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Investigating The Initial Detection Stage Of Meiotic Silencing By Unpaired Dna In The Model Organism Neurospora Crassa

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INVESTIGATING THE INITIAL DETECTION STAGE OF MEIOTIC SILENCING

BY UNPAIRED DNA IN THE MODEL ORGANISM

NEUROSPORA CRASSA

Pegan A. Sauls

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In an attempt to neutralize transposable elements or retrovirus invasions *Neurospora crassa* will rely on one of its many genome defense mechanism, Meiotic Silencing by Unpaired DNA (MSUD). MSUD works in a two-step process that first detects unpaired sequences between homologous chromosomes followed by downstream silenced expression of the sequence. The ultimate silencing stage of MSUD is widely accepted to operate through an RNAi-like system. However, the mechanics of the detection step of MSUD remains elusive.

The research presented attempts to elaborate on how the initial stage of MSUD occurs and its specifics. First, a genetic approach is utilized to answer what kind of genomic distance limitations are placed on the homology searching procedure of this mechanism. In these experiments, I have inserted a genetic phenotypic marker at different locations on *N.crassa*'s chromosome VII. Many combinations of strains were crossed to create varying distances of the marker between homologs during a sexual cross. Interestingly, I observed mixed phenotypes when markers were physically unpaired by as

small a distance as 13.9 kb. This suggested that MSUD was only partially detecting the unpairing events. Overall, the experimental crosses expressed an interestingtrend that illustrated a positive correlation between increasing marker distance and MSUD activity. I concluded that the searching process is effected by distance and may not search in a linear manner.

Secondly, biochemical attempts were conducted to purify the first recognized nuclear MSUD protein, SAD-5. I inserted the *sad-5* gene into a pET15b expression vector and attempted to express the protein in Lemo21 *E.coli* cells. After multiple rounds of unsuccessful purification using many techniques to try to alleviate the protein from the insoluble fraction, I decided to move the vector to a different cell line. The new cell line, ArcticExpress, was believed to be more suitable because of its modified ability to produce charperonin proteins to aid folding of the recombinant protein. ArcticExpress cells are also adapted to grow at lower temperatures which is thought to support proper protein folding as well. However, expressing the recombinant protein in this cell line failed to solubilize the SAD-5 protein. It is unfortunate that all attempts were unsuccessful, but many alternative methods have still yet to be tested. One potential alternative would be to move the eukaryotic protein to a eukaryotic system. Once the protein is successfully purified, protein binding assays can be accomplished to determine SAD-5's binding preferences to different substrates allowing insight into the protein's function.

The research outlined is only the beginning of our understanding of how MSUD's detection processes operates. As our lab continues to investigate this phenomenon, we may find that the characteristics of MSUD that we discover may elaborate on current

problems in biological research and medicine such as RNAi treatments for cancer or

retroviral detection.

INVESTIGATING THE INITIAL DETECTION STAGE OF MEIOTIC SILENCING

BY UNPAIRED DNA IN THE MODEL ORGANISM

NEUROSPORA CRASSA

PEGAN A. SAULS

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NEUROSPORA CRASSA

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P.A.S.

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CHAPTER I

MEIOTIC SILENCING BY UNPAIRED DNA

Introduction to Meiotic Silencing by Unpaired DNA

Meiotic Silencing by Unpaired DNA (MSUD) describes a fundamental pathway for regulating expression of unpaired DNA. This process is considered one of the model organism *Neurospora crassa*'s genome defense mechanisms and is found to function in similar to RNAi (SHIU *et al.* 2001, 2006; SHIU and METZENBERG 2002; LEE *et al.* 2003; HAMMOND *et al.* 2011, 2013a; b). MSUD is believed to occur in two separate stages: the detection stage and the silencing stage. While there has been increasing information discovered about the silencing stage, the detection stage of MSUD has remained elusive. It has therefore been our goal to elucidate on this initial stage through the experiments outlined in the following chapters.

It is hypothesized that the initial stage of MSUD must utilize a type of homology search for detecting unpaired DNA. It has therefore been suggested that homologous recombination or a similar mechanism may be the homology searching process of choice (SAMARAJEEWA *et al.* 2014). The timing of MSUD suggests that it coincides with meiotic homologous recombination activity making it a worthy candidate for MSUD's homology searching machinery (COHEN and POLLARD 2001). Thus, it is important to investigate these processes for multiple reasons. Homologous recombination plays multiple roles in protecting our genome. This fundamental process is needed during

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meiosis for genetic exchange, DNA repair of double-stranded breaks (DSB) (also occurs during mitosis), and chromosomal synapsis (QIAO *et al.* 2012). If MSUD follows a pathway similar to homologous recombination, any information gained about this process will also enhance our understanding of meiosis. Further, these results may prove to be particularly important for additional understanding of homologous recombination activity as it has become a target for cancer therapies (HOEIJMAKERS 2001; BINDRA *et al.* 2004; HELLEDAY *et al.* 2005). Cancer therapies like radiation treatment can induce DSB breaks in the DNA. However, the therapy can be less effective with a functioning DSB repair process like homologous recombination (ROTHKAMM and LOBRICH 2002). Learning more about how HR functions can produce better specific targets for disrupting repair, leading to more successful eradication of cancer cells.

The overall purpose of MSUD is its contribution to the genome defense system in *N. crassa*. Specifically, it is believed that MSUD helps keep the *N. crassa* genome free of foreign genetic invaders, such as transposable elements or retrovirus that are capable of incorporating their genetic material into a genome (SELKER *et al.* 1987; SELKER 1990; GLASS *et al.* 2000; SHIU *et al.* 2001; DANG *et al.* 2011). As a result of such successful defenses, *N. crassa*'s genome has an extremely low level of repetitive DNA and a relatively small genome (GALAGAN *et al.* 2003; KELLY and ARAMAYO 2007). Studying these defense mechanisms in *N. crassa* may clarify why other eukaryotes are not as successful at identifying and silencing foreign DNA. Investigation of these systems offers the potential to gain more information about detection of human retroviruses such as HIV/AIDS. As a result, any knowledge gained could lead to advances in combating such diseases.

The results of these experiments are also important to give insight into general RNAi-like pathways and their applications. RNAi systems such as co-suppression in plants, quelling in fungi, and RNAi in mammals have already contributed significant advances in science. For example, the knowledge of RNAi pathways has produced common laboratory techniques such as gene knockdowns for experimental research. Researchers have discovered that they are able to reproduce the same effects that the endogenous cellular process does by introducing synthetic small interfering RNAs (siRNA) into a system (SCHERER and ROSSI 2003). The increase in understanding of RNAi pathways has also allowed further advancement in potential medicinal uses for cancer, permitting specific targeting of disease related genes (AMARAVADI *et al.* 2011; UCHINO *et al.* 2013). However, the mechanisms of these systems are not completely understood, and medicinal therapies that utilize this system still have adversities to overcome. For instance, improvement upon problems such as off-target effects, a triggered immune response, and effective delivery are still underway (SCHERER and ROSSI 2003; AAGAARD and ROSSI 2007). Therefore, any further investigation into these processes are not only beneficial for gaining insight into the mechanism but can also create substantial downstream uses in many biological areas.

The research presented in the following chapters illustrates two very different approaches to elaborate on the detection process using genetic manipulation as well as biochemical methods. The first experiment presented outlines fungal sexual crosses between parent strains carrying a genetic marker at different locations. My ultimate goal was to test the distance sensitivity of MSUD's homology search on the homolog's unpaired genes. The second experiment introduced will explain attempts at isolation of a

MSUD protein, SAD-5, believed to be active during the detection stage. My goal was to effectively purify the recombinant protein to later test its affinity for different nucleic acids or other proteins. With this information, we can gain insight into the protein's function.

However, before the experiments can be described in further detail, an introduction and literature review of pertinent information is provided below. This will cover background information *N. crassa* and its main genome defense pathways. The discussion will finally lead into specifics about the MSUD mechanism and the genes involved.

Literature Review

Neurospora crassa: A Model Organism for Fungal Genetics. The MSUD mechanism has been identified in fungi such as *Gibberella zeae* (SON *et al.* 2011) and similar mechanisms called Meiotic Silencing of Unsynapsed Chromosomes (MSUC) and Meiotic Sex Chromosome Inactivation (MSCI) have been discovered in mice and nematodes (KELLY *et al.* 2002; TURNER *et al.* 2005; CLOUTIER and TURNER 2010). However, the model organism used for our studies, *Neurospora crassa*, was the original organism in which a meiotic silencing mechanism (MSUD) was discovered (ARAMAYO and METZENBERG 1996; SHIU *et al.* 2001).

The filamentous fungus *Neurospora crassa* (named due to its characteristic grooved spores (SHEAR and DODGE 1927)) belongs to the phylum Ascomycota, meaning 'sac fungi' (WEBSTER and WEBER 2007). Including at least 64,000 different species, Ascomycota are by far the largest fungal phyla (DEACON 2005). Characteristic of Ascomycota, *N. crassa* produce elongated sac-like asci that form during its sexual phase

(**Figure I-1**). When mature, these asci will contain eight black spores (ascospores) that will be ejected from the fruiting body for further propagation (WEBSTER and WEBER 2007).

N. crassa, once referred to as a "bakery pest" in the 1900s (PAYEN 1843; PERKINS 1992), is now considered a model organism for genetic investigations. *N. crassa* is a suitable organism for genetic work because its entire genome is sequenced (GALAGAN *et al.* 2003). It is easy to culture and has rapid growth in the lab. Another benefit of this orange mold revolves around the fact that it spends most of its lifecycle as haploid. As a direct consequence of this haploid state we can easily determine the parental origin of all expressed traits.

Neurospora crassa's Genome Defense Mechanisms. Considering this organism is coenocytic, meaning multiple nuclei share the same cytoplasm, *N. crassa* is vulnerable to invasions from viruses and transposons that could lead to compromised genome integrity (SELKER 1990, 1997; GLASS *et al.* 2000; DANG *et al.* 2011). Interestingly, despite that vulnerability, *N. crassa* maintains a small genome with a very low repetitive content (GALAGAN *et al.* 2003; KELLY and ARAMAYO 2007). This sustainability can be attributed to what some call *Neurospora's* 'genome paranoia' (KELLY and ARAMAYO 2007) which is illustrated by multiple defenses that allow protection over its genome throughout the course of its lifecycle (**Figure I-1**). *N. crassa* protects its DNA with the use of processes like Repeat-Induced Point mutation (RIP), quelling, and the defense system our lab examines labeled Meiotic Silencing by Unpaired DNA (MSUD) (CAMBARERI *et al.* 1989, 1991; SELKER 1990; ROMANO and MACINO 1992; ARAMAYO and METZENBERG 1996;

SHIU *et al.* 2001; GALAGAN and SELKER 2004; KELLY and ARAMAYO 2007; CHANG *et al.* 2012).

The other pathways used by *Neurospora* to defend the genome are important to understand because they provide a context for defining MSUD. Paving the way for the other processes, RIP was not only the first genome defense mechanism to be discovered in *Neurospora* but was also the first genome defense mechanism to be described in any eukaryote (SELKER *et al.* 1987; SELKER 1990, 2002). Like MSUD, RIP can occur during the sexual phase of *N. crassa*'s lifecycle. However, RIP is believed to occur right before karyogamy (fusing nuclei of opposite mating type) which is before the stage in which MSUD happens (SELKER *et al.* 1987; SELKER 1990; SHIU *et al.* 2001). Interestingly, the mechanism of RIP is not similar to that of MSUD. RIP creates mutations at inserted DNAs (SELKER *et al.* 1987; SELKER 1990). Nonetheless, RIP does have the same end goal as MSUD to eliminate expression of these sequences at certain lifecycle stages.

The gene silencing mechanism, quelling, is important to describe because it is most similar to MSUD and even shares some of the same proteins (**Figure I-2)** (CATALANOTTO *et al.* 2004; MAITI *et al.* 2007; ALEXANDER *et al.* 2008; LEE *et al.* 2010b; XIAO *et al.* 2010). Although the trigger for action is slightly different in quelling due to a haploid genome stage, it could be described as MSUD's counterpart in the vegetative stage. Quelling was first discovered in fungi soon after the discovery of cosuppression, a related process in plants (NAPOLI *et al.* 1990; ROMANO and MACINO 1992). It was observed while inserting extra copies of the *albino-1* (*al-1*) or *albino-3* (*al-3*) genes into the *N. crassa* genome. Under normal expression, the albino genes account for *Neurospora's* bright orange color. However, when increasing amounts of ectopic

transgenes were added, the fungi became more white in color. This suggested a silencing effect of the endogenous *al* genes by the transgenes (ROMANO and MACINO 1992; CARATTOLI *et al.* 1994; COGONI *et al.* 1996; FULCI and MACINO 2007). Further investigation showed that gene silencing during quelling does not alter transcript precursor levels of the silenced endogenous gene. This suggested that the route of silencing was not occurring before transcription. However, a sense RNA produced specifically from the transgene was observed and was absent when the strain was reverted back to w.t. (COGONI *et al.* 1996). With this data it was reasoned that quelling was effecting gene expression after transcription and it is now referred to as an RNAi-like pathway for posttranscriptional gene silencing (PTGS).

The final genome defense process to be described, MSUD, will be the central focus of the experiments outlined later. MSUD was first discovered during manipulation of the ascospore maturation gene (*asm-1$^+$*) in *N. crassa*. When expressed correctly this gene is responsible for a mature spore's black color along with its ability to develop into the vegetative stage. Surprisingly, all progeny of an *asm-1$^+$* x *asm-1^Δ* cross were immature and white. Thus, the a*sm-1^Δ* mutant phenotype was labeled "ascus-dominant" indicating the knock-out allele was controlling the phenotype. Interestingly, in an attempt to rescue the knockout strain with an inserted copy of the *asm-1$^+$* at a non-native location, a cross to a wild-type (wt) strain still produced immature spores. It is only when both parental strains have inserted ectopic copies at the same location that all spores produced were mature and fertile. Together, these findings suggested that genes must be located in close proximity to their homologs for proper expression (ARAMAYO and METZENBERG 1996).

Like quelling, MSUD is a posttranscriptional gene silencing mechanism utilized by *N. crassa* (SHIU *et al.* 2001; HAMMOND *et al.* 2013a). However, MSUD occurs during meiosis (ARAMAYO and METZENBERG 1996; SHIU *et al.* 2001). Following karyogamy the organism becomes transiently diploid. At this time *N. crassa* will normally carry a copy of each gene or DNA fragment on each homologous chromosome. During meiotic prophase I, genomes from both parental strains will be aligned in preparation for crossing over events that can generate genetic diversity in the progeny. While the exact timing of MSUD activity is unclear, its expected to act at this point in meiosis (SHIU *et al.* 2001). Its activity is triggered during detection of heterologous or unpaired regions as small as 700 bp between the two homologs, ultimately silencing any expression from the unpaired DNA (LEE *et al.* 2004).

Meiotic Silencing by Unpaired DNA Mechanism. MSUD is believed to occur in two distinct stages; recognition and silencing. During the recognition step, regions of unpaired DNA between homologous chromosomes are detected. The proposed theoretical mechanism posits that a protein complex initially scans and compares sets of homologous chromosomes. Unmatched sequences that are detected are likely flagged to promote the recruitment of polymerases that produce aberrant RNA (aRNA) (SHIU and METZENBERG 2002).

In comparison to the recognition stage, the silencing stage of MSUD is better characterized. Specifically, an RNA interference-like (RNAi) pathway is thought to act on the generated aRNA following recognition (SHIU *et al.* 2001, 2006; ALEXANDER *et al.* 2008; XIAO *et al.* 2010; HAMMOND *et al.* 2011, 2013a). This process is ultimately responsible for silencing the expression of the unpaired region. The initial step in a

typical RNAi pathway is the production of a double-stranded RNA (dsRNA). If the targeted pre-RNA is single-stranded, an RNA-directed RNA polymerase (RdRP) synthesizes its complement to make it a double-stranded molecule. Next, an RNase-III-like Dicer enzyme binds to and cleaves the dsRNA into smaller sequences. These cleaved dsRNAs are then bound by an RNA-induced silencing complex (RISC). The Argonaute protein in RISC removes the passenger strand (anti-sense) of the dsRNA. The guide strand (sense) is left associated with the RISC complex and directs it to a complementary mRNA to trigger degradation or block translation (CHANG *et al.* 2012; BILLMYRE *et al.* 2013).

It was first proposed in 2001 by Shiu *et al.* that silencing of gene expression in MSUD could occur through an RNAi pathway. This was concluded based on MSUD's activity dependence on the discovered SAD-1 (suppressor of ascus dominance) protein. Mutated or deleted SAD-1 caused MSUD silencing to be suppressed at different levels (SHIU *et al.* 2001). SAD-1 is believed to be an RdRP as it shows homology to other RdRP proteins such as those found in *Arabidopsis thaliana* and *Caenorhabditis elegans* (SMARDON *et al.* 2000; DALMAY *et al.* 2000; SHIU *et al.* 2001; SHIU and METZENBERG 2002). It also seems to have homology with *N. crassa's* QDE-1 protein that functions as an RdRP protein for quelling (COGONI and MACINO 1999; SHIU *et al.* 2001; SHIU and METZENBERG 2002). After SAD-1's discovered role in MSUD, many other necessary proteins were also found to be involved that supported the idea that MSUD works through RNAi. These proteins include SMS-2, SAD-2, DCL-1, QIP, and SAD-3 which were recently found to all form a complex in the perinuclear region (SHIU *et al.* 2001; LEE *et al.* 2003; SHIU *et al.* 2006; ALEXANDER *et al.* 2008; XIAO *et al.* 2010; HAMMOND

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et al. 2011, 2013a; D<small>ECKER</small> *et al.* 2015). **Figure I-3** illustrates the MSUD proteins and their expected roles.

SAD-2 was initially found to be necessary for SAD-1 localization to the perinuclear region; however, it has proved to be dissimilar to any protein previously described for an RNAi pathway. Thus, its specific function is not clear (S<small>HIU</small> *et al.* 2006). Although recently it was shown that in the absence of SAD-2, all necessary MSUD proteins lack the ability to localize to the perinuclear region. However, this is not true for the reverse statement (D<small>ECKER</small> *et al.* 2015). This suggests that perhaps SAD-2 recruits and is potentially a scaffolding component for the other involved proteins (S<small>HIU</small> *et al.* 2006; D<small>ECKER</small> *et al.* 2015). During MSUD screening of knock-out strains, the third *suppressor of ascus dominance* gene was established. SAD-3 was found to have orthologs in many organisms such as *C. elegans*, *Drosophila melanogaster*, and even *Homo sapiens*. Although, none of the orthologs are very well characterized. However, further examination of SAD-3 revealed two helicase domains and localization in the perinuclear region (H<small>AMMOND</small> *et al.* 2011).

A protein labeled SMS-2 (<u>s</u>uppressor of <u>m</u>eiotic <u>s</u>ilencing-2) is believed to be the Argonaute protein involved in MSUD (L<small>EE</small> *et al.* 2003). While searching the genome of *N. crassa, sms-2* was found and shown to be a paralog to *qde-2* a previously established Argonaute protein involved in quelling (C<small>ATALANOTTO</small> *et al.* 2000; L<small>EE</small> *et al.* 2003).

There are a few proteins involved in a related pathway that may be important for MSUD. The *dcl-1* (dicer-like) gene was originally discovered to be involved in quelling alongside *dcl-2.* The putative protein sequence of both showed homology to the *Drosophila* Dicer-1 protein (C<small>ATALANOTTO</small> *et al.* 2004). Interestingly, it was later

discovered that DCL-1 was essential for MSUD activity although DCL-2 was not. This suggested some overlap and similarities between quelling and MSUD (ALEXANDER *et al.* 2008). Another protein found to be necessary for both quelling and MSUD is QIP (QDE-2-interacting protein) (MAITI *et al.* 2007; XIAO *et al.* 2010). QIP was initially discovered during a biochemical purification of QDE-2 (Argonaute protein for quelling) and was found to have an endonuclease domain involved in passenger strand removal (MAITI *et al.* 2007).

