Identification of Sfi1 protein interactions through yeast two-hybrid analysis

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Abstract

The most notable eukaryotic microtubule organizing center, the centrosome, forms the mitotic spindle required for mitosis and meiosis[3]. Problems with centrosomal duplication and function can lead to chromosomal missegregation, which has been linked to numerous human diseases and cancer[3]. Though the structure has been closely studied in different organisms, the mechanism by which it duplicates is still not well understood[5]. In the budding yeast *Saccharomyces cerevisiae* the spindle pole body is analogous to the human centrosome, and has numerous highly conserved components, making it an excellent model to study to better understand centrosome duplication and function[5]. This study aimed to identify protein interactions of Sfi1, one essential and highly conserved SPB component, in order to characterize its role in SPB duplication. Through a yeast two-hybrid screen, the C terminus of Sfi1 was found to interact with RNA II polymerase mediator complex protein Cse2. This interaction provides more evidence for Cse2 being involved at the spindle, and further analysis of the interaction will elucidate the role of Sfi1 in SPB duplication.

Introduction

Microtubule organizing centers (MTOCs) are structures in eukaryotes from which the minus ends of microtubules are anchored and nucleate[1]. These structures function to organize cilia and flagella and also to create the mitotic spindle for segregation of the chromosomes during cell division. There are various forms of MTOCs that range from basal bodies, which form cilia and flagella used for cellular motility and signaling, to the more notable eukaryotic centrosomes, which form the mitotic spindle[1]. Cells can have numerous MTOCs floating in the cytoplasm or embedded in the nuclear or plasma membrane. They may also have just one MTOC, as is the case in the budding yeast *Saccharomyces cerevisiae*[1]. Though the structure of MTOCs is varied among organisms, many of the components and regulators are highly conserved[5].

The main animal MTOC, the centrosome, is involved specifically in chromosome segregation and is duplicated once per cell cycle in a highly regulated process to ensure formation of a bipolar spindle and progression through the cell cycle[2]. Despite the critical role centrosomes play in many eukaryotic cells, centrosomes are not essential for cell division in some cell types that are able to go through the cell cycle in centrosome-independent pathways as well[3].

Studying the function of centrosomes has been an ongoing area of research due to the severe impacts that have been observed in cells from alterations to the centrosome. Eukaryotes that go through embryogenesis are particularly reliant on proper function of their centrosomes. One example of this comes from mimicking fertilization in *Xenopus leavis* and allowing the embryo to attempt to divide without being provided the centrosome from the sperm, the egg fails to cleave after cell division and ceases to develop[3]. However, lacking centrosomes during embryogenesis is not the only concern. In embryos implanted with extra centrosomes, multipolar rather than bipolar spindles are observed and chromosomes are segregated between them, leading to genomic instability and cellular defects[3]. The idea from these and many other studies is that the centrosome allows formation of the mitotic spindle and ensures appropriate separation of genetic material into the daughter cells, and defects with this process can lead to severe phenotypes.

Faulty duplication and function of the centrosome has been implicated in many human diseases, including cancer. Genetic instability, which can be caused by missegregation of the

chromosomes by the centrosomes, can lead to aneuploidy[3] It can manifest as extra copies of chromosomes, chromosomal translocations, deletions, or breaks in the DNA[3]. Genetic instability is also one of the hallmarks of cancer cells that contribute to their erratic behavior, and can occur prior to or after cellular transformation[4]. Cancer cells have frequently been observed to have extra centrosomes. Extra centrosomes might cause cells to go through a brief multipolar spindle phase before completing division into two daughter cells, which disrupts the normally even pull of chromosomes into the daughter cells and leads to chromosomal missegregation[4]. Understanding the mechanisms by which these structures duplicate and function is critical to better understanding cancer and other diseases caused by centrosomal defects and how to treat them.

The only MTOC in *S. cerevisiae* is called the spindle pole body (SPB), and it nucleates cytoplasmic microtubules that orient the mitotic spindle during cell division and spindle microtubules that separate the chromosomes[17]. Animal centrosomes also nucleate both cytoplasmic and spindle microtubules[3]. The cytoplasmic microtubules from centrosomes orient the spindle during mitosis, and in animal cells there are other sources of cytoplasmic microtubules for purposes unrelated to the spindle as well[3]. Variability of MTOCs can be seen when comparing the structure of the SPB to that of the human centrosome. The human centrosome is made of a pair of centrioles, which are complex microtubule structures, surrounded by a matrix of proteins. It does not associate with the nuclear membrane until mitosis, then after nuclear envelope breakdown the centrosomes may form the mitotic spindle[1]. Rather than being made of the barrel shaped centrioles, the SPB is a tri-laminar structure embedded in the nuclear envelope throughout the entire cell cycle. The structure of the SPB can be seen in Fig. 1. It is composed of an outer plaque, on the cytoplasmic side of the nuclear envelope, a central plaque that lies in conjunction with the nuclear envelope, an inner plaque that faces the nucleoplasm, and a specialized region of the nuclear envelope called the half-bridge sits on one side of the central plaque[5]. The SPB is an excellent model to study centrosome duplication and function because all of the components of this simpler centrosome have been identified, and it shares many of the same components and regulators as the human centrosome[11]. Sixteen of the eighteen core SPB components are essential genes[11]. The fact that so many of the SPB genes are essential makes it a difficult but interesting centrosome to study, as many unique phenotypes can be observed.

A model for SPB duplication (Fig. 2) has been developed based on observational electron microscopy data of wild type SPBs and SPBs with mutated components. Duplication begins during early G1 phase of the cell cycle with the elongation of the half-bridge [6]. The half-bridge elongates from about 90nm to twice it's original size, 180nm[6]. After the half-bridge has elongated, a satellite of four core SPB components, Spc42, Spc29, Cnm67, and Nud1, is then deposited on the cytoplasmic side of the half-bridge, distal to the mother SPB[5]. Next the satellite expands to a layered structure that resembles the central plaque of the mother SPB[5]. As it expands, the duplication plaque is inserted into the nuclear envelope[5]. Finally the nuclear components and gamma tubulin complexes, which are anchored to both the inner and outer plaque and serve as the site for microtubules to bind and nucleate, are assembled to create an identical daughter SPB[5]. The duplicated SPBs sit side by side at the end of G1/beginning of S phase, but they must separate and migrate to opposite ends of the nucleus to form the mitotic spindle.

Sfi1 is an essential component of the SPB that localizes to the half-bridge, and is highly conserved among organsims[6]. It binds the yeast centrin, Cdc31, another SPB component that localizes to the half-bridge, along DNA sequence repeats in the central, alpha-helical domain of the protein[6]. Sfi1 has 21 of these repeats and binds one molecule of Cdc31 per repeat[6]. Kilmartin and colleagues (2006) found unique localization of the termini of Sfi1 by immuno-electron microscopy, with the amino (N) terminus next to the mother SPB and the carboxy (C) terminus at the end of the half bridge before duplication and in the center of the full-length bridge. Kilmartin (2006) constructed an Sfi1 molecule with 15 of the centrin binding repeats and found it to be a 60 nm filament. This suggests that one 90nm filament of Sfi1 may span the half-bridge with it's C terminus at the distal end, and then half-bridge elongation is due to the addition of another molecule of Sfi1 with both C termini together and the N terminus free for the satellite to be deposited at the site of new SPB assembly[6]. It is thought that the free N terminus of Sfi1 may play a role in recruiting the satellite components to the distal end of the half-bridge[2].

