# THE ROLE OF RNAi GENES IN *NEUROSPORA CRASSA* POST-TRANSCRIPTIONAL GENE SILENCING

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Master of Arts

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by

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The undersigned, appointed by the dean of the Graduate School,

have examined the thesis entitled

THE ROLE OF RNAi GENES IN *NEUROSPORA CRASSA* POST-TRANSCRIPTIONAL GENE SILENCING

Presented by Lindsay A. Reustle,

A candidate for the degree of Master of Arts,

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Patrick Shiu, Chair

Dr. Elizabeth Bryda

Dr. Troy Zars

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# ABSTRACT

Post-transcriptional gene silencing is a fascinating mechanism that regulates gene expression and protects the genome from invaders. In *Neurospora crassa*, genes that are unpaired during meiosis are silenced by a process known as Meiotic Silencing by Unpaired DNA (MSUD). Utilizing the genetic advantages of this filamentous fungus, we are able to further characterize this RNAi pathway. Previously, we have developed a suppressor screen to identify mutants defective in this process. We are currently characterizing two newly identified MSUD genes, *sad-a'* and *sad-c*. Homology searches reveal that these genes encode proteins related to known RNA interference factors. Our analyses suggest that they are essential for sexual development but not vegetative growth. Like many other known MSUD proteins, SAD-A' and SAD-C preferentially localize in the perinuclear region, a presumed center of meiotic silencing activity. Furthermore, SAD-A´ and SAD-C interact with one another and with other well-known MSUD components. Identification and characterization of these two new components will add significantly to our understanding of the MSUD process. We propose that SAD-A´ and SAD-C form a complex with the Argonaute SMS-2 and are important for MSUD.

# CHAPTER I - RNA INTERFERENCE IN *NEUROSPORA CRASSA*

## History of *Neurospora crassa*

The first documented account of *Neurospora* was recorded as a bread mold infestation occurring in French bakeries in 1843 (DAVIS and PERKINS 2002). This orange powdered substance was later named *Neurospora* by Shear and Dodge in 1927 for its nerve-like markings on the ascospore. Dodge also identified three different species within the genus and established a role for *Neurospora* in genetics (PERKINS 1992). In 1941, Beadle and Tatum utilized *Neurospora* for its advantages as a model system to study if and how genes regulate biochemical processes (BEADLE and TATUM 1941). This concept, commonly known as the "one-gene-one enzyme hypothesis," laid the foundation for modern molecular biology and later resulted in the shared award of the Nobel Prize in Physiology or Medicine 1958. After a visit to Beadle's lab at Stanford, Barbara McClintock identified the seven chromosomes of *Neurospora* and described the phases of meiosis and post-meiotic mitosis (PERKINS 1992). These significant early discoveries using *Neurospora* set a strong foundation for the fungal biology community that exists today.

The genus *Neurospora* is a part of the Kingdom Fungi and has been classified under the phylum Ascomycota due to the spore sac, the ascus. *Neurospora* is further classified into the class Sordariomycetes because of the formation of the female fruiting body, the perithecia. The genome of the species *Neurospora crassa* was fully sequenced in 2003. *N. crassa* has a genomic size of 43 megabases and about 10,000 genes

(GALAGAN *et al.* 2003). Because of the public access to the genome and the relative ease to which *N. crassa* can be obtained and grown, this species is the most widely used in laboratory research.

## Life Cycle of *Neurospora crassa*

One advantage of using *N. crassa* as a model organism is that there are two distinct phases, vegetative and sexual, that give rise to differentiated cell types. The three most common cell types are macroconidia, microconidia, and ascospores.

In nature, *N. crassa* is found in tropical, subtropical, and temperate forests on burned vegetation. The heat of the fire causes ascospores (sexual spore) to germinate (JACOBSON *et al.* 2004) and allows this haploid filamentous fungus to grow over and under its substrate to form a highly branched network of hyphae (filamentous cells) known as mycelium. Hyphae have incomplete crosswalls through which nutrients, organelles, and cytoplasmic contents flow freely. Aerial hyphae then grow away from the substrate towards the air. Large multi-nucleate asexual spores, macroconidia, bud from the macroconidiophore and are dispersed by wind to repeat the cycle. Microconidia are uninucleated asexual spores that bud from the mycelia and are important for sexual reproduction (Figure 1-1) (SHIU *et al.* 2001; BISTIS *et al.* 2003).

Under stress conditions, such as carbon or nitrogen starvation, *N. crassa* can undergo a sexual cycle. This begins with the development of a female structure called the protoperithecium. Once fertilized by a male conidial cell, the nuclei from each mating type fuse, creating a transient diploid cell housed within the perithecium (fruiting body). After two rounds of meiosis and a mitotic division, eight haploid nuclei form and mature

within the spore sac known as an ascus. Once matured at about fourteen days post fertilization, these ascospores are ejected out of the perithecia (Figure 1-1) (SPRINGER 1993; SHIU *et al.* 2001). Once exposed to a heat source, such as a forest fire, these ascospores will germinate (JACOBSON *et al.* 2004).

## History of RNA Interference (RNAi)

RNA Interference (RNAi) is a gene silencing mechanism that is found in plants, animals, and fungi, and its purpose is to provide genomic defense and gene regulation. The first organism most scientists recall when discussing RNAi is *Caenorhabditis elegans*; however, before Fire and Mellow conducted their Nobel-prize winning research defining RNAi, co-suppression was first characterized in plants. In an attempt to generate a darker violet petunia plant, NAPOLI *et al.* (1990) inserted the chalcone synthase (CHS) gene which resulted in pale or white, not purple, petunias. When further examined, they noticed that both endogenous and ectopic levels of CHS were dramatically reduced compared to wild type and named this phenomenon co-suppression. A few years later, ROMANO and MACINO (1992) obtained a similar result when they inserted the carotenoid pathway gene, *al-1*, in multiple copies within the *Neurospora* genome and ended up with a range in vegetative colors from a dark yellow to white and a decreased *al-1* mRNA level. This phenomenon was termed quelling. Finally in 1998, Fire and Mellow explained the reason for these results and others like them. Through elegant experimentation, they showed that it was the production of the double-stranded RNA (dsRNA) that led to the silencing action (FIRE *et al.* 1998). It was this groundbreaking result that led to the discovery of the RNAi machinery components.

## RNAi Machinery

There are three main components in RNAi systems: Dicer, Slicer, and RNA-dependent RNA polymerase (RdRP); although, it has been shown that some systems do not require the classical RdRP for silencing (MATZKE and BIRCHLER 2005). Researchers in the *Drosophila* field first hypothesized that dsRNA had to be converted into small interfering RNAs (siRNAs). These siRNAs act as guides to cleave the target mRNA (SEN and BLAU 2006). The next step was to determine what enzyme(s) were responsible for this process. Dicer was first identified in *Drosophila* and later found to be conserved across organisms with RNAi. In addition to RNaseIII domains, Dicer typically has a helicase and a Piwi-Argonaute-Zwille (PAZ) domain. The RNaseIII cleaves the dsRNA into 20-25 nucleotide siRNAs while the helicase presumably acts to unwind the dsRNA. The PAZ domain is not necessarily required (CATALANOTTO *et al.* 2004).

The next discovery was the Slicer proteins, called Argonautes. This group is very diverse with many functions. Argonautes are characterized by their PAZ and PIWI domains. The PAZ domain acts to bind the 3′ end of a small RNA molecule while the PIWI domain acts to cleave the RNA. There are two main subgroups of Argonautes: AGO and PIWI. The AGO group mediate siRNA generation, and the PIWI group mediates piRNAs as first described in the *Drosophila* germline (CASTEL and MARTIENSSEN 2013).

Lastly, a role for RNA-dependent RNA polymerase (RdRP) in RNAi has been described in organisms such as *Neurospora, C. elegans*, and *Arabidopsis*. These proteins are important for initiating RNAi by converting aberrant RNA (aRNA), a single-stranded

RNA template, into double-stranded RNA (dsRNA). Once the dsRNA is constructed by the RdRP, then Dicer can cleave the molecule into siRNAs. Argonautes slice the double-stranded siRNA into single-stranded siRNA (ss siRNA). These ss siRNAs are loaded onto the RNA-induced Silencing Complex (RISC) by Argonaute which destroys the target mRNAs. Not all organisms require an RdRP for silencing activity (MATZKE and BIRCHLER 2005).

## Applications of RNAi

Since its discovery, the RNAi mechanism has been exploited for use in the laboratory. The first step for using RNAi knock down is to decide the method of delivery. Long dsRNAs or synthetic siRNAs can be injected into the studied organism to trigger the RNA-induced Silencing Complex (RISC) to degrade target mRNAs. Alternatively, small hairpin RNA (shRNA) can be delivered via transformation or a viral particle to be converted into siRNAs by Dicer proteins. Different mechanisms for small RNA delivery are dependent upon the model organism used and the transgene being studied (PERRIMON *et al.* 2010). RNAi-based knockdown has been utilized in many model organisms to demonstrate a role for genes that otherwise could not be studied. Many genes are essential for development and must be expressed for viability. This is true for the *Neurospora* gene *frh*. A shRNA under the control of an inducible promoter was transformed in to knock down *frh* allowing researchers to determine its important role in circadian clock regulation (CHENG *et al.* 2005). RNAi can also be used as a high throughput genome screening method as in *C. elegans* and *Drosophila*. In an effort to understand cardiac development, KIM *et al.* (2004) used RNAi knockdown in *Drosophila* embryos to identify heart loss-of-function mutant phenotypes. Over 5,000 genes were

identified using this method which led to an important understanding of cardiac development.

The uses of RNAi in clinical research and as a therapeutic agent are also being developed. Many human diseases can be studied and possibly treated using RNAi such as cancer, immunity, neurodegenerative disorders, developmental disorders, and aging. Synthetic siRNAs, like anti-*bcl-2* siRNA, have been shown to have antitumor properties in mouse cancer models. When injected, this siRNA decreased *bcl*-2 levels and tumor growth *in vitro* and *in vivo* (YANO *et al.* 2004). While a promising technique, more studies are needed to evaluate the effectiveness of RNAi-mediated knockdown as a therapeutic agent. Many of the delivery vehicles are not yet efficient enough to induce long-lasting RNAi activity, thus silencing is temporary without further injections. This is a painful proposition for an individual seeking treatment. Getting the siRNA to the appropriate cells in target tissues also poses a challenge. Modifications to the delivery particles or to the RNAs themselves are being developed to overcome these concerns (PERRIMON *et al.* 2010).

Benefits of RNAi knock down are not limited to the animal kingdom. RNAi can also be utilized in agricultural pursuits. RNAi-mediated modifications can lead to improved nutritional value, reduction of toxins, pest resistance, and increased plant productivity. For example, lysine content in the corn plant has been increased by the RNAi-mediated knockdown of enzymes that reduce lysine levels (HEBERT *et al.* 2008). Corn is a staple for both human consumption and for livestock feed. By increasing lysine levels through RNAi, there is no need to supplement diets with this essential amino acid thus reducing costs. This aspect is particularly exciting for those in developing countries.

With all of these emerging applications, it makes sense to continue to identify new components of the RNAi machinery in all model organisms to better understand this particularly useful phenomenon.

## RNAi Mechanisms in *Neurospora crassa*

### Quelling

Because vegetative *N. crassa* has incomplete crosswalls that allow the exchange of cytoplasmic contents between cells, it is susceptible to harmful agents such as transposons and viral invasion. Quelling is one mechanism designed to protect the genome integrity (DANG *et al.* 2011). Quelling is a post-transcriptional gene silencing mechanism that can become activated during the vegetative phase of the *N. crassa* life cycle. In essence, target mRNAs are reduced in response to the presence of a transgene. *al-1* encodes phytoene dehydrogenase, an important enzyme in the production of *N. crassa*'s vegetative orange coloring. Without *al-1*, *N. crassa* becomes albino, thus *al-1*can be used as a phenotypic marker for silencing. ROMANO and MACINO (1992) discovered that when they ectopically inserted the *al-1* gene in many copies, it resulted in a decreased *al-1* mRNA level. Furthermore, it was shown in subsequent experiments that this phenomenon was not unique to the albino genes, but many genes could be silenced in this way (PICKFORD *et al.* 2002). Gene expression was inactivated by introduction of a repeated homologous sequence. This mechanism is *trans*-acting in that it affects both transgenes and the endogenous gene. In another study, COGONI *et al.* (1996) revealed that quelling is a dominant trait and transgenes are required for its establishment and maintenance.