More recently discovered MSUD proteins include SAD-4, SAD-5, and SAD-6 (HAMMOND *et al.* 2013b; SAMARAJEEWA *et al.* 2014). Interestingly, homozygous knockouts of SAD-4 and SAD-5 were not necessary for sexual development (HAMMOND *et al.* 2013b). All other MSUD proteins recognized previously, could only be used in a heterozygous gene knockout crosses because homozygous crosses produced barren perithecia (fruiting bodies). This indicates that all previously described MSUD proteins are linked to the sexual phase of *N. crassa* as well as MSUD activity (SHIU *et al.* 2001, 2006; ALEXANDER *et al.* 2008; XIAO *et al.* 2010; HAMMOND *et al.* 2011, 2013b). Therefore, SAD-4 and SAD-5 seemed to have specific MSUD function not linked to sexual development. This ability to do homologous knockout crosses with the two genes allowed a clear examination of the different RNAs produced. Manipulation of *sad-4* and *sad-5* genes proved to not affect particular RNAs made during the vegetative stage of *N. crassa*; however, knockouts of these genes did decrease available MSUD-associated small-interfering RNAs (masiRNAs) verifying both genes involvement in MSUD (HAMMOND *et al.* 2013b).

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SAD-4's specific role in MSUD is difficult to speculate because it carries no previously characterized motifs, but it was found to localize to the perinuclear region along with all other MSUD proteins previously discovered (HAMMOND *et al.* 2013b). Considering this characteristic along with its effect on masiRNA production, it was speculated that SAD-4 is active upstream of masiRNA formation and possibly aides the Argonaute protein (HAMMOND *et al.* 2013b).

Interestingly, SAD-5 was the first MSUD protein found to localize inside the nucleus. However, because it does not show homology to any known protein motifs its function will be challenging to predict (HAMMOND *et al.* 2013b). Although, since it is found in the nucleus it is enticing to think that SAD-5 is involved in the early processes of MSUD such as aiding in scanning the DNA for unpaired regions.

The most recently published MSUD component is SAD-6. SAD-6 is also a nuclear protein, and it contains a well-established helicase domain that is similarly found in S*accharomyces cerevisiae*'s Rad54 protein (SAMARAJEEWA *et al.* 2014). Rad54-like proteins aid in double-stranded break repair of DNA during homologous recombination, specifically assisting scanning the genome for a homologous sequence. The only other Rad54-like protein found in *N. crassa* is MUS-25 (FLAUS *et al.* 2006). *sad-6* and *mus-25* produce relatively low transcript levels in both vegetative and sexual states in *N. crassa* (SAMARAJEEWA *et al.* 2014). This suggests that SAD-6 has other activities separate from MSUD.

The experiments I present in the following chapters help to characterize the MSUD pathway by elucidating aspects of the early detection process that have not previously been investigated. I will first describe my genetic assays involved in

examining the distance sensitivity during unpaired DNA detection. In the later chapter, I will explain the steps I have taken in my biochemical investigation of the nuclear MSUD protein, SAD-5.

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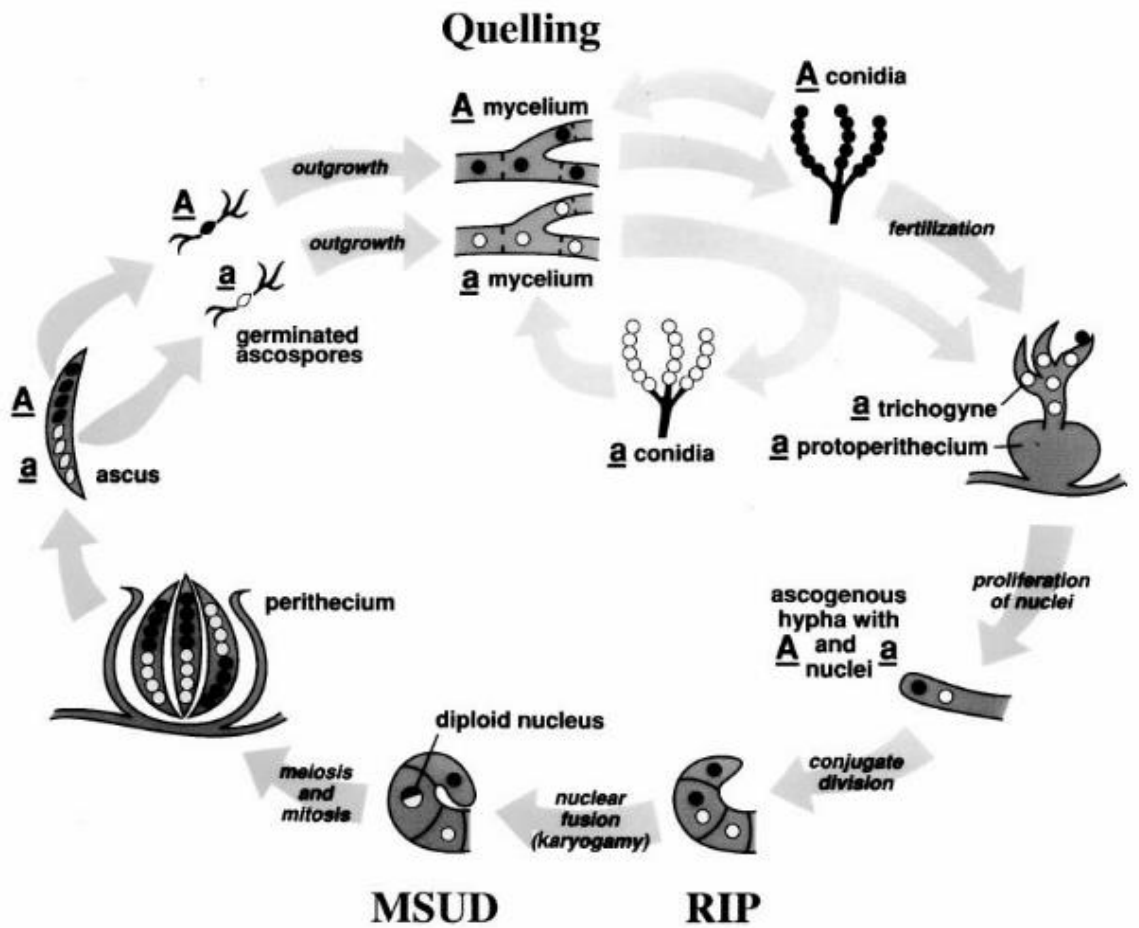
Figure I-1: Genome Defense Activities During the Lifecycle of *Neurospora crassa*
Source: Shiu P. K., Raju N. B., Zickler D., Metzenberg R. L., 2001 Meiotic silencing by unpaired DNA. Cell **107**: 905–16. Reprinted with permission from Elsevier Limited: *Cell.* Copyright 2001.
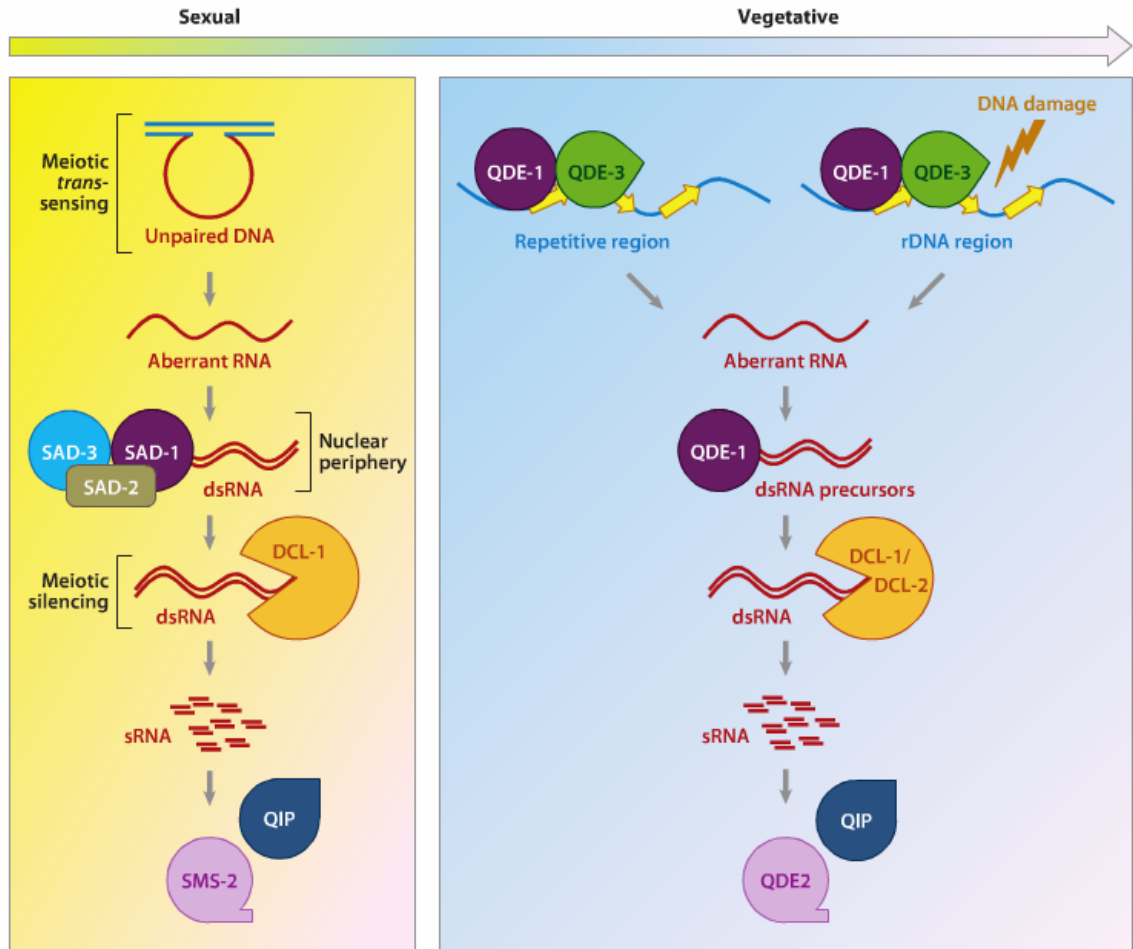
Figure I-2: Genome Defense Mechanisms in the Sexual Stage (MSUD) and Vegetative Stage (Quelling)
Source: Chang S.-S. S., Zhang Z., Liu Y., 2012 RNA interference pathways in fungi: mechanisms and functions. Annu. Rev. Microbiol. **66**: 305–23. Reprinted with permission from Elsevier Limited: *Annu. Rev. Microbiol.* Copyright 2012.
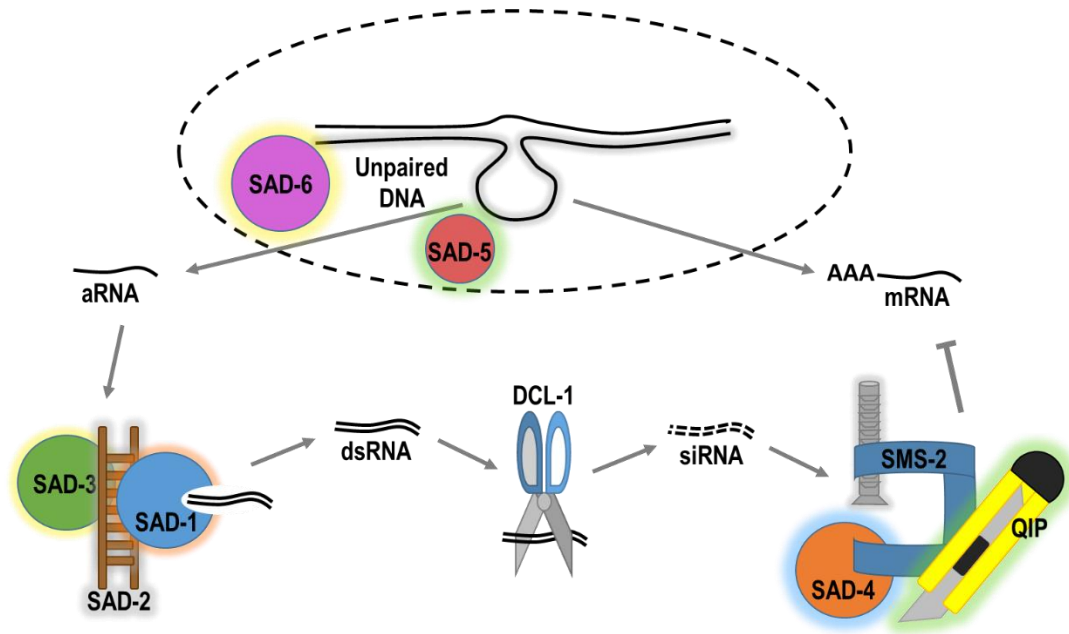
Figure I-3: MSUD Mechanism and Components
Adapted from the Source: Hammond, T.M., Xiao, H., Boone, E.C., Perdue, T.D., Pukkila, P.J., and Shiu, P.K. (2011). SAD-3, a Putative Helicase Required for Meiotic Silencing by Unpaired DNA, Interacts with Other Components of the Silencing Machinery. G3 (Bethesda) *1*, 369–376. Reprinted with permission from Creative Commons: Attribution 2.5 Generic. http://creativecommons.org/licenses/by/2.5/

CHAPTER II

MSUD'S GENE DETECTION PROCESS AND DISTANCE PARAMETERS

Abstract

Background. Unlike many other eukaryotes, the fungus *N. crassa* is especially susceptible to invasions from threatening entities such as viruses or transposable elements primarily because multiple nuclei share the same cytoplasm (SELKER 1990, 1997; DANG *et al.* 2011). To circumvent such problems, *N. crassa* has multiple defense mechanisms (SELKER *et al.* 1987; CAMBARERI *et al.* 1991; ROMANO and MACINO 1992; CHANG *et al.* 2012). One in particular is called Meiotic Silencing by Unpaired DNA (MSUD) (SHIU *et al.* 2001). When homologous chromosomes pair up during meiosis, MSUD searches for any DNA that is unpaired between the two homologs. If heterologous regions are found, these areas will be silenced by MSUD's RNAi-like pathway (ARAMAYO *et al.* 1996; ARAMAYO and METZENBERG 1996; SHIU *et al.* 2001; ALEXANDER *et al.* 2008; XIAO *et al.* 2010; HAMMOND *et al.* 2011, 2013a; b; SAMARAJEEWA *et al.* 2014). Currently, the way in which unpaired regions are detected between homologous chromosomes is unknown. To gain further understanding about this topic, I decided to examine the distance constraints of the MSUD detection method. The goals of this study are outlined in two aims, below.

  AIM 1: Construct and select strains with necessary genotypes for experimental crossing.

AIM 2: Determine if the amount of MSUD recognition and gene silencing is dependent upon distance between genes on homologous chromosomes.

Methods. To test MSUD's detection method, many strains were developed to carry an ectopic copy of a DNA fragment of the *Roundspore* (r^+ or *rsp*) gene in varying locations on the seventh chromosome. Each strain was crossed with another strain carrying the r^+ ectopic fragment (r^{ef}) insert at a different location. These crosses allowed particular distances between the r^{ef} of each strain. Around 14 days after fertilization, images of the ascospore rosettes found in the fruiting bodies produced by the cross were taken. This allowed a pre-result visual for each cross. After day 24, all spores were collected from the plate lids and counted to get a ratio of American football-shaped spores versus round-shaped spores. The ratio observed signified a quantitative result for MSUD detection activity.

Results. Through my experiments I discovered that crosses between parental strains with slightly offset r^{ef} barely triggered MSUD detection while very widely separated r^{ef} almost always activated MSUD silencing.

Discussion. Based on my results, it is clear that there is a positive correlation between distance separating r^{ef} and MSUD silencing activity. Thus, MSUD recognition is spatially constrained and less effective when homologous sequences are increasingly closer in proximity between homologous chromosomes. Results also indicate that recognition is not exact and does not seem to have the need for precise sequence position. Thus, we will discuss theoretical alternative mechanisms for the search and recognition process as well as possible future experiments.

Background

Meiotic Silencing by Unpaired DNA (MSUD) is believed to be a defense mechanism used by *N. crassa* to silence any unwanted genome insertions (ARAMAYO and METZENBERG 1996; SHIU *et al.* 2001). If an appropriate-sized insertion is incorporated into a strain, this insert can be post-transcriptionally silenced during meiosis when crossed to another strain (ARAMAYO and METZENBERG 1996; SHIU *et al.* 2001). How *N. crassa* is able to compare the entire genomes of both crossed strains and how unpaired sequences are detected is still unknown. My goal was to gain insight into the MSUD detection method. Thus, I used a phenotypic marker that will only be expressed during meiosis. This marker was derived from the r^+ gene [also known as *Roundspore* (MITCHELL 1996)] that is expressed naturally in *N. crassa* and is located on chromosome I. The r^+ gene is expressed to allow any ascospores (Ascomycete fungal spore) produced to be the shape of an American football (MITCHELL 1996). If this gene is not expressed, the spores will be round. When wild-type (wt) strains are crossed, the gene is at the same location on homologous chromosomes and will appear paired during meiosis (**Figure II-1A**). We decided to incorporate an r^+ ectopic copy fragment (r^{ef}) coupled with a selectable marker onto chromosome VII of certain strains. When these experimental strains are crossed to wt, chromosome VII will have a physical unpairing of the r^{ef} (**Figure II-1B**). r^+ on chromosome I will be paired between both homolog pairs; however, because chromosome VII does not have paired sequences, all copies should be silenced by MSUD. With this in mind, we asked ourselves what would happen between two ectopic strains that have the r^{ef} located at increasing distances from each other on

chromosome VII (**Figure II-1C**). **Figure II-1D** illustrates examples of strains crossed with varying distances between the r^{ef} on chromosome VII.

AIM 1: Construct and select strains with necessary genotypes for experimental crossing.

AIM 2: Determine if the amount of MSUD recognition and gene silencing is dependent upon distance between genes on homologous chromosomes.

Methods

DNA Insert Construction. Each DNA insert was designed to carry a 2.5 kilobase fragment of *N. crassa's Roundspore* gene (r^+), the hygromycin B phosphotransferase (*hph*) gene, and flank sequences homologous to various regions along *N. crassa's* chromosome VII. Each construct, depicted in **Figure II-2C**, was assembled by double-joint PCR (DJ-PCR) (YU *et al.* 2004). Primers were strategically designed to allow fusion of each section to one another. Once fused through double-joint PCR, the construct was amplified as a whole piece to be used for transformation. The entire DNA insert spans 4.1 kilobases. **Table II-1** displays all the primers names and sequences used for construct assembly.

Fungal Strains and Transformation. All strains used in this experiment are listed in **Table II-3**. For vegetative growth, each strain was cultured in Vogel's minimal media (VMM) (VOGEL 1956). All sexual crosses were completed using synthetic crossing medium (SCM) (WESTERGAARD and MITCHEL 1947). Each fungal transformation was principally performed as described by (MARGOLIN *et al.* 1997), and all vectors were transformed into strain P8-43 after 7-14 days of growth in VMM. Exceptions to this protocol include separating conidial (asexual spores) growth away from the mycelial (vegetative) growth using a 100-µm nylon filter (Steriflip; Millipore, Billerica, MA). Each

experimental transformation contained 100 µl of properly diluted conidial cells and ~500 ng of DNA insert. Dilutions were determined by the following calculation: [(O.D.) x (Volume) x (100 Units)]/2 = Amount of 1M sorbitol added to the pelleted conidia. Immediately after electroporation, each transformation was suspended in 750 µl of 1M sorbitol and diluted with VMM. Each was then incubated and cultured at 28 °C, 800 rpm for 3 hours. Transformations were aliquoted to 10 ml of molten VMM top agar and then poured on top of VMM plates that contained antibiotic, hygromycin B. After 3-5 days at room temperature, individual areas of fungal growth on each plate were transferred to VMM slants containing histidine and hygromycin B. Transformants that showed growth on the slants were then backcrossed to the wild-type strain, F2-26. Backcrossing consists of first making water suspensions with the transformant's conidia and then fertilizing SCM plates previously inoculated with strain F2-26. Over a 24-day period all sexual crosses produced fruiting bodies containing ascospores. After 24 days, ascospores ejected onto the lids of the SCM plates were collected and incubated for 24 hours at 4 °C. Spores were eventually heat shocked at 60 °C and plated on VMM plates containing histidine and hygromycin B. After one day of incubation, ascospores were observed under a microspore, and spores that showed promising germination were transferred to VMM hygromycin B slants for further selection. Slants that showed healthy fungal growth were chosen for further genotyping. This process included determining mating type, vegetative growth classification, *his-3* genotype, and PCR insertion verification. All strains used for each experiment were verified by PCR amplification of each r^{ef}. Primers used for PCR are listed in **Table II-1**. All PCR products were analyzed on a 1% agarose gel to confirm genotypes.

Figure II-2 and **Table II-2** illustrates and states each insertion location on chromosome VII of all strains.