There is no published data specifically on the N terminus of Sfi1 and limited data on the other domains of the protein. Mutants in the repeat domain of Sfi1 arrest prior to duplication of the SPBs, indicating the protein is required for duplication[5]. Additionally, four alleles of Sfi1 with different point mutations in the C terminus caused arrest of the cell cycle in mitosis[7]. Electron microscopy found a novel phenotype in which the cells had fully duplicated side-by-

side SPBs[7]. Another paper found through a synthetic lethal screen that *SFI1* and *BIK1* genetically interact[17]. Bik1 is a homolog of the mammalian CLIP-170 and is a microtubule-associated protein that stabilizes microtubules during mitosis[17]. A mutant in the C terminus of Sfi1 in a Bik1 null background also showed the arrest in mitosis with duplicated side by side SPBs[17]. These findings with mutations in the repeats and C terminus of Sfi1 indicate that it may have an important role in initiating duplication of the SPBs and in splitting of the bridge after duplication of the SPBs as well.

Based on the data discussed above, it would be interesting to determine whether the termini of Sfi1 play distinct roles in SPB duplication and whether this would give a better understanding of the function of Sfi1. The yeast two-hybrid method is a powerful tool for identifying protein-protein interactions and has been used to identify interactions of many other SPB components already. The two-hybrid system uses the modularity of transcription factors to determine protein interactions[8] (Fig. 3). One protein is fused to the DNA binding domain of a transcription factor and the other is fused to the activation domain of the same transcription factor. If the proteins interact, the transcription factor is brought together, therefore activating transcription of a reporter gene that it regulates[9].

In addition to Sfi1 interactions identified with fellow half-bridge component Cdc31, many genetic interactions involving Sfi1 have been found in synthetic lethal screens[6]. Full-length Sfi1 was found to interact with another SPB component, Spc110, in a complex in an affinity capture experiment[6]. *SFI1* exists within a genetic network of interactions with the genes encoding SPB components *BBP1, NDC1, MPS2* and *KAR1* as determined by a screen for suppression of the synthetic lethality of a *sfi1-65, mad1* Δ double mutant, with the Sfi1 mutation in the C terminus[7]. Kar1 is also part of the specialized region of the nuclear envelope called the half-bridge, while Bbp1, Ndc1, and Mps2 are involved in inserting the newly duplicated SPB into the nuclear envelope[7]. The results from that screen suggest that mutations in the C terminus of Sfi1 do not result from defective interaction with Cdc31 because Cdc31 overexpression could not rescue the synthetic lethality, but possibly from disrupted interactions of proteins involved in the process of insertion at the nuclear envelope[7]. Genetic interactions of *SFI1* have been identified with motor associated protein *CIK1*, and kinetochore protein *BUB3*[12]. *SFI1* showed genetic interaction with genes encoding motor associated proteins Cin8 and Kar3, and Bub1, which forms a complex required for the spindle checkpoint[7,17]. The use of genetic screens has

also identified some protein interactions for Sfi1 that would likely not be anticipated otherwise and exemplify the complexity of protein interactions in the cell[12]. Though all of the data on interactions does give more evidence for the importance of Sfi1 to the SPB, more research is still needed to fully elucidate Sfi1s critical function.

Studying distinct domains of a protein separately is a useful way to understand how a protein functions, and that idea drives this study of Sfi1. The model for SPB duplication and localization data of Sfi1 described above suggest that the N terminus of Sfi1 may interact with core SPB components, specifically, the satellite proteins Spc29, Spc42, Cnm67 and Nud1, while the C terminus may interact with itself or other half-bridge proteins, such as Kar1 or Mps3. Based on that model, this study tested the N terminus for interaction with SPB satellite components Spc29 and Spc42, and the C terminus was tested for interaction with itself and screened against a library containing approximately all of the 6000 open reading frames (ORFs) in the S. cerevisiae genome[18]. The C terminus of Sfi1 was also tested for interaction with itself including the two Sfi1 repeats that directly precede the C terminus because those repeats bind Cdc31 and may play a critical role in the function of the protein.

The C terminus was not found to interact directly with itself or with itself when two Sfi1 repeats were included before the C terminal domain begins, indicating some other proteins may be involved in duplication and addition of additional molecules of Sfi1 at the half-bridge. The C terminus of Sfi1 was found to interact with RNA polymerase II mediator complex component Cse2 through a screen of the *S.cerevisiae* genome ORFs. A genetic interaction between SFI1 and CSE2 was confirmed previously, but no data on the C terminus of Sfi1 being sufficient for this interaction has been shown until now[12].

Results

Confirming expression of the BDSfi1C construct

Expression of the bait construct BDSfi1C (plasmids constructed are listed in Table 1) was confirmed through western blot analysis as described in the methods section. Western blot analysis showed the empty pOBD2 construct and BDSfi1 constructs were expressed at similar levels (Fig. 4).

Directed Two-hybrid

Prior to actual tests for interaction, the bait strains transformed with BDSfi1N, BDSfi1C and BDSfi1C2rep were tested for autoactivation. Only diploid strains with bait and prey constructs that interact should be able to grow on media lacking histidine because the interaction brings together the BD and AD pieces of GAL4 and activates transcription of the HIS3 reporter[16]. The autoactivation test tests the bait strains on media lacking tryptophan and histidine, and with increasing concentrations of the drug 3-amino 1,2,4-triazole (3-AT), which inhibits an enzyme required for hisitidine biosynthesis, in order to eliminate any autoactivaiton of the reporter to allow isolation of strong two-hybrid interactions. All BD constructs grew normally on media lacking tryptophan (Fig. 5). The negative control, the empty BD plasmid, also grew normally on media lacking tryptophan, and showed no growth on media lacking histidine. The BDSfi1N construct showed high levels of autoactivation that required 50mM concentrations of 3-AT to eliminate what? (Fig. 5A).

Sequence analysis of the N terminus of Sfi1 showed numerous regions with a net negative charge per 30 amino acids as well as many hydrophobic amino acids, which have both been shown to be important for minimal activating domains and cause autoactivation of the reporter gene[13]. From amino acids 147 to 185 there are nine negatively charged residues and only two positively charged residues, and there are also 17 hydrophobic residues in that region. From amino acids 148 to 178 there is a net negative charge of 6, and from amino acid 154 to 185 there is a net negative charge of 5. To determine if the highly acidic and hydrophobic amino acids at the end of the N terminus of Sfi1 were causing the high levels of autoactivation two truncations of the terminus were created at amino acids 154 and 175. The BDSfi1N175 truncation did not decrease autoactivation (Fig. 5A), and growth was still observed on media containing 50mM 3-AT. The BDSfi1N154 truncation did decrease autoactivation (Fig. 5A) from 50mM to 5mM 3-AT. The BDSfi1C and BDSfi1C2rep constructs showed no autoactivation of the reporter so the lowest concentration, 1.5mM, of 3-AT was used (Fig. 5B).

