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MIG-10, an Adapter Protein, Interacts with ABI-1, a Component of Actin Polymerization Machinery

Jennifer Gosselin and Sean O'Toole

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MIG-10 is a protein known to be involved in axon guidance and neuronal migration in early development in C. elegans. In an effort to better understand the protein's function, this project used the yeast two hybrid system to screen a cDNA library (representing the entire C. elegans genome) for proteins with which MIG-10 interacts. The idea is if we can associate it with proteins of known function, we'll have a better idea as to what MIG-10 itself does. Our research revealed that MIG-10 interacts with (among other proteins) ABI-1, which is a component of actin polymerization machinery.

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Introduction

Mig-10

In general, development of the nervous system requires an extraordinarily dynamic array of signaling molecules and transduction pathways. This stems from the fact that proper nervous system function relies on the formation of specific connections between varying neuronal cell types (Bear et al., 2007). The migration of axons and cell bodies necessary to achieve these connections will occur during and beyond embryonic development. The migration path of each neuron is dependent on a variety of extra cellular cues, providing information about the immediate environment. Biomolecules, such as netrin, slit, ephrin and semaphorin, have already been indicated as signaling compounds in vertebrates, *C. elegans* and *D. melanogaster* (Yu and Bargman, 2001). These signals then act on a variety of receptors, eliciting a signal cascade that may lead to actin polymerization, depolymerization, or other fundamental cellular processes.

In some cases signal molecules can have a variety of responses (Disanza et al, 2005). Depending on the cell, receptor, or cellular localization, these signals can alter the migration pattern of a cell or axon in a concentration gradient dependent manner. In *C. elegans* there are a number of important players involved in these processes; one of them is *mig-10* (Quinn et. al, 2006). It was a screen for defective neuronal migration that led to *mig-10*'s discovery (Manser, 1990). Several recessive mutations of the *mig-10* gene have been shown to cause abnormal cell migration in multiple types of neurons and deformities in excretory cell outgrowth (Manser et al., 1997). It is currently thought that the MIG-10 protein mediates the anterior posterior guidance/migration system for these cells. Additionally, it has been found that axonal guidance can be enhanced by the over-expression of *mig-10* (Quinn, Ryder, et al, 2006).

When MIG-10 is expressed in HEK293 cells, it co-localizes with filamentous actin and creates fillipodial protrusions (Quinn et al., 2006). Additionally, when *mig-10* is over-expressed in *C. elegans* in the absence of guidance cues netrin or slit, it causes multi-polar axonal outgrowth. This suggests that at the cellular level, MIG-10 is creating actin filamentation mediated outgrowth, wherever it is expressed. *In vivo,* MIG-10 mediated outgrowth is controlled by cellular polarization of slit and netrin guidance cues (Quinn et al., 2006). However, the link between guidance cues and MIG-10, as well as MIG-10 and the actin polymerization machinery, is somewhat elusive. What is known is that MIG-10 most likely interacts

with UNC-34, an Ena/Vasp protein directly involved with actin polymerization machinery (Chang et al., 2006). Still, other potential interactors for *mig-10* remain speculative.

The MIG-10 protein contains a pleckstrin homology (PH) domain (Manser et al., 1997). PH domains in eukaryotic cells are usually involved with intracellular signaling. More specifically, they often assist proteins in the binding of inositol phosphates. The presence of this domain suggests association of MIG-10 to the inner leaflet of the plasma membrane. There are also several proline rich regions within MIG-10. These regions typically imply an interaction with an SH3 domain, commonly a part of effector proteins downstream of transmembrane receptors. Another important feature of the MIG-10 protein is the presence of a Ras Association (RA) domain. These domains allow for binding of small monomeric GTPases, which are also involved in intracellular signaling. All of these domains are highly suggestive of involvement in signal transduction pathways.