Experimental Crosses. **Figure II-3A** depicts different steps of the crosses throughout the experiment. All sexual crosses were completed using SCM. All strains involved in experimental crosses were first inoculated to new VMM slants and grown at 28 °C to allow consistent age of the tissue. SCM 60 mm plates were inoculated on the same day with selected female (strains fertilized by the opposing mating type strain) fungal strains. After three days of growth at 28 °C and three at room temperature, 33 ul of water-suspended conidia from the male fungal strains were used to fertilize three different areas on the 6-day-old plates growing the female strains. Conidial suspensions were concentrated to 1 million conidia per 1 ml. Each cross was performed in four replicates. The duration of experimental strain crossing extended until day 24. Once crossing was complete, the lids of each replicate were moved to new plate bottoms.

Cross Imaging. **Figure II-3B** illustrates steps of imaging. Approximately day 14 after fertilization, the fourth plate (Replicate D) of each cross was utilized for rosette (cluster of ascospore containing asci) imaging to predetermine progeny phenotype. Perithecia (fruiting body that contain spores) from each cross were dissected in 25% glycerol and ascospore rosettes were transferred to wet mount slides also supplied with 25% glycerol. Images were taken using light microscopy at 20x magnification. **Figure II-4** shows examples of rosette images.

Spore Collection and Cross Phenotyping. Refer to **Figure II-3A** for a visual of the spore analysis steps. Spores from the same replicate of all crosses were collected in the same 24 four hour period to inhibit any spore counting bias between days. Using the lid's

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1 ml of allotted water, each lid was washed with 330 μl three times to collect spores and move them to an empty microcentrifuge tube. Spores were then stored at 4 °C until spore counting and phenotyping began. This entire protocol was carried out for each cross and each replicate involved in the experiment.

A representative sample of each spore suspension was transferred to a hemocytometer and viewed under a microscope for spore counts. For each replicate, at least a hundred spores were counted and characterized for each cross. Spores were categorized into two groups and labeled as "round" shape or "football/spindle". Using this method each replicate was given a percentage of round spores vs. football spores.

Results

Physically Unpaired r^{ef}s Cause Mixed Phenotypes in the Progeny. Crosses between WT N. crassa strains will predominately produce American football-shaped ascospores when r^+ is being regularly expressed. However, if MSUD is activated due to an unpaired r^{ef}, the fragments will be silenced along with each endogenous r^+, ultimately producing rounded spores. Preliminary crosses between two strains with r^{ef} separated by approximately 13.9 kilobases were predicted to show 100% silencing of r^+ expression resulting in 100% round spores produced. This result was expected considering the r^{ef} marker between these two strains was separated by approximately 13.9 kilobases producing a physical unpairing of this gene between homologous chromosomes. As seen in **Figure II-4A**, this cross (labeled CPS06) gave an unexpected mixed phenotype of football and round-shaped spores. After spore counting, 34% of the spores from CPS06 were round (**Table II-4**). This result suggested that the MSUD mechanism recognized this

unpairing 34% of the time compared to the predicted 95% to 100%. This led to further inquiry about distance restrictions of MSUD's scanning and detection process.

Nineteen strains were designed to have the r^{ef}-*hph* marker inserted along chromosome VII of *N. crassa* at varying locations (**Figure II-2A, B**). These different strains allowed multiple crosses to be performed across a range of distances between the r^{ef} of each strain. **Table II-2** states the insertion location along chromosome VII. All crosses performed, round spore percentages, and standard deviations can be viewed in **Table II-4.**

Positive Control Unpaired Crosses. All positive control crosses consist of a WT strain crossed to any strain designed to carry a r^{ef} on chromosome VII (**Figure II-5A**). These crosses have completely unpaired r^{ef} on chromosome VII. Therefore, I predicted complete silencing of all Roundspore expression and predicted outcomes of these crosses should be 95% or higher counts of round spores. As seen in **Figure II-5B**, nearly all positive controls fall within the 95% or higher round spore range among all experiments. Cross wt x r6 was the only cross to show a fairly low percentage of rounded spores. Examining **Table II-4**, the wt x r6 cross was only performed in two experiments. One experiment reveals a ~70% round spores count with a high standard error (SE). The other experiment produces a 98% round spore count with a very low SE. Counts such as this for the controls that fall slightly out of range can most likely be attributed to differences in environmental aspects such as fluctuation in room temperature, humidity, and sunlight.

Negative Control Paired Crosses. All negative control crosses consist of any strain designed to carry a r^{ef} on chromosome VII crossed to a strain carrying a r^{ef} at the same location but of opposing vegetative growth and mating type (**Figure II-6B**). **Figure II-6A**

illustrates the paired genes of crossed wt strains as well. These crosses have fully paired fragments between homologous chromosomes therefore allowing normal expression of the r^+ and no MSUD activity. All negative control crosses were predicted to provide 5% or lower counts of round-shaped ascospores. Shown in **Figure II-6C**, all six negative control crosses fell way below the 5% threshold indicating MSUD activity is working properly in these strains.

Unpaired Experimental Crosses. All experimental crosses consist of multiple cross combinations between any two strains carrying a r^{ef} on chromosome VII. Refer to **Figure II-1D** for a visual example. These crosses allowed for a range of distances between r^{ef} allowing for various percentages of silencing. **Figure II-7** shows all six experiments performed, and **Figure II-8** shows all plots overlain on each other to compare all experiments. Interestingly, as seen in **Figure II-8**, the smallest distance between two strains at 4.1 kb showed extremely low levels of silencing. However, as the distance difference became greater, MSUD activity seemed to also increase. Remarkably, even at a difference of ~42 kb (CPS65), there stilled seem to be recognition of "paired" r^{ef}. **Table II-4** shows CPS65 to have 83.67% round spores. It was not until a distance of over 1000 kb during CPS66 that 99% round spores were produced (always detected by MSUD as unpaired).

In all plots shown in **Figure II-8** a positive correlation between distance and average percent round spores observed is shown. **Figure II-8** also shows that variance can be attributed to individual experiments. For example, the data points in experiment CPS25-39 drop slightly compared to the other experiments; however, it is consistent within that experiment that all points drop slightly. Again, this could be due to environmental factors such as temperature or humidity.

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Discussion

Thus far, much has been discovered about the second stage of MSUD. Many proteins have been examined that suggest the second stage involves an RNAi-like pathway that can target transcripts of unpaired DNA regions (ARAMAYO and METZENBERG 1996; ARAMAYO *et al.* 1996; SHIU *et al.* 2001, 2006; LEE *et al.* 2003, 2010; MAITI *et al.* 2007; HAMMOND *et al.* 2011, 2013a, b). However, the initial activity that takes place inside the nucleus is still unknown. How can an organism efficiently scan two sets of an entire genome of seven chromosomes that carry approximately 40Mb and 10,000 protein coding genes and discover unpaired sequences?

The strong positive correlation acquired throughout these experiments as seen in **Figure II-8** suggests that an increasingly larger distance of unpairing between genes on homologous chromosomes allows progressively stronger MSUD activity. These results suggest that detection relies on gene proximity and depicts certain distance sensitivity. Physically unpaired genes do not always seem to be detected by MSUD. This suggests that MSUD searching is not exact, and that the mechanism does not use a linear comparison but perhaps samples DNA regions semi-randomly. One idea is that MSUD's homology search could be happening alongside *N. crassa's* homologous recombination activity that is already occurring during meiosis. As it states in its descriptive name, homologous recombination relies on a homology search as well (COHEN and POLLARD 2001). Meiotic homologous recombination (HR) starts with intentional double-stranded breaks (DSBs) in the DNA. Strands are processed to produce an extended 3' single-stranded DNA end. Multiple Rad51 proteins are then loaded onto the single-stranded DNA that ultimately directs the strand to the opposite homolog. Once in proximity, the Rad51 protein/DNA

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filament is thought to rapidly bind and unbind the homolog's DNA in a test for homology. Once homology is found, repair of the broken ends occurs and can ultimately lead to genetic exchange and diversity among progeny if crossing-over occurs (FILIPPO *et al.* 2008; CRISMANI and MERCIER 2012; QIAO *et al.* 2012).

Another HR protein, Rad54, is believed to work alongside Rad51 doing such activities as allowing Rad51 to disassociate from sequences that do not show homology (BUGREEV *et al.* 2007). Recently, a Rad54 homolog in *N. crassa*, SAD-6, was found to localize to the nucleus during meiosis and is necessary for efficient MSUD (SAMARAJEEWA *et al.* 2014). These findings could suggest that MSUD is utilizing homologous recombination or using a similar system for homology searching.

If MSUD is using a homologous recombination style homology search, this could mean that the percentages I observed during the unpaired crosses could actually be representing the successfulness of homologous recombination. For example, in the initial crosses between r2 and r3 (**Figure II-4A**) I saw approximately 34% round spores and 66% football-shaped spores. This indicated that 66% of the time the r^{ef} insertions were not detected as unpaired by MSUD even when they were physically separated by 13.9 kb. However, if homologous recombination is the process utilized for homology searching in MSUD, I could conclude that 66% of all unpaired instances were not necessarily missed by MSUD but were successfully found by the homologous recombination homology-searching proteins. This would suggest that 34% of the unpairing events were targeted by MSUD because they were unsuccessful in the homology search. If this is found to be true then maybe observations of increasing distance between r^{ef}s results in increasing MSUD activation because of homologous recombination limitations. Perhaps, MSUD activation

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is triggered by unsuccessful homologous recombination homology searching caused by the limited length of the ssDNA Rad51 protein/filament in combination with placement of the DSB. As distances increase it is possible that the filament cannot reach such homologous areas or because it becomes ineffective when trying to lengthen for a wider search.

If this happens to be the detection method used then it would suggest that the homologous recombination proteins could initiate the "targeting" of the unpaired region for aRNA production as well. For instance, it is possible that when HR fails to find homology on the homolog the presence of the unresolved double-stranded break or the lingering ssDNA Rad51 filament will eventually recruit other repair mechanisms, modifying agents, or potentially a MSUD-associated RNA polymerase. This could possibly add another layer on the genome integrity role that homologous recombination plays in the nucleus. We know that during meiotic homologous recombination there are crossover events that cause genetic diversity in the progeny. However, in eukaryotes crossover events happen very rarely, usually anywhere from 1-4 times between homologous chromosomes (CRISMANI and MERCIER 2012; CRISMANI *et al.* 2012). This is interesting considering there is a considerable amount of DSB at the beginning of meiosis (CRISMANI and MERCIER 2012; CRISMANI *et al.* 2012). In *N. crassa* the majority of DSBs that are resolved through non-crossover events could be attributed to simple homology testing for unwanted foreign DNA.

There is also the alternative idea that homologous recombination is not the homology searching method used by MSUD. It was recently shown that in repeat-induced point mutation (RIP), another genome defense mechanism in *N. crassa*, homologous recombination proteins *mei-3* and *spo-11* were not necessary for RIP function (GLADYSHEV

and KLECKNER 2014). These results indicated that homologous recombination was not involved in RIP. However, in my investigation of these implications in MSUD, I used *Δmei-3 Δspo-11* strains derived from those used in the paper and saw that homologous sexual crosses were very poor (unpublished observation). Limited spores were produced and detection of MSUD activity was very mixed and unreliable. Separate from the *Δmei-3 Δspo-11* results, these researchers did find an interesting phenomenon during homology detection in RIP. They discovered that as little as three bases of sharing homology to a homolog every period of 11-12 bases was sufficient for RIP homology detection. We are currently investigating if MSUD homology searching works in a similar manner. It is difficult to speculate if this method could be utilized for homology searching in MSUD because RIP and MSUD seemed to be very different mechanisms that are activated during two different stages of ploidy.

We are only beginning to understand how MSUD detects unpaired regions in the genome. However, the genetic assays outlined here and future work can help us to further investigate. I am currently testing MSUD's detection abilities when faced with large DNA deletions. Specifically, I am examining the effect of large deleted regions of the DNA (30 and 50 kb in size) that are replaced by a r^{ef}. We are interested to determine if MSUD will still recognize the inserted ectopic fragment when such a strain is crossed to wt. If I observe that football-shaped spores are predominantly produced, I could infer that the machinery may be overly occupied with the large deleted segments to recognize a 4.1 kb r^{ef} insertion. If I were to observe predominantly round-shaped spores produced, it would suggest that the r^{ef} insertion was detected and the large deletion did not pose a problem for the MSUD machinery.

Future experiments can be done with the MSUD assay in regions such as the centromere or the telomere. These areas of the chromosome would be interesting to consider because each region is very tightly compacted leaving one to wonder how homology detection is effected by such compaction. If a r^{ef} was incorporated into either of these areas, would MSUD be able to detect it?

Perhaps, other future experiments that can be done should more specifically examine homologous recombination's role in the MSUD detection process. However, this proves to be difficult because deleting key homologous recombination genes such as *mei-3* (Rad51 homolog) or *mus-25* (Rad54 homolog) in strains results in unproductive crosses (unpublished; SAMARAJEEWA *et al.* 2014). Nevertheless, our lab is already considering and conducting yeast-two-hybrid interactions and biochemical binding assays with a focus on nuclear-localizing MSUD proteins. It would prove very advantageous to eventually incorporate the key homologous recombination proteins into these assays to determine if there are any protein-protein interactions between them and the MSUD associated proteins.

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Table II-1: Transformation Vector Primers

Primer Name	Sequence
r^{ef} fragment amplification	
Eco-RSP-F	CAGAATTCAGTCGAGGACAGAACGCAGCA
Eco-RSP-R	TTGAATTCTTGGACCTCTTCCGCAGTTTCC
hph marker amplification	
APAI-HPH-F	AAGGGCCCAACTGATATTGAAGGAGCAT
APAI-HPH-R	AAGGGCCCAACTGGTTCCCGGTCGGCAT
r^{ef}-*hph* amplification (center fragment for DJ-PCR)	
Rsp-center-A	AGGACAGAACGCAGCAGCAGAGC
Rsp-center-B	ACAGCGAACGAAACCCCTGAAAC
r^{ef1}-*hph* insertion between *ncu09443* and *ncu09444*	
Rsp-040613-C	TAGTGGAGGGGCTTGGGATGGT
Rsp-040613-D	AGAGAAGCTCTGCTGCTGCGTTCTGTCCTCTGCTGAACGAACACCCCTGCT
Rsp-040613-E	TAACGGGTTTCAGGGGTTTCGTTCGCTGTCGTCCACTGATCTTCGCTAGAATTT
Rsp-040613-F	TCACCGCCCGTCCCTACTATCA
Rsp-040613-G	GCCTTGGACTGGTGATGGTGCT
Rsp-040613-H	GGAGGAGTCGGTTTGCTTTGGTG
r^{ef2}-*hph* insertion between *ncu09444* and *ncu09445*	
Rsp-040613-I	ATGAGGGAGGTGCCGTGTCC
Rsp-040613-J	AGAGAAGCTCTGCTGCTGCGTTCTGTCCTCCATTCTGCCATTTCCCATGC
Rsp-040613-K	TAACGGGTTTCAGGGGTTTCGTTCGCTGTCCGCACACTTTCTCCACCCATC
Rsp-040613-L	GCAATCCACCTCTGGCATCGAC
Rsp-040613-M	AGCCAATCCTTTACCGACTCCAACA
Rsp-040613-N	GTGGTTCTCGCCCGCTTTCAAC
r^{ef3}-*hph* insertion between *ncu09449* and *ncu09450*	
RSP-042613-A	CGAGGGCCGAGTCTGGTGGTTA
RSP-042613-B	AGAGAAGCTCTGCTGCTGCGTTCTGTCCTGTACTAGCGTTTGCGCGGGACA
RSP-042613-C	TAACGGGTTTCAGGGGTTTCGTTCGCTGTAGGTGGGAAAGTGTTAGTGGTGGA
RSP-042613-D	GTTGAGGGTCTTGAGGGCGAAG
RSP-042613-E	TCTCACACGTTGCTTCGGCTGT

RSP-042613-F   GAGGTTCTGGTTGGCTGGTTGG

r^{ef4}-*hph* insertion between *ncu09451* and *ncu17161*

RSP-042613-A	AAGTGGGCGTTGAAGGAGGATG
RSP-042613-B	AGAGAAGCTCTGCTGCTGCGTTCTGTCCTCGGAGGTCGGAGACGAGATG
RSP-042613-C	TAACGGGTTTCAGGGGTTTCGTTCGCTGTCCAAGTCCTCCATCCGTCCATC
RSP-042613-D	TTCATCCAGCAATCCACCACCA
RSP-042613-E	CCTCTTCACCCTCTACCCAAACGA
RSP-042613-F	AGCGACCATCCCAAACCAACAA

r^{ef5}-*hph* insertion between *ncu09455* and *ncu09456*

RSP-050213-A	CAGACAGTGGTGGGAAGGTGGTC
RSP-050213-B	AGAGAAGCTCTGCTGCTGCGTTCTGTCCTCAGTGCGGAAATGGAAGGGAGAG
RSP-050213-C	TAACGGGTTTCAGGGGTTTCGTTCGCTGTCGGCCATCACGGTCAAAGAAAC
RSP-050213-D	ATGGTGCCGACGCTAAAGGAGA
RSP-050213-E	CGTTCCGTCATTCGGGTATTGC
RSP-050213-F	ACGCAGGGAGGGAGATTGCCTA

r^{ef6}-*hph* insertion between *ncu06068* and *ncu06067*

RSP-061813Y	AGCTCGTTTGGGTATCAGCAGTCC
RSP-061813Z	AGAGAAGCTCTGCTGCTGCGTTCTGTCCTGGATGCAAGGGCGAGAGTCAA
RSP-061813AA	TAACGGGTTTCAGGGGTTTCGTTCGCTGTGCGAAACCCTGGAGATAACGGAAG
RSP-061813AB	GGCGTCGGCAACTGAAGGAC
RSP-061813AC	CGTGGGAAGCGAGGTGAGAGG
RSP-061813AD	CGTGGTCTGTGTGTGTGTGGTCTG

r^{ef7}-*hph* insertion between *ncu02357* and *ncu02356*

RSP-061813AE	GAACGGGAATGGGTGCATAGGA
RSP-061813AF	GAGAAGCTCTGCTGCTGCGTTCTGTCCTCCACTCGCAAGATCAGTCAGCA
RSP-061813AG	AACGGGTTTCAGGGGTTTCGTTCGCTGTGTGGCAGTTGTGGGTTGCAGGT
RSP-061813AH	AGCGGCCAGACGAAGTGGAAG
RSP-061813AI	TGTGGGTTCGCAATGTGCCTTT
RSP-061813AJ	CCCTTCCCATGACCCTTTGTCC

r^{ef8}-*hph* insertion between *ncu02258* and *ncu02257*

RSP-061813AK	AATCCTCACCACCACCACACCA

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RSP-061813AL	GAGAAGCTCTGCTGCTGCGTTCTGTCCTGACAGCCGCAGACTCAGGTCAA
RSP-061813AM	AACGGGTTTCAGGGGTTTCGTTCGCTGTGCTTGGTTCGTTTGGCTTGAACA
RSP-061813AN	GACCGCCTTTGCCTGCACATA
RSP-061813AO	ATCGGCACTCGGCATCACAATC
RSP-061813AP	TACCACACCCACCAACAACCAC

*r*ef9-*hph* insertion between *ncu10080* and *ncu08174*

RSP-061813AQ	GCCGTGGTGCTCTGTGGAGAC
RSP-061813AR	GAGAAGCTCTGCTGCTGCGTTCTGTCCTGAGGAGGAGTCTGGGCCTCTGG
RSP-061813AS	AACGGGTTTCAGGGGTTTCGTTCGCTGTTCAATGGTGGGATCGACGGAAC
RSP-061813AT	GGATTCGACGCGCCTTATGTCT
RSP-061813AU	CGATGTGTGGGAAGGTGAAAGG
RSP-061813AV	CGATGTGGAGATGGCGGTGT

*r*ef10-*hph* insertion between *ncu05856* and *ncu05858*

RSP-061813AW	CGTTGGCTGTGGGTTGAGGTTG
RSP-061813AX	GAGAAGCTCTGCTGCTGCGTTCTGTCCTTTGGTAGGGTCGGTGCTGGAGT
RSP-061813AY	AACGGGTTTCAGGGGTTTCGTTCGCTGTGACCAGGACAAACACGCCCAAA
RSP-061813AZ	TCGGAGCCCTTCACATTCAACC
RSP-061813BA	GGCGACTACAACCACGGCACCT
RSP-061813BB	AGCGTTCCACCTCTGCTCCAAA

*r*ef1-*r*ef10 primer names and primer sequences are listed. The first four primers of each r^ef are used for DJ-PCR and construction of fragment. The last two primer sequences of each r^ef are the nested primers used for PCR during strain verification.