Directed two-hybrid tests with BDSfi1N as bait were performed on media lacking leucine, tryptophan and histidine, and with 50mM 3-AT, as determined by the autoactivation tests, and on media without leucine and tryptophan as a control. The following three negative controls were used: BDSfi1N: AD, BD: ADSpc29 and BD: ADSpc42, and BD: AD. All of the negative controls grew normally on diploid selective media (media without leucine and tryptophan; Fig. 6A). Of these negative controls, only BDSfi1N: AD showed a small amount of

growth on media lacking histidine with 50mM 3-AT. The BDSfi1N: ADSpc29 test did show a small amount of growth on the media lacking histidine with 50mM 3-AT, but it was consistently equal to or less than that seen from the BDSfi1N: AD negative control, indicating no interaction. There was no growth seen on media lacking histidine for the BDSfi1N: ADSpc42 diploids, indicating there is no interaction between these proteins (Fig. 6A). The expression of the BD and AD constructs was not confirmed via western blot analysis for any of the constructs used in this test.

The C terminus of Sfi1 was tested for interaction with itself, and the same negative controls were performed (BDSfi1C: AD, BD: ADSfi1C, and BD:AD). All combinations grew normally on diploid selective media (Fig. 6B). No growth was seen on media lacking histidine with 1.5mM 3-AT, as determined by the autoactivation test (Fig. 6B), indicating the C terminus of Sfi1 does not interact with itself . Expression of the BDSfi1C construct was confirmed with western blot analysis, however the ADSfi1C expression was not confirmed.

The BDSfi1C2rep construct was also tested for interaction with the ADSfi1C2rep construct to determine if the Sfi1 repeats may be required for interaction. No growth was seen on media lacking histidine with 1.5mM 3-AT, and normal growth was seen on diploid selective media (Fig. 6C). The same negative controls were performed and showed normal growth on diploid selective media but no growth on media lacking histidine also. Expression of these constructs was not confirmed by western blot.

Library Screen

In this study a yeast two-hybrid screen was performed with the BDSfi1C construct as the bait to find protein interactions specifically with this terminus. The screen was performed against an ORF pool containing approximately all of the ORFs of the *S. cerevisiae* genome fused in frame to the AD of GAL4. The large scale liquid mating was grown on SC media lacking leucine, tryptophan and histidine, with 1.5mM 3-AT and allowed to grow at 30°C. In this screen 4.84 million clones were screened and there was a mating efficiency of 5.37%.

The first two potential positive interaction colonies (called positive 17 and positive 45) were selected from the screen on day two, and the other two potential positives (positive 24 and positive 32) found in this study were selected on day 7 (Fig. 7A). Sequence analysis determined the ORF for positive 17 to be a fragment of GAL4, but not the BD or AD regions. The sequence analysis of positive 45 also matched a fragment of GAL4. Therefore, these were not true

positive interactions but false positives. The ORF fused to the GAL4 AD in positives 24 and 32 was Cse2.

The isolated plasmids from positives 24 and 32 were retransformed in to pJ694 mating type A and a directed two-hybrid with BDSfi1C was performed to confirm interaction. The same negative controls were performed in previous two hybrid tests (BDSfi1C:AD, BD:ADCse2, and BD:AD) and showed normal growth on the diploid selective media, and no growth on media lacking leucine, tryptophan, and histidine with 1.5mM 3-AT. The BDSfi1C and ADpositive24 and 32 diploids were able to grow on media lacking leucine, tryptophan and histidine with 1.5mM 3-AT, while no growth was observed from any negative controls, indicating an interaction (Fig. 7B).

Discussion

This study used the yeast-two hybrid method to identify an interaction of Cse2 with the C terminus of Sfi1. The N terminus of Sfi1 was also tested for interaction with SPB satellite components Spc29 and Spc42, and no interactions were found. The C terminus of Sfi1 was not found to interact with itself through directed two-hybrid, nor did the C terminus including the two Sfi1 repeats preceding the C terminal domain interact with itself. These preliminary results need to be further verified through the methods discussed below.

It has been known for some time that transcription activating domains do not frequently share much sequence homology, but share regions of many acidic and hydrophobic amino acids[13]. A net negative charge in a region of approximately 30 amino acids has been shown to be a minimal transcription activating domain[13]. The yeast two-hybrid system commands the modularity of the domains of a transcription factor in order to find proteins that interact enough to bring the DNA binding and transcription activating domains together and activate a reporter gene, without the domains physically touching. A problem with this system can be that a minimal activating domain within the sequence of a protein being studied through yeast two-hybrid is able to activate transcription without truly interacting with the prey protein. This was observed in this study with the N terminus of Sfi1, which contains many stretches of net negative charges in its sequence. Truncating the protein and removing a total of seven negatively charged residues decreased the autoactivation of the HIS3 reporter from requiring 50mM concentrations of 3-AT, to only 5mM concentrations (Fig. 5A). These new truncations could be tested in future

two-hybrid experiments, however it is not known if they would result in the same interactions *in vivo*.

The directed two-hybrid of the N terminus of Sfi1 with SPB satellite components Spc29 and Spc42 showed no interaction (Fig. 6). Growth was observed on media lacking histidine with 50mM 3-AT for BDSfi1N:AD, which is consistent with the high level of autoactivation of the reporter that the BDSfi1N construct showed in the autoactivation tests. The small amount of growth seen on media lacking histidine with 50mM 3-AT for the test of BDSfi1N with ADSpc29 was most likely a result of autoactivation because the growth observed was less than that seen from the negative control. Ultimately a repeat of the experiment with confirmation of expression of the constructs could give more concrete evidence that those proteins do not interact.

The C terminus of Sfi1 was not found to interact with itself in this two-hybrid analysis (Fig. 6B). When two Sfi1 repeats were included in the C terminal constructs, the HIS3 reporter gene was still not activated (Fig. 6C). This indicates that some other factors may be necessary for the C termini to interact with each other on the half-bridge. Potentially other half-bridge components Kar1 and Cdc31 are involved. There is also the possibility that the proteins do not directly interact at all, but are merely in close proximity. The results of this two-hybrid test need to be confirmed by verifying expression of the constructs. Once expression of the other constructs has been confirmed, the results of this yeast two-hybrid study provide evidence that the C terminus of Sfi1 or the C terminus of Sfi1 with two Sfi1 repeats do not interact with themselves, and other interactions or requirements for their interaction on the half-bridge of the SPB can be explored. A pull-down assay or variations of directed two-hybrid assays with other half-bridge components could be tested to find C terminal interactions. It would be interesting to test some of the C terminal Sfi1 mutant alleles previously identified that give the duplicated side-by-side SPB phenotype in two-hybrid. The phenotype of these mutant alleles may enhance some interactions and allow for detection of an interaction in this system, or give another interesting result.

The yeast two-hybrid screen of Sfi1 came up with four positive hits, and of those two were false positives and two could be true positives but more analysis is needed to confirm that they are not false positives(Fig. 7A). Positive 17 was found by sequence analysis to be a fragment of GAL4, and positive 45 matched a region of chromosome 16 with no annotated gene. The other two positives found were actually the ORF Cse2. The fact that interaction was found

with the same protein twice in the screen is good support that this is not a false positive. One survey of false positives from the two-hybrid system found the most common ones to be heat shock proteins, then ribosomal proteins, or mitochondrial proteins, and Cse2 fits none of these categories[22].

