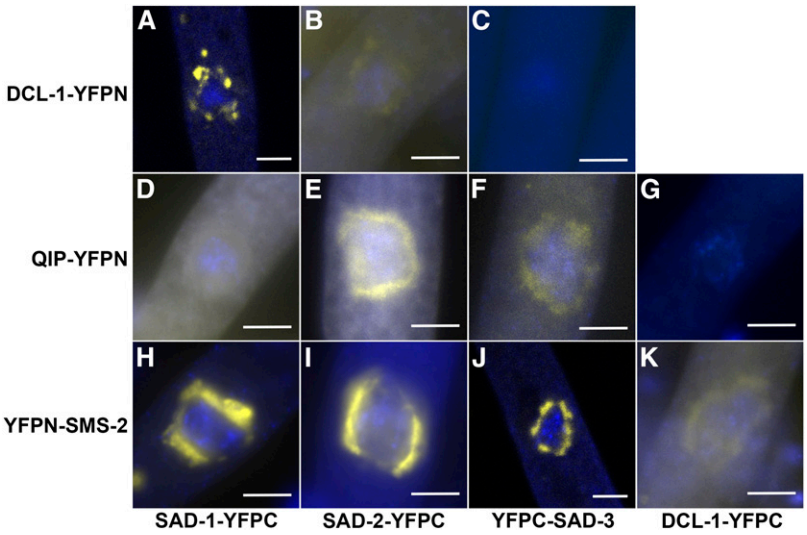

Figure 1 Assay of interactions among MSUD proteins using BiFC. As demonstrated by the reconstituted YFP signal in prophase asci (spore sacs), the six MSUD proteins are closely associated with each other with the exception of DCL-1, which has intimate interaction with only SAD-1, SAD-2, and SMS-2 (and not SAD-3 or QIP; see C and G). The BiFC temporal expression patterns are similar to those observed in individual MSUD proteins. Construction of YFP tagging vectors was as described by Bardiya *et al.* (2008) and Hammond *et al.* (2011b). Preparation of asci from perithecia (fruiting bodies) and visualization of YFP/DAPI (using Olympus BX61 and Zeiss LSM710) were performed as previously reported (Alexander *et al.* 2008; Xiao *et al.* 2010; Hammond *et al.* 2011a). (A) P7-26 × P13-34. (B) P18-29 × P18-52. (C) P21-18 × P21-19. (D) P22-05 × P22-06. (E) P16-37 × P16-38. (F) P21-20 × P21-21. (G) P18-37 × P18-39. (H) P18-19 × P18-21. (I) P16-35 × P16-36. (J) P15-37 × P16-06. (K) P22-03 × P22-04. See Table 2 for full genotypes. Bars, 5 μm.

interactions among six MSUD proteins formerly shown to localize at the nuclear periphery, a presumptive center for silencing activity. In addition, we explored the role of SAD-2 in the complex formation of these MSUD factors.

Physical Associations Among MSUD Factors

In this work, N-terminal (YFPN) or C-terminal (YFPC) fragments of the yellow fluorescent protein (YFP) were linked to six individual MSUD proteins, and each possible interaction was examined. Our results, as shown in Figure 1 and Table 1, indicate close associations among various perinuclear MSUD proteins. One noticeable exception is DCL-1, which appears to show affinity for SAD-1, SAD-2, and SMS-2 only.

The six proteins examined include those that are common in many organisms (SAD-1, SAD-3, DCL-1, and SMS-2) and those that are seemingly found only in fungi (QIP and SAD-2). These proteins are localized in the perinuclear region regardless of the presence/absence of artificially unpaired genes (Xiao *et al.* 2010; Hammond *et al.* 2011a,b). QIP was first isolated as a QDE-2-interacting protein (Maiti *et al.* 2007). Since QDE-2 is the Argonaute responsible for vegetative silencing, it is not surprising that QIP also interacts with the meiotic phase Argonaute (SMS-2). The fact that DCL-1 and QIP are important for both vegetative and sexual silencing (Alexander *et al.* 2008; Xiao *et al.* 2010) suggests that there is a cross-talk between the two genome surveillance systems.