Table II-2: r^{ef} Locations

Vector	Insertion Location	Sequence Deletion Size
r1	VII: 214,752–214,773	20 b.p.
r2	VII: 218,848–219,011	161 b.p.
r3	VII: 232,895–232,984	88 b.p.
r4	VII: 241,034–241,159	124 b.p.
r5	VII: 256,797–256,822	24 b.p.
r6	VII: 1,918,140–1,918,315	175 b.p.
r7	VII: 2,765,237–2,765,587	350 b.p.
r8	VII: 3,156,619–3,157,078	459 b.p.
r9	VII: 3,631,984–3,632,008	24 b.p.
r10	VII: 4,074,801–4,075,045	244 b.p.

Location on *N. crassa's* chromosome VII where each r^{ef} was inserted for each vector. WT sequence deletion size is also indicated. Each "r" insert is associated with two strains of opposing vegetative growth and mating type except for r6 and r10.

Table II-3: Genotype of Each Experimental Strain

Original Strain Name	ISU Strain Name	Abridged Strain Name	Strain Genotype
P8-43	P8-43	-	*rid his-3*; *mus-52^Δ::bar* A
1005.2	F2-26	wt	*rid; fl* a
P10-15	P10-15	wt	*rid his-3* A
RTPS7.2.1	ISU 3118	r1	*rid his-3; mus-52^Δ::bar; VIIL::r^{ef1}-hph* A
RPS7F1	ISU 3143	r1	*rid; fl;* VIIL::r^{ef1}-hph a
RTPS6.1.2B	ISU 3116	r2	*rid; fl; mus-52^Δ::*bar;VIIL::r^{ef2}-hph a
RTPS6.1.3	ISU 3117	r2	*rid his-3; VIIL::r^{ef2}-hph* A
RTPS13.2.2	ISU 3119	r3	*rid his-3; mus-52^Δ::bar; VIIL::r^{ef3}-hph* A
RSP13F3	ISU 3138	r3	*rid fl ;r^{ef3}::hph* a
RKS10.1.8	ISU 3114	r4	*rid his-3; mus-52^Δ::bar; VIIL::r^{ef4}-hph* A
RKS10.1.9	ISU 3115	r4	*rid; fl; VIIL::r^{ef4}-hph* a
RZS1.2	ISU 3124	r5	*rid his-3; mus-52^Δ::bar; VIIL::r^{ef5}-hph* A
RZS1.20	ISU 3127	r5	*rid; fl; VIIL::r^{ef5}-hph* a
RKS18.3.2	ISU 3141	r6	*rid his-3; VIIL::r^{ef6}-hph* A
RKS19.2.7	ISU 3128	r7	*rid his-3; r^{ef7}::hph* A
RKS19.2.3	ISU 3129	r7	*rid ; fl; r^{ef7}::hph* a
RKS20.5.4	ISU 3131	r8	*rid his-3; r^{ef8}::hph* A
RKS20.5.6	ISU 3132	r8	*rid ; fl; r^{ef8}::hph* a
RKS21.2.4	ISU 3133	r9	*rid his-3; r^{ef9}::hph* A
RKS21.4.1	ISU 3134	r9	*rid ; fl; r^{ef9}::hph* a
RKS22.4.4	ISU 3135	r10	*rid his-3; r^{ef10}::hph* A

The *rid* genotype refers to strains that are defective in RIP. "fl" indicates that the strain's vegetative state is "fluffy" and cannot produce conidia. These strains are useful as females for crossing. The *mus-52^Δ::bar* notation indicates that *mus-52*, the non-homologous end joining gene, has been replaced with the bialaphos resistance gene. This genotype occurred due to the parent strain, P8-43. *mus-52* was knocked out to aid in insertion using homologous recombination into the correct location. Any *VIIL::r^{ef#}-hph* notation indicates that on chromosome VII an r^{ef}, including the hygromycin resistance gene, was inserted at a specific location. The number indicates the vector and these correspond to the abridged strain names. Each location can be found in Table II-2 and visualized in Figure II-2A,B. The uppercase and lowercase "A"s represent mating type.

Table II-4: All Experimental Crosses Performed including Rsp % and SE

Cross Name	Cross Strains	University Cross Strains	r^{ef} locations	Round Spore (%)	SE
CPS01	1005.2 x P10-15	F2-26 x P10-15	wt x wt	0.71	0
CPS02	1005.2 x 6.1.3	F2-26 x ISU 3117	wt x r2	94.41	0.03
CPS03	1005.2 x13.2.2	F2-26 x ISU 3119	wt x r3	87.22	0.15
CPS04	RTPS6.1.2B x P10-15	ISU 3116 x P10-15	r2 x wt	92.36	0.06
CPS05	RTPS6.1.2B x RTPS6.1.3	ISU 3116 x ISU 3117	r2 x r2	0.46	0
CPS06	RTPS6.1.2B x RTPS13.2.2	ISU 3116 x ISU 3119	r2 x r3	33.74	0.06
CPS07	1005.2 x P10-15	F2-26 x P10-15	wt x wt	0.18	0
CPS08	1005.2 x RTPS6.1.3	F2-26 x ISU 3117	wt x r2	96.4	0.03
CPS09	1005.2 x RTPS7.2.1	F2-26 x ISU 3118	wt x r1	99.21	0
CPS10	RTPS6.1.2B x P10-15	ISU 3116 x P10-15	r2 x wt	97.93	0.01
CPS11	RTPS6.1.2B x RTPS6.1.3	ISU 3116 x ISU 3117	r2 x r2	0.28	0
CPS12	RTPS6.1.2B x RTPS7.2.1	ISU 3116 x ISU 3118	r2 x r1	4.77	0.01
CPS13	1005.2 x P10-15	F2-26 x P10-15	wt x wt	0.03	0.05
CPS14	1005.2 x RTPS6.1.3	F2-26 x ISU 3117	wt x r2	89.64	4.61
CPS15	1005.2 x RZS1.2	F2-26 x ISU 3124	wt x r5	96.84	0.62
CPS16	1005.2 x RTPS13.2.2	F2-26 x ISU 3119	wt x r3	88.78	4.57
CPS17	RTPS6.1.2B x P10-15	ISU 3116 x P10-15	r2 x wt	100	0
CPS18	RTPS6.1.2B x RTPS6.1.3	ISU 3116 x ISU 3117	r2 x r2	0	0
CPS19	RTPS6.1.2B x RZS1.2	ISU 3116 x ISU 3124	r2 x r5	68.12	2.03
CPS20	RTPS6.1.2B x RTPS13.2.2	ISU 3116 x ISU 3119	r2 x r3	19.01	2.81
CPS21	RZS1.20x P10-15	ISU 3127 x P10-15	r5 x wt	99.36	0.55
CPS22	RZS1.20 x RTPS6.1.3	ISU 3127 x ISU 3117	r5 x r2	54.45	3.98
CPS23	RZS1.20x RZS1.2	ISU 3127 x ISU 3124	r5 x r5	0.2	0.35
CPS24	RZS1.20 x RTPS13.2.2	ISU 3127 x ISU 3119	r5 x r3	42.29	5.74
CPS25	1005.2 x P10-15	F2-26 x P10-15	wt x wt	0	0
CPS26	1005.2 x RTPS 6.1.3	F2-26 x ISU 3117	wt x r2	80.27	0.15
CPS27	1005.2 x RKS10.1.8	F2-26 x ISU 3114	wt x r4	94.82	0.01
CPS28	1005.2 x RZS1.2	F2-26 x ISU 3124	wt x r5	96.56	0.04
CPS29	1005.2 x RTPS 7.2.1	F2-26 x ISU 3118	wt x r1	97.85	0.02
CPS30	RTPS6.1.2B x P10-15	ISU 3116 x P10-15	r2 x wt	97.36	0.02
CPS31	RTPS6.1.2B x RTPS6.1.3	ISU 3116 x ISU 3117	r2 x r2	0.43	0.01
CPS32	RTPS6.1.2B x RKS10.1.8	ISU 3116 x ISU 3114	r2 x r4	28.61	0.06
CPS33	RTPS6.1.2B x RZS1.2	ISU 3116 x ISU 3124	r2 x r5	66.78	0.04
CPS34	RTPS6.1.2B x RTPS7.2.1	ISU 3116 x ISU 3118	r2 x r1	4.97	0.02
CPS35	RKS10.1.9 x P10-15	ISU 3115 x P10-15	r4 x wt	100	0
CPS36	RKS10.1.9 x RTPS6.1.3	ISU 3115 x ISU 3117	r4 x r2	20.84	0.06
CPS37	RKS10.1.9 x RKS10.1.8	ISU 3115 x ISU 3114	r4 x r4	0.29	0
CPS38	RKS10.1.9 x RZS1.2	ISU 3115 x ISU 3124	r4 x r5	20.65	0.08
CPS39	RKS10.1.9 x RTPS7.2.1	ISU 3115 x ISU 3118	r4 x r1	34.32	0.07
CPS40	1005.2 x P10-15	F2-26 x P10-15	wt x wt	0.3	0.35
CPS41	1005.2 x RTPS7.2.1	F2-26 x ISU 3118	wt x r1	96.73	1.96
CPS42	1005.2 x RTPS13.2.2	F2-26 x ISU 3119	wt x r3	90.73	0.25
CPS43	1005.2 x RKS18.3.2	F2-26 x ISU 3141	wt x r6	70.44	6.01
CPS44	RPS7F1 x P10-15	ISU 3143 x P10-15	r1 x wt	95.25	4.32

Cross Name	Cross Strains	University Cross Strains	r^{ef} locations	Round Spore (%)	SE
CPS45	RPS7F1 x RTPS7.2.1	ISU 3143 x ISU 3118	r1 x r1	0.35	0.1
CPS46	RPS7F1 x RTPS13.2.2	ISU 3143 x ISU 3119	r1 x r3	33.77	6.74
CPS47	RPS7F1 x RKS18.3.2	ISU 3143 x ISU 3141	r1 x r6	96.02	1.43
CPS48	RPS13F3 x P10-15	ISU 3138 x P10-15	r3 x wt	91.12	9.16
CPS49	RPS13F3 x RTPS7.2.1	ISU 3138 x ISU 3118	r3 x r1	34.04	2.62
CPS50	RPS13F3 x RTPS13.2.2	ISU 3138 x ISU 3119	r3 x r3	0	0
CPS51	RPS13F3 x RKS18.3.2	ISU 3138 x ISU 3141	r3 x r6	95.6	3.29
CPS52	1005.2 x P10-15	F2-26 x P10-15	wt x wt	0	0
CPS53	1005.2 x RTPS7.2.1	F2-26 x ISU 3118	wt x r1	97.85	0.89
CPS54	1005.2 x RTPS6.1.3	F2-26 x ISU 3117	wt x r2	95.33	3.42
CPS55	1005.2 x RTPS13.2.2	F2-26 x ISU 3119	wt x r3	96.61	1.44
CPS56	1005.2 x RKS10.1.8	F2-26 x ISU 3114	wt x r4	98.55	0.98
CPS57	1005.2 x RZS1.2	F2-26 x ISU 3124	wt x r5	99.17	1.01
CPS58	1005.2 x RKS18.3.2	F2-26 x ISU 3141	wt x r6	98.23	0.82
CPS59	RPS7F1 x P10-15	ISU 3143 x P10-15	r1 x wt	98.57	1.43
CPS60	RPS7F1 x RTPS7.2.1	ISU 3143 x ISU 3118	r1 x r1	0.1	0.17
CPS61	RPS7F1 x RTPS6.1.3	ISU 3143 x ISU 3117	r1 x r2	4.81	0.23
CPS62	RPS7F1 x RTPS13.2.2	ISU 3143 x ISU 3119	r1 x r3	42.93	5.36
CPS63	RPS7F1 x RKS10.1.8	ISU 3143 x ISU 3114	r1 x r4	49.24	5.84
CPS64	RPS7F1 x RZS1.2	ISU 3143 x ISU 3124	r1 x r5	83.67	7.5
CPS65	RPS7F1 x RKS18.3.2	ISU 3143 x ISU 3141	r1 x r6	99.05	0.14
CPS66	1005.2 x P10-15	F2-26 x P10-15	wt x wt	0.08	0.14
CPS67	1005.2 x RKS19.2.7	F2-26 x ISU 3128	wt x r7	95.96	0.5
CPS68	1005.2 x RKS20.5.4	F2-26 x ISU 3131	wt x r8	96.57	1.75
CPS69	1005.2 x RKS21.2.4	F2-26 x ISU 3133	wt x r9	98.77	2.13
CPS70	1005.2 x RKS22.4.4	F2-26 x ISU 3135	wt x r10	98.36	0.44
CPS71	RKS19.2.3 x P10-15	ISU 3129 x P10-15	r7 x wt	98.67	2.31
CPS72	RKS20.5.6 x P10-15	ISU 3132 x P10-15	r8 x wt	98.19	0.88
CPS73	RKS21.4.1 x P10-15	ISU 3134 x P10-15	r9 x wt	100	0

This table is a list of all crosses that were performed. Average round spore percentage was calculated from three replicate crosses. Standard Error (SE) was calculated by obtaining the standard deviation divided by the square root of the number of replicates. Each cross had three replicates used for the SE.
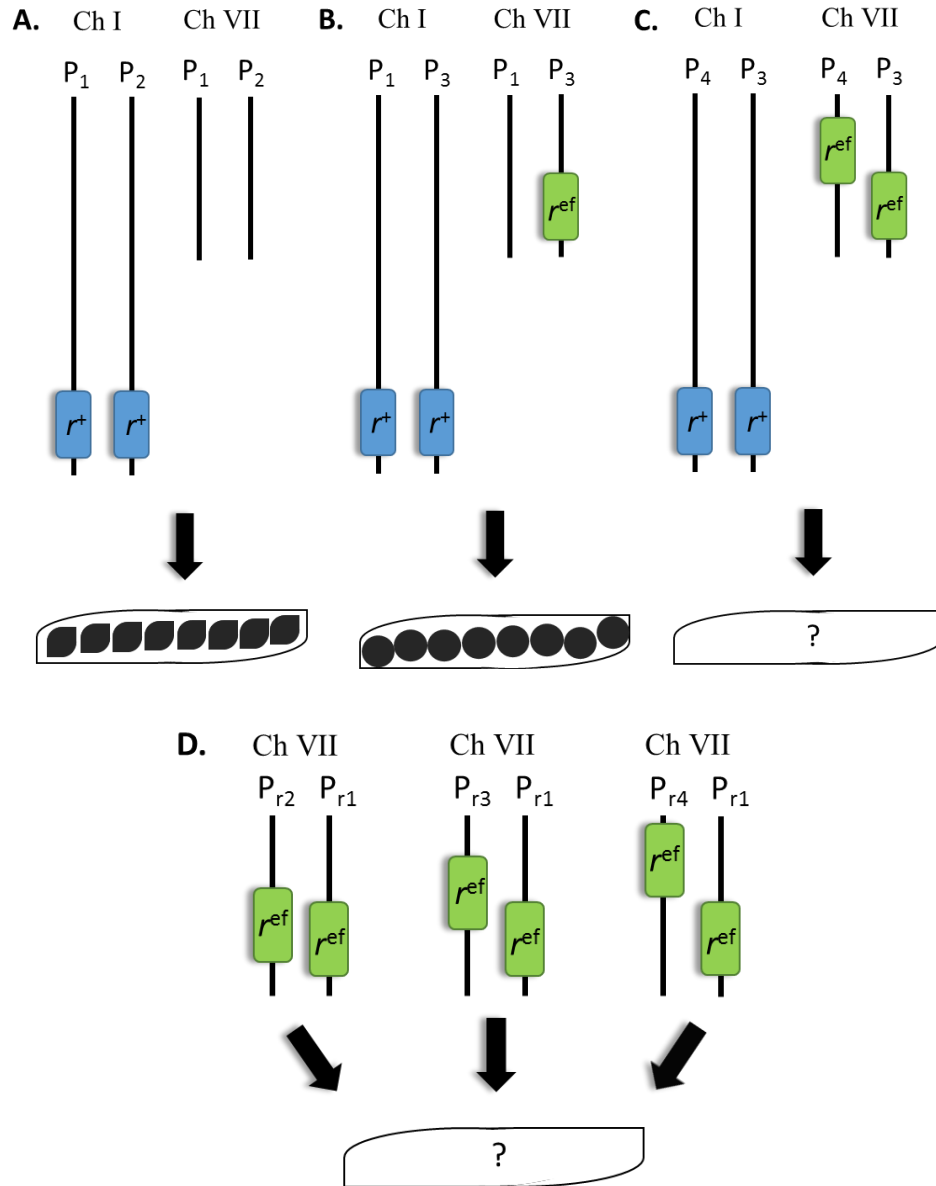
Figure II-1: MSUD Activation is Dependent on Sequence Pairing.
(A) Normal wt strains that both contain the endogenous r^+ gene on chromosome I. Illustration depicts pairing of the endogenous gene during meiosis leads to normal expression allowing production of football shaped ascospores. (B) A wt strain crossed to a strain carrying an ectopic r^+ gene on chromosome VII. All copies will be silenced because the r^{ef} on chromosome VII is not paired. This will produce round spores. (C) Two strains with r^{ef} insertions on chromosome VII at different locations had not been currently investigated. The spore phenotype of the progeny is unknown. (D) Illustration of three crosses with the r^{ef} of each strain separated by varying distances. "P#" represents different parent strains. Arrows point to illustrations of ascospore phenotype.
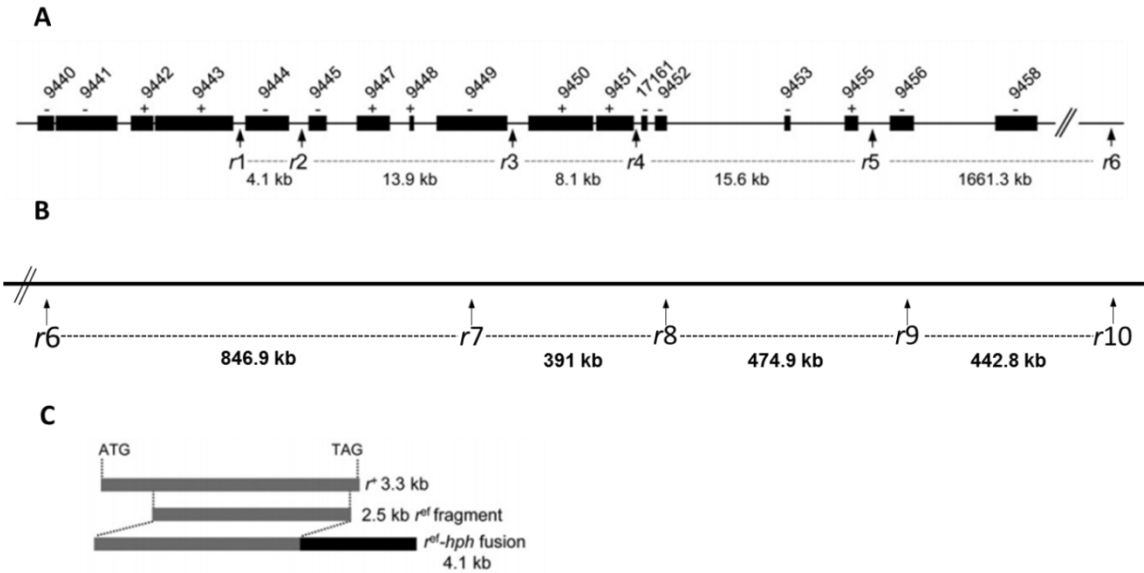
Figure II-2: r^{ef} Locations Across Chromosome VII of *Neurospora crassa*.
The round spore ectopic fragment coupled to the hygromycin resistance marker (r^{ef}-*hph*) was inserted all along chromosome VII of the *N. crassa* genome. (A-B) r1-r10 strains used in experimental crosses are represented according to where the r^{ef}-*hph* marker is located on chromosome VII. Numbers below each dotted line signify the distance in kilobases between each strain's inserted r^{ef}-*hph* marker. In panel A, the numbers above the solid line indicate the NCU0 gene number found on the Broad Institute *Neurospora crassa* Database website. Panel B does not show the relation to gene number due to such large distances between fragment insertions. (C) The r^{ef} consists of 2.5 kb of the native 3.3 kb r^+ open reading frame. Coupled to the *hph* gene, the r^{ef}-*hph* insert in total spans 4.1 kb.
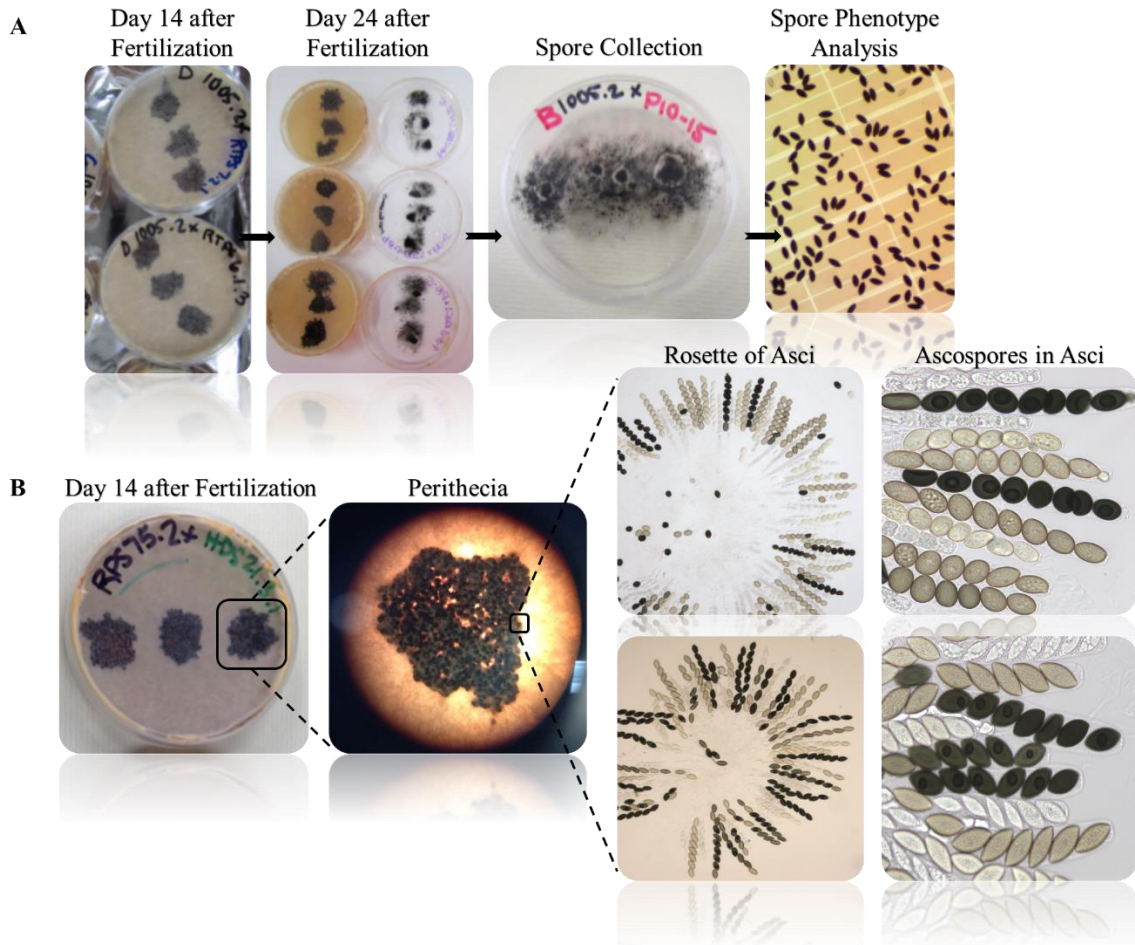
Figure II-3: Spore Phenotype Analysis and Replicate "D" Imaging.
(A) Day 14 after fertilization shows formation of perithecia before spores shoot. Day 24
after fertilization represents the end of the experiment when most of the spores have been
shoot to the lids. Lids are moved to new bottoms to stop collection. Spores are collected
from the lids. Spores are then analyzed on a hemocytometer to determine percentage of
round vs. football spores. (B) Replicate plate "D" is used only for imaging. Around day
14 after fertilization individual perithecia are cut open to observe the rosettes (10x
magnification). A preliminary phenotype can be observed by examining the ascospores.
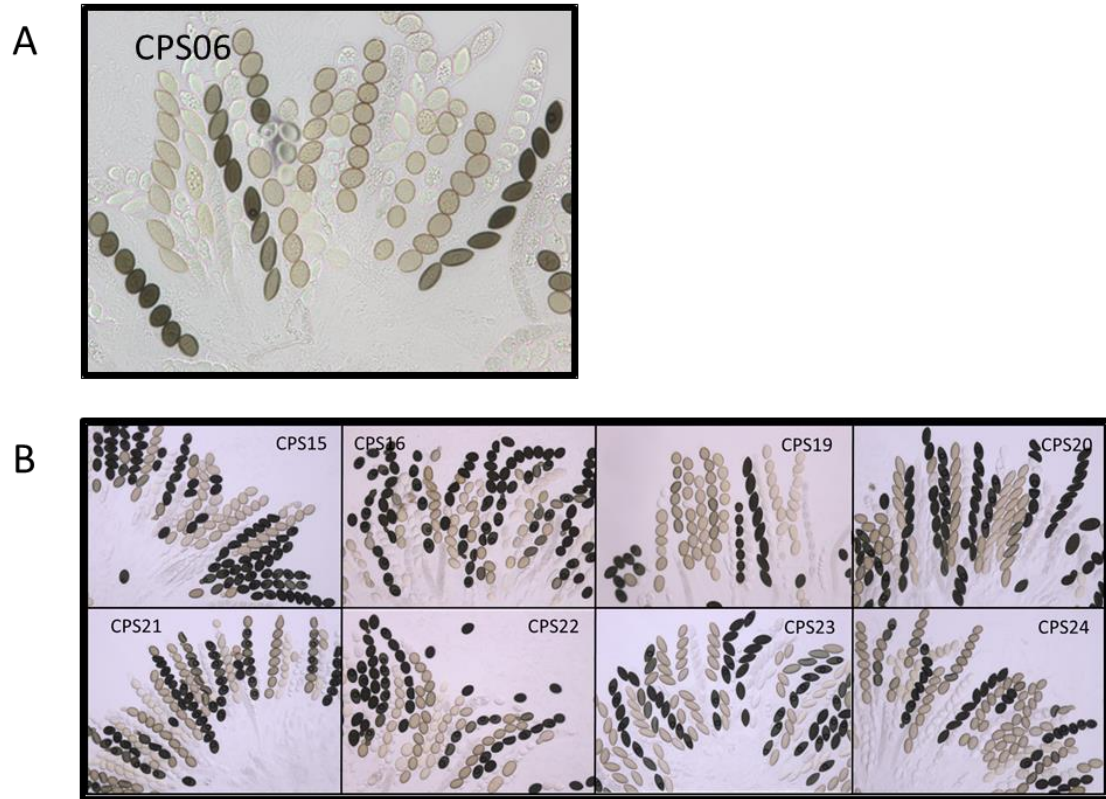
Figure II-4: Rosette Images of Preliminary Phenotypic Results of Crosses.
Images are asci from dissected perithecia (fruiting bodies) of varying crosses. (A) CPS06
(**Table II-4**) cross was the product of r2 x r3 (Figure II-2A) separated by a distance of
13.9 kb. Phenotypic results show a mix of round and football spores. (B) CPS15-24
(**Table II-4**) are examples of other phenotypes observed among crosses. Black spores are
mature and lighter tan spores are immature.