Cse2 is an essential part of the RNA polymerase II mediator complex, which is required for transcription of nearly all genes with class II gene promoters in yeast[23]. Though it is an unexpected interaction for Sfi1, there are some aspects of Cse2 that lead to the conclusion that this may be a true interaction. First, in a synthetic lethal screen for protein interactions in *S. cerevisiae*, *CSE2* and *SFI1* were found to have genetic interaction[12]. This interaction was found with the *sfi1-3* allele, which contains a mutation in the central domain that binds Cdc31[12]. Secondly, conditional mutants of Cse2 show abnormal chromosome segregation, a phenotype also observed as a result of mutations of SPB components Cin8 and Ndc1[24,27,28]. Cse2 null cells show delayed progression through mitosis and chromosomal abnormalities as well[24]. Chromosomal nondisjunction was also observed when mutants in Cse2 were combined with mutants in proteins at the centromere[24]. This indicates that Cse2 has a role in chromosome segregation, and means it would possible for Cse2 to be at the spindle interacting with SPB components at some point during the cell cycle . Lastly, *CSE2* has also been found to interact genetically with both *BIM1* and *CIK1* through synthetic genetic arrays[12,25]. Bim1 is a microtubule binding protein that helps capture cortical microtubules at the cortex of the bud and prevent progression through mitosis in the event of abnormal spindle orientation[26]. Cik1 targets Kar3, a microtubule motor that localizes to the SPB, to the microtubules and aids in proper orientation of the mitotic spindle, and again these interactions provide support for Cse2 being at the spindle[11]. The fact that these genetic interactions have been found and that mutants in Cse2 show spindle related defects indicates that the two-hybrid interaction identified in this study should be studied further.

This, taken together with the defects in chromosomal segregation of Cse2 mutants, led to the hypothesis that Cse2 could either be involved in interactions between chromosomes and microtubules, such as at the kinetochore, or that Cse2 could be important for expression of proteins involved in chromosomal segregation[24]. Sfi1 does not localize to kinetochores or microtubules, but its role in SPB duplication and separation at the half-bridge has been shown[6,7]. The genetic interaction of *CIK1* with both *CSE2* and *SFI1* provides an interesting link at the

SPB. It has been suggested that Cik1 may play a role with Kar3 in separation of the SPBs, and is conceivable, because of the genetic interactions observed, that Cse2 could be actively involved in SPB separation as well[17]. It is also possible that Cse2 may exist both at the SPB during separation and at the kinetochore interacting with chromosomes or microtubules during segregation. This would give some explanation of why Sfi1 would interact with Cse, however, the mechanisms by which the SPB functions are still not fully clear.

Despite the support for Cse2 truly interacting with Sfi1, there is still the possibility that it could be a false positive and this needs to be ruled out through further testing. Analysis of Cse2 shows it has a potential leucine zipper domain, which is a common structural motif in proteins that is usually found in DNA binding domains of transcription factors. It is possible that the bZIP domain could bring the AD Cse2 fusion to the DNA and close enough to the BDSfi1C construct to allow activation of transcription inappropriately, therefore making the interaction a false positive.

The interaction of the C terminus of Sfi1 and Cse2 needs confirmation on media lacking leucine, tryptophan and adenine, with adenine as the second reporter that GAL4 can activate. To further confirm these interactions the bait and prey can be swapped, making BDCse2 and ADSfi1C, and again tested for direct two-hybrid interactions. It would also be interesting to test BDSfi1N or full length Sfi1 for direct two-hybrid interaction with Cse2. To rule out random two-hybrid interaction with any SPB components, numerous different components could be tested directly with Cse2. Finally, the *in vivo* test of protein interaction, co-immunoprecipitation, could be performed. After more evidence is gathered that supports this interaction, then further analysis can be done to understand why Cse2 would interact with a SPB half-bridge component. It would be interesting to study the phenotype of a double mutant of the C terminus of Sfi1 and Cse2 as that may give insight into the interaction. Further analysis can also be done to understand what may control these interactions, such as phosphorylation.

Sfi1 is a highly conserved protein involved in centrosome duplication and function, which are imperative cellular processes that need to be better understood. This study identified one novel interaction between the C terminus of Sfi1 and mediator complex protein Cse2. This interaction provides another interesting link to Cse2 and the SPB, and further analysis of it can potentially advance the knowledge of Sfi1s role in SPB duplication.

Materials and Methods

Yeast strains and plasmids

The plasmids used in this study were pOBD2 (BD), which contains genes encoding ampicillin resistance, tryptophan and the DNA binding domain of GAL4 (amino acids 1-147 of GAL4), and pOAD (AD), which contains genes encoding ampicillin resistance, leucine, and the activating domain of GAL4 (amino acids 768-881 of GAL4)[18]. These are centromeric plasmids[18]. The bait and prey genes for this study were cloned in translational frame to the C terminus of the GAL4 region of the appropriate plasmid by generating EcoRI restriction sites at the 5' ends and Sal1 sites at the 3' ends using Polymerase Chain Reaction (PCR) with primers containing the restriction sites[15]. The N terminus of Sfi1 included amino acids 1-185 of the full length protein, the C terminus included amino acids 802-946, and the C terminus plus two Sfi1 repeats included amino acids 735-946, and these were all cloned into the BD plasmid. Full length Spc29, Spc42, the C terminus of Sfi1, and the C terminus of Sfi1 plus two repeats were cloned into the AD plasmid. Constructs were confirmed using sequencing. Table 1 shows the constructs made for this study. All cloned genes were from the w303 sequence.

The *Saccharomyces cerevisiae* strain used for yeast two-hybrid experiments was pJ694, trp1-901 leu2-3,112 ura3-52 his3-200 gal4 Δ gal80 Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ[18]. The binding domain plasmids were transformed into mating type alpha and grown in synthetic complete media (SC) lacking tryptophan (TRP in figures), and the activating domain plasmids were transformed into mating type A and grown in SC media lacking leucine (LEU in figures) in order to select for the plasmids as described previously[19]. Strains containing each plasmid designated in Table 1 will be referred to by their plasmid names. The yeast transformation protocol used was the lithium acetate high efficiency transformation method[19]. The mating type A strain was also transformed with an AD *S. cerevisiae* genome ORF library used for two-hybrid screening[19].

Confirming expression of the BDSfi1C construct

Western blot analysis was used to determine expression of the BDSfi1C construct. Protein samples were collected as previously described by using the rapid protein preparation method[20]. Samples were resolved on a 12% gel by SDS-polyacrylamide gel electrophoresis and

transferred to a PVDF membrane for analysis. The antibody to detect expression of the GAL4 DNA binding domain fusion proteins was a mouse monoclonal IgG_{2a} from Santa Cruz Biotechnology (RK5C1) at a concentration of 1µg/mL 5% skim milk emulsion in TBS plus .25% Tween, and the membrane was incubated in the primary antibody overnight at 4°C. The secondary antibody used was a goat anti-mouse IgG antibody (Pierce), and Thermo Scientific Supersignal West Femto Chemilluminescent substrate was used for detection.