In yeast's RNAi-mediated heterochromatin formation, the model for silencing complex assembly involves an RdRP-helicase-Argonaute association (Motamedi *et al.* 2004). These three proteins (SAD-1-SAD-3-SMS-2) also have interactions in *Neurospora*, suggesting that the complex formations in MSUD and RNAi-induced heterochromatin assembly may be evolutionarily related. The interaction between SAD-1 RdRP and DCL-1 Dicer is also not surprising, as the latter functions to process dsRNAs produced by the former. This finding is consistent with those observed in other organisms, in which the two common RNAi proteins conglomerate (Duchaine *et al.* 2006; Lee and Collins 2007). The affinity between these two proteins may be of special importance, as their physical association actually stimulates Dicer activity on dsRNA substrates in *Tetrahymena* (Lee and Collins 2007). Finally, the observed interaction between Dicer and Argonaute is not unexpected (Dueck and Meister 2014), as the small RNA has to be transferred from the site of origin (DCL-1 in this case) to the effector protein (SMS-2).

Table 1 Matrix examination of BiFC interactions among MSUD proteins

	SAD-1-YFPC	SAD-2-YFPC	YFPC-SAD-3	DCL-1-YFPC	QIP-YFPC	YFPC-SMS-2
SAD-1-YFPN						
SAD-2-YFPN	+[a]					
YFPN-SAD-3	+[b]	+[b]				
DCL-1-YFPN	+ (A)	+ (B)	− (C)			
QIP-YFPN	+ (D)	+ (E)	+ (F)	− (G)		
YFPN-SMS-2	+ (H)	+ (I)	+ (J)	+ (K)	+[c]	

A positive interaction between two proteins (*i.e.*, one that leads to the reconstitution of the yellow fluorophore) is denoted by +. A negative result (−) may indicate that either the two proteins do not have intimate interaction or the BiFC assay is not sensitive enough to detect their weak/transient interaction. A–K in parentheses refer to Figure 1, A–K.

[a] Bardiya *et al.* (2008).

[b] Hammond *et al.* (2011a).

[c] Hammond *et al.* (2011b).