Using *al-1* as a marker in a mutagenesis screening, several *qde* (*quelling-defective*) mutants were identified. Three *qde* genes were further characterized: *qde-1*, *qde-3*, and *qde-2*. *qde-1* shared homology with the tomato plant RdRP. *qde-3* was homologous to a RecQ DNA helicase. *qde-2* shared homology with *Arabidopsis* argonaute (PICKFORD *et al.* 2002). Over time, more components of the quelling pathway were identified and characterized. The current model for quelling begins with the recognition of a tandemly repeated gene (ZHANG *et al.* 2013). Aberrant RNA (aRNA) is recognized and made into dsRNA by QDE-1, a DdRP/ RdRP, with the assistance of the DNA helicase QDE-3. Two Dicer-like proteins, DCL-1 and DCL-2, cleave the dsRNA into ~25 nt siRNAs. These ds siRNAs then bind to the argonaute protein, QDE-2, and are sliced. QDE-2-interacting Protein (QIP) acts as an exonuclease, removing the passenger strand (Figure 1-2) (LI *et al.* 2010).

**Meiotic Silencing by Unpaired DNA (MSUD)**

Meiotic Silencing by Unpaired DNA (MSUD) is another RNAi mechanism that *N. crassa* utilizes to protect the genome from mobile elements. Originally considered a form of transvection, ARAMAYO and METZENBERG (1996) showed that when the wild-type ascospore maturation gene, *asm-1*, was crossed to *asm-1*$^{\Delta}$, it resulted in white, inviable spore production instead of the usual mature black spores. In 2001, this unique phenomenon was renamed Meiotic Silencing by Unpaired DNA (MSUD). During prophase I of meiosis, one unpaired copy of a gene anywhere within the genome silences all homologous DNA copies through MSUD (SHIU *et al.* 2001).

To date, nine components of MSUD have been identified and characterized: SAD-1, SAD-2, SMS-2, DCL-1, QIP, SAD-3, SAD-4, SAD-5, and SAD-6. The first of these identified through a mutagenesis screening was SAD-1 (suppressor of ascus dominance). SAD-1 shares homology with other RdRPs, and was suggested to replicate aberrant RNA (aRNA) into dsRNA (SHIU *et al.* 2001; SHIU and METZENBERG 2002). The second gene involved was named *sad*-2. This gene is interesting in that it shared similarity with the proteins from many RNAi mechanism-containing organisms; however, no functions of the protein were known. SHIU *et al.* (2006) were the first to tag any MSUD genes with the reporters, green fluorescent protein (GFP) and red fluorescent protein (RFP). When *sad-1* and *sad-2* were independently tagged with GFP, they localized to the perinuclear region during prophase I of meiosis. Furthermore, it was shown that these two proteins co-localized in this region. In a *sad-2*$^{\Delta}$ background, SAD-1-GFP could no longer localize to the perinuclear region; however, this was not the case for SAD-2-GFP in a *sad-1*$^{\Delta}$ background suggesting that SAD-2 recruits SAD-1to the site of MSUD activity (SHIU *et al.* 2006). A gene called *sms-2* (suppressor of meiotic silencing) was found to be paralogous to the quelling argonaute *qde-2* and also played a role in MSUD. This suggested that an argonaute protein was required for MSUD (LEE *et al.* 2003). Next, it was shown that *dcl-1*, but not *dcl-2*, was also required for MSUD. DCL-1-GFP also localized to the perinuclear region and co-localized with the other established MSUD components (ALEXANDER *et al.* 2008).

Identifying the three main components, RdRP, Argonaute, and Dicer, suggested that MSUD was indeed a bona fide RNAi pathway. Recently, additional genes were identified as also playing a role in MSUD. QIP (QDE-2 interacting protein), which also

functions for quelling, acts as an exonuclease that removes the passenger strand, an important step in the silencing process (LEE *et al.* 2010; XIAO *et al.* 2010). With the construction of the *N. crassa* knockout library (COLOT *et al.* 2006), a new approach to genetic screening was utilized to identify more members of this pathway starting with *sad*-3. SAD-3 encodes a putative helicase and may serve to assist SAD-1 in dsRNA production. Like the other MSUD components, SAD-3 localizes to the perinuclear region and co-localizes with the other MSUD proteins (HAMMOND *et al.* 2011a). *sad-4* and *sad-5* are fungal specific genes that do not encode known functional protein domains. SAD-5 is the first identified MSUD protein that localizes to the nucleus. While functions for SAD-4 and SAD-5 in MSUD are unclear, it is known that they both are upstream of siRNA production (HAMMOND *et al.* 2013). SAD-6, a Rad54-like homology search protein, is the most recent nuclear MSUD factor identified and may be the key to understanding how unpaired DNA is recognized (SAMARAJEEWA *et al.* 2014).

The current model for MSUD begins in the nucleus with the production of an aberrant RNA (aRNA) from an unpaired gene identified by an unknown mechanism, possibly involving SAD-5 and/or SAD-6. Once in the perinuclear region, SAD-1 acts to convert the aRNA to dsRNA. SAD-3 may serve to assist SAD-1 by unwinding nucleic acids. SAD-2 acts to recruit SAD-1, and possibly other MSUD components, to the perinuclear region, an important location for meiotic silencing activity. DCL-1 cleaves the dsRNA into siRNAs where then SMS-2 acts as an argonaute-like protein guided by the siRNAs to target mRNAs for degradation. QIP acts as an exonuclease that removes the passenger strand. This working model is depicted in Figure 1-3 (HAMMOND *et al.* 2011a).

## Figures

**Figure 1-1. Life cycle of *Neurospora crassa*.**

Macroconidiation (vegetative) pathway produces multinucleated, genetically-identical macroconidia through aerial hyphae whereas the microconidiation pathway produces uninucleated, genetically-identical microconidia through mycelia. The sexual cycle begins with the development of the female structure, the protoperithecium. After fertilization by a male conidium, the diploid cell gives rise to eight haploid ascospores within the ascus. Figure reprinted from SHIU *et al.* (2001).

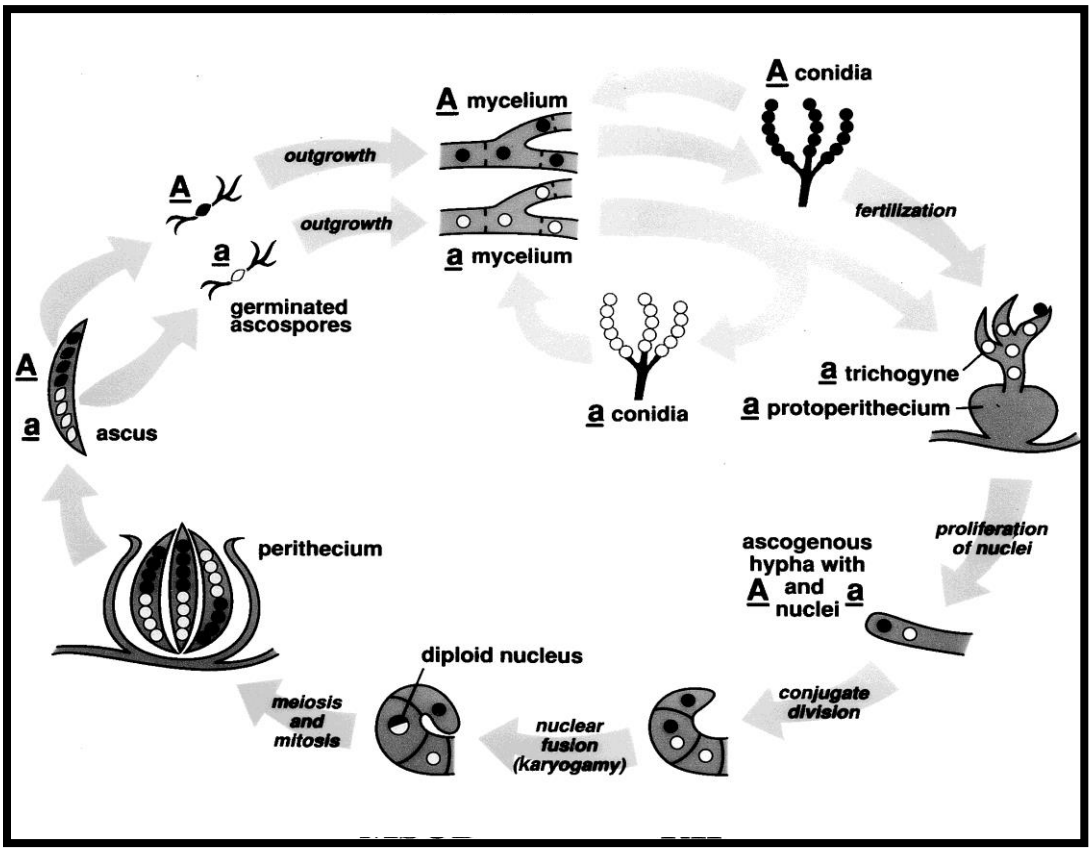


**Figure 1-2. Quelling in *Neurospora crassa*.**

Diagram illustrating the quelling pathway, a post-transcriptional gene silencing mechanism that occurs during the vegetative phase of the *N. crassa* life cycle. Tandemly repeated genes are recognized and converted to an aberrant RNA. The aRNA is made into dsRNA by QDE-1. That dsRNA is diced by DCL-1/DCL-2. With the help of QIP, the duplex siRNA is converted to a single-stranded siRNA which guides QDE-2 to target homologous mRNA.