Directed two-hybrid

Directed two-hybrid tests were performed as previously described, employing the HIS3 reporter as a primary test for interaction[9,16]. The yeast strain used for this study has three possible reporter genes fused to the GAL promoter, HIS3, ADE2, and lacZ. For this study only the HIS3 reporter was used. HIS3, which codes for the enzyme imidazoleglycerol-phosphate dehydratase, involved in step 6 of histidine biosynthesis, was the primary reporter used[16]. The drug 3-amino1,2,4-triazole (3-AT, purchased from Sigma-Aldrich, catalog number 61-82-5) competitively inhibits that enzyme, therefore limiting histidine biosynthesis and growth[16]. Using predetermined concentrations of 3-AT in the growth media can select for strong yeast two-hybrid interactions that are able to overcome the inhibitory effects of the drug. To determine the concentration of 3-AT to use for each bait construct, an autoactivation test was performed[16]. The bait strain was tested for growth at 30°C for up to 7 days on media lacking tryptophan and histidine with increasing concentrations of 3-AT. The concentration that best eliminated autoactivation of the HIS3 reporter and growth on the media was used in all two-hybrid assays with that bait.

The media used for the two-hybrid tests lacked leucine and tryptophan to select for both the bait and prey plasmids, and histidine (HIS in figures) to test for interaction and 3-AT. The media was also supplemented with additional adenine, 80mg/mL, for optimal growth in tests employing the HIS3 reporter gene. Plating for single colonies and replica plating techniques were used. Directed two-hybrid assays went for up to 7 days at 30°C, with observation for interaction beginning at day 2. The directed two-hybrid tests were as follows: BDSfi1N for interaction with ADSpc29 and ADSpc42, BDSfi1C for interaction with ADSfi1C, and BDSfi1C2rep for interaction with ADSfi1C2rep. All directed two hybrid tests, including negative controls, were performed by mating an alpha mating type of the pJ694 strain that was

transformed with a BD plasmid, to an A mating type strain that was transformed with an AD plasmid. BD:AD indicates the resultant diploids. All directed two-hybrid tests were done in duplicate, so that multiple transformants of each plasmid strain were tested. Primarily, one representative example of each test is shown in this paper.

Library Screening

A screen of the BDSfi1C construct was performed against an AD ORF pool containing approximately all of the ~6000 ORFs in the genome created by PCR from the Fields lab[18]. The screen was performed as outlined in the *BD Biosciences Clontech Matchmaker Pretransformed Libraries User Manual*[14], but with the above mentioned plasmids and media. Large scale liquid mating was used, and positive single colonies that grew at 30°C on media with 3-AT lacking leucine, tryptophan and histidine were selected up to 14 days after plating, beginning on day 2. The positive diploids were streaked onto a master plate lacking leucine, tryptophan and histidine with 1.5mM 3-AT and streaked for single colonies to confirm expression of the HIS3 reporter. The plasmids from each potential positive were isolated from yeast and restriction digests were performed to determine if the plasmids were BD or AD[21]. All unique AD plasmids were sent for sequencing to determine what ORF gave the positive interaction. AD plasmids giving interaction were isolated as described previously and retransformed into pJ694 to confirm interaction through the directed two-hybrid approach[21].

Acknowledgements

I would like to thank Dr. Mark Winey for his invaluable mentorship for the last two years, Jennifer Avena for her patience in teaching me far more than protocols, as well as the rest of the Winey lab for their support, insight and assistance. I would like to thank the Fields lab for the ORF library and reagents, the Odorizzi lab for the equipment and plasmids, the Espinosa lab for the equipment, and the Yi lab for the strains. I would like to thank Lynn Wolfe and the Bioscience Undergraduate Skills and Training Program and May Penuela and the Undergraduate Research Opportunities Program for funding this project.

Figures

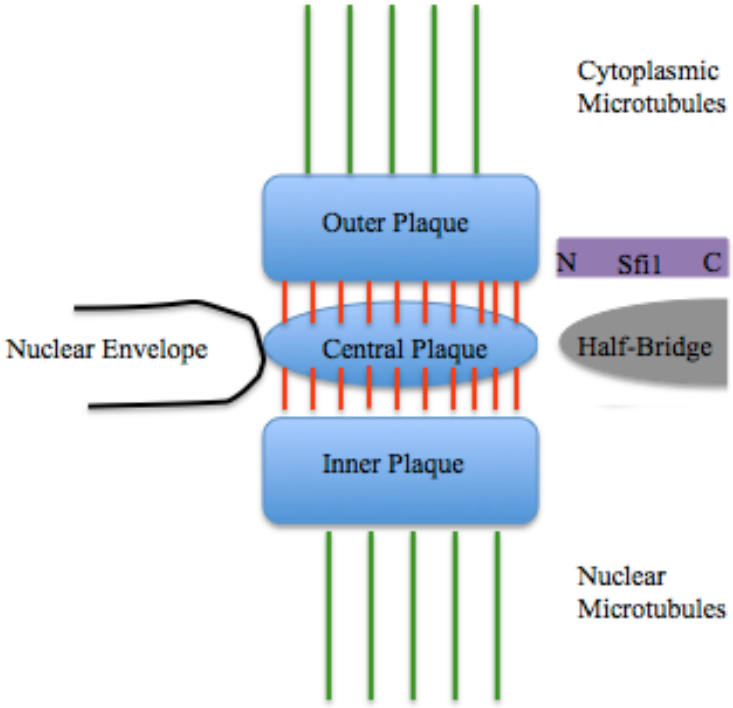


Figure 1. The Spindle Pole Body. The SPB is composed of a central plaque embedded in the nuclear envelope. There is an outer plaque on the cytoplasmic face, and an inner plaque on the nuclear side that nucleate cytoplasmic and nuclear microtubules, respectively, from gamma tubulin complexes anchored to the plaques. A dense structure called the half-bridge emanates off one side of the central plaque and elongates during SPB duplication. The SPB component Sfi1 localizes to the half-bridge structure with its N terminus next to the SPB and the C terminus at the distal end of the cytoplasmic face of the half-bridge.

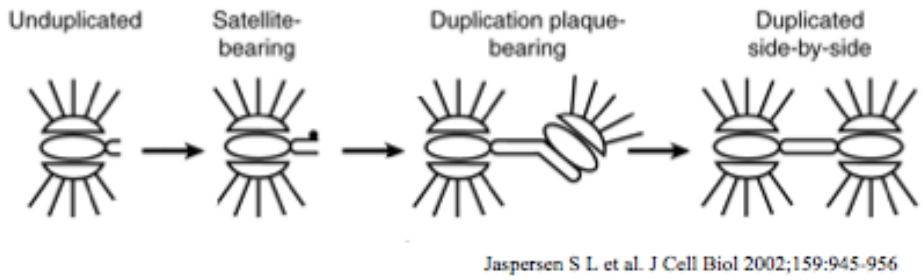


Figure 2. SPB Duplication. Duplication of the SPB begins in G1 of the cell cycle with the mother SPB. The half-bridge of the SPB elongates to twice its original size and then a satellite of the 4 core SPB components (SPC29, SPC42, Cnm67, and Nud1) is deposited on the distal cytoplasmic tip of the bridge. The satellite then expands to a duplication plaque. Finally the duplication plaque is inserted into the nuclear envelope and assembly of the nuclear SPB components and gamma tubulin complexes occurs.