Table 2 *Neurospora* strains used in this study

Strain	Genotype
F2-23	*rid; fl A*
F4-30	*sad-3-gfp::hph rid; fl; mus-52$^{\Delta}$::bar a*
F5-06	*fl; gfp-sms-2::hph a*
F6-30	*rid his-3; fl; sad-2$^{\Delta}$::hph; gfp-sms-2::hph A*
F6-31	*sad-3-gfp::hph rid his-3; fl; sad-2$^{\Delta}$::hph A*
P7-26	*sad-1$^{\Delta}$::hph rid his-3^{+}::sad-1-yfpc a*
P13-14	*sad-1-gfp::hph; mus-51$^{\Delta}$::bar a*
P13-15	*sad-1-gfp::hph A*
P13-34	*rid his-3^{+}::dcl-1-yfpn; mus-52$^{\Delta}$::bar; dcl-1$^{\Delta}$::hph A*
P14-59	*sad-3-gfp::hph rid his-3 A*
P15-14	*rid his-3; mus-52$^{\Delta}$::bar; gfp-sms-2::hph A*
P15-37	*rid; yfpn-sms-2::hph A*
P15-62	*rid; mus-52$^{\Delta}$::bar qip-gfp::hph a*
P15-63	*rid his-3; mus-52$^{\Delta}$::bar qip-gfp::hph A*
P16-06	*yfpc-sad-3::hph rid; sms-2-yfpn::hph a*
P16-35	*rid his-3^{+}::sad-2-yfpc; inv sad-2^{RIP}; yfpn-sms-2::hph A*
P16-36	*rid his-3^{+}::sad-2-yfpc; inv sad-2^{RIP}; yfpn-sms-2::hph a*
P16-37	*rid his-3^{+}::sad-2-yfpc; qip-yfpn::hph; inv sad-2^{RIP} a*
P16-38	*rid his-3^{+}::sad-2-yfpc; qip-yfpn::hph; inv sad-2^{RIP} A*
P18-19	*rid his-3^{+}::sad-1-yfpc; yfpn-sms-2::hph A*
P18-21	*sad-1$^{\Delta}$::hph rid his-3^{+}::sad-1-yfpc; yfpn-sms-2::hph a*
P18-29	*rid his-3^{+}::sad-2-yfpc; mus-51$^{\Delta}$::bar dcl-1-yfpn::hph; inv sad-2^{RIP} a*
P18-37	*rid his-3; qip-yfpn::hph; dcl-1-yfpc::hph A*
P18-39	*rid; qip-yfpn::hph a*
P18-52	*rid his-3^{+}::sad-2-yfpc; mus-51$^{\Delta}$::bar dcl-1-yfpn::hph; inv sad-2^{RIP} A*
P21-18	*yfpc-sad-3::hph rid; mus-52$^{\Delta}$::bar a*
P21-19	*yfpc-sad-3::hph rid his-3^{+}::dcl-1-yfpn; mus-52$^{\Delta}$::bar A*
P21-20	*yfpc-sad-3::hph rid his-3; mus-52$^{\Delta}$::bar qip-yfpn::hph a*
P21-21	*yfpc-sad-3::hph rid his-3; qip-yfpn::hph A*
P21-26	*rid his-3; mus-52$^{\Delta}$::bar qip-gfp::hph; sad-2$^{\Delta}$::hph a*
P21-27	*rid his-3; mus-52$^{\Delta}$::bar qip-gfp::hph; sad-2$^{\Delta}$::hph A*
P21-28	*rid his-3; sad-2$^{\Delta}$::hph; gfp-sms-2::hph a*
P21-30	*rid his-3; mus-51$^{\Delta}$::bar gfp-dcl-1::hph; sad-2$^{\Delta}$::hph A*
P21-31	*rid his-3; mus-51$^{\Delta}$::bar gfp-dcl-1::hph; sad-2$^{\Delta}$::hph a*
P21-32	*sad-3-gfp::hph rid his-3; sad-2$^{\Delta}$::hph a*
P21-33	*sad-3$^{\Delta}$::hph rid A*
P21-34	*sad-3$^{\Delta}$::hph; sad-2-gfp::hph a*
P21-35	*sad-1-gfp::hph sad-3$^{\Delta}$::hph A*
P21-36	*sad-1-gfp::hph sad-3$^{\Delta}$::hph a*
P21-37	*sad-1-gfp::hph rid his-3; sad-2$^{\Delta}$::hph a*
P21-38	*sad-1-gfp::hph; sad-2$^{\Delta}$::hph A*
P21-39	*rid his-3; mus-51$^{\Delta}$::bar gfp-dcl-1::hph A*
P21-40	*rid; mus-51$^{\Delta}$::bar gfp-dcl-1::hph a*
P21-41	*rid; sad-2-gfp::hph a*
P22-03	*rid; dcl-1-yfpc::hph; yfpn-sms-2::hph a*
P22-04	*rid his-3; dcl-1-yfpc::hph; yfpn-sms-2::hph A*
P22-05	*sad-1$^{\Delta}$::hph rid his-3^{+}::sad-1-yfpc; qip-yfpn::hph a*
P22-06	*rid his-3^{+}::sad-1-yfpc; qip-yfpn::hph A*

Genetic markers and knockout mutants are originated from the Fungal Genetics Stock Center (FGSC; Mccluskey *et al.* 2010) and the Neurospora Functional Genomics Group (Colot *et al.* 2006), and their descriptions can be found in the e-Compendium (http://www.bioinformatics.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm). Strains used in this study were constructed essentially as described (Bardiya *et al.* 2008; Hammond *et al.* 2011b). Fungal manipulations were performed as described in the *Neurospora* protocol guide (http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm).