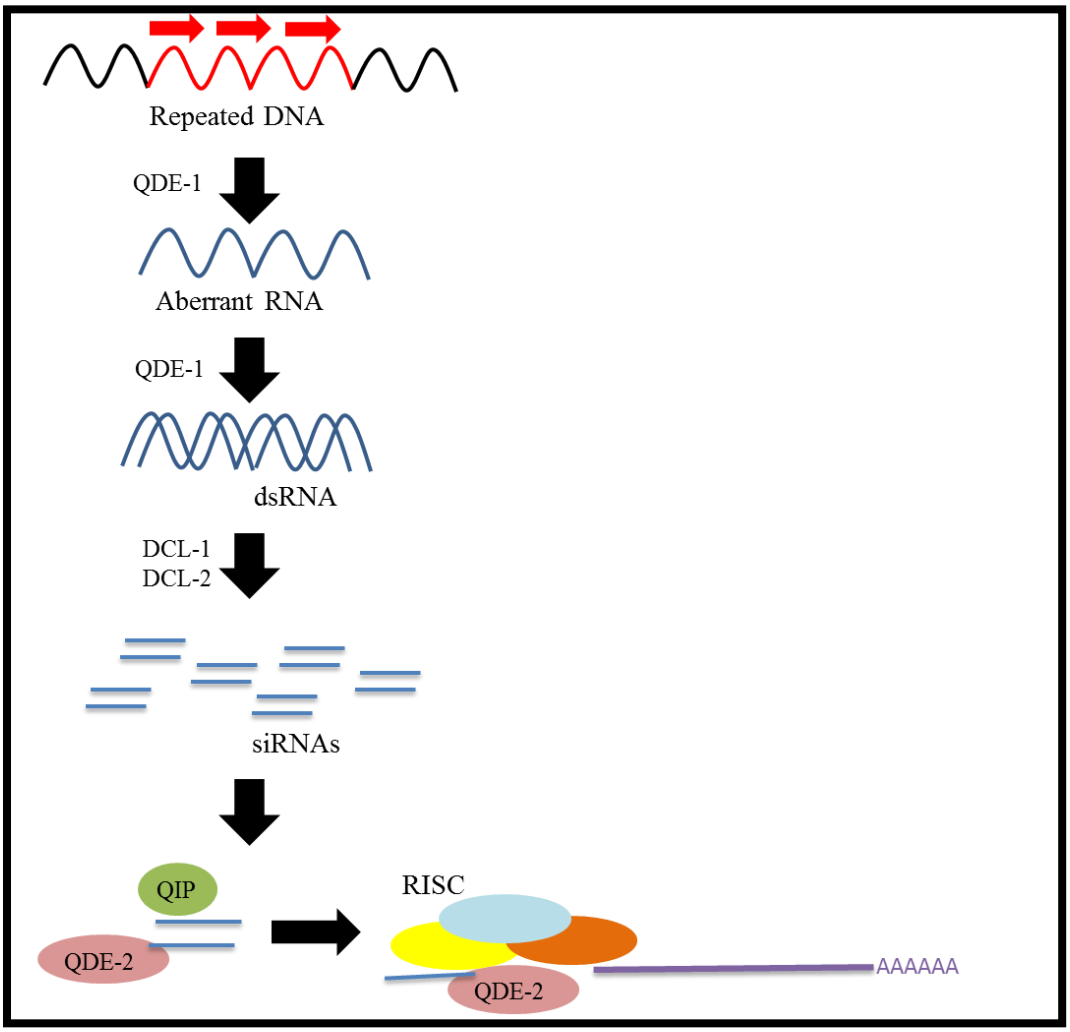
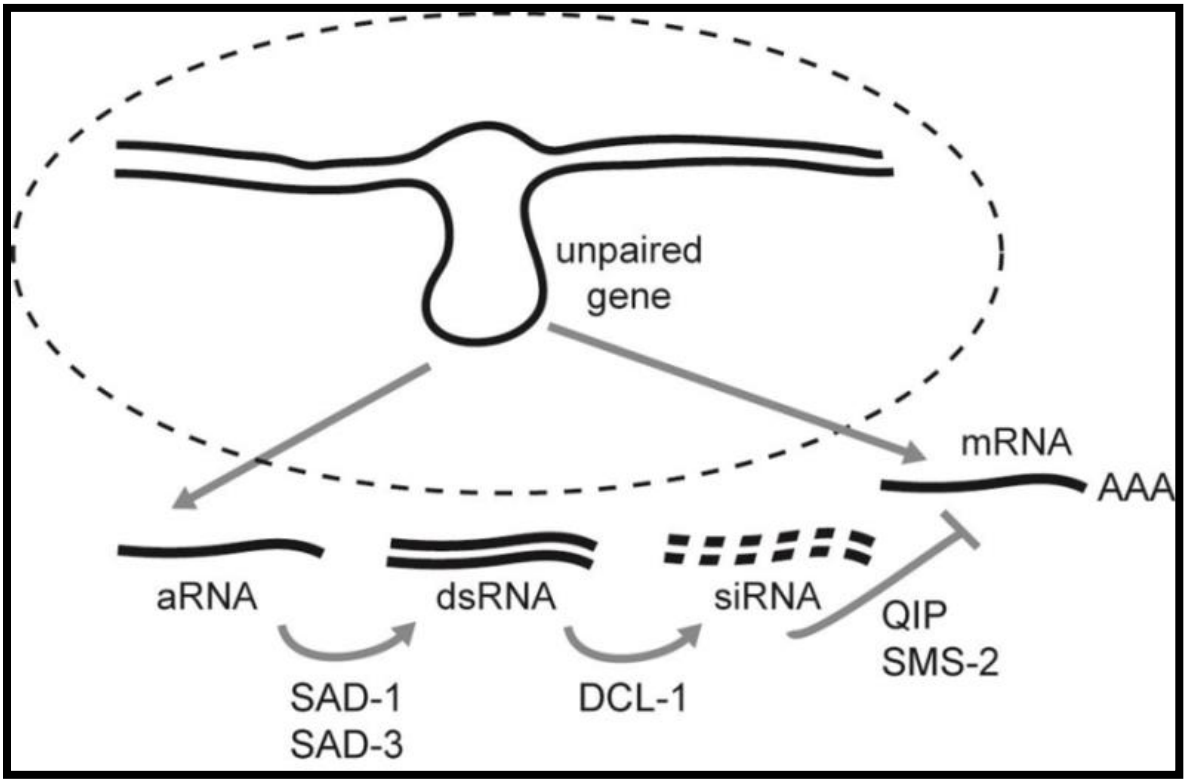


**Figure 1-3. MSUD in *Neurospora crassa*.**

Diagram illustrating the MSUD pathway, a post-transcriptional gene silencing mechanism that occurs in *N. crassa* during prophase I of meiosis. Unpaired genes are recognized and converted to an aberrant RNA. The aRNA is made into dsRNA by SAD-1. That dsRNA is diced by DCL-1. With the help of QIP, the duplex siRNA is converted to single-stranded siRNA which guides SMS-2 to target homologous mRNA. Figure reprinted from HAMMOND *et al.* (2011a).



# CHAPTER II - IDENTIFICATION OF SAD-A´$^{\Delta}$ AND SAD-C$^{\Delta}$ AS SEMIDOMINANT SUPPRESSORS OF MSUD

## Introduction

In an attempt to identify additional MSUD candidates, HAMMOND *et al.* (2011a) employed a high-throughput reverse genetic screen using the *N. crassa* knockout library. The library is made up of knockout strains for the entire annotated genome provided on 96-well plates (COLOT *et al.* 2006). Conidia from the library are transferred to MSUD suppression tester strains cultured on 96-well plates. Once spores are produced, the plates can quickly be examined under the microscope for evidence of MSUD suppression. This method provides an efficient way to screen the entire *N. crassa* genome for candidates of MSUD. The two MSUD suppression tester strains used in the screening process contain unpairing of *ascospore maturation-1 (asm-1)* or *round spore (r)* genes. When *asm-1* is unpaired, white, inviable spores are produced whereas when *r* is unpaired, round spores are produced instead of the wild-type, American football-shaped spores (SHIU *et al.* 2001). Any candidates displaying the wild-type phenotype are considered MSUD-deficient and most likely play a role in this process. Many MSUD candidates have been identified using this method, including *sad-a´* and *sad-c*.

A search using the NCBI conserved domain database for the *sad-a´* and *sad-c* sequences identified two argonaute binding proteins, Arb1 and Arb2, respectively. Arb1 and Arb2 have been identified and characterized in the fission yeast

*Schizosaccharomyces pombe*. Arb1 and Arb2 form a complex with *S. pombe* Ago1 (Argonaute) known as the ARC (Argonaute siRNA chaperone). ARC contains mostly double-stranded siRNA, and it has been proposed that ARC may function to transfer the double-stranded siRNA from Dcr1 (Dicer) to Ago1 (BUKER *et al.* 2007). It is possible that SAD-A´ and SAD-C form a complex with the *N. crassa* argonaute protein SMS-2 in MSUD and/or QDE-2 in quelling and perform a similar biological function.

Loss of any of the published MSUD genes leads to a defect in MSUD efficiency, according to the "silencing the silencer" model (SHIU *et al.* 2001). When an MSUD gene is unpaired by deletion in one of the parents, it triggers MSUD to silence that gene. This negative feedback mechanism severely impedes the MSUD process, and other unpaired genes within the genome can no longer be silenced. Accordingly, we can use this "silencing the silencer" phenomenon to test new genes for a role in MSUD. Four tester strains are used to specifically test for MSUD suppression: *ascospore maturation-1*$^{\Delta}$ *(asm-1*$^{\Delta}$*), round spore* $^{\Delta}$ *(r*$^{\Delta}$*),* ectopic *β-tubulin (::bml*$^{R}$*),* and ectopic *actin (::act*$^{+}$*).* Whereas *asm-1*$^{\Delta}$ and *r*$^{\Delta}$ are assayed by a spore phenotype, *bml* and *act* are essential genes and cannot be deleted. These genes are ectopically inserted at the *his-3* locus, generating an unpaired copy of that gene. When *::bml*$^{R}$ or *::act*$^{+}$ is silenced by MSUD, there is a significant reduction in ascopore production. To determine if *sad-a´* and *sad-c* are indeed components of MSUD, we tested their ability to suppress MSUD when crossed to these four unpaired tester strains.

Roles for the newly identified *sad* genes may not be limited to MSUD. Six of the nine previously well-characterized MSUD components have also been shown to be required for sexual development (SHIU *et al.* 2001; LEE *et al.* 2003; SHIU *et al.* 2006;

ALEXANDER *et al.* 2008; XIAO *et al.* 2010; HAMMOND *et al.* 2011a; 2013; SAMARAJEEWA *et al.* 2014). Of these six, homozygous crosses of *dcl-1*$^{\Delta}$, *qip*$^{\Delta}$, and *sms-2*$^{\Delta}$ produced no recognizable asci and were found to be required at an early stage in sexual development. The relationship between MSUD components and sexual development is not yet well understood. In this study, we tested the effect of *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ in homozygous/heterozygous crosses to wild-type to determine if they also play a role in sexual development.

While some MSUD genes are unique to silencing during the sexual phase only, there does seem to be some redundancy between the quelling and MSUD pathways. Both silencing mechanisms require *dcl-1* and *qip* (ALEXANDER et al. 2008; XIAO et al. 2010). *sad-6* RNA transcripts are expressed in both vegetative and sexual tissue samples, suggesting a role in both phases of the life cycle (SAMARAJEEWA *et al.* 2014). In an effort to identify additional roles for these *sad* genes, it is important to understand if and how these genes affect all phases of the *N. crassa* life cycle. Vegetative assays have been developed and utilized to examine all MSUD components for linear growth, branching, and conidiation patterning defects. To date, none of the published MSUD components display any vegetative growth defects. We also included these assays in the characterization of *sad-a´* and *sad-c*.

## Methods

Most of the methods described herein are modified from standard *Neurospora* protocols (http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm).

**Table 2-1. Strains used in this study**

| Strain | Genotype |
|---|---|
| F2-01 | *fl* A (FGSC 4317) |
| F2-29 | *rid r*$^{\Delta}$*::hph*; *fl* A |
| F2-35 | *his-3::his-3*$^{+}$ *actin*$^{+}$; *fl* A |
| F2-36 | *his-3::his-3*$^{+}$ *Bml*; *fl* A |
| F3-23 | *rid his-3*$^{+}$:: *asm-1*; *fl*; *asm-1*$^{\Delta}$*::hph* A |
| F4-17 | *sms-2*$^{\Delta}$*::hph*; *fl* A |
| F5-24 | *sad-1*$^{\Delta}$*::hph*; *fl* A |
| F6-36 | *sad-a'*$^{\Delta}$*::hph sad-c*$^{\Delta}$*::hph fl* A |
| P3-08 | Oak Ridge wild type A (FGSC 2489) |
| P3-25 | mep *sad-1*$^{\Delta}$*::hph* a |
| P8-44 | *sms-2*$^{\Delta}$*::hph* a (FGSC 1160) |
| P9-42 | Oak Ridge wild type a |
| P13-06 | *sad-c*$^{\Delta}$*::hph* a |
| P13-07 | *sad-c*$^{\Delta}$*::hph* A |
| P15-25 | *sad-a'*$^{\Delta}$*::hph* a |
| P15-26 | *sad-a'*$^{\Delta}$*::hph sad-c*$^{\Delta}$*::hph* a |
| P15-28 | *sad-a'*$^{\Delta}$*::hph* A |
| P16-21 | *sad-1*$^{\Delta}$*::hph* a |

## Growth Media

Vogel trace elements- 5 g citric acid hydrate, 5 g zinc sulfate septahydrate, 1 g ferrous ammonium sulfate hexahydrate, 250 mg cupric sulfate pentahydrate, 50 mg manganese sulfate hydrate, 50 mg boric acid, and 50 mg sodium molybdate dihydride were dissolved in 95 mL of nanopure water, then sterilized into 50 mL conical vials via a 0.45 μm pore filter (Vogel 1964). The solution was stored at room temperature.