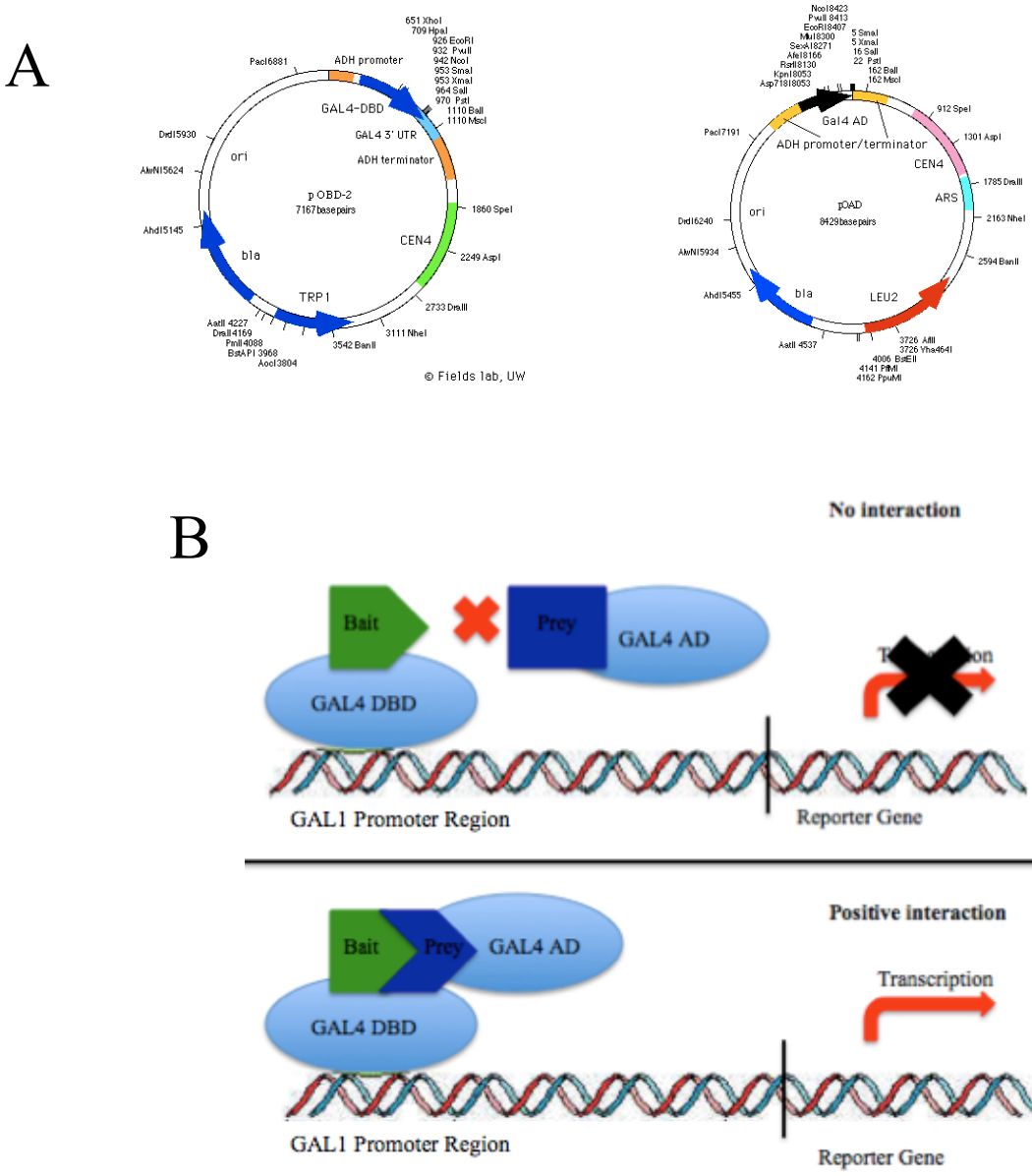


Figure 3. The yeast two-hybrid system. Part A) The plasmids pOBD2 (BD) and pOAD (AD) are used for yeast two-hybrid[18]. The BD plasmid contains the DNA binding domain of GAL4 under the expression of the ADH1 promoter and the selectable marker TRP1, and the AD plasmid contains the transcription activating domain of GAL4 under the expression of the ADH1 promoter and the selectable marker LEU2. The bait and prey genes are fused in frame to the BD and AD domains of these plasmids via general cloning procedures. The plasmids are transformed into separate mating types of a yeast strain containing the GAL1 promoter fused to a reporter gene, HIS3. Part B.) If the proteins interact in a diploid strain containing both plasmids as shown, then the binding domain and activating domain of the GAL4 transcription factor activate transcription of the reporter gene and the cells are able to grow on selective media lacking the selectable markers and the reporter gene.

Plasmid	Insert, Amino Acids used	Name of plasmid
pOBD2 (BD), Selectable marker TRP1	Sfi1 N terminus, 1-185	BDSfi1N
	Sfi1 N terminus, 1-154	BDSfi1N154
	Sfi1 N terminus, 1-175	BDSfi1N175
	Sfi1 C terminus, 801-946	BDSfi1C
	Sfi1C terminus plus two repeat domains, 735-946	BDSfi1C2rep
pOAD (AD), Selectable marker LEU2	Spc29, entire gene	ADSpc29
	Spc42, entire gene	ADSpc42
	Sfi1 C terminus, 801-946	ADSfi1C
	Sfi1 C terminus plus two repeat domains, 735-946	ADSfi1C2rep

Table 1. Constructs generated for two-hybrid experiments. Genes were inserted into the appropriate plasmids by generating restriction sites at the 5' and 3' ends of the gene through polymerase chain reaction. All constructs were verified by DNA sequencing.

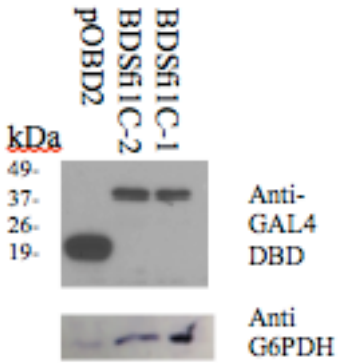
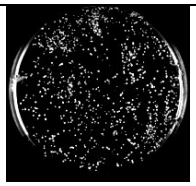
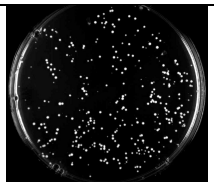
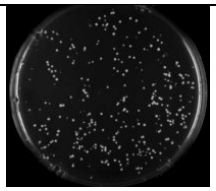
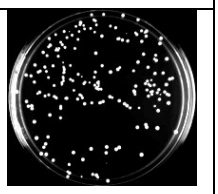
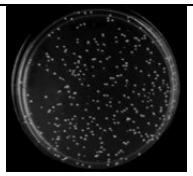
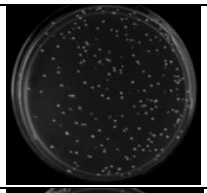
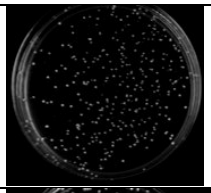
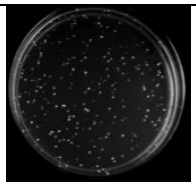
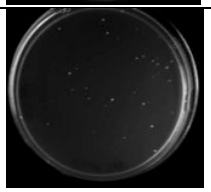
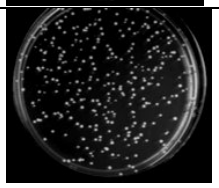
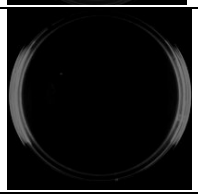
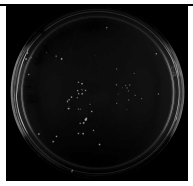
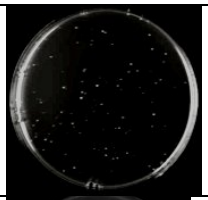
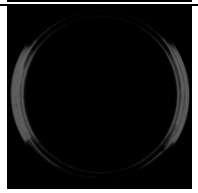
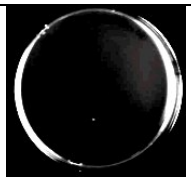
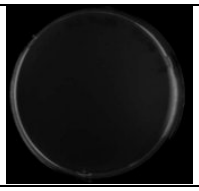
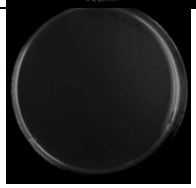
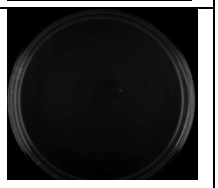