Figure II-5: Average Silencing of Positive Control Crosses Across All Experiments. (A) Positive control crosses consist of every r^{ef} strain crossed to a wt strain causing a physical unpairing of the r^{ef} on chromosome VII. (B) Graph of all positive control crosses in relation to percentage of round spores produced (degree of silencing). All abridged names labeled first in each cross are strains with "fluffy" vegetative states while the second strain listed has conidiating vegetative state. Each bar is the average of at least three replicates. Error bars represent standard error among all replicates of each cross.
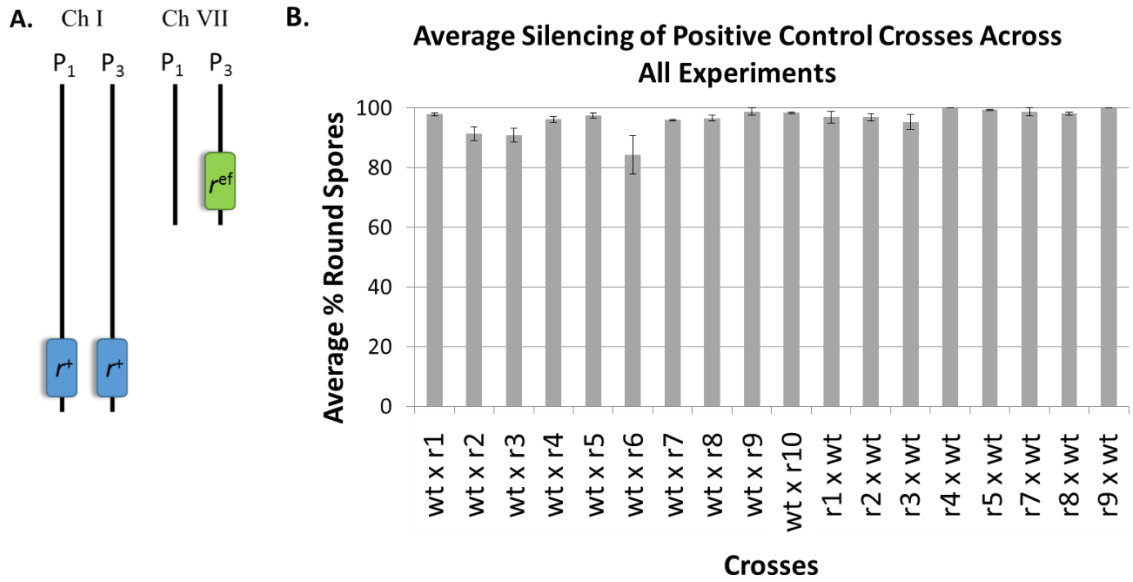
Figure II-6: Average Silencing of Negative Control Crosses Across All Experiments. Negative control crosses consist of strains carrying genes of interest at the same locations. (A) Example of paired genes between two wt strains. (B) Example of paired genes between two r^{ef} strains. (C) Graph of all negative control crosses in relation to percentage of round spores. Each bar is the average of at least three replicates. Error bars represent standard error among all replicates of each cross.

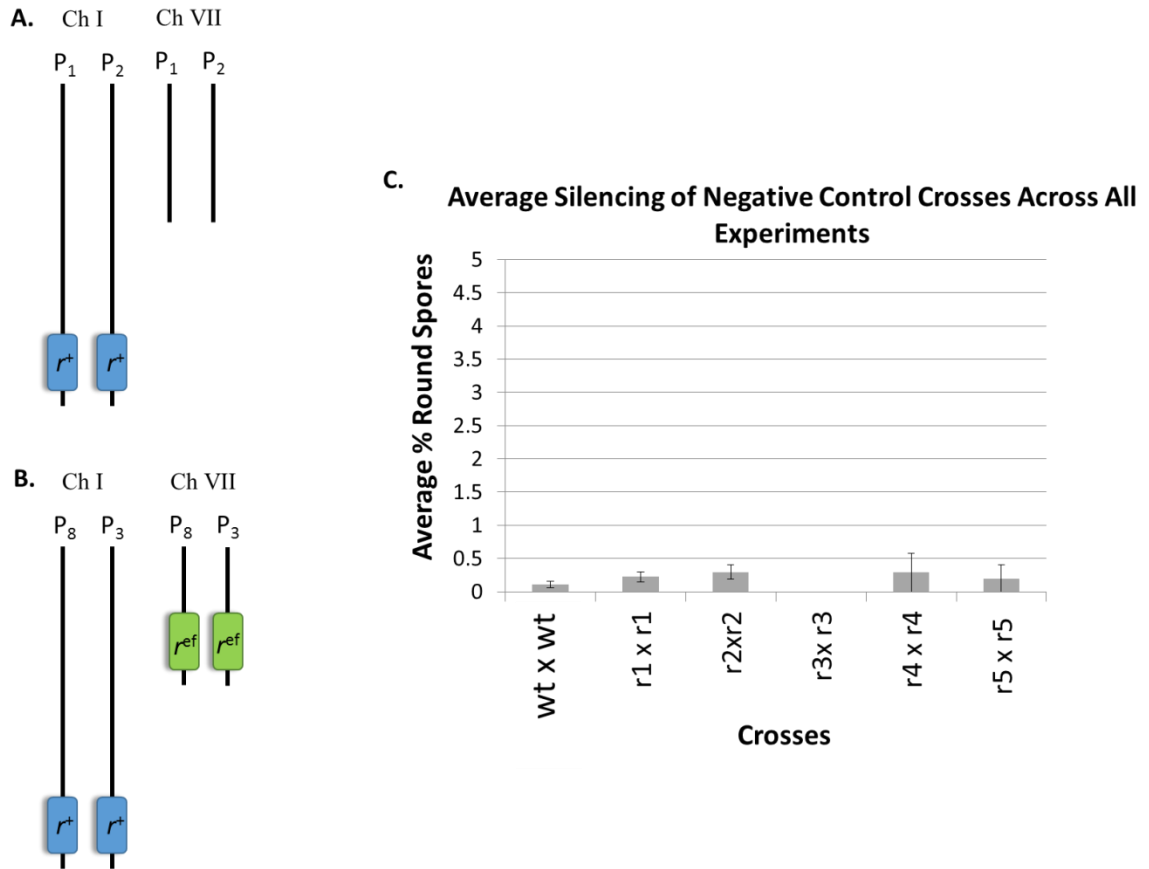**Figure II-7: Average % Round Spores in Relation to r^{ef} Distance on Homologous Chromosomes between Each Experiment.**

Each graph in the figure represents one full experiment of experimental crosses. Titles of each plot represents the cross names and numbers. Positive control crosses are not included in each experiment because r^{ef}s were separated across chromosomes and no linear distance could be stated. All distances are not represented on all plots due to limited strain availability at the time of each experiment. Each data point is the average of three plate replicates. Error bars represent standard error among all replicates of each cross.

GENE SILENCING VS DISTANCE BETWEEN EXPERIMENTS

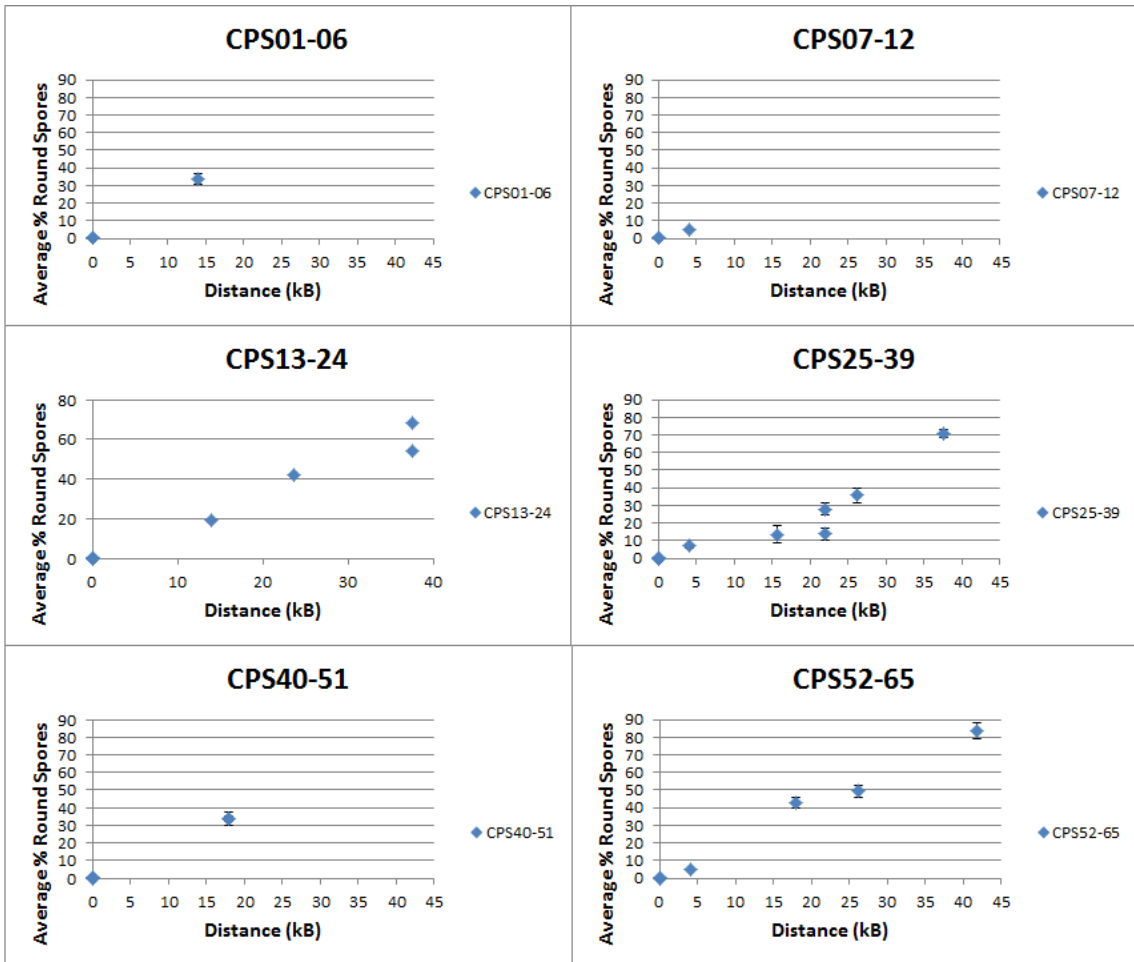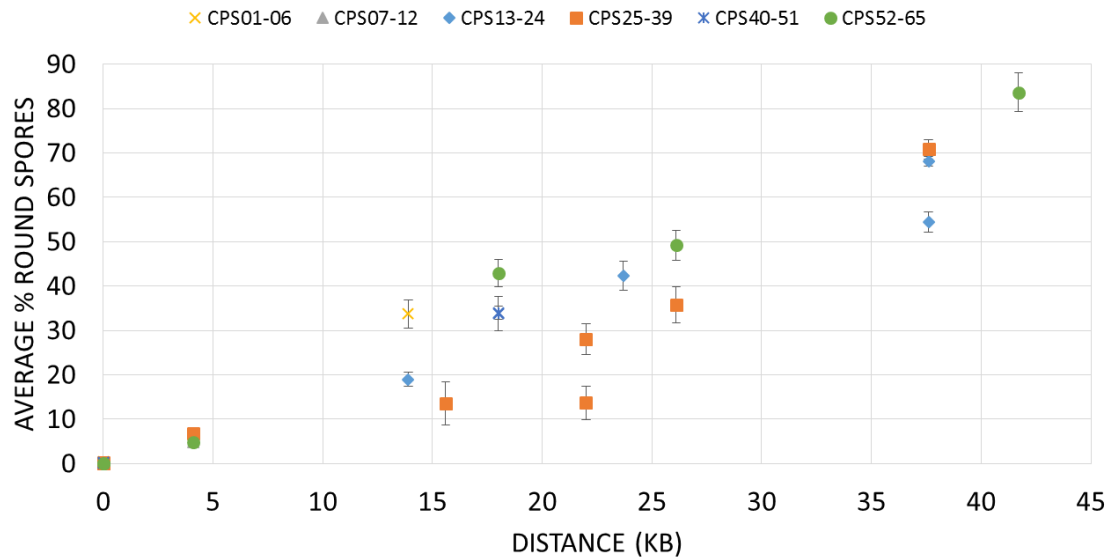**Figure II-8: Average % Round Spores in Relation to r^{ef} Distance on Homologous Chromosomes between All Experiments.**

Different colored and shaped points are associated with specific experiments crossed during the same time. Each data point is the average of three plate replicates or more. Error bars represent standard error among all replicates of each cross. Data from crosses utilizing strains r6-r10 were not included in the figure due to a marker distance of 1703.2 kb or greater. This data can be found in **Table II-4**.

CHAPTER III

SAD-5: A NUCLEAR PROTEIN OF MSUD

Abstract

Background. Meiotic Silencing by Unpaired DNA (MSUD) is one of the many genome defense mechanisms in model organism *N. crassa.* It is partially responsible for detecting invasions by transposable elements or retroviruses in the *N. crassa* genome during its sexual phase. MSUD is believed to occur in two stages including a recognition stage that is active in the nucleus and a silencing stage that happens in the perinuclear region of the cell. A recent discovery of a novel gene involved in MSUD, *sad-5,* was found to localize in the nucleus when expressed. This is the first MSUD protein found to have activity in this particular part of the cell. Therefore, it is possible that SAD-5 is involved in MSUD's initial detection stage.