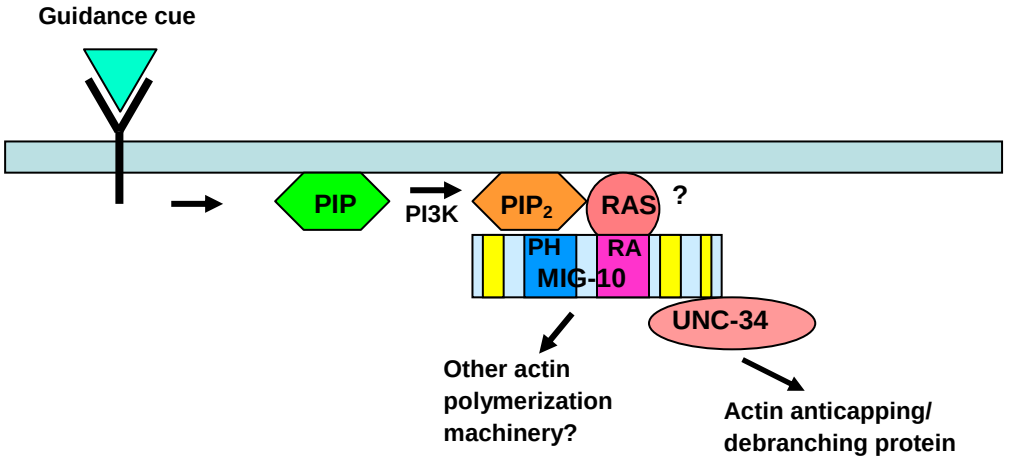


Figure 1: Speculative Model for MIG-10 signaling cascade (Ficociello and Ryder 2007). Extracellular guidance cues recruit a P13 kinase along with a Ras family GTPase which localize MIG-10, and hence the actin polymerization machinery. RA, ras association domain; PH, pleckstrin homology domain. Ras is a monomeric GTPase; PI3K, Phosphatidylinositol-3 kinase; PIP (phosphatidyl inositol); PIP2 (a phosphorylated version of PIP); PIP2 is recognized specifically by MIG-10's PH domain. UNC-34 is an ENA/VASP protein.

The model shown in Figure 1 demonstrates what the MIG-10 pathway might look like. At the beginning of the pathway extracellular guidance cues activate a Ras family GTPase as well as a Phosphatidylinositol-3 kinase (a signaling enzyme with dual catalytic function, able to act as a lipid and protein kinase). However, the identity of these proteins remains unknown. It is likely that MIG-10 localization is caused by association with a Ras family member through its Ras Association (RA) domain

(it is known that RIAM binds RAP-1, a Ras family member). Additional localization might be provided by $PI(3,4)P_2$, a phospholipid created through the catalytic action of a P13 kinase, most likely in the form of AGE-1 (Chang et al., 2006). This might occur via MIG-10's Pleckstrin Homology (PH) domain's binding of a $PI(3,4)P_2$ phospholipid. This is based on the properties of lamellipodin, a MIG-10 vertebrate ortholog (Krause et al., 2004).

It is likely that MIG-10's localization would then recruit one or more types of actin polymerization machinery. This would explain MIG-10's role in axonal migration as well as excretory cell outgrowth. One possibility is that MIG-10 recruits Ena/Vasp, thought to be involved with actin bundling, which has been shown to co-localize, in vertebrates, with lamellipodin, a MIG-10 ortholog (Chang et al., 2006). It has also been shown that MIG-10 associates with UNC-34; this has been shown in both vertebrates and *C. elegans.* One proposal for Ena/Vasp's molecular function is that it acts as an anti-capping complex (Krause et al, 2003). This allows for increased f-actin extension in regions of actin polymerization where Ena/Vasp is localized. Such a function fits well with MIG-10's role in actin polymerization. If MIG-10 increases site directed f-actin outgrowth, then it might need to cooperate with protein complexes that promote generation of new filaments. Another likely candidate is the WAVE-Abi1-Nap1-PIR121 complex, which is thought to act as an activator of the Arp2/3 complex, a piece of cellular machinery responsible for actin polymerization (Chang et al., 2006). The evidence behind a Wave complex/MIG-10 interaction will be elaborated on later in this paper.

Currently, three different isoforms of MIG-10 (A, B and C) have been identified (Wormbase). MIG-10A is comprised of 667 amino acids, while MIG-10B is only 650 amino acids long. The transcripts of these two isoforms are highly identical. The recently identified Mig-10 C is 779 amino acids. The most marked difference between these three isoforms is that they contain a variable number of initial exons and different promoter regions. Both MIG-10A and MIG-10C contain additional proline rich regions when compared with MIG-10B. The MIG-10A protein is found in the pharynx, as well as neurons such as ALM and CAN (Wormbase). The MIG-10B protein may also be involved with axonal guidance; it is expressed in six head neurons as well as the intestine. However, Mig-10A has been studied to a greater extent (Quinn et al., 2006). For this reason it was decided to focus on elucidating MIG-10A's role in an intracellular pathway. To accomplish this goal, a yeast two hybrid library screen was performed using MIG-10A as bait.

The Yeast Two Hybrid System

The yeast-two-hybrid system is a relatively straight-forward way to visually search for and confirm protein-protein interactions. It makes the task of screening an entire cDNA library possible, while at the same time minimizes the incidence of false positives with fairly stringent selective media. It also allows for plasmid extraction from the system, so that possible interactors can be singled out and sequenced for further analysis and characterization.