Vogel minimal media- 25× Vogel's salts was made as follows: 100 g sodium citrate septahydrate, 166.5 g anhydrous monobasic potassium phosphate, 66.5 g ammonium

nitrate, 6.65 g magnesium sulphate septahydrate, 3.34 g calcium chloride dehydrate, 1.67 mL of 100 μg/mL biotin solution, and 3.34 mL of Vogel's trace elements were mixed and brought to a total volume of 1 L with nanopure water. 5 mL of choloroform was added as a preservative.

Westergaard (WG) trace elements- To 1 L of water the following was added: 57 mg boric acid, 396 mg copper (II) sulfate pentahydrate, 72 mg manganese chloride hexahydrate, 4.2 g zinc chloride, 100 g sodium choloride, and 100 g calcium chloride dehydrate. The resulting solution was autoclaved for 30 minutes and stored at room temperature (Westergaard and Mitchell 1947).

Westergaard (WG) media- 20× WG salts were made as follows: 20 g potassium nitrate, 20 g potassium phosphate monobasic, 10 g magnesium sulfate septahydrate, 2 g sodium chloride, 2 g calcium chloride dihydrate were added to 1 L of nanopure water and autoclaved. To make WG plates, an appropriate amount of 20× WG salts was diluted with water and 1.5% (w/v) sucrose. Next, 1.5% (w/v) Bacto Agar (BD), 1 mL WG Trace Elements per liter of media, and 5 μg/mL biotin were added. The media was autoclaved and 45 mL was added to 100 x 100 x 15 mm square petri dishes (Westergaard and Mitchell 1947).

Brockman and de Serres (BDS) 20× solution- 200 g sorbose, 10 g fructose, and 10 g glucose were added to 1 L of nanopure water, autoclaved for 30 minutes, and stored at room temperature.

Brockman and de Serres (BDS) media- An appropriate amount of 25× Vogel's salts and 1.5% (w/v) Bacto Agar (BD) was added to water and autoclaved for 30 minutes. After

sterilization, an appropriate amount of 20× BDS was added; the solution was mixed, and stored at room temperature until needed.

**Genomic DNA Isolation**

25 mL of Vogel's liquid media was added to a 125 mL Erlenmeyer flask and then autoclaved for 30 minutes. Flasks were then inoculated with conidia from a strain of interest and placed in an incubator at 30°C. After 2-4 days, the resulting vegetative tissue was blotted dry with paper towels and then put in a desiccator containing sodium hydroxide. After the tissue was dried, it was ground using a glass rod in a test tube and a vortexer. The resulting powder was then used with the DNeasy Plant Mini Kit from Qiagen.

**Long Template PCR**

All PCR was set up according to the protocol enclosed with the Roche Expand Long dNTP Pack (Roche Applied Science, Indianapolis, IN).

**DNA gel electrophoresis**

0.8% (w/v) agarose in 1× TAE with 0.5 μg/mL of ethidium bromide was utilized in both analytical and purification procedures. 50 mL gels were used for sample groups of ten or fewer, while samples greater than ten were run on a 200 mL gel. TAE buffer was made in accordance to Sambrook and Russell (2001), page A1.17. Gels were run at varying voltages depending on the size of the DNA bands in question; 120 V for one hour was the most common setting for both analytical and purification applications.

**Quantitative Analysis of MSUD Suppression and Ascospore Production**

*r*$^{\Delta}$, *asm-1*$^{\Delta}$, *::β-tubulin, ::actin,* and wild-type fluffy strains were inoculated on the center of 60 mm WG plates and incubated at room temperature for 6 days. Conidia from each male of mating type *a* were adjusted to a concentration of 1000 counts per microliter. Fluffy plates were fertilized with 3 × 33µL aliquots of male conidial suspension. Each cross was replicated in triplicate. Ascopores were collected from the lids at 21 days post-fertilization and analyzed using a hemocytometer under the microscope.

**Microscopy and photography**

Rosettes of asci were dissected from 11-14 day old perithecia in 25% glycerol using a VanGuard 1231CM Trinocular Microscope. A Canon Power Shot S3 IS digital camera with a Canon conversion lens adapter was attached to this microscope and used to photograph the rosettes.

**Vegetative Assays**

Plate Assay- Conidial strains of mating type *a* were adjusted to 1000 counts per microliter. 4 µL of each strain was inoculated on the center of 150 mm Vogel's + his plate and incubated for 7 days at room temperature. Two replicates per strain were used.

Race Tube Assay- Conidial strains of mating type *a* were adjusted to 1000 counts per microliter. 4 µL of each strain was inoculated at one end of race tubes containing 15 mL of Vogel's + his media. Position of the leading hyphae was marked every 12 hours, starting at 18 hours post-inoculation. Three race tubes per strain were used.

**Permanent Stocking of Strains**

Conidial strains were inoculated to agar/milk tubes (with appropriate nutrients) and incubated at 30°C for 2-4 days. After sufficient conidiation, tubes were vortexed to allow the sterile milk and conidia to mix. One mL of conidia mix was then pipetted into sterile silica stock tubes, vortexed and placed in the 4°C refrigerator. Another 0.9 mL of the conidia mix was added to cryogenic vials containing 0.9 mL of 50% sterile glycerol, vortexed, and then placed in the -80°C freezer.

## Results

**_sad-a´_$^{\Delta}$ and _sad-c_$^{\Delta}$ semi-dominantly suppress meiotic silencing**

To determine if _sad-a´_$^{\Delta}$ and _sad-c_$^{\Delta}$ are in fact suppressors of MSUD, we crossed these strains and the double mutant to our four MSUD suppression assay testers. Crosses of _sad-a´_$^{\Delta}$ to an _asm-1_$^{\Delta}$ tester yielded ~87.50% white spores. Crosses of _sad-c_$^{\Delta}$ to an _asm-1_$^{\Delta}$ tester yielded ~75.76% white spores. Crosses of wild-type, MSUD-proficient strains to an _asm-1_$^{\Delta}$ tester yielded 99.24% white spores (Figure 2-1). Similar to the suppressors found in _Sk-2_ and _Sk-3_ (RAJU _et al._ 2007), _sad-a´_, _sad-_c, and the double mutant did not suppress _r_$^{\Delta}$ (Figure 2-2). On the other hand, crosses to _::bml_$^{R}$ or _::act_$^{+}$ were suppressed (Figures 2-3; 2-4). The double mutant suppressed _asm-1_$^{\Delta}$ nearly the same as _sad-a´_$^{\Delta}$; however, suppression of ::_bml_$^{R}$ and ::_act_$^{+}$ was significantly higher in the double mutant than the single mutants combined. _sad-a´_$^{\Delta}$ and _sad-c_$^{\Delta}$ suppression levels

were low compared to *sad-1*$^{\Delta}$ and *sms-2*$^{\Delta}$; therefore, these two candidates are considered semi-dominant suppressors of MSUD.

### *sad-a´* and *sad-c* are required for sexual development

Many of the previously characterized MSUD genes are required for sexual development. Quantitative analysis shows that crosses heterozygous for *sad-a´*$^{\Delta}$ or for *sad-c*$^{\Delta}$ produce a normal level of progeny (Table 2-2); however, homozygous crosses for *sad-a´*$^{\Delta}$ or for *sad-c*$^{\Delta}$ produce perithecia without normal beaks, and no ascospores are produced (Figure 2-5). Because single *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ knockout strains are barren in homozygous crosses, we expected that the *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ homozygous cross would also be barren. We tested the effect of *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ in a heterozygous cross by quantifying the ascospores from a cross to wild-type (Table 2-2). Like the single mutants, the double mutant was also barren (Figure 2-5). When examining the contents of the perithecia, no recognizable asci were present in homozygous crosses for *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, or *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$. These results resembled what has been previously described for *dcl-1*, *qip*, and *sms*-2 (LEE *et al.* 2003; ALEXANDER *et al.* 2008; XIAO *et al.* 2010) and suggest that *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ are required for the early stages of sexual development.

### *sad-a´* and *sad-c* are not required for vegetative growth

To monitor the linear growth rate of *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$, each strain was inoculated into a set of race tubes. None of the *sad* deletion strains had a linear growth rate significantly different from the wild-type strain (Figure 2-6 B). In addition, no obvious morphological differences were seen in the vegetative plate assay between the *sad* deletion strains. Branching and conidiation patterns were all comparable to wild-type

(Figure 2-6 A). In contrast to the sexual phase, *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ are normal during the vegetative phase. This is true for all of the well-characterized MSUD genes.

## Discussion

In a previous study, HAMMOND *et al.* (2011a) set out to identify new components of the Meiotic Silencing by Unpaired DNA (MSUD) pathway. *sad-a´* and *sad-c* were identified as potential candidates through this high-throughput reverse genetic screen. In this study, we used the "silencing the silencer" approach to confirm that *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ are semi-dominant suppressors of MSUD. The strongest suppressor of MSUD to date has been *sad-1*$^{\Delta}$ (SHIU *et al.* 2001; SHIU and METZENBERG 2002). While all of the characterized *sad* genes are needed for MSUD, it is unclear why some appear to be semi-dominant suppressors when knocked out. It is possible that they are less efficient at self-silencing compared to *sad-1*$^{\Delta}$. Perhaps the proteins produced from these genes may have a high expression or a long protein half-life. Despite the fact that *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ are weaker suppressors of MSUD, there is no indication that they are any less important to this process than other *sad* genes.

Double mutants had not yet been exhaustively explored for MSUD, and this experiment aimed to examine the effect of relationships between components of the silencing machinery on MSUD suppression. Interestingly, there was no effect on silencing of *r*$^{\Delta}$ in either the single or double mutants. This is reminiscent of the case seen in *Sk* strains (RAJU *et al.* 2007). For MSUD suppression using the *asm-1*$^{\Delta}$ tester, the double mutant did not seem to be stronger than the individual knock outs. Interestingly,

the effect of the double mutant using *::bml*$^{R}$ or *::act*$^{+}$ testers was nearly twice as strong as in the single mutants. These results support the idea of a cumulative effect of *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ on MSUD suppression.

*sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ are barren in homozygous crosses and do not form any recognizable asci, thus these *sad* genes must play a role in early sexual development. Perhaps, MSUD may act as part of a meiotic checkpoint for cell cycle progression (SHIU *et al.* 2006). Alternatively, certain *sad* genes may have other important roles independent of MSUD activity. The latter seems more plausible considering the recent identification of *sad* genes not required for sexual development (HAMMOND *et al.* 2013; SAMARAJEEWA *et al.* 2014). Both quelling and MSUD mechanisms require *dcl-1* and *qip.* Our data show that homozygous crosses for *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ resemble those of *dcl-1*$^{\Delta}$, *qip*$^{\Delta}$, and *sms-2*$^{\Delta}$ (LEE *et al.* 2003; ALEXANDER *et al.* 2008; LEE *et al.* 2010; XIAO *et al.* 2010). No other *arb-1* or *arb-2* homologs have been discovered in the *N. crassa* genome; therefore, it is reasonable to suggest that they might also play a role in the quelling pathway. Analysis of mRNA for *sad-a´* and *sad-c* from vegetative and sexual tissues would lend stronger support to this hypothesis.

## Figures

**Figure 2-1. MSUD is semi-dominantly suppressed in *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ × *asm-1*$^{\Delta}$.**

Wild-type (WT) strain included as MSUD-proficient control, and two MSUD-deficient controls are *sms-2*$^{\Delta}$ and *sad-1*$^{\Delta}$. Experimental strains are *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$. When MSUD is suppressed, the percentage of white spores decreases in a cross to *asm-1*$^{\Delta}$. *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ act as semi-dominant suppressors of MSUD, allowing the production of black spores in a cross to *asm-1*$^{\Delta}$. Bar represents the mean for three replicates. Error bars represent the standard deviation.