Methods. The *sad-5* coding sequence was inserted in the pET15b expression vector that adds a histidine tag when the gene is expressed. Our recombinant protein was initially expressed in a Lemo21 *E. coli* cell line where optimal conditions for growth were determined. I attempted to purify the recombinant protein many times using different lysing techniques and other protein solubilizing procedures. I eventually moved the expression vector into a potentially more suitable *E. coli* cell line, ArcticExpress. This

cell line grows at lower temperatures and is engineered to express certain chaperone proteins. Both of these qualities are to aid in solubilizing insoluble proteins. The SAD-5 rotein was expressed in this cell line and optimal conditions for growth were determined. Lastly, I attempted to purify the SAD-5 protein from this cell line.

Results. All attempts at purifying the soluble SAD-5 protein in both cell lines were unsuccessful. No technique applied was able to remove the SAD-5 protein from the insoluble fractions.

Discussion. Although all attempts of SAD-5 purification failed, there are numerous other variations of the procedures used here as well as completely new approaches. One solution that could have great success would be to move from a prokaryotic system to a eukaryotic organism for purification. This move may allow a better environment for expressing a eukaryotic gene. Once SAD-5 is successfully purified, biochemical binding assays can be used to determine SAD-5's substrate specificity and ultimately more about its function.

Background

The filamentous fungus *N. crassa* proves to be particularly 'paranoid' when it comes to protecting its genome. This characteristic is highlighted by the fact that *N. crassa* possesses several genome defense mechanisms activated in many parts of its lifecycle (WATTERS *et al.* 1999; GALAGAN and SELKER 2004; DANG *et al.* 2011; BILLMYRE *et al.* 2013) . Meiotic Silencing by Unpaired DNA (MSUD) is one of the mechanisms involved in this tight security. MSUD occurs during the sexual stage of *N. crassa* and is triggered when a sequence on one homologous chromosome cannot locate its partner on the opposing homolog (SHIU *et al.* 2001; SHIU and METZENBERG 2002). This unpairing event

signals a breach in the system and is somehow tagged for further action. Ultimately, the sequence is silenced and is not allowed expression during meiosis.

Many components of the silencing stage of MSUD have been discovered; however, the initial recognition portion of unpairing events in the nucleus has stayed a mystery. A newly found MSUD protein, SAD-5, could potentially be able to shed some light on the elusive first stage of MSUD (HAMMOND *et al.* 2013).

Interestingly, SAD-5 is one of the first MSUD proteins that is not required for sexual development (HAMMOND *et al.* 2013). This characteristic alone has allowed for successful homozygous knockout crosses that have not been possible with other MSUD proteins (SHIU *et al.* 2001, 2006; HAMMOND *et al.* 2013). Homozygous knockout crosses of *sad-5* show a complete inactivation of MSUD. Interestingly, heterozygous crosses of *sad-5Δ* to wt show weak suppression of MSUD. This might suggest one copy is sufficient for MSUD activity due to higher levels of *sad-5* expression (HAMMOND *et al.* 2013). However, it was observed that *sad-5* expresses relatively low transcript levels during the sexual stage and has no expression in the vegetative phase (SAMARAJEEWA *et al.* 2014). Alternatively, *sad-5* may produce a protein with low turnover rates. *sad-5Δ's* ability to be crossed homozygously also permitted further investigation into SAD-5's involvement in masiRNA (MSUD associated small interfering RNA) production. These experiments have shown SAD-5 is required for masiRNA production (HAMMOND *et al.* 2013).

Most importantly, however, SAD-5 was the first MSUD protein found to localize inside of the nucleus (HAMMOND *et al.* 2013). Due to its location, our lab believes it could be interacting with unpaired DNA or associated with other steps of the recognition

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process of MSUD. Examination of SAD-5 and its activities could prove to be particularly exciting as it might lead to a breakthrough towards understanding this initial recognition stage. Here we show the first biochemical approaches applied to the SAD-5 protein. The goals of this study are outlined in two aims below.

AIM 1: Purify the SAD-5 protein from *E. coli* cells.

AIM 2: Preform binding assays to determine SAD-5 binding affinity to different substrates.

Methods

Initial Plasmid Construction and Transformation. The oligonucleotide primers used can be viewed in **Table III-1**. The *sad-5* gene was amplified from *N. crassa* wt strain F2-01 cDNA using New England Biolab's (NEB) Phusion® High-Fidelity DNA Polymerase kit. Primers for this amplification were designed to contain restriction sites for restriction enzymes *XhoI* and *NdeI* on the flank ends to allow cloning to various plasmids. The *sad-5* PCR product was ligated into the pJET1.2 plasmid from Thermo Scientific's CloneJET PCR Cloning Kit and transformed into NEB Turbo Competent *E. coli* cells using NEB's High Efficiency Transformation Protocol. Exceptions to this protocol include substituting 1 ml of lysogeny broth (LB) in place of super optimal broth (SOC).

After 24 hours incubation at 37 °C, individual colonies growing on selective ampicillin media were selected and grown in LB liquid cultures overnight. Plasmids were then isolated from the cultures using a Qiagen Miniprep kit and protocol. All isolated plasmids were analyzed for gene insert by a diagnostic restriction digest using *XhoI* and *NdeI* enzymes from NEB. Correct plasmid, VPS02, was chosen and sequenced by UIUC Core DNA Sequencing Facility in Urbana, Illinois for verification. Sequencing results were

compared to the *sad-5* predicted coding sequence provided by the Broad Institute's *Neurospora crassa* Database.

Expression Vector Construction and Transformation. Invitrogen expression vector pET15b and VPS02 (pJET1.2 plasmid with *sad-5* insert) were both digested with *XhoI* and *NdeI* NEB restriction enzymes. Expression vector pET15b carries an ampicillin resistance gene, an IPTG inducible promoter system, and allows placement of a histidine tag on the inserted gene when transcribed to allow for His-Tag purification. The *sad-5* insert and cut pET15b vector were isolated using an IBI gel extraction kit. The *sad-5* gene insert was then ligated into pET15b using T4 ligase from NEB. For insert verification, the construct was transformed into NEB Turbo Competent *E. coli* cells again using NEB's High Efficiency Transformation Protocol. After 24 hours at 37 °C, growing colonies were transferred to 3 ml of LB broth containing 1x ampicillin/carbenicillin antibiotic solution and grown overnight at 37 °C. Plasmids were then isolated using a Qiagen Miniprep kit. Verification of a correctly inserted plasmid was carried out by means of a diagnostic restriction digest again using NEB enzymes *XhoI* and *NdeI*. Plasmids p288.2 and p289.7 were sent off for sequencing for further confirmation.

SAD-5 Protein Expression in LEMO21 cells. **Table III-2** depicts all solution and buffer recipes. Plasmid p289.7 was transformed into NEB's LEMO21 (DE3) Competent *E. coli* cells following NEB's Transformation Protocol (C2528). This transformation was aliquoted to a 25 ml suspension of LB inoculated with 1x ampicillin/carbenicillin antibiotic solution and then cultured overnight at 37 °C, 200 rpm. The culture was added to 500 ml of LB liquid containing 1x ampicillin/carbenicillin antibiotic solution and cultured at 37 °C, 200 rpm until the cells reached an O.D. of approximately 0.3. A sample of uninduced

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culture was retrieved for gel analysis. Protein expression was then induced by culture inoculation of 1M IPTG to a total concentration of 0.4 mM IPTG. The cells were cultured at 37°C, 200 rpm for an additional 3.5 hours. A sample of induced culture was retrieved for gel analysis. After incubation, the culture was placed on ice for 10-15 minutes and then pelleted for 15 minutes at 8100 rpm, 4 °C. The pellet was resuspended in a rinse buffer and the suspension was pelleted as before. The supernatant was discarded and the pellet was stored at -80 °C until the purification step.

SAD-5 Optimal Protein Expression in LEMO21 Cells. The optimization time expression experiment was carried out in the same manner as the regular expression protocol outlined above. However, multiple samples of induced culture were taken at different time intervals, up to 5.5 hours. The volume of each sample collected was determined by each sample's optimal density. Therefore, consistent cell density across samples was accounted for. **Figure III-2B** lists each time sample's O.D. and also the corresponding volumes that were retrieved for each sample. The sample volumes listed were pelleted and prepared for gel analysis.

I also determined optimal IPTG concentration to induce production of the SAD-5 protein. Cultures were prepared as previously stated. However, once the culture reached an O.D. of 0.3 before induction, the culture was divided into seven flasks of 30 ml samples. At this time, each flask was inoculated with varying concentrations of 0-1 mM IPTG to induced protein expression. After samples were cultured for 3.5 hours, two 1 ml aliquots were collected for O.D. readings and gel analysis (**Figure III-2D**).

SAD-5 Native Protein Purification in LEMO21 cells. The pelleted culture was removed from storage and placed on ice to thaw allowing cells to lyse. Exceptions include

French pressure cell press (French press) and sonication cell lysis during cell lysis experiments. The pellet was resuspended in 15-20 ml of Wash buffer (See **Table III-2** for all buffer components) and pelleted for 30 minutes at 8100 rpm, 4 °C. The supernatant and pellet were separated and aliquots were taken for gel analysis. Following, 750 μl of equilibrated high affinity nickel-charged resin from Genscript (Catalog # L00223) was added to the supernatant. This was mixed at 4 °C for 15-30 minutes. The solution was centrifuged for 2 minutes at 1500 rpm, 4 °C to pellet the resin and collect the "flow-through" supernatant. The nickel resin was then washed with 10 ml of Wash buffer and allowed to incubate on ice for approximately 2 minutes. The sample was again centrifuged as before and the protocol was repeated for Wash II (**Table III-2A**). The nickel resin was incubated on ice with 1.5 ml of Elution Buffer in 3-5 rinses of 300-500 μl. All elutions were frozen at -80 °C. A 20 μl aliquot from each fraction was taken for gel analysis.

Cell Lysis Alternatives. Expression for both cell lysis alternatives is carried out as stated above. The French Press lysis protocol called for a 500 ml culture pelleted and then resuspended in 20 ml of Wash buffer. The machine was set at 1000 psi and the cells were subjected to this pressure twice. Another alternative lysing method used was sonication. Again, a 500 ml cell culture was pelleted and resuspended in 20 ml of Wash buffer. The cells were stored in ice and the sonication probe was inserted into the culture. Cells were sonicated 7 times at 10 second intervals and 30 second rests. Purification was carried out as stated above.

SAD-5 Denaturing Protein Purification and Dialysis in LEMO21 cells. Protein expression is the same as the native purification expression except a 5 ml overnight culture and a 100 ml expression culture were used in place of a full 500 ml culture and 25 ml

overnight. The denaturing purification protocol parallels that of the native protocol except for substitution of denaturing buffers (**Table III-2B**) and scaled down volumes due to the smaller starting culture volume.

Concentrated elutions were inserted into Spectra/Por dialysis tubing made by Spectrum Laboratories with a molecular weight cut off (MWCO) of 3500 Da. Elutions were initially dialyzed in 8M urea dialysis buffer at room temperature (**Table III-2B**). Dialysis buffer missing 8M urea was then added gradually to dilute the urea concentration to 6.9 M urea. Elutions dialyzed for 4-5 hours. Over a course of approximately 20 hours, the elutions were diluted to a concentration of 4 M urea. At this point the apparatus was moved to 4 °C.

SAD-5 Protein Expression with Ethanol in LEMO21 cells. Expression of SAD-5 protein was carried out primarily as stated above during native protein expression. Exceptions include inducing protein expression at an O.D. of 0.7 and adding a 2% final volume of ethanol at the time of induction.

SAD-5 Protein Expression with Varying Temperatures in LEMO21 cells. Cultures were prepared as outlined in the native protein expression in Lemo21 cells. However, a 300 ml culture was initially used and antibiotic volumes were adjusted accordingly. After cells reached an O.D. of ~0.3, a 2 ml uninduced culture was collected and the remaining culture was separated into three flasks with 100 ml of culture in each. All three cultures were then induced with 0.4 mM IPTG. One flask was cultured at 37 °C for 4 hours. The second flask was cultured at 29 °C for 9 hours, and the third flask was cultured at 4°C for approximately 21.5 hours. Sample volumes taken from each flask were determined based on the each culture's O.D. reading after final collection. These were compared to the

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uninduced O.D. reading and volume. The following equation was used [(2 ml)(0.36)]=[(x Sample volume)(Sample O.D.)]. This allowed cell densities to be equivalently represented on the PAGE gel. **Figure III-4D** shows a table of those values.

SAD-5 Protein Expression in ArcticExpress DE3 RP Cells. The plasmid p289.7 containing the SAD-5 gene was transformed into ArcticExpress DE3 RP *E.coli* cells supplied by Agilent Technologies. This cell line was designed to carry an extra plasmid with the ability to code for a chaperonin protein and is selected for with gentamicin. The transformation protocol was completed as stated previously when describing transformation into Lemo21 cells. One exception is the addition of gentamycin to selection media.

Once transformation was verified, a 50 ml sample of LB inoculated with the ArcticExpress transformed cells and antibiotics (ampicillin and gentamicin) was cultured at 37 °C, 200 rpm overnight. A 350 ml LB culture with antibiotic was then inoculated with the overnight and grown at 30 °C, 200 rpm for 3 hours. The culture was next cooled to 13 °C while being continuously stirred. An uninduced 1 ml aliquot was retrieved for gel analysis before inducing expression with inoculation of 0.4 mM IPTG. Expression continued for the next 48-72 hours.

Optimal Time Expression of SAD-5 in ArcticExpress Cells. The culture was prepared and expressed as previously stated. However, after inoculation of IPTG to induce expression, 2 ml aliquots were taken at every 24 hours for 96 hours. The same volume was also retrieved for the uninduced sample. The optical densities of each time interval was determined at a wavelength of 600 nm. All densities were compared to the uninduced sample O.D. to determine the volume of culture to pellet. This allowed consistent cell

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density amongst all samples. **Figure III-5B** indicates the volumes and O.D. of each sample pelleted.

SAD-5 Protein Purification in ArcticExpress DE3 RP Cells. After approximately 60 hours after the previously described expression protocol, the culture was separated into three autoclaved plastic centrifuge bottles and centrifuged at 4000 rpm for 20 minutes at 4 °C. The supernatant was discarded and the pellets were resuspended in 30 ml of purification buffer (See **Table III-2C** for recipe). The suspension was then put through the French press twice at slightly over 1000 psi and the movement of the chamber was set to the high setting. The solution was divided into two small centrifuge tubes and centrifuged for 20 minutes at 15000 rpm. The supernatant was transferred to a small flask and kept cold at 4 °C after a 200 μl sample was taken. The supernatant was diluted by adding purification buffer to equal a total volume of 75 ml of solution. Next, a final volume of 1 mM ATP and 1 mM Mg^{2+} was added to the diluted supernatant to constitute the first ATP wash allowing chaperonin proteins to release the protein of interest. This was incubated at 28 °C, 50 rpm for 30 minutes. Approximately 5 ml of HisPur cobalt resin made by Thermo Scientific was added to a chromatography column and equilibrated with 50 ml of purification buffer. After incubation, the supernatant was added to the column and 1 ml of the final flow through was collected. The column was then washed with 50 ml of 10 mM imidazole purification buffer and 1 ml was collected from the final pool. A second ATP wash consisted of adding 10 ml of 10 mM imidazole purification buffer with 5 mM ATP, 5 mM Mg^{2+} gently to the column and incubating again at 28 °C, 50 rpm for 30 minutes. After collection of the 2nd ATP wash, 50 ml of 20mM imidazole purification buffer was added and collected. A 1 ml sample was keep for gel analysis. Ten milliliters of 200 mM imidazole purification buffer

(elution buffer) was added to the column and approximately ten fractions of 1 ml elutions were collected.

Protein Determination of SAD-5 in ArcticExpress cells. Protein concentration of each fraction was initially determined by a Bradford Assay. The samples were prepared by adding 200 μl of Bio-Rad Protein Assay Dye Reagent, a preliminary small sample of the fraction being tested, and an amount of water that would total to 1 ml. The blank sample contained 200 μl of Bio-Rad Protein Assay Dye Reagent and 800 μl of water. Each mixture was then compared to a previously determined BSA protein standard curve, and the absorbance was detected at a wavelength of 595 nm. Each fraction was diluted with water and load dye to get a final concentration 1 μg/μl (See **Table III-3** for volumes). Some fractions however were not concentrated enough to reach such a value.

SDS-PAGE Gels. ExpressPlus 8-16% PAGE gels from Genscript were used for sample analysis. All aliquots from each fraction collected were mixed with 6x SDS-PAGE load dye and then boiled for 3 minutes in approximately 90°C before being loaded onto the gel. Uninduced, induced, and pellet fractions were additionally ran through filter columns for optimal loading conditions. Columns were constructed using 0.5ml microcentrifuge tubes stuffed with DMCS treated glass wool made by Ohio Valley and placed in empty 2 ml tubes. Each 0.5ml microcentrifuge tube was cut on the bottom with a razor blade to allow solutions to flow through. All gels were ran with prepared MOPS running buffer as outlined in Genscript's Technical Manual for ExpressPlus PAGE gels. Gels were ran at 150V for approximately an hour. Gels were then stained overnight. The stain was removed from the gel the next day and destain was added. Gels were destained for approximately 3

to 4 hours. Destain was then rinsed off with water and the gel was imaged using an iPhone 5c camera courtesy of Jonathan Williams.

Results

Sequencing Results of Cloned Sad-5 cDNA. *sad-5* cloned cDNA sequencing results were aligned to the Broad Institute's *Neurospora crassa* Database provided *sad-5* sequence. Results revealed a six base pair difference, indicating that the sequence "TTACAG" was missing from the beginning of the second exon in the sequenced plasmid when compared to the database. Considering alternative sequenced replicate plasmids confirmed this result, this could indicate that these six bases are actually apart of the intron. **Figure III-1** shows the *sad-5* sequence retrieved from the Broad Institute.

Optimal Expression for SAD-5 in the Lemo21 Cell Line. Before regular protein isolation could begin, optimal expression conditions were determined. **Figure III-2A** gel depicts optimal expression time for SAD-5 in the Lemo21 cell line. In the pET15b expression vector used, protein expression is regulated by the presence of IPTG. Lanes 3-8 represent individual samples of isolated culture at increasing time points that were taken after IPTG inoculation. Lane 2 shows protein expression of the Lemo21 *E.coli* cell line without expression of SAD-5 due to the absence of IPTG. After induction, a protein that runs just below 55 kD starts to appear with increasing intensity as time progresses. The Broad Institute's *Neurospora crassa* Database amino acid sequence of endogenous SAD-5 predicts a 47.78 kD sized protein. Therefore, I predicted that the HIS-SAD-5 fusion protein to be approximately 49.96 kD in size which would coincide with the size of the expressed protein that appears in **Figure III-2A.** It appears that the intensity of the predicted SAD-5 fusion protein product does not increase past 3.5 hours in lane 6 when

compared to the two later time points. This suggests that the SAD-5 fusion protein can be optimally expressed in the Lemo21 cell line at a minimum of 3.5 hours after IPTG inoculation.

I also investigated at what IPTG concentration SAD-5 would be ideally expressed. **Figure III-2C** shows the results of this experiment. Each lane shows an increasing amount of IPTG added to a 30 ml culture. Except for the uninduced sample (lane 1), all other samples expressed a relatively similar O.D. for the samples that were prepared for gel analysis (**Figure III-2D**) indicating comparable cell densities across samples. Compared to the uninduced (No IPTG; lane 1), all induced samples (lane 2-7) provide the expressed SAD-5 protein near the 55 kD mark on the protein ladder (lane 8). All induced samples appear to have comparable SAD-5 expression. Cells grown with 0.1 mM IPTG (lane 2) seem to show slightly more expression of SAD-5; however, this could be attributed to a small increase in cell density (**Figure III-2D**). Overall, it appears there is minimal difference in expression based on IPTG concentration. Therefore, I decided to use 0.4 mM IPTG for induction as this was similar to most expression protocols.

SAD-5 in Lemo21 Cells is Expressed in the Insoluble Fractions during Purification. After optimal expression conditions were established, ongoing purification trials of the SAD-5 protein began. **Figure III-3A** depicts fractions of a nondenaturing purification trial using the freeze thaw cell lysis method typical of this type of *E. coli* cell line. Lane 7 and 8 show proteins produced under uninduced and induced expression conditions respectively. Again, there is the appearance of a band around 55 kD in the induced lane (lane 8) indicating expression of our SAD-5 protein. When compared to the supernatant (Lane 1) and the pellet (Lane 5) fractions, it is apparent that much of the SAD-5 protein is

being expressed in the insoluble pellet fraction and little if any SAD-5 protein is present in the soluble supernatant fraction.