SAD-2 Is Required for the Localization of Perinuclear Silencing Proteins

SAD-2 appears to be restricted to the fungal kingdom, and it has not been shown to mediate RNAi other than in *Neurospora*. SAD-2 is absolutely required for the silencing of all unpaired

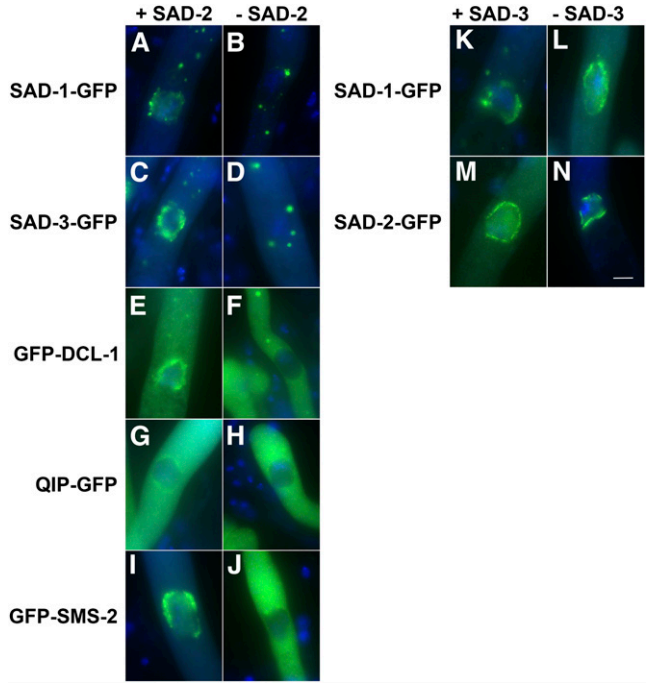

Figure 2 SAD-2 is required for the localization of other perinuclear MSUD proteins. (A–J) In the absence of SAD-2, other MSUD proteins lose their affinity for the nuclear periphery. (K–N) As with SAD-1 (Shiu *et al.* 2006), SAD-3 does not appear to affect the localization of others. GFP constructs were made according to Hammond *et al.* (2011b). (A) P13-14 × P13-15. (B) P21-37 × P21-38. (C) F4-30 × P14-59. (D) F6-31 × P21-32. (E) P21-39 × P21-40. (F) P21-30 × P21-31. (G) P15-62 × P15-63. (H) P21-26 × P21-27. (I) F5-06 × P15-14. (J) F6-30 × P21-28. (K) P13-14 × P13-15. (L) P21-35 × P21-36. (M) F2-23 × P21-41. (N) P21-33 × P21-34. Bar, 5 μm. [Note that a perinuclear MSUD protein first emerges as aggregates around the nuclei at the binuclear stage (Shiu *et al.* 2006). These aggregates coalesce after karyogamy and eventually become two opposite crescents during leptotene. The crescents spread into a ring-like structure during zygotene and pachytene. The ring becomes more patchy and irregular during the diffuse stage, and the perinuclear localization can no longer be seen after diplotene. A perinuclear MSUD protein may also appear as cytoplasmic foci between karyogamy and metaphase I. The exact nature of these foci is still unknown, although they have been speculated as some kind of endomembrane body.]

markers tested, suggesting its importance in the MSUD pathway (Shiu *et al.* 2006). It was first identified as a silencing protein required for the proper localization of SAD-1. Without SAD-2, SAD-1 RdRP mislocalizes in the cytoplasm. In this study, we asked whether SAD-2 is also crucial for the localization of SAD-3, DCL-1, QIP, and SMS-2. As shown in Figure 2, A–J, their affinity for the perinuclear region is also hampered by the absence of SAD-2.