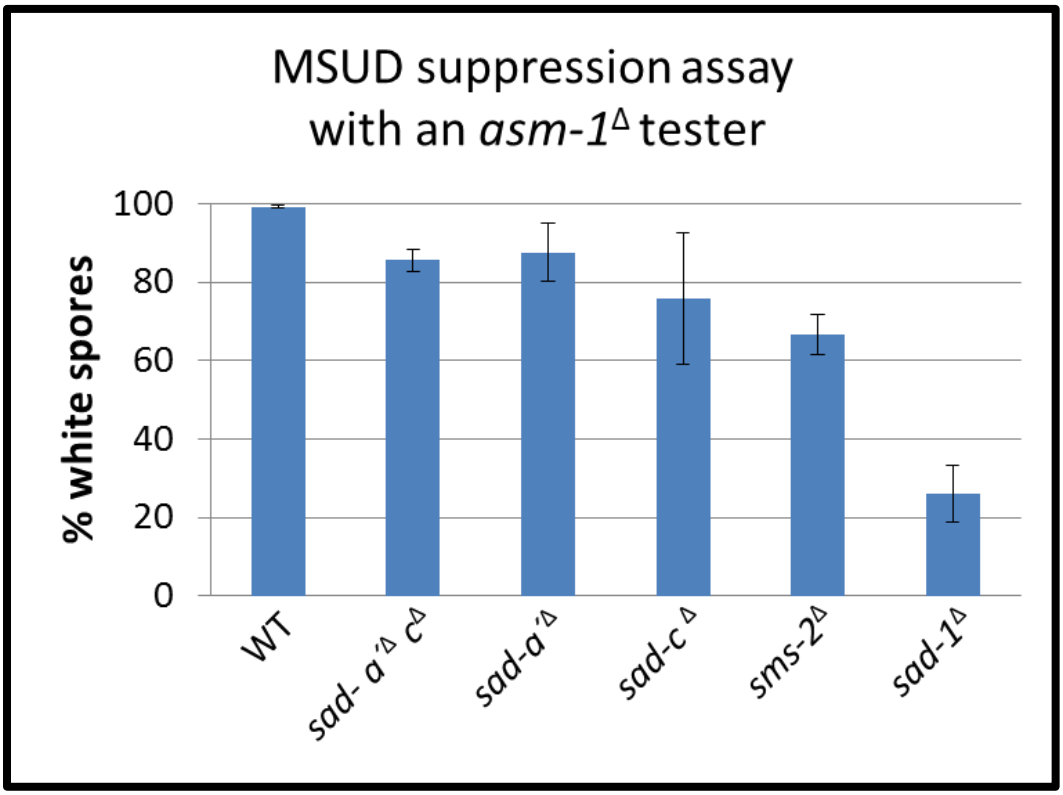


**Figure 2-2. MSUD is not affected in *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ × *r*$^{\Delta}$.**

Wild-type (WT) strain included as MSUD-proficient control, and two MSUD-deficient controls are *sms-2*$^{\Delta}$ and *sad-1*$^{\Delta}$. Experimental strains are *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$. When MSUD is suppressed, the percentage of round-shaped spores decreases in a cross to *r*$^{\Delta}$. *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ produce a wild-type level of round-shaped spores when crossed to *r*$^{\Delta}$. Bar represents the mean for three replicates. Error bars represent the standard deviation.

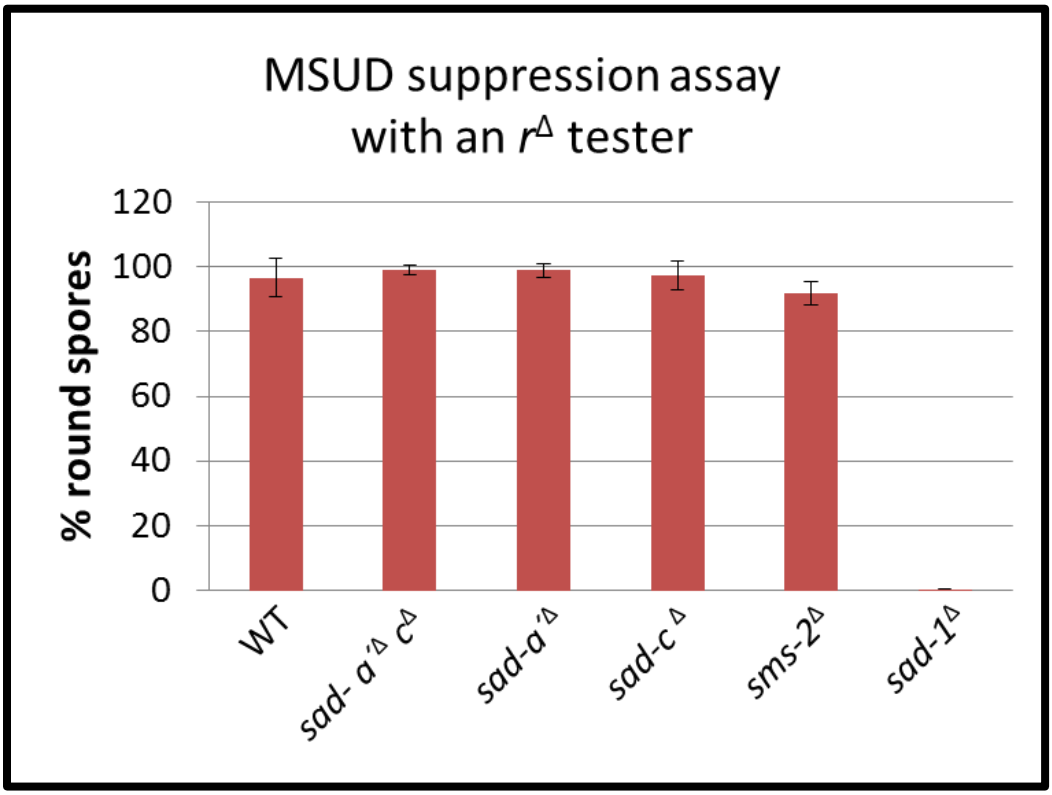


**Figure 2-3. MSUD is semi-dominantly suppressed in *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ × *::actin*$^{+}$.**

Wild-type (WT) strain included as MSUD-proficient control, and two MSUD-deficient controls are *sms-2*$^{\Delta}$ and *sad-1*$^{\Delta}$. Experimental strains are *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$. When MSUD is suppressed, the amount of ascospores produced increases in a cross to *::actin*$^{+}$. *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ act as semi-dominant suppressors of MSUD, increasing the production of ascospores in a cross to *::actin*$^{+}$. MSUD suppression in *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ is higher than in the single mutants.

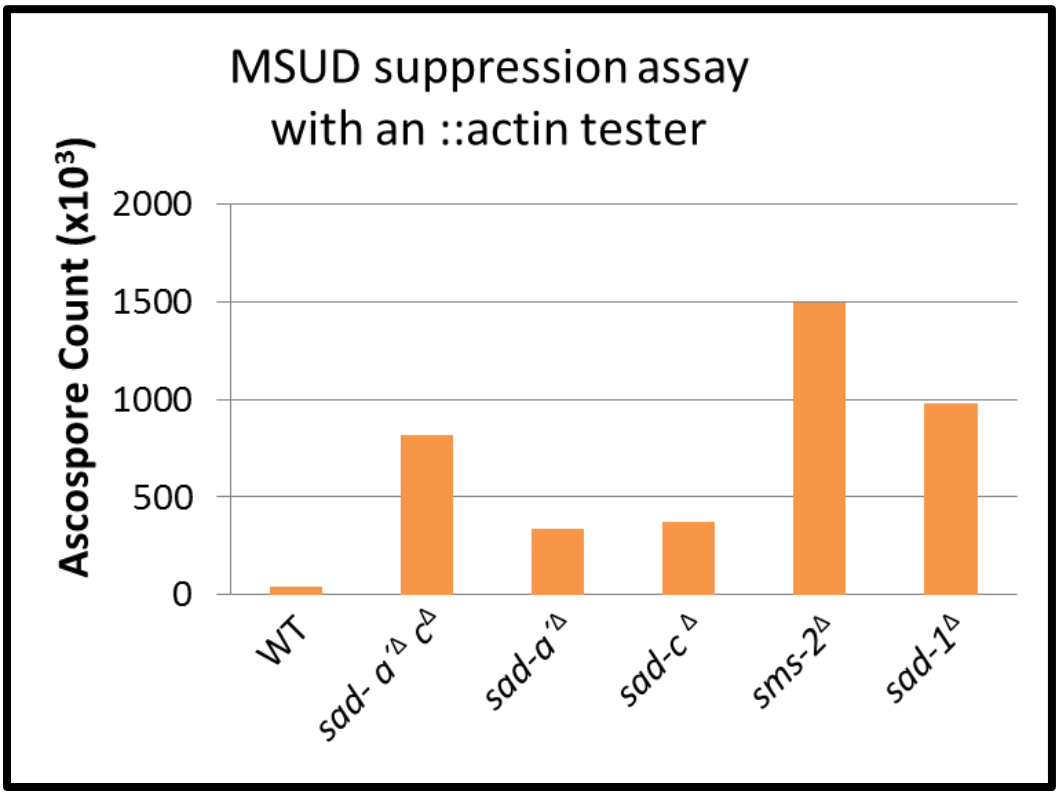


**Figure 2-4. MSUD is semi-dominantly suppressed in *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ × *::bml*$^{R}$.**

Wild-type (WT) strain included as MSUD-proficient control, and two MSUD-deficient controls are *sms-2*$^{\Delta}$ and *sad-1*$^{\Delta}$. Experimental strains are *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$. When MSUD is suppressed, the amount of ascospores produced increases in a cross to*::bml*$^{R}$. *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ act as semi-dominant suppressors of MSUD, increasing the production of ascospores in a cross to *::bml*$^{R}$ (*β-tubulin*). MSUD suppression in *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ is higher than in the single mutants.

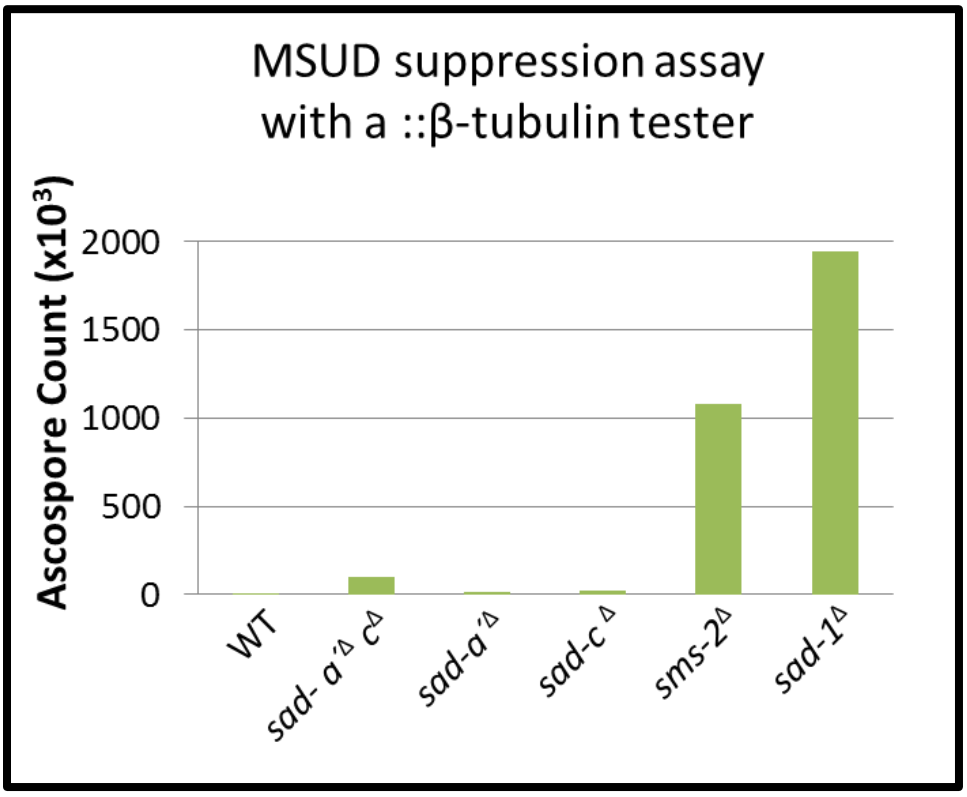


**Table 2-2. Quantitative analysis of *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ in sexual development.**

Quantitative analysis of shot ascospores. Heterozygous crosses produce a normal level of progeny; however, homozygous crosses are completely barren for *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$.

| Parent 1 (*mat* a) | Parent 2 (*mat* A) | Ascospore Count (in millions) |
|---|---|---|
| **Wild-type** | Wild-type | 2.06 |
| ***sad-a´*$^{\Delta}$** | Wild-type | 1.76 |
| | *sad-a´*$^{\Delta}$ | 0 |
| ***sad-c*$^{\Delta}$** | Wild-type | 1.86 |
| | *sad-c*$^{\Delta}$ | 0 |
| ***sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$** | Wild-type | 1.98 |
| | *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$ | 0 |

**Figure 2-5. *sad-a´*$^{\Delta}$ and *sad-c*$^{\Delta}$ are required for sexual development.**

Normal perithecia (left) and rosettes (right) can be seen in the wild-type cross compared to the undersized beaks and absence of asci in the homozygous cross for *sad-a´*$^{\Delta}$, *sad-c*$^{\Delta}$, and *sad-a´*$^{\Delta}$ *sad-c*$^{\Delta}$. Arrow shows the beak on wild-type perithecia.