The basic idea behind the yeast two-hybrid system is that two hybrid proteins are made: one bait and one prey (Figure 2). The bait is composed of the GAL4 DNA binding domain (DBD) and the gene of interest, which in our case is *mig-10*. The bait vector also includes the LEU2 gene, which allows for selection on –Leu dropout media. The prey consists of the GAL4 activation domain (AD) and a putative interactor DNA of interest; in our case, a member of a cDNA library. The prey vector also contains the TRP1 gene, which allows for selection on –Trp dropout media. The library is composed of cDNA corresponding to transcripts of many different *C.elegans* genes that were expressed by a mixed-stage culture of worms; the hope is that the cDNA library will represent all the expressed genes of the organism.

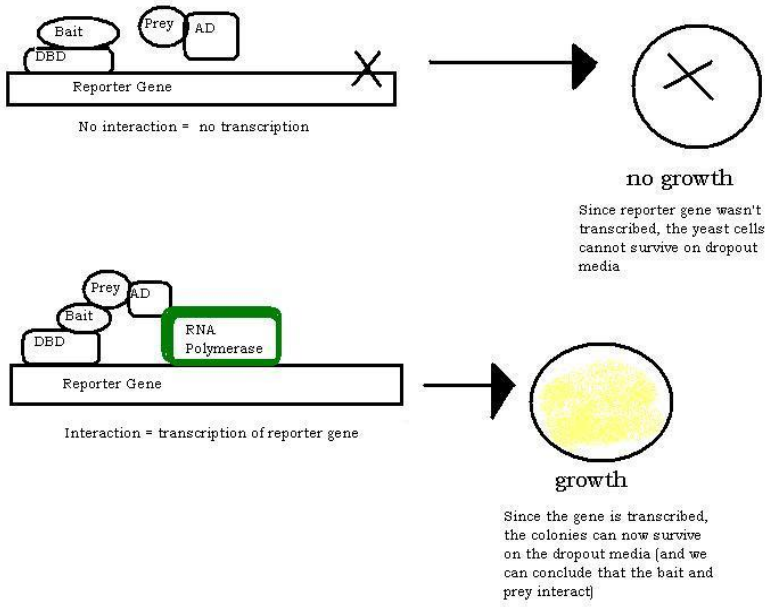


Figure 2: Schematic showing how interaction influences transcription of reporter gene. If the bait and prey do not interact, there can be no transcription of the reporter gene, and no growth will be observed. DBD, GAL4 DNA binding domain; AD, GAL4 activation domain.

If the bait and prey interact, the GAL4 activation and DNA binding domains will be brought into close proximity to each other. The DNA binding domain will bind the upstream activating sequence of several reporter genes (see below), which will be transcribed. If there is no interaction, the activation domain remains distant from the DNA binding domain, and the reporter genes cannot be transcribed.

The host strain, Mav203, is an auxotrophic mutant, normally unable to synthesize several amino acids. Normally when plated on dropout media (lacking uracil or histidine, as well as leucine and tryptophan) this auxotrophic mutant will not grow. However, when this mutant strain contains both prey and bait plasmids, containing a pair of interacting fusion proteins, the strain becomes capable of normal growth on dropout media. The LEU2 and TRP1 on the bait and prey plasmids allow growth on media lacking leucine and tryptophan, whether or not interaction occurs. Interaction of the bait and prey proteins induces transcription of the reporter genes, URA3 and HIS3. These genes encode enzymes necessary in the biosynthesis of pyrimidine ribonucleotides and histidine, respectively, allowing growth on medium lacking uracil and histidine. If there is no interaction, no growth will be observed on this medium.

The incidence of false positives is minimal, due to fairly stringent reporter genes, low-copy plasmids, and other precautionary measures, such as replica cleaning (see below). The reporter genes also have unrelated promoters, a factor that reduces the chance of non-specific transcription. The URA3 gene can also be paired with 5FOA (5-fluoroorotic acid) for a negative selection. If there is interaction, URA3 will be transcribed, but it will convert the 5FOA to 5-fluorouracil, a toxin that will kill any growth on the plates. Therefore, no growth is indicative of interaction, while growth shows that the URA3 gene was not transcribed.

The yeast strain used in the two-hybrid system expresses a basal level of HIS3, whether or not interaction occurs. 3AT (3-Amino-1,2,4-Triazole) inhibits the enzyme encoded by the HIS3 gene, and is included in the media when testing for HIS3 transcription. Since the strength of the interaction between bait and prey directly influences the level of expression of the HIS3 gene (which in turn must be able to overcome the resistance threshold to 3AT), it is important to choose a concentration of 3AT that reflects the strength of interaction one is seeking. Because even the smallest levels of expression are detected, HIS3 is the most sensitive of the reporter genes, since even weak interactors will grow on the plates.