Figure 4. Confirmed expression of BDSfi1C in pJ694. Rapid protein preparations of strains transformed with pOBD2 and BDSfi1C (-1 and 2 are separate transformants) were loaded onto a 10% SDS-PAGE gel. Proteins were detected using anti-GAL4 DBD (Santa Cruz Biotechnology) and anti-mouse HRP conjugated secondary antibody (Pierce). Blots were reblotted with a mouse anti-glucose-6-phosphate dehydrogenase primary loading control.

A	BDSfi1N	BDSfi1N154	BDSfi1N175	pOBD2
SC-TRP Control				
SC-TRP-HIS 0mM 3-AT				
SC-TRP-HIS +5mM 3-AT				
SC-TRP-HIS +50mM 3-AT				
SC-TRP-HIS +100mM 3-AT				

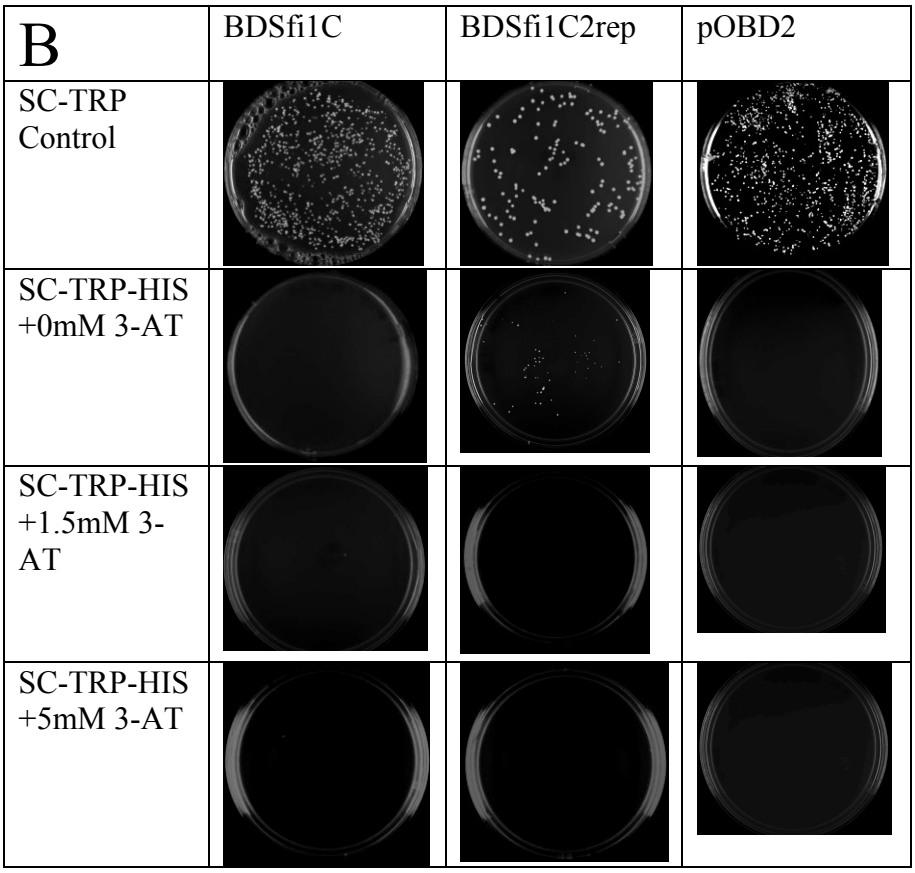
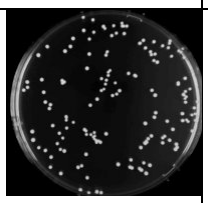
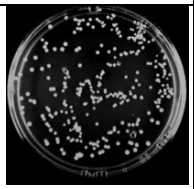
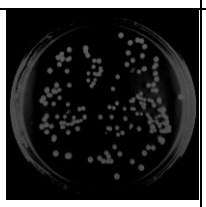
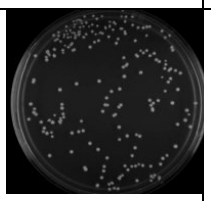
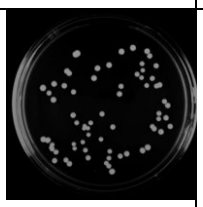
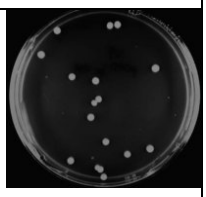
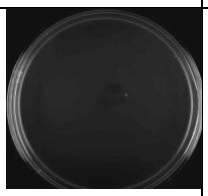
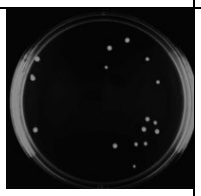
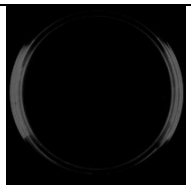
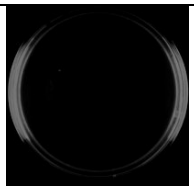
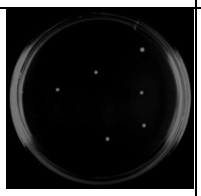
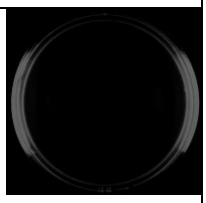
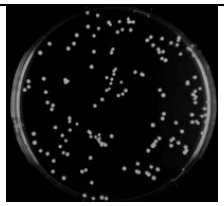
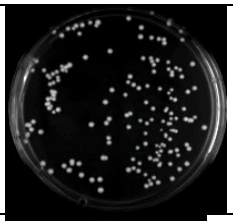
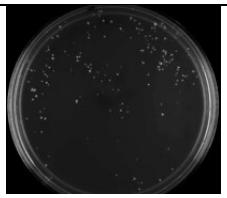
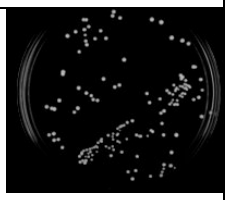
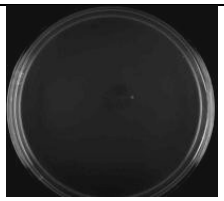
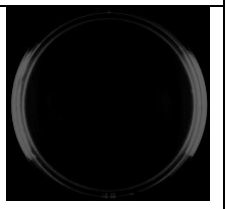


Figure 5. Autoactivation of bait constructs. The bait constructs were tested for autoactivation of the HIS3 reporter gene by testing them for growth on media lacking TRP and HIS with increasing concentrations of the drug 3-AT, and comparing this to growth on media lacking TRP, which selects for the BD plasmid. The name of the construct being tested is listed at the top of each column, and media and 3-AT concentration is listed at the start of each row. Part A) shows the autoactivation of the full length N terminus of Sfi1, which required 50mM 3-AT to control autoactivation, and the two truncations, N154 and N175, which required 5mM and 50mM concentrations of 3-AT respectively. The empty pOBD2 plasmid was also tested and showed no autoactivation. Part B) shows the autoactivation test of the C terminus of Sfi1 and the C terminus plus two repeat domains. Both of these showed no significant level of autoactivation so the lowest concentration of 3-AT, 1.5mM, was used.