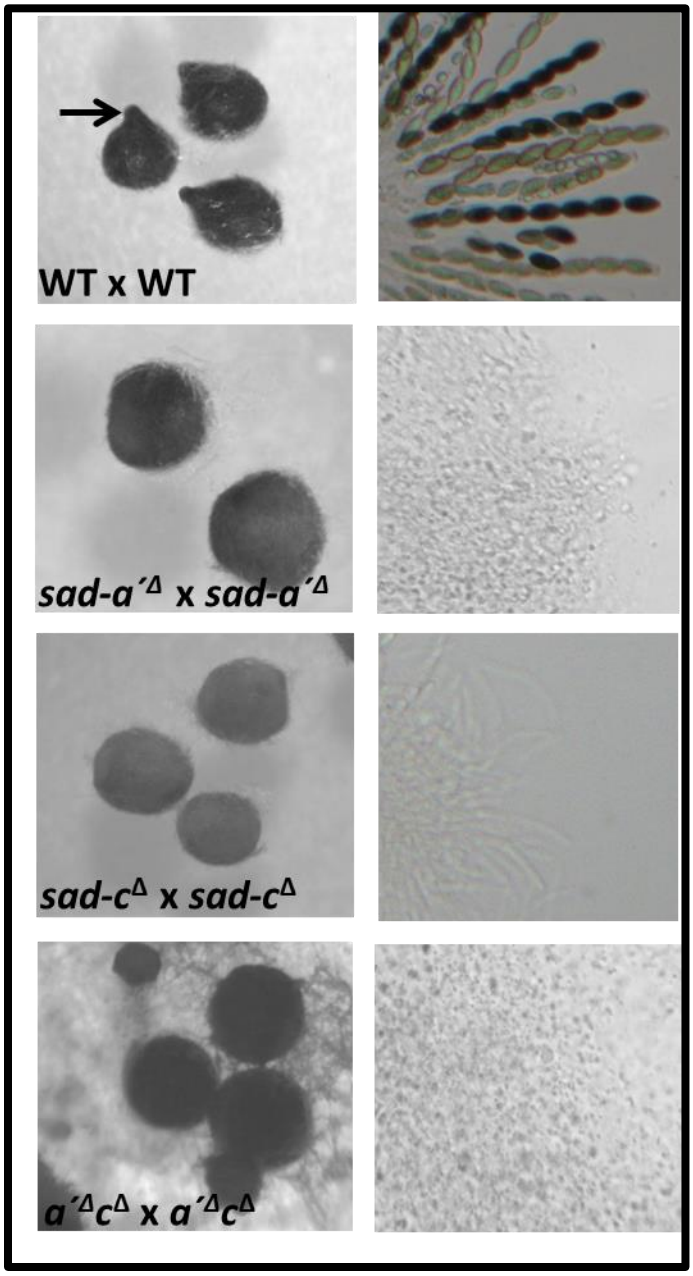
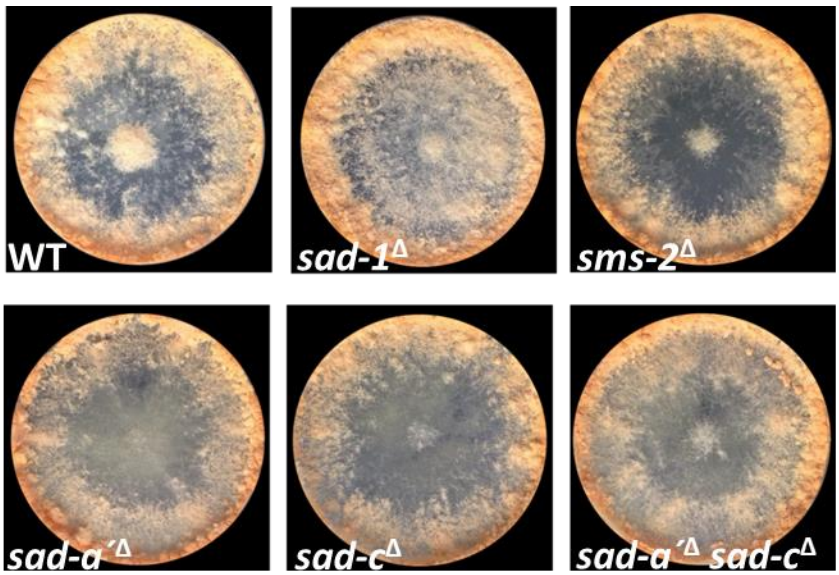


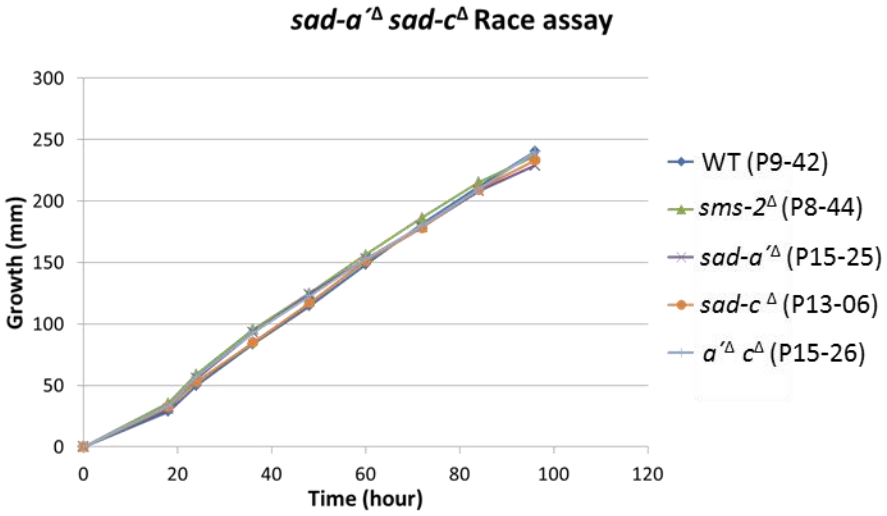
**Figure 2-6.** ***sad-a´*** **and** ***sad-c*** **are not required for vegetative growth.**

(A) Plate Assay**.** No obvious morphological differences are visible between WT and *sad* deletion strains in the vegetative phase. (B) Race Tube Assay. Linear growth of *sad* deletion strains is normal. The identification number for each strain is included.

A




B



# CHAPTER III - LOCALIZATION AND PHYSICAL INTERACTION OF MSUD PROTEINS

## Introduction

It is possible that MSUD proteins form a complex and process aberrant RNA molecules as they exit the nucleus. It has already been shown that SAD-1, SAD-2, DCL-1, SMS-2, QIP, SAD-3, and SAD-4 all localize to the perinuclear region. Furthermore, many of these MSUD proteins colocalize in this region during prophase I of meiosis (SHIU *et al.* 2006; ALEXANDER *et al.* 2008; XIAO *et al.* 2010; HAMMOND *et al.* 2011a; HAMMOND *et al.* 2013). We asked if we could also tag *sad-a´* and *sad-c* with GFP to determine their subcellular localization.

A variety of genetic tools have been developed to understand relationships among different genes and proteins. Bimolecular fluorescence complementation (BiFC) is one technique used to directly visualize protein-protein interactions *in vivo* (HU *et al.* 2002). The yellow fluorescence protein (YFP) is first split into two molecules. Each protein of interest is tagged with one half of the YFP molecule. If these two proteins of interest physically interact, then the two halves of the YFP molecule will be in close enough proximity to fluoresce under the microscope. If the two proteins of interest do not interact, then there will be no fluorescence emitted due to the fact that the split YFP molecules cannot fluoresce on their own. It has been shown through BiFC that SAD-1 and SAD-2 (BARDIYA *et al.* 2008) as well as QIP and SMS-2 (HAMMOND *et al.* 2011b)

have direct interactions. In addition, SAD-3 interacts with SAD-1, SAD-2, SMS-2, and QIP (HAMMOND *et al.* 2011a). Because Arb1 and Arb2 form a complex in the fission yeast *S. pombe* (BUKER *et al.* 2007), it is likely that SAD-A´ and SAD-C will also directly interact in *N. crassa*.

siRNAs cannot be detected in *S. pombe arb1*$^{\Delta}$ and *arb2*$^{\Delta}$ cells, indicating their role in siRNA generation. Arb1 and Arb2 localize to the same region as Ago1 (Argonaute) and form a complex with it known as the ARC (Argonaute siRNA chaperone) (BUKER *et al.* 2007). It is possible that SAD-A´ and SAD-C interact with the *N. crassa* argonaute SMS-2 during MSUD activity. In this study, we set out to determine if SAD-C has direct interaction with SMS-2 in *N. crassa*.

Until recently, the use of biochemical methods in *N. crassa* was quite challenging, but improvement of these techniques provides another valuable tool for researchers to investigate biological processes. HONDA and SELKER (2009) constructed an expression vector containing the HAT-FLAG epitope for use in verifying a histone protein complex through a protein purification mechanism. The standard biochemical protocols established for *N. crassa* have been improved and expanded upon by GUO *et al.* (2010). Co-immunoprecipitation is a technique using antibodies for a specific target protein to identify interacting proteins within a protein complex. Following protein extraction, FLAG-tagged samples are incubated with anti-FLAG-coupled beads. Any unbound proteins are washed away, and the bound complexes are eluted from the beads. The eluate is analyzed on a Western blot to detect protein complexes bound to the anti-FLAG-coupled beads. In this study, we utilized the HAT-FLAG epitope tag provided by

HONDA and SELKER (2009) and the protocols described by GUO *et al.* (2010) to perform biochemical analysis on SAD-C and SMS-2.

## Methods

Most of the methods described herein are modified from standard *Neurospora* protocols (http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm).

**Table 3-1. Strains used in this study**

| Strain | Genotype |
|---|---|
| F2-23 | *rid fl* A |
| F4-36 | *rid sad-c-gfp::hph fl* a |
| F4-37 | *rid sad-a´-gfp::hph fl* a |
| F5-06 | *gfp-sms-2::hph fl* a |
| F6-37 | *rid his-3; flag-hat-sms-2::hph fl* a |
| P6-59 | *rid sad-1*$^{\Delta}$*::hyg; his-3*$^{+}$*::yfpn* A |
| P8-25 | *rid his-3*$^{+}$*::yfpc; inv sad-2*$^{RIP}$ a |
| P9-42 | Oak Ridge wild type a |
| P15-12 | *rid his-3; sad-c-gfp::hph* A |
| P15-13 | *rid his-3; sad-a´-gfp::hph* A |
| P15-37 | *rid yfpn-sms-2::hph* A |
| P21-63 | *rid his-3; flag-hat-sad-c::hph* A |
| P21-64 | *rid mus52::bar; yfpn-sad-a´::hph* A |
| P21-65 | *rid yfpc-sad-c::hph* a |
| P21-66 | *rid flag-hat-sad-c::hph; gfp-sms-2::hph* a |
| P21-67 | *rid his-3; flag-hat-sad-c::hph; gfp-sms-2::hph* A |
| P21-68 | *rid; flag-hat-sms-2::hph* A |

### Media

10× FIGS- 100 g sorbose, 2.5 g fructose, 2.5 g glucose, and 1 g inositol were added to 500 mL nanopure water and autoclaved for 30 minutes. The solution was stored at room temperature.