Although SAD-2 is required for the localization of other perinuclear MSUD proteins, the reverse is not true. In the absence of SAD-1 (Shiu *et al.* 2006) or SAD-3 (Figure 2, K–N), SAD-2 localizes normally (we were not able to perform the same assay for DCL-1, QIP, or SMS-2, as their presence is required for ascus formation). Since (1) SAD-2 has close interaction with all other perinuclear MSUD factors and (2) it is required for their localization (but not *vice versa*), it is possible that it functions as a scaffold protein that unites silencing

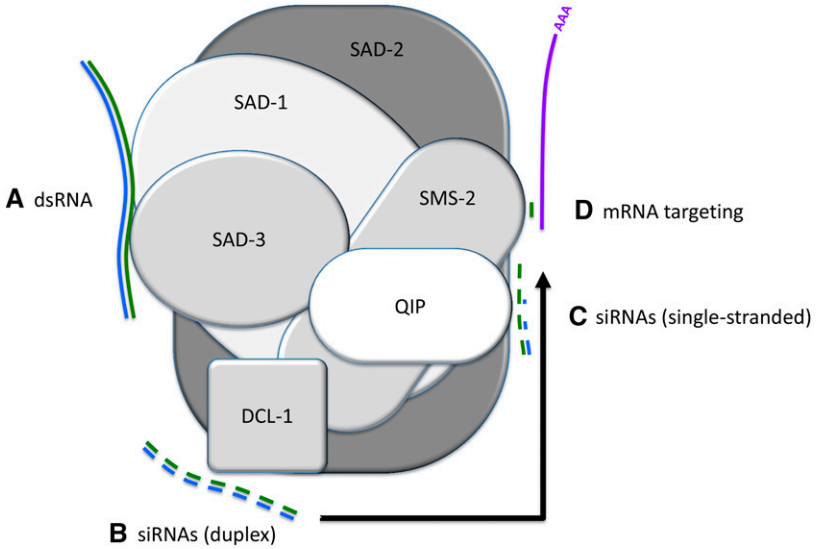

Figure 3 A model for the reactions performed by the meiotic silencing complex (MSC). (A) Exported aberrant RNA (blue) is turned into double strands by SAD-1 RdRP and SAD-3 helicase. (B) DCL-1 Dicer processes the dsRNA into siRNAs, (C) which (with the help of QIP exonuclease) become single-stranded (green) and (D) are utilized by SMS-2 Argonaute to target homologous mRNAs (purple). SAD-2, a presumptive scaffold protein, holds these silencing factors in the perinuclear region.

factors at the nuclear periphery. A working model for the MSUD activity is shown in Figure 3.

Concluding Remarks

Our results show that the aforementioned MSUD components form a silencing complex in the perinuclear region (the "surveillance checkpoint") during prophase I of meiosis. In addition, we have established the role of SAD-2 as the likely scaffold protein for this complex. In higher eukaryotes, the perinuclear region in germ cells contains an organelle known as chromatoid body in mammals, nuage in flies, and P-granules in worms (Meikar *et al.* 2011; Voronina 2013; Kloc *et al.* 2014). By providing an environment in which RNAs can meet up with their developmental regulators, these perinuclear organelles play an important role in silencing, translation, and/or RNA processing/regulation/organization. Certain silencing proteins (*e.g.*, Argonaute) have been shown to congregate in these perinuclear regions, and their presence is required for specific RNA regulation and meiotic progression (Updike and Strome 2010; Meikar *et al.* 2011; Kloc *et al.* 2014). Conglomeration of related proteins may allow the coupling of consecutive reactions, thereby increasing the efficiency of the biochemical process. To our knowledge, this is the most comprehensive examination of RNAi protein interactions in a single organism. Results from this and future studies could be useful in determining if silencing complex formation is conserved among various RNAi processes.

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