A	BD:AD	BDSfi1N: AD	BD:ADSpc29	BD:ADSpc42	BDSfi1N: ADSpc29	BDSfi1N: ADSpc42
SC-LEU-TRP Diploid selective						
SC-LEU-TRP-HIS +50mM 3-AT						

B	BD: AD	BDSfi1C: AD	BD: ADSfi1C	BDSfi1C: ADSfi1C
SC-LEU-TRP Diploid Selective				
SC-LEU-TRP-HIS +1.5mM 3-AT				

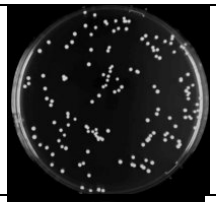
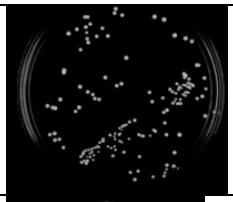
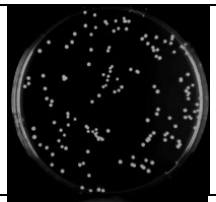
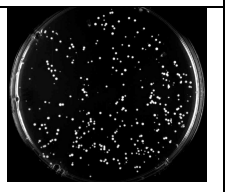
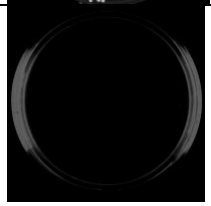
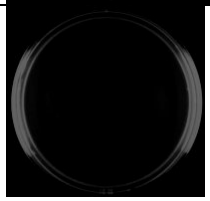
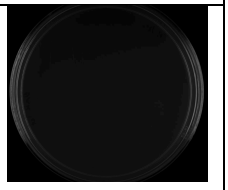
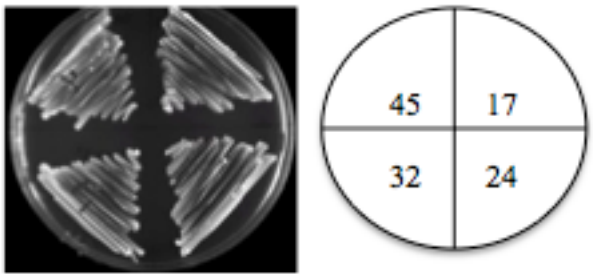
C	BD: AD	BDSfi1C2rep: AD	BD: ADSfi1C2rep	BDSfi1C2rep: ADSfi1C2rep
SC-LEU-TRP Diploid Selective				
SC-LEU-TRP-HIS +1.5mM 3-AT				

Figure 6. Directed two-hybrid tests of the N and C termini of Sfi1. Part A) the BDSfi1N and ADSpc29 and ADSpc42 constructs were transformed into separate mating types of pJ694 and diploids were selected for (diploids indicated by colon) and tested for interaction through yeast two-hybrid. Negative controls were BDSfi1N:AD, BD:ADSpc29 and BD:ADSpc42, and BD:AD. All strains grew normally on diploid selective media. None of the negative controls or BDSfi1N: ADSpc42 grew on diploid selective media, but BDSfi1N:AD did show some growth. BDSfi1N: ADSpc29 showed a small amount of growth on media selecting for diploids and lacking HIS, but at levels equal to or less than the negative control BDSfi1N:AD. Part B) In part B the BDSfi1C and ADSfi1C constructs were transformed into separate mating types of pJ694 and tested for two-hybrid interactions. Negative controls were BDSfi1C: AD, BD:ADSfi1C, and BD:AD. Normal growth was seen for all combinations on media selecting for diploids. No growth was seen for any negative controls or tests for interaction on media selecting for diploids and lacking HIS. Part C) The BDSfi1C2rep and ADSfi1C2 rep constructs were transformed into separate mating types of pJ694 and tested for two-hybrid interactions. The negative controls were BDSfi1C2rep:AD, BD:ADSfi1C2rep, and BD:AD. Normal growth was seen on media selecting for diploids, and no growth was seen for the negative controls or test for two-hybrid interaction on plates selecting for diploids and lacking HIS.

A



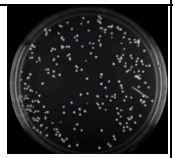
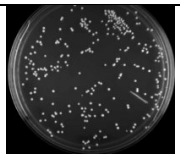
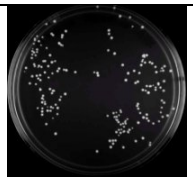
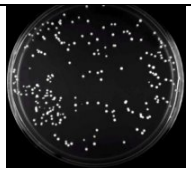
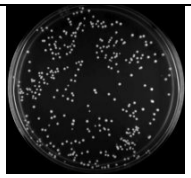
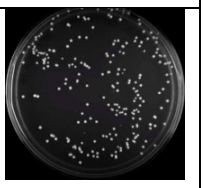
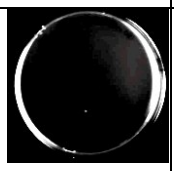
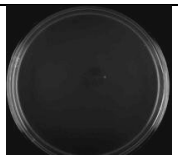
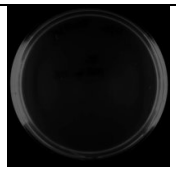
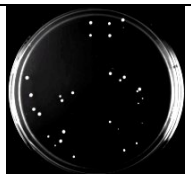
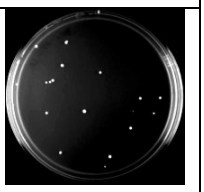
B	BD: AD	BDSfi1C: AD	BD: ADpositive24	BD: ADpositive32	BDSfi1C: ADpositive24	BDSfi1C: ADpositive32
SC-LEU-TRP Diploid Selective						
SC-LEU-TRP-HIS +1.5mM 3-AT						

Figure 7. Confirmation of true positive from a yeast two-hybrid screen of BDSfi1C. Part A) All four positives, 17, 24, 32, and 45, that came up in a yeast two-hybrid screen for interaction with BDSfi1C were streaked out for single colonies then a single colony for each positive was patched on to a master positive plate lacking LEU, TRP and HIS with 1.5mM 3AT to confirm expression of the HIS3 reporter. Part B) True positives 24 and 32 were isolated and retransformed in to pJ694 mating type A, and tested for direct two-hybrid interaction with BDSfi1C. Negative controls performed were empty pOBD2 to empty pOAD, BDSfi1C to empty pOAD, empty pOBD2 to ADpositive24 and empty pOBD2 to ADpositive32. All combinations, including tests for interaction, showed normal growth on diploid selective media. None of the negative controls grew on media selecting for diploids and lacking HIS. Both positive tests were able to grow on media lacking HIS also, with less colonies observed than on the diploid selective plates but more than the negative controls.

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