Top agar- 2 g Bacto Agar (BD), 36.4 g sorbitol, 8 mL 25× Vogel's salts, and 172 mL nanopure water were added to 500 mL bottle and autoclaved. After sterilization, 20 mL of 10× FIGS was added. The agar was kept molten at 50°C until it was needed.

Bottom agar- 24 mL 25× Vogel's salts, 9 g Bacto Agar (BD), and 516 mL nanopure water were added to a 1 L bottle and autoclaved for 30 minutes. After sterilization, 60 mL 10× FIGS was added to the molten mix. Twenty mL was poured into 100 x 15 mm round petri dishes to solidify.

**Fungal Transformation**

Recipient strains were inoculated to 100 mL of Vogel's agar (plus any additional necessary supplements) in a 500 mL flask. Flasks were then incubated at 30°C for 5-7 days to allow for robust conidial growth. Conidia from the flasks was collected to 30 mL of 1M sorbitol, in 50 mL tubes, and then shaken. Next, conidia were passed through a 100 μm vacuum filter column into another 50 mL tube. 100 μL of filtered conidia were diluted to 1000 μL by adding 900 μL of 1M sorbitol. Absorbance was measured at 420 nm on a spectrophotometer. The remaining filtered conidia were then spun down at 3200 rpm for 10 minutes. The supernatant was then gently removed and the conidial pellet re-suspended in 1M sorbitol at 1 unit/μL. Transformation DNA (50 ng) was diluted to 10 μL with water and 90 μL of the conidia/sorbitol mix was added to the DNA. The entire mix was then added to a 1 mm gap cell and placed on ice. When all of the samples were ready, an electroporator was set to 1500 volts. Post-electrocution, 750 μL of 1M sorbitol was added to the gap cell before the entire mix was transferred to a new 50 mL tube and placed on ice. To aid in the recovery of the conidial cells, 4.2 mL of Vogel's liquid media

was added to the cell mix and the entire solution was placed in a shaker for 3-4 hours at 30°C and 80 rpm. Finally, 500 µL of the conidia mix was mixed with 20 mL of top agar and then poured onto a bottom agar plate and incubated at 37°C for ~3 days.

**Double-Joint PCR**

Double-joint PCR was conducted in the manner described in (HAMMOND *et al.* 2011b). This process requires the production of 5′ and 3′ regions flanking a gene of interest and a center fragment containing the fluorescent or epitope tag (-*gfp*, -*yfpn*, -*yfpc*, or -*hat-flag*). The first round of PCR amplifies the three components (5′ and 3′ flanking fragments and the center fragment). Reactions from the first round are combined into one tube without any specific primers for the second round of PCR as the overhanging chimeric extensions in the flanking regions act as primers. In the third round of PCR, nested primers amplify the final product. The final product is subsequently transformed into a *N. crassa* strain by electroporation. *gfp*-tagged *sad-a´* and *sad-c* strains used in this study were tagged at the C-terminus while all other strains were tagged at the N-terminus.

**Sample Preparation and Fluorescence Microscopy**

Perithecia were fixed in a fresh solution of 4% paraformaldehyde, 90 mM PIPES pH 6.9, 10 mM EGTA, and 5 mM $MgSO_4$ for 20 minutes at room temperature. After a brief rinse with phosphate buffered saline (PBS), perithecial contents were dissected out into a drop of 90% glycerol, 10% 100 mM $K_2HPO_4$ pH 8.7, 10 mg/mL DAPI, and 100 mg/mL 1,4-diazabicyclo[2,2,2]octane. Once the perithecial contents were dispersed under a cover slip, they were sealed with clear nail polish and imaged after storage at -20° C overnight. All samples were imaged under an Olympus BX61 fluorescence microscope using FITC,

Cy5, and DAPI filter cubes. Images were captured using Metamorph (Molecular Devices) software.

**Fungal Protein Extraction**

*N. crassa* strains were grown at 32°C in 250-mL flasks containing 50 ml Vogel's minimal medium and supplements required based on their genotypes. After 48 hours, the resulting vegetative tissue was blotted dry with paper towels and ground into a fine powder using a mortar and pestle then quickly placed in liquid nitrogen. Sexual tissue was prepared by crossing strains on WG plates overlaid with a single layer of Miracloth (Calbiochem) and incubated at room temperature for 6 days. Perithecia were then scraped from the Miracloth using a razor blade and ground into a fine powder using a mortar and pestle then quickly placed in liquid nitrogen.

Frozen samples were suspended in 1 ml of ice-cold lysis buffer (50 mM HEPES [pH 7.5], 137 mM NaCl, 10% glycerol, 1mg/ml pepstatin A, 1 mg/ml leupeptin, and 100 mM phenylmethylsulfonyl fluoride [PMSF]). Extracts were incubated on ice for 10 minutes followed by centrifugation at 15,000 g for 15 minutes at 4°C. Protein concentrations were measured by Bradford Assay.

**Co-Immunoprecipitation and Western Blots**

Protein samples (1 mg each) were incubated with ANTI-FLAG M2 affinity gel (Sigma) at 4°C overnight. Immune complexes were washed three times in PBS and suspended in 2× LDS sample buffer. Samples were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes in transfer buffer (Invitrogen) for 1 hour. Membranes were blocked in 1× PBS and 0.05% Triton X-100

containing 5% skim milk powder for 1 hour and incubated for 1 hour at room temperature with anti-FLAG M2 antibody (Sigma) in PBST containing 5% skim milk; rabbit anti-GFP antibody (Abcam) in PBST containing containing 5% skim milk. Immunoprecipitates were detected by using horseradish peroxidase-conjugated secondary antibodies and ECL chemiluminescent substrate (GE Life Sciences) as described in the manufacturer's instructions.

**Immunoprecipitation with Mass Spectrometry**

Protein samples (1 mg each) were incubated overnight at 4° with anti-FLAG M2 affinity gel (Sigma) on a rotator. Mass spectrometry analysis was done at the University of Missouri Proteomics Core. The supplied beads were washed once with 25 mM ammonium bicarbonate pH 7.8, centrifuged and the supernatant removed, and then resuspended in 20 µL of 6M urea/100mM HEPES pH 7.8. Proteins (Cys residues) were reduced with DTT and alkylated with IAA, and then digested overnight with trypsin (0.6 µg per reaction). Digestion was halted by adding formic acid to 1% final concentration (v/v) and then peptides were pre-enriched using a large-format 100 µL C18 tip (Pierce). Peptides were eluted from the tips in 70% acetonitrile (ACN)/1% formic acid (FA), lyophilized, and resuspended in 21 µL of 5/1 % ACN/FA.

A full-loop injection (18 µL) was loaded onto a C8 trap column (Pepmap 100 C8, Dionex/Thermo) and then eluted onto a 25 cm long, 150 µm inner diameter, pulled-needle analytical column packed with HxSIL C18 reversed phase resin (The Hamilton Co.). Peptides were separated and eluted from the analytical column with a gradient of acetonitrile as follows: initial conditions (during trap load) and for the first 2 min was 5%

B (A: 0.1% formic acid in water; B: 99.9% acetonitrile, 0.1% FA), followed by a gradient from 5 to 20% B over 20 min, gradient from 20-30% B over 30 min, ramp to 90% B over 10 min, hold at 90% B for 22 min, ramp to 5% B over 1 min, hold at 5% B for 5 min prior to loading next sample. The Proxeon Easy nLC system is attached to an LTQ Orbitrap XL mass spectrometer. Following a high-resolution (30,000 res, profile) FTMS scan of the eluting peptides (300-1800 m/z range), each cycle, the 9 most abundant peptides (reject porcine trypsin autolysis ions, reject +1 ions, >2000 counts) were subjected to ion-trap CID peptide fragmentation (NCE of 35%, centroid, isolation width of 2). Dynamic exclusion was enabled with repeat count of 1, repeat duration of 30 sec, exclusion list of 500, and exclusion duration of 180 sec. Data across a total of 90 minutes of elution were collected.

Raw data were copied to the Sorcerer2 IDA and searched against the NCBI-Ncrassa-only database. A scaffold (.sfd) file was outputted by the Sorcerer and imported into the Scaffold software V3.4.8 and examined for hits. Full details of data processing and search parameters are available on request.

## Results

### SAD-A´ and SAD-C are preferentially localized in the perinuclear region

To determine the subcellular localization of SAD-A´ and SAD-C, their genes were tagged with GFP using double-joint PCR (HAMMOND *et al.* 2011b). Microscopic analysis indicates that both SAD-A´-GFP and SAD-C-GFP are present diffusely throughout the cytoplasm, but intense expression is visualized as a ring around the nucleus during prophase I of meiosis (Figure 3-1). Both fusion constructs are fully

functional in that they allow production of ascospores when put through a homozygous cross. These results are consistent with the other perinuclear MSUD components.

**Bimolecular complementation reveals interaction between MSUD components**

*yfpn-sad-a´* and *yfpc-sad*-c strains were constructed using double-joint PCR (HAMMOND *et al.* 2011b). These two strains were crossed, and the resulting asci were examined using fluorescence microscopy. We observed intense yellow fluorescence in the perinuclear region, indicating an interaction (Figure 3-2 A). YFPN-SAD-A´ (Figure 3-2 C) or YFPC-SAD-C (data not shown) does not fluoresce on their own. When co-expressed, the untagged split-YFP molecules do not fluoresce on their own (Figure 3-2 D), confirming that the protein-protein interaction above is genuine.

The previously constructed *yfpn-sms-2* (HAMMOND *et al.* 2011b) strain was crossed to the *yfpc-sad-c* strain, and fluorescence was observed under the microscope. A direct interaction between the protein products of these constructs was visualized (Figure 3-2 B). Taken together, these data suggest that SAD-A´ and SAD-C are a part of the MSUD complex and interact in the perinuclear region.

**Co-immunoprecipitation suggests SAD-C and SMS-2 are part of an MSUD protein complex**

SAD-C encodes a 450 amino acid protein that includes an Arb2 domain. The *flag-hat-sad-c* strain was constructed using double-joint PCR (HAMMOND *et al.* 2011b) and crossed to a *gfp-sms-2* strain to confirm the interaction between SAD-C and SMS-2. Co-IP assays were performed on *flag-hat-sad-c*; *gfp-sms-2* and control protein samples. A Western blot for anti-FLAG detected a protein at the expected size of approximately 58

kDa in the *flag-hat-sad*-c and *flag-hat-sad-c; gfp-sms-2* samples. A Western blot for anti-GFP detected a protein for *gfp-sms-2* in the input samples and for *flag-hat-sad-c; gfp-sms-2* in the IP samples at the expected size of 145 kDa. (Figure 3-3). No signal was detected in strains without the FLAG epitope. These data strongly support the BiFC results in which SAD-C and SMS-2 form a protein complex.

**Immunoprecipitation by FLAG-affinity with Mass Spectrometry identifies potential protein interactions for SMS-2**

Mass spectrometry (MS) is a sophisticated tool for measuring characteristics of individual molecules. Coupling MS with immunoprecipitation provides a sensitive and accurate way to identify new protein complexes. In this work, we set out to detect novel interacting partners for the MSUD Argonaute SMS-2. SMS-2 encodes a 990 amino acid protein containing both PAZ and PIWI domains. The *flag-hat-sms-2* strain was constructed using double-joint PCR (HAMMOND *et al.* 2011b). In a preliminary work, mass spectrometry analysis of immunoprecipitated FLAG-HAT-SMS-2 from vegetative tissues identified several previously unknown interacting partners (Table 3-2). Interestingly, but perhaps not surprising, no known interacting partners of SMS-2 were pulled down in this assay, since they are not highly expressed during the vegetative phase (SAMARAJEEWA *et al.* 2014). Ongoing work in our laboratory is aimed at determining if these proteins interact with SMS-2 during the sexual phase and if they are important for MSUD.