I hypothesized that perhaps the cells were not being completely lysed during freeze thaw leaving most of the proteins stuck in the pellet fraction. Therefore, alternative lysing methods were utilized to prevent this problem. **Figure III-3B, and C** indicate sonication and French press cell lysis purification alternatives. In panel B (sonication), the uninduced and induced cultures (lanes 7 and 8, respectively) can again be compared to the supernatant and the pellet (lanes 2 and 1). Yet again, the SAD-5 protein is expressed in the pellet and the supernatant shows very little protein. Panel C (French press cell lysis) seems to represent a similar outcome with respect to SAD-5 expression in the pellet; however, the supernatant fraction shows more protein. The initial loss of protein in the supernatant fractions for the first two lysis methods are most likely attributed to over-dilution of the pelleted cells during resuspension. However, all lysing methods still resulted in SAD-5 expression in the insoluble pellet fraction and never in the soluble supernatant fraction. These results suggest that the SAD-5 protein could potentially be folded improperly, ultimately leading to protein aggregation. This effect can cause proteins to fall out of solution and is referred to in the cell as inclusion body formation (HAASE-PETTINGELL and KING 1988).

Efforts to Solubilize the SAD-5 Protein in the Lemo21 Cell Line. Three different techniques were performed for resolving or avoiding potential formation of inclusion bodies. I first decided to try a denaturing purification of the *E. coli* cells in hopes of disrupting the hydrophobic interactions that can develop during the formation of inclusion bodies (ZANGI *et al.* 2009). Ultimately, this may allow increased solubility; however, it

will most likely cause the purified protein to be inactive. However, many studies have shown that there is a potential to refold some denatured proteins (THOMAS and BANEYX 1996; CLARK 1998; BANEYX and MUJACIC 2004; PALMER and WINGFIELD 2004, 2012; YANG *et al.* 2011). **Figure III-4A** represents all fractions of a denaturing purification of SAD-5. Again, the SAD-5 protein appears in the pellet fraction (lane 2) and does not seem to appear in the supernatant (lane 3). Interestingly, the protein seems to appear in the elution fractions (lanes 8-12) especially in the initial elution wash (lane 8). However, attempts at renaturing the protein through removal of urea from the concentrated elutions were unsuccessful. Attempts to remove such a high concentration of urea caused the protein to come out of solution.

Figure III-4B depicts all expression and purification fractions of an initial culture that was grown with ethanol. Previous studies have suggested that different external stimuli can induce a heat shock response in *E. coli* that improves recovery of active recombinant proteins (JONES *et al.* 1987; NEIDHARDT *et al.* 1987). In a paper from 1996, it was found that an addition of 3% ethanol (v/v) to growth medium elicits a response ultimately aiding in protein misfolding (THOMAS and BANEYX 1996). Therefore, we decided to try this technique to eliminate the potential inclusion body formation of SAD-5 and allow it to collect in the soluble fractions during purification. **Figure III-4B** depicts an SDS-PAGE gel of the results. Lane 7 (uninduced SAD-5 culture) is comparable to lane 8 (induced culture) indicating no expression of the protein of interest. Thus, expectedly, there was no concentrated band that appeared in pellet or supernatant lanes (lane 5 and 4 respectively). This would suggest that addition of ethanol to these cells inhibits the expression of SAD-5. Thus, implying that this method was not successful. Interestingly, I used 2% ethanol for

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the final culture volume and this percentage was actually lower than the optimal percentage

of 3% found in the Thomas and Baneyx 1996 paper (THOMAS and BANEYX 1996). Lower

percentages such as the one I used in the study was previously found to have no effect on

protein recovery while higher than 3% ethanol has been shown to inhibit the growth rate

of *E. coli* (JONES *et al.* 1987; THOMAS and BANEYX 1996, 1997). This discrepancy can

most likely be attributed to difference in *E.coli* cell lines as well as the sensitivity of the

recombinant protein being expressed in each line.

We hypothesized that if the cells were allowing improper folding of the SAD-5

protein then perhaps slowing the cells down in colder temperatures would allow sufficient

time for correct folding. This approach has previously proved to be advantageous because

it also allows a reduction in strength of hydrophobic interactions (VASINA and BANEYX

1997). **Figure III-4C** illustrates expression fractions of cultures grown at three different

temperatures. It is clear in lane 4 (uninduced culture for all temperatures) that the SAD-5

protein is not being expressed when compared to the concentrated 55 kD band represented

in the protein ladder (lane 5). In induced lanes 3 and 6 (37 °C and 29 °C respectively) the

SAD-5 protein is expressed and appears near the 55 kD mark; however, lane 9 (4 °C

induced) does not seem to show apparent SAD-5 expression. Furthermore, the pellet

fractions of both higher temperatures (lanes 2 and 8) unfortunately still withhold the SAD-

5 protein. The 4 °C pellet fraction (lane 5) did not show SAD-5 expression which is

attributed to poor expression in the induced fraction. These results suggest that lower

temperatures for expression of the SAD-5 protein in Lemo21 cells are not adequate to aid

in solubilizing the recombinant protein. Conversely, these results may be limited based on

the fact that the 4 °C culture was only expressed for 21.5 hours. This time frame may not

have been sufficient for SAD-5 expression at this temperature. Also, this does not rule out the possibility that temperatures between 4 °C and 29 °C could be effective in eliminating inclusion bodies.

SAD-5 Optimal Expression in ArcticExpress Cells. As an alternative of trying to further manipulate the SAD-5 protein in the Lemo21 cell line, I decided to move the expression vector to another *E. coli* cell line referred to as ArcticExpress DE3 RP. This cell line is known to be more appropriate for expressing eukaryotic genes because it carries a plasmid that codes for a chaperonin protein along with the added effect of preferred growth at lower temperatures. These characteristics will aid in helping the protein to fold properly. Therefore, by moving our construct to this cell line I hoped to alleviate the problematic formation of inclusion bodies.

Figure III-5A depicts an optimal time expression test after induction of the SAD-5 fusion protein in ArcticExpress cells. Lane 2 of the gel depicts the uninduced culture (No IPTG added) at time zero, while lanes 3-6 show different time points after induction. In the induced samples a protein right below the 55 kD mark appears that does not appear in the uninduced sample. It is evident that this must be expression of the SAD-5 fusion protein. Also, it appears that lane 5 shows the most concentrated production of SAD-5. Therefore, these results suggest that the SAD-5 fusion protein can be expressed in the ArcticExpress cell line, and it is optimally expressed at 72 hours after induction.

SAD-5 Purification in ArcticExpress Cells. **Figure III-6A** depicts an SDS-PAGE gel of SAD-5 purification in ArcticExpress cells. As expected, SAD-5 production (~50 kD in size) in induced fractions (lane 1) is apparent when compared to the uninduced fraction (lane 2). However, again when both the pellet (lane3) and supernatant (lane 4) have been

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concentrated to 1 μg/ml (data shown on **Table III-3**) it is obvious that most of the SAD-5 protein is still accumulating in the insoluble pellet fraction. Interestingly, there does seem to be a small portion of SAD-5 in the supernatant that carries over into the flow through (lane 5). Although in Wash 1, only a faint chaperonin product around 70 kD is present while the product indicative of SAD-5 is missing. Not only is there nothing visible in the elutions from **Figure III-6A**, the Bradford Assay results in **Table III-3** show very low levels of overall protein in these fractions. Ultimately, this approach was not successful in purifying the SAD-5 protein.

Discussion

A main question of our genetics laboratory is to determine if the detection process of MSUD uses homologous recombination process/machinery to aid in its homology search. The discovery of the nuclear MSUD protein, SAD-5, has allowed us to attempt some of the first biochemical experiments on a protein that may have a role in this mechanism.

In these early experiments I have gained valuable information on SAD-5 stability and solubility in recombinant expression attempts in *E. coli*. Largely, SAD-5 expressed in the bacterial systems here resulted in the insoluble fractions. This may indicate that the *E. coli* cell line is not equipped to accommodate for the over-expression of the recombinant protein. The denaturing purification was the only successful attempt in purifying SAD-5; however, efforts to renature the protein failed. It was surprising that the SAD-5 protein purification conducted in the ArcticExpress cell line was not successful because it was effective in purifying another nuclear-localizing MSUD protein, SAD-6.

There are numerous alternative approaches that have yet to be explored to help us move closer to the purification of SAD-5. All the above approaches have varying protocols that could be investigated. However, perhaps the most valuable alternative may be to move the expression vector to a eukaryote organism such as yeast. It has been shown previously that expressing eukaryotic genes in a prokaryotic system can be problematic (MARSTON 1986; GUAN and DIXON 1991; MAKRIDES 1996; KHOW and SUNTRARACHUN 2012).

Eukaryotic systems rely on post-translational modifications such as methylation, phosphorylation, glycosylation or even removal or cutting of amino acids like seen in insulin to regulate and produce mature functioning proteins (BELL *et al.* 1980; WESTERMANN and WEBER 2003; ULRICH 2009; CAIN *et al.* 2014). Most prokaryotic systems such as *E. coli* are usually not equipped with the machinery to correctly modify the recombinant protein of choice (WACKER *et al.* 2002; IHSSEN *et al.* 2010). It also not advantageous for mature proteins containing disulfide bonds to be produced due to *E.coli*'s reducing environment (BESSETTE *et al.* 1999). Perhaps the difference between SAD-6 and SAD-5 success in protein purification is due to the lack or presence of post-translational modifications. There is the possibility that SAD-5 needs a different modification compared to SAD-6 that our *E. coli* strain cannot provide. For instance, if a modification allows certain areas of the SAD-5 protein to be protected from hydrophobic interactions, without those modifications it might be contributing to the formation of inclusion bodies and unsuccessful purifications. Thus, two alternatives would be to move the expression vector to a eukaryotic organism such as yeast that would be more equipped to add the appropriate modifications or use a modified *E. coli* strain that can co-express enzymes with the recombinant protein that can potentially add these modifications.

Once a successful alternative method for purifying SAD-5 is achieved, it is exciting to think about the activities that this novel protein could have. Mobility shift assays can be conducted to determine the proteins preferred substrate. Initially, substrates such as double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and structured DNA (Holliday junctions, branched DNA, hairpins) can be used for analysis. It is difficult to hypothesize what SAD-5 might show affinity for because it possesses no known protein domains. However, if MSUD does use homologous recombination for recognition of unpairing events, we could compare its substrate preference to other homologous recombination protein activities. For example, if SAD-5 were to bind to ssDNA, we could investigate if SAD-5 has similar activities to RAD51 or BRCA2 both of which are homologous recombination proteins that bind ssDNA (OGAWA *et al.* 1993; SEITZ *et al.* 1998; LIU *et al.* 2010). If SAD-5 favors structured DNA, we could investigate its similarities to RAD54 or BRCA1. RAD54 or BRCA1 are homologous recombination proteins that prefer Holliday junctions and branched DNA, respectively (PAULL *et al.* 2001; BUGREEV *et al.* 2006).

If no favored DNA substrate is observed, this would potentially mean that SAD-5 does not directly interact with DNA. Perhaps, SAD-5 interacts with something else such as another protein like SAD-6 or even RNA. Preferences for these substrates may be more likely considering previously discovered HR proteins show no homology to SAD-5 (HAMMOND *et al.* 2013). Perhaps, SAD-5 could be a loading protein for SAD-6 or it could potentially be interacting with the uncharacterized aRNA.

The recently discovered SAD-6 protein interestingly also localizes to the nucleus (SAMARAJEEWA *et al.* 2014). It shows promise in being associated with the recognition phase of MSUD because it possesses a Rad54-like domain. Rad54 contributes to the

homology search during homologous recombination that occurs during double-strand break repair. Another member of our lab, Zach Smith, has been successful in purifying the SAD-6 protein using the ArcticExpress cell line and protocol outlined above

Unfortunately, the previously explained experiments were unsuccessful in purifying SAD-5. Nevertheless, with the success of the SAD-6 purification and multiple future alternative approaches it is just a matter of time before active SAD-5 is purified. Along with biochemical assays, our lab is also conducting genetic assays as well as exploring SAD-5's protein interactions with other MSUD proteins using the yeast-two hybrid system. With all this upcoming information the potential for results is exciting, and I look forward to future outcomes of mobility shifts and other biochemical assays potentially connecting the two nuclear MSUD proteins and adding further evidence to the link between homologous recombination and the detection process of MSUD.

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Table III-1: Oligonucleotide Primers

Primer Name	Sequence
sad-5 cDNA amplification from F2-01	
SAD-Q-230613F	TTTTTTCATATGAGTCCCAAAAAGGCCGAGCCTG
SAD-Q-230613R	AAAAAACTCGAGCTATGGAGACAGGTCTGACTGGTTC
VPS02 sequencing primers (pJET1.2)	
SO501-pJET1.2 forward sequencing primer	CGACTCACTATAGGGAGAGCGGC
SO511-pJET1.2 reverse sequencing primer	AAGAACATCGATTTTCCATGGCAG
p289.7 sequencing primers (pET15b)	
T7 promoter	TAATACGACTCACTATAGGG
T7 terminator	GCTAGTTATTGCTCAGCGG

sad-5 cDNA amplification from F2-01 primers were used for the initial amplification of the *sad-5* gene. VPS02 sequencing primers (pJET1.2) are forward and reverse primers of the pJET1.2 multiple cloning site. These primers were used during sequencing to verify *sad-5* in the pJET1.2 plasmid. p289.7 sequencing primers (pET15b) are forward and reverse primers of the pET15b multiple cloning site. These primers were used during sequencing to verify *sad-5* in the pET15b plasmid.

Table III-2: Buffer Recipes

A

Native Purification Buffers For Lemo21 Cells			
Rinse Buffer	**Wash Buffer**	**Wash II Buffer**	**Elution Buffer**
10mM HEPES	25mM HEPES	25mM HEPES	25mM HEPES
10 % Sucrose	200mM NaCl	200mM NaCl	0.5M NaCl
1x PSMF	10mM Imidazole	40mM Imidazole	300mM Imidazole
	5% Sucrose	5% Sucrose	5% Sucrose
	1x PMSF	1x PMSF	1x PMSF

C

Arctic Express Purification Buffer
20mM Tris-Cl, pH 7.5
100mM NaCl
1x PMSF

B

Denaturing Purification Buffers For Lemo21 Cells			
Denaturing Rinse/Wash Buffer	**Denaturing Wash II Buffer**	**Denaturing Elution Buffer**	**Dialysis Buffer**
8M Urea	8M Urea	25mM HEPES	8M Urea
100mM NaCl	100mM NaCl	0.5M NaCl	100mM NaCl
10mM Tris pH8	10mM Tris pH8	250mM Imidazole	10mM Tris pH8
	10mM Imidazole	5% Sucrose	1x PMSF
		1x PMSF	

D

SDS-PAGE Gel Solutions		
6x SDS Load Dye	**Coomassie Gel Stain**	**Gel Destain**
375mM Tris pH 6.9	45% H20	50% H20
60% Glycerol	45% EtOH	40% EtOH
Brilliant Blue Powder	10% Acetic Acid	10% Acetic Acid
SDS (1.2g/10mL)	Brilliant Blue Powder	
0.1M DTT		

(A) Recipes for all buffers used in the native purification using the Lemo21 cell line. (B) Recipes for all buffers used in the denatured purification protocol using the Lemo21 cell line. (C) Recipe for the base buffer used in the purification protocol for the ArcticExpress cell line. (D) Recipes for all solutions using in running and staining the GenScript PAGE gels.

Table III-3: Bradford Assay Results for SAD-5 Purification in ArcticExpress Cells

Sample	Sample (µL)	Bio-Rad (µL)	H$_2$O (µL)	Abs	Protein (µg)	Protein (µg/µL)	Protein (µL)	4x SDS dye (µL)	H$_2$0 (µL)	[Final] (µg/µL)
Pellet	1.5	200	798.5	0.325	6.44	4.293	23	25	52	1
Supernant	2	200	798	0.39	7.7955	3.898	26	25	49	1
Flow through	3	200	797	0.59	11.627	3.876	26	25	49	1
W1-10	5	200	795	0.12	2.3478	0.470	75	25	0	0.35217
W2-ATP	20	200	780	0.11	2.1709	0.109	75	25	0	0.08140875
W3-20	25	200	775	0.04	0.72896	0.029	75	25	0	0.0218688
E1	25	200	775	0.03	0.57011	0.023	75	25	0	0.0171033
E2	30	200	770	0.02	0.38128	0.013	75	25	0	0.009532
E3	30	200	770	0.10	1.9302	0.064	75	25	0	0.048255
E4	30	200	770	0.09	1.8289	0.061	75	25	0	0.0457225
E5	30	200	770	0.07	1.4581	0.049	75	25	0	0.0364525
E6	30	200	770	0.07	1.3263	0.044	75	25	0	0.0331575
E7	30	200	770	0.03	0.62418	0.021	75	25	0	0.0156045
E8	30	200	770	0.10	1.9558	0.065	75	25	0	0.048895
E9	30	200	770	0.04	0.70245	0.023	75	25	0	0.01756125

This table lists all raw values used and observed during the Bradford Assay of the SAD-5 purification in ArcticExpress cell. The first column lists all the names or abbreviations of all fractions used. The next three columns (column 2-4) list all volumes and reagents added to the make the total 1 ml Bradford Assay reaction. The Abs column represents the absorption observed when samples were subjected to a wavelength of 595 nm. The following column gives predicted amount of protein when compared to a BSA standard curve. This value was divided by the sample volume added to the reaction to get the total protein concentration (column 7). The next three columns indicate the volumes of protein sample, dye, and water to dilute to sample to 1 µg/µL for loading onto the polyacrylamide gel. All fractions listed after the flow through were not concentrated enough to dilute to the preferred 1 µg/µL. The highest volume of protein allowed for dilution was 75 µL (column 8). Therefore, all fractions after the flow through were very low in protein concentration.