## Discussion

Like many of the other MSUD components, SAD-A´ and SAD-C preferentially localize in the perinuclear region. Presumably, aberrant RNAs are exported from the nucleus to be processed immediately by the MSUD machinery in this region. Previous experiments suggest that SAD-2 acts as a scaffold protein in MSUD. Functional *sad-2*$^{+}$ is required for the localization of SAD-1 to the perinuclear region; however, the reverse is not true (SHIU et al. 2006; BARDIYA et al. 2008). Other lab members are currently determining if SAD-2 recruits other MSUD components to the perinuclear region. Recently, it was shown that SAD-2 is required for SAD-A´ and SAD-C's localization in the perinuclear region (BOONE et al., unpublished results). This supports the idea that SAD-A´ and SAD-C are being recruited to the perinuclear region and are critical to the production of siRNAs.

Using BiFC, we revealed the interaction of SAD-A´ and SAD-C *in vivo*. Additionally, SAD-C and SMS-2 also directly interact. This SAD-A´, SAD-C, and SMS-2 complex is currently being examined further. Experiments for YFPN-SMS-2 and YFPC-SAD-A´ are currently underway. If these three proteins are forming a complex similar to that in *S. pombe*, it is possible that they are performing a similar function. In *S. pombe*, Arb1, Arb2, and Ago1 form a complex known as ARC. It is proposed that Arb1 and Arb2 act to shuttle dsRNAs from Dicer to Ago1. Once Arb1 and Arb2 dissociate from the ARC, Ago1 can then cleave the dsRNAs into ssRNAs. This is supported by the fact that Arb1 and Arb2 are only associated with dsRNAs (BUKER *et al.* 2007). Additional studies exploring SAD-A´ and SAD-C's interactions with DCL-1 (and others)

may define the link between DCL-1 and SMS-2 and would provide more insight into their proposed function for MSUD.

This work marks the first time biochemical methods have been used to examine MSUD interactions. The results of our experiments confirmed the SMS-2 and SAD-C interaction and revealed potential new interactions for SMS-2. While it would be ideal to examine MSUD interactions using sexual tissue, these studies were conducted using vegetative tissue containing constitutive protein expression for tagged constructs. The problem with using sexual tissue in biochemical analysis is the difficulty in obtaining sufficient amounts for detection. Several attempts were made at protein extraction from sexual tissue during the course of this work; however, a signal was never detected by Western blot or by Mass Spectrometry analysis (data not shown). We anticipate that over time the development of more efficient extraction and/or more sensitive detection methods will allow proteins from sexual tissue to be investigated by these assays; however, the results in this study provide additional insight into MSUD protein-protein interactions. Co-IP can be used as a tool to support BiFC analysis. IP with Mass Spectrometry may identify other MSUD components not detected through our "silencing the silencer" screenings. It will be interesting to learn if these previously unknown SMS-2-interacting proteins identified by MS are important for meiotic silencing. For now, we propose that SAD-A´ and SAD-C form a complex with the Argonaute SMS-2 and are important for MSUD.

## Figures

**Figure 3-1. SAD-A´ and SAD-C are preferentially localized in the perinuclear region.**

Asci expressing SAD-A´-GFP (left) and SAD-C-GFP (right) during prophase I of meiosis. Both proteins are localized throughout the cytoplasm with a preference for the perinuclear region. Chromatin was stained with DAPI.

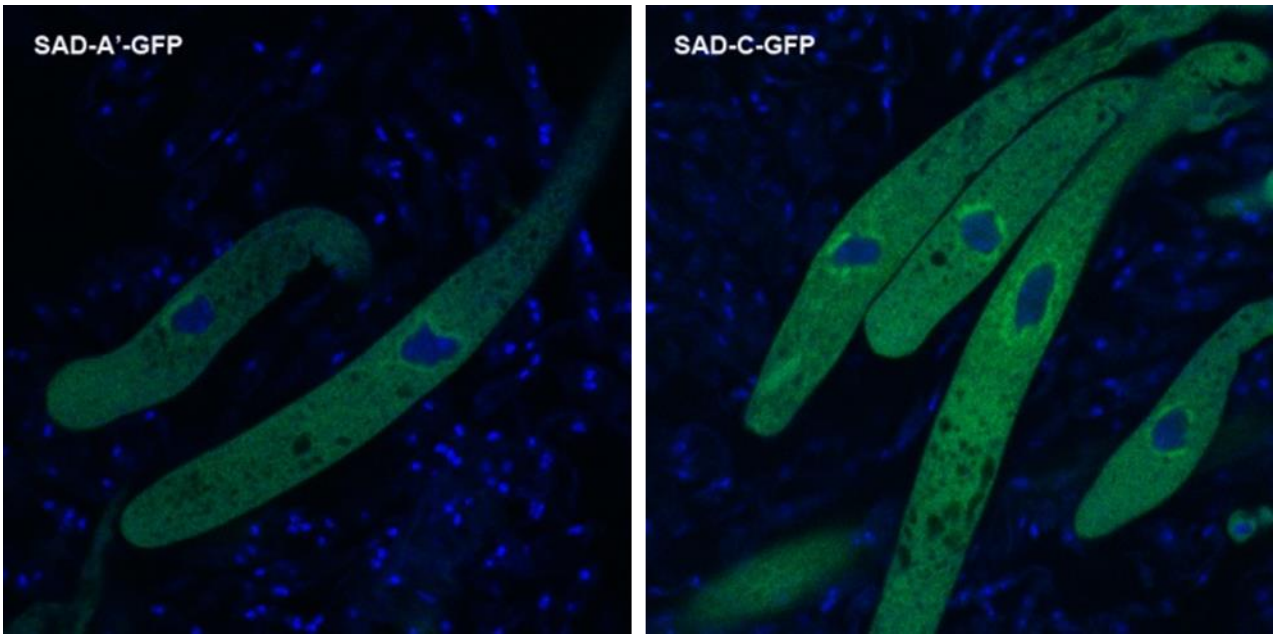


**Figure 3-2. *in vivo* interactions among MSUD proteins**

Asci expressing (A) YFPN-SAD-A´;YFPC-SAD-C, (B) YFPN-SMS-2;YFPC-SAD-C, (C)YFPN-SAD-A´; YFPN-SAD-A´ (D) YFPN; YFPC during prophase I of meiosis. Chromatin was stained with DAPI.

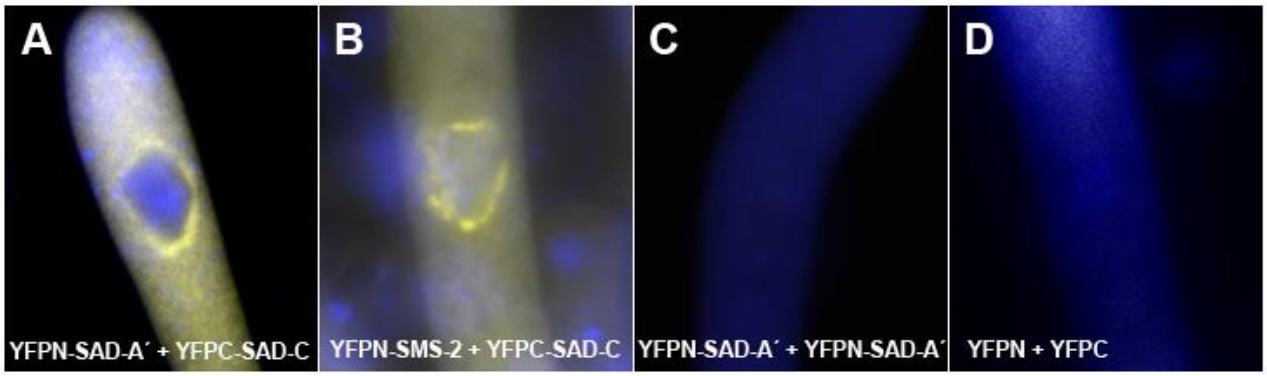


**Figure 3-3. Co-IP assays of epitope-tagged SAD-C and SMS-2 confirm interaction.**

Extracts from strains with or without FLAG-HAT-tagged SAD-C and/or GFP-tagged SMS-2 were immunoprecipitated with ANTI-FLAG M2 affinity gel. Input (total protein lysate) and immunoprecipitation (IP) samples were separated by SDS-PAGE and transferred to nitrocellulose membranes and immunoblotted with anti-FLAG antibodies or anti-GFP antibodies, as indicated.

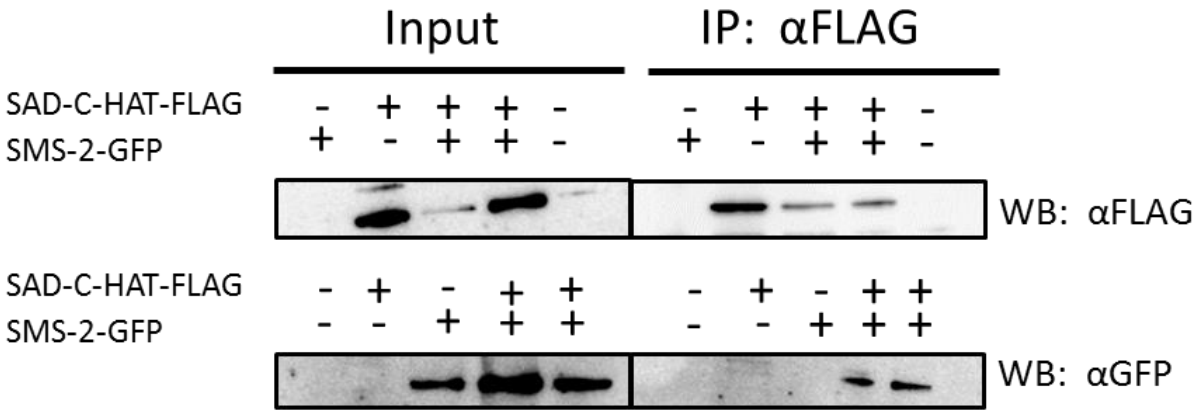


**Table 3-2. Summary of identified proteins detected in FLAG-HAT- SMS-2 affinity-purified sample**

| Identified Proteins | Molecular Weight (kDa) | Fold Change* | Exclusive Unique Spectral Count† | |
|---|---|---|---|---|
| | | | FLAG-HAT-SMS-2 | WT |
| SMS-2 (#09434) | 112 | INF | 12.33 | 0.00 |
| RNA binding domain-containing protein (#01793) | 26 | INF | 2.33 | 0.00 |
| Eukaryotic Translation Initiation Factor-3 (#06279) | 54 | 7.0 | 4.67 | 0.67 |
| Hypothetical protein with hyaluronan/mRNA-binding family domain (#00225) | 34 | 2.3 | 7.00 | 3.00 |
| Subunit of the Arp2/3 complex (#03050) | 49 | 2.5 | 9 | 3.67 |
| Hypothetical protein with RNA recognition motifs & Nuclear transport factor 2 domain (#07574) | 56 | 2.7 | 5.33 | 2.00 |

* Fold change provides a relative quantification measure between two sample sets. It is defined as the ratio between the average of FLAG-HAT-SMS-2 samples versus the average of wild-type samples. Fisher's Exact Test of spectral counts for the differentially-abundant proteins show $p \leq 0.05$; therefore, fold-changes $\geq 2$ indicate a statistically significant change between sample sets. If a zero appears in the denominator, an INF will appear.

† Spectral count is the primary measure of protein abundance. Exclusive Unique Spectral Count refers to the total number of distinct spectra associated only with a single protein, averaged over three replicates. Spectra are considered distinct when they identify different amino acids or peptides that were observed in the MS experiment. A minimum of 2 spectral counts are needed for identification; however, a minimum of 4 spectral counts are needed for quantification.

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