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ATGAGTCCCAAAAAGGCCGAGCCTGACGCAGTGGCATGGCCGCTTTTCGGGCGGCTCCCCGCGGAAATACAAATCATGATCTTTCA
CGAGGCGCTCCGGAAGCCTCAGGTTCACTTCGTTAACGCGTCACGTCGTGAGGTTGACCCGGCCACATGGAAGCTGGTCCTGAAAT
CCAGGAACAAGAAAGGCGACACTTCTGGCTACCGCCTTATAGAAAATATCCAGGATGTTGCGCGCGATTCTCCTGTCGCGGCTGAA
GCGATCCTCAAAAGCGCCCTTCAGCCACTTCTTCTGCCTATTCCAGTCCGCCCGAACGACTGGTGCAAGATTGACGCCGCCACGGA
TCTCGTGGTCTTCGAATTCGGTGTCGACAAATCGGGCAAACTGTGGCTTTGGCACCCACGAAACCAACTCGTCGATTTGAACCCTC
TCGCCAGTCACATCCACTCCAGGCGGGTCCGTGAAGACCTGAAAGGGATCCGAAAGGTCGCTTTCGTTTACGGTGGGAACAAACAA
CCTCAGGCTGGGTCGAGCGAGAGTGTATTTCAGTGCTTGCAACATCACAATCGCGGACATGCGAGGCAAAAATTCTGCCCGGAGGA
GCTGCTGGGATTTATTTACCAGCTATCTGACATCGAAACGGTCTATTTCATTCTCCACGACAGAGTAAATGCAAAAGCCGTGACAA
ACTATGCGGCCAGCTACTTTTCTGGTAAGTTGCTCACCCCATGAATTCCCCCTCGGCCCACATGGACCGGCACGTGTTTCCTTCAC
CTTGCTTTTATCCCCGTTCGCCGGACGAGTATCTCTCGCCTAACCAACAGTTACAGTCCCTGCCGCTACACGAAACACATTTGGC
CTCAAGACTTTCTACTCCACAACAAGGAGCTACATTACTGTTCCCCTTCCTGAGTATCAGATAGGACCGGTACTCCACGCGACCTG
GAAGACAAAGGATCGCATCTGGCCACATGGCCCATCACCGCCACTTGTCAACGCAGCCCGTGAAGCGGCTATAGCCCGTCCCCCGG
ATTCCAGAGAGAGACTTTATGCGGAGCATGTGCGCAAACTATCAGATCTAGCTGACGCACTGCACCATTGTAAAGAGGTCTGGCGC
TTGGTTTTGGAGATGATTCAGCAAATACGAGCAGACCAAGACACCCCTCTGTCGCCGGACTGGCCCTTGCCAATCCCACCTGAAGT
CAACACCGCCCAGTTCAGGACTCTCAAACAGCATCACAGGCAGCGCTACAACCTTGAGAACTTCACCAAGGAAAGGAGAGATAATT
TGGAGTTCGGCATGTTGCTCATGGTGGATGAGACGGCTGTCTACAAGAGCGGGAAGAACCAGTCAGACCTGTCTCCATAG
```

Figure III-1: Opening Reading Frame of the *sad-5* Gene Including Introns
Green represents the start codon while red indicates the stop codon. The sequence highlighted in blue is considered exon sequences and dark gray is representing the only intron of *sad-5* as illustrated by Broad Institute's *Neurospora crassa* Database. Experiments suggest that the bold, underlined 6 base sequence at the beginning of the second exon is actually a part of the intron in the sequencing results.
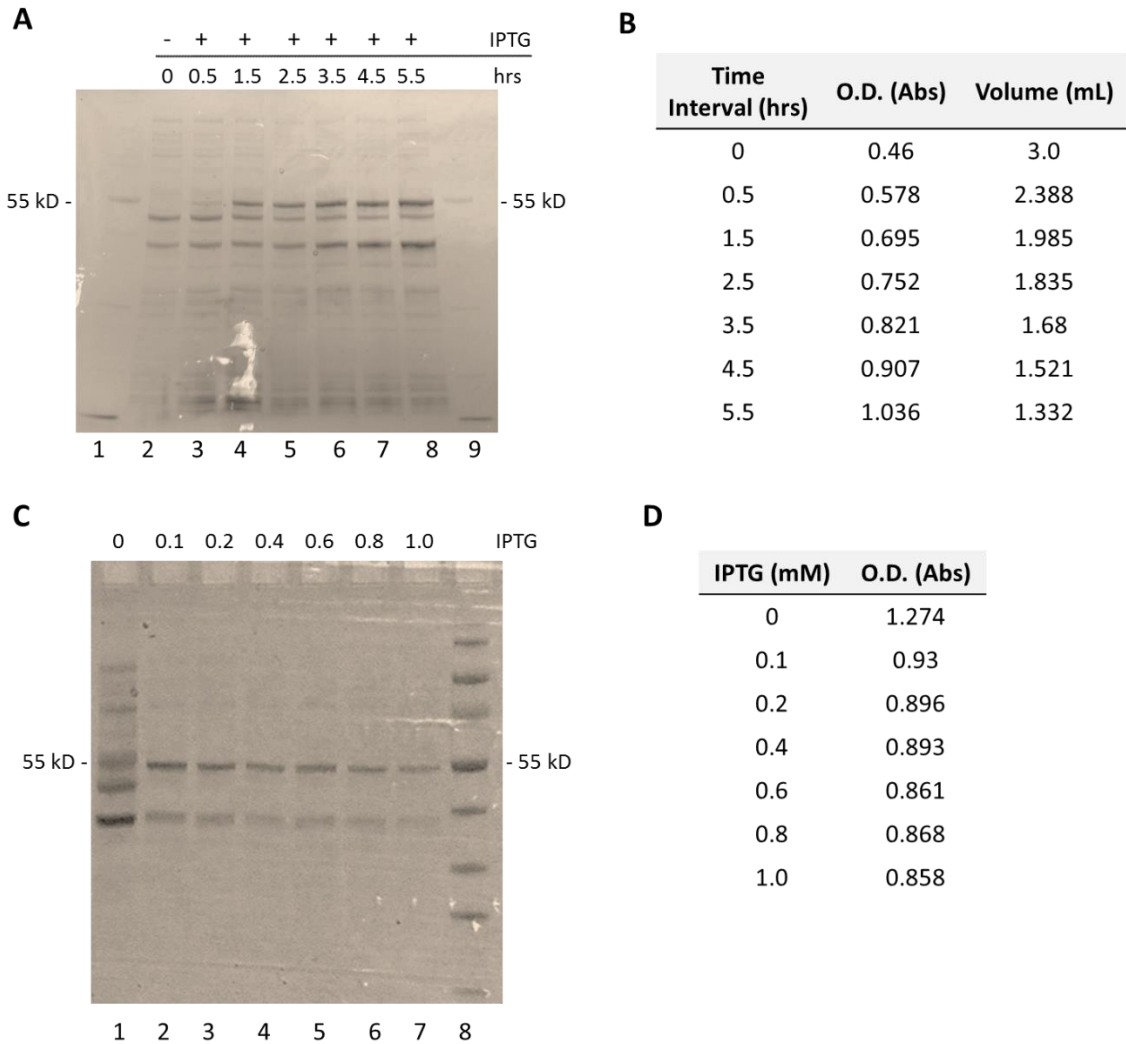
A

| | - | + | + | + | + | + | + | IPTG |
| | 0 | 0.5 | 1.5 | 2.5 | 3.5 | 4.5 | 5.5 | hrs |

55 kD - ... - 55 kD

Lanes: 1 2 3 4 5 6 7 8 9

B

Time Interval (hrs)	O.D. (Abs)	Volume (mL)
0	0.46	3.0
0.5	0.578	2.388
1.5	0.695	1.985
2.5	0.752	1.835
3.5	0.821	1.68
4.5	0.907	1.521
5.5	1.036	1.332

C

| 0 | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | IPTG |

55 kD - ... - 55 kD

Lanes: 1 2 3 4 5 6 7 8

D

IPTG (mM)	O.D. (Abs)
0	1.274
0.1	0.93
0.2	0.896
0.4	0.893
0.6	0.861
0.8	0.868
1.0	0.858

Figure III-2: Optimal Expression of SAD-5 Protein in Lemo21 Cells
(A) SDS-PAGE gel of increasing time after IPTG inoculation for SAD-5 expression. Lanes 1 and 9 contain GeneRuler Plus Unstained Protein Ladder. The SAD-5 fusion protein is estimated to be approximately 50 kD in size. Lanes 3-8 contain samples of culture with IPTG added. Amount of hours at when the sample was retrieved are represented above each lane in the row labeled "hrs" (B) Table of O.D.s and volumes of each time interval sample retrieved. Volumes were calculated based on culture O.D. to keep cell density consistent among samples. (C) SDS-PAGE gel of increasing amounts of IPTG concentrations added to each culture. Lane 8 represents the GeneRuler Plus Unstained Protein Ladder. (D) Table of IPTG concentrations for each sample and O.D.s. Illustrates approximate consistency of cell density between cultures.
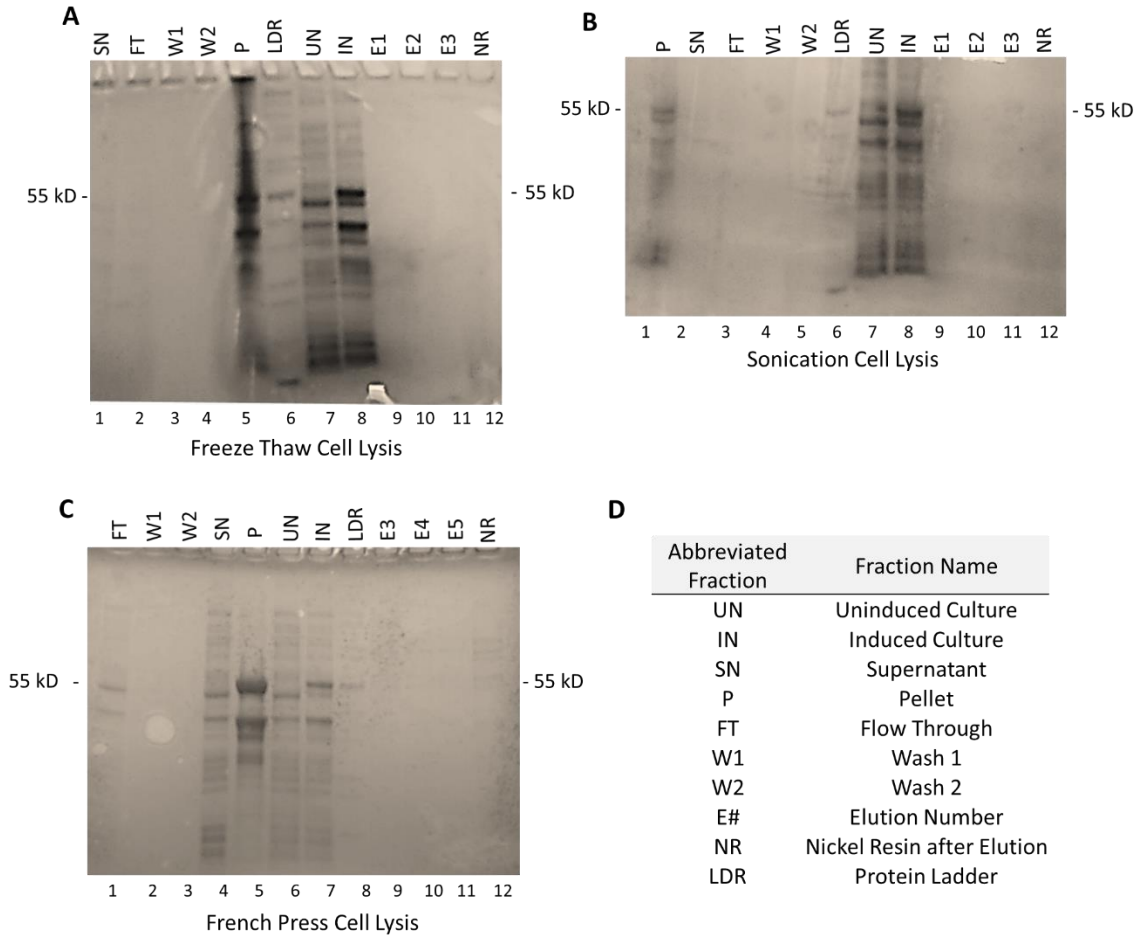
Figure III-3: Cell Lysis Approaches
(A) SDS-PAGE gel of fractions taken during a SAD-5 purification using the freeze thaw method to lyse cells. (B) SDS-PAGE gel of fractions taken during a SAD-5 purification using sonication to lysis cells. (C) SDS-PAGE gel of fractions taken during a SAD-5 purification using the French Press method to lyse cells. "55 kD" represents were on the gel the ladder indicates that particular size. SAD-5 protein is expected to be ~50 kD in size. (D) Table of abbreviated fractions and their full fraction names.
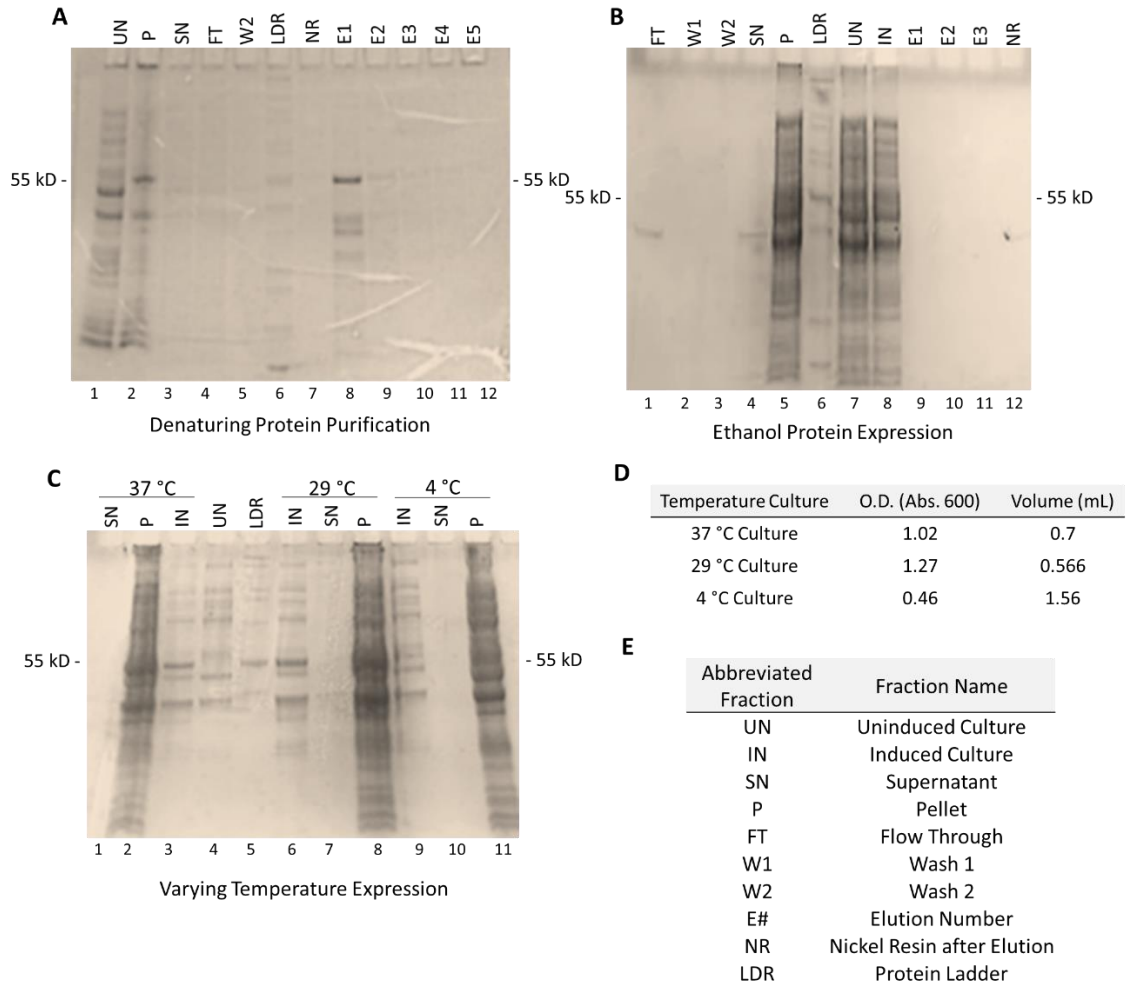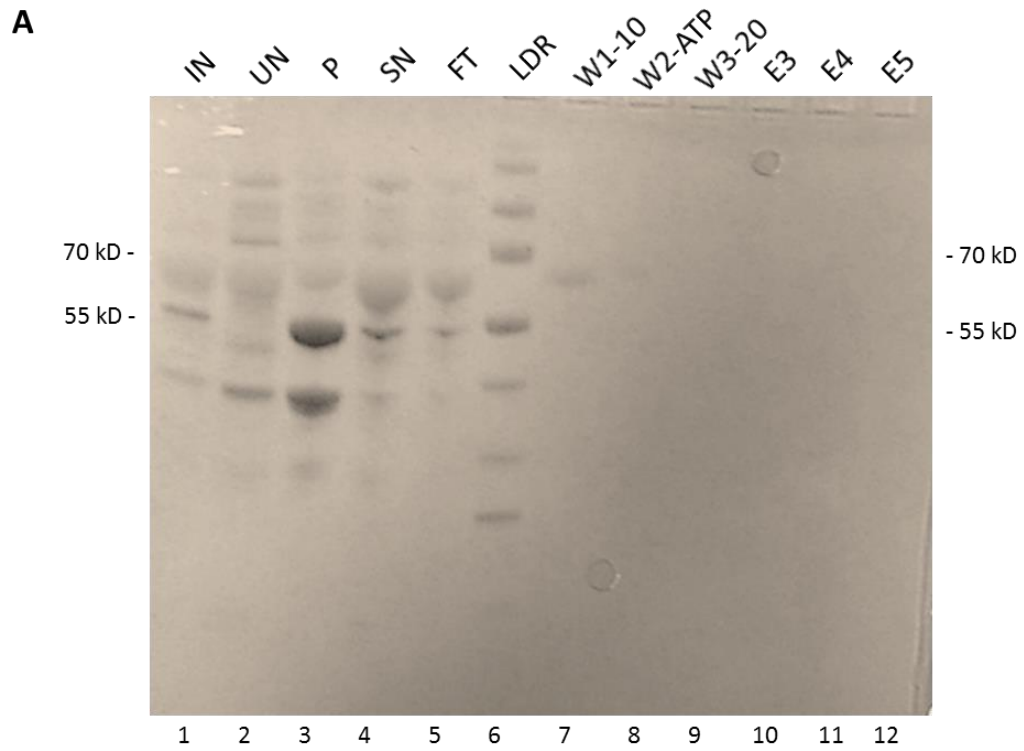
Figure III-4: Efforts to Solubilize the SAD-5 Protein in the Lemo21 Cell Line
(A) SDS-PAGE gel of SAD-5 purification fractions under denaturing purification conditions. (B) SDS-PAGE gel of SAD-5 expression under addition of ethanol. (C) SDS-PAGE gel of fractions cultures of varying temperatures and their effects on SAD-5 expression. (D) Table of temperature cultures and their O.D. readings at the time of sample retrieval. Also included is volume, which is calculated for that culture's sample for gel analysis. This calculation keeps the cell densities consistent.

A
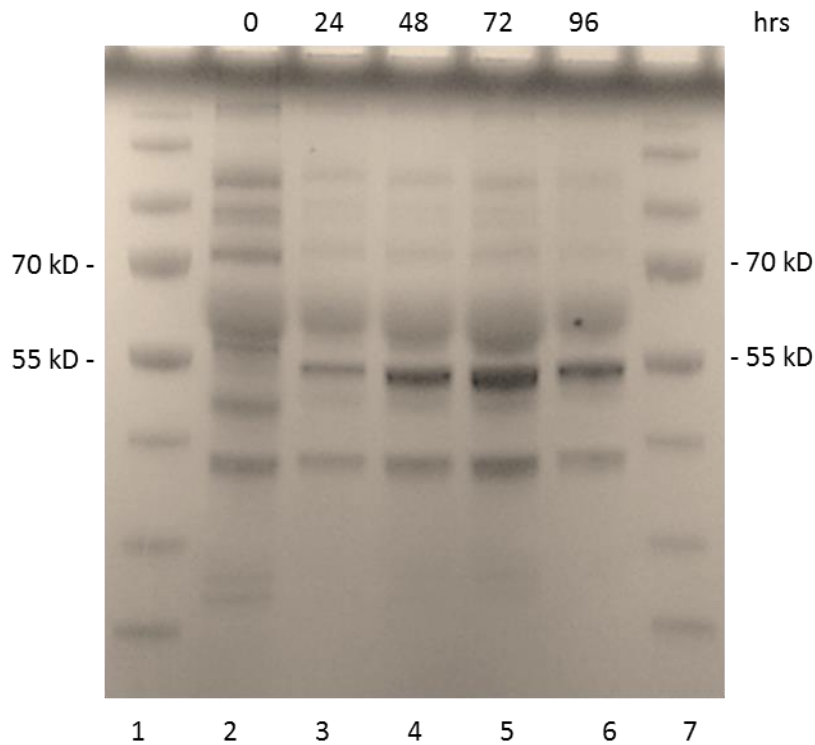
B

Time Interval (hours)	0	24	48	72	96
O.D. (600 nm)	1.227	1.496	1.535	1.592	1.373
Volume (mL)	2	1.64	1.598	1.541	1.787

Figure III-5: Optimal Time for SAD-5 Expression After Induction in ArcticExpress Cells
(A) Lanes 1 and 7 represent the protein ladder standard and on each side of the gel the sizes for 70 and 50 kD are denoted. The cell lines expressed chaperonin proteins that are approximately 57 kD. These are likely the large products that run in between the 55 kD and 70 kD marks. Numbers above the lanes represent at which hour of expression the sample was taken. (B) Table of O.D.s taken at each time point of expression as well as the volume that was pelleted for gel analysis. Allowed sample consistency of cell densities.

A

	IN	UN	P	SN	FT	LDR	W1-10	W2-ATP	W3-20	E3	E4	E5

70 kD -

55 kD -

- 70 kD

- 55 kD

1   2   3   4   5   6   7   8   9   10   11   12

B

Abbreviated Fraction	Fraction Name
UN	Uninduced Culture
IN	Induced Culture
SN	Supernatant
P	Pellet
FT	Flow Through
W1-10	Wash 1 - 10mM Imidazole Wash
W2-ATP	Wash 2 - 5mM ATP, 5mM Mg^{2+} 10mM Imidazole Wash
W3-20	Wash 3 - 20mM Imidazole Wash
E#	Elution Number
LDR	Protein Ladder

Figure III-6: Purification of SAD-5 in ArcticExpress Cells
(A) SDS-PAGE gel of SAD-5 purification in the ArcticExpress cell line. (B) Table of fraction abbreviations and full fraction name for reference when interpreting panel A.