Typically, the initial screening of cDNA libraries for interactors of the gene of interest is done using transcription of the HIS3 reporter gene. Once potential interactors have grown on the SC-Leu-Trp-His+3AT media, the next recommended step is to replica clean the plate. Pressing the plate to an autoclaved velvet and removing all visible colonies strongly suggests that if colonies grow back on the plate, they are interacting as a result of reporter gene expression. This reduces the risk that cells might grow on the media because they are being influenced by the presence of so many other cells, and not

because they are expressing the necessary reporter gene. After replica cleaning, the colonies can be replica-plated to further validate interaction. Transferring the colonies from the original plate to a velvet, then pressing that against other plates to transfer the colonies onto additional selective media, is a highly conservative way to execute additional experiments. If the original plate is SC-Leu-Trp–His+3AT, the same colonies that appeared as interactors on that plate would then be replica-plated onto SC-Leu-Trp-Ura and SC-Leu-Trp+Ura+5FOA, and so would be tested on those selective media as well. If the candidates for interaction perform as expected on all media, there will be strong evidence for interaction.

Once candidates have proven themselves as possible interactors, the next step can be to get the yeast strain to drop the bait plasmid, so that the prey DNA can be isolated and extracted for sequencing. Determining the identity of the prey plasmid protein, already shown to interact with the bait, can provide a great deal of useful information. If it can be shown that the bait interacts with a known protein, this places the bait in a specific pathway, possibly giving clues about its function.

Materials and Methods

Library Transformation

All library transformations were done using an MAV203 auxotrophic mutant, previously transformed with a pDEST™32 bait plasmid. Bait plasmid strains were grown in 5 ml cultures (-Leu media) overnight at 30° C and then transferred to a 50 ml –Leu culture for five to six hours at 30° C. The cells from the 50 ml culture were then concentrated, using centrifugation (5,000 G) for five minutes, and placed in one ml of a 1X LiOac (0.1M LiOAc pH 7.5) solution. After resuspension, cells were pelleted top speed in a microfuge for one minute and resuspended again, but in 600 µl of 1X LiOac. 50 µl of suspension were then aliquoted to several eppendorf tubes. To each tube, 5 µl of single stranded salmon sperm DNA (10 mg/ml) was added along with one microgram of the cDNA library DNA. 300 µl of a solution containing Polyethelene Glycol (9 ml), 10 X LiOac(1M LiOAc pH 7.5) and DMSO(330 µl) were added to each eppendorf tube. Then transformation tubes were incubated for 30 minutes at 30° C, followed by 20 minute incubation in a 42° C water bath. Then each transformation tube was centrifuged at 4,000 RPM in a microcentrifuge for one minute and the supernatant was removed. The pellet was resuspended in a one ml of a 0.9% saline solution. In the final step 100 µl from each tube was spread onto –His-Leu-Trp+65 mM 3AT (referred to as 65 mM 3AT) plates and allowed three and a half to four days to grow at 30° C. For each transformation set, a dilution series was prepared to judge transformation efficiency.

Assessment of Library Transformation Efficiency

A dilution series from one of the transformation tubes (1:10, 1:100 and 1:1,000) was made and plated on -Leu-Trp medium. Then, based on the number of colonies that grew on these plates, the total number of library clones screened for the transformation was calculated. In order for a library screen to be successful for any individual bait plasmid, a benchmark of 330,000 colonies had to be screened. This estimate was based on the following equation.

$$N = \frac{\ln(1-P)}{\ln(1-\frac{l}{G})}$$

P is the probability that a given DNA sequence is present in a collection of N colonies. L is the length of insert in the plasmid, or in this case the cDNA library. Lastly, G is the size of the genome the library covers. For the purposes of this study a P value of 0.99 was selected, G is equal to 100 * 10^6, L is equal to 1.4 kB. Given these values it was determined that a benchmark of 330,000 clones was needed to be screened.

Replica Cleaning

After the recommended time in the incubator, the plates showing growth should be replica-cleaned to ensure that only true interactors are growing on the plates. If too much time elapsed, overgrowth could occur and invalidate the procedure. The plates were removed from the incubator and an autoclaved velvet was placed over a press. Then one plate was pressed agar-side down on the velvet and finger-pressed gently but firmly to remove cells. This sometimes had to be done again on a clean piece of velvet to ensure that no visible evidence of cells was left on the plate, but to avoid pressing too firmly and destroying the surface of the medium. This was done for all the plates, using a new, clean, autoclaved velvet each time, and then the plates were placed back in the incubator overnight at 30^{o}C.

Replica Plating

First, any possible strong interactors (those colonies that grew on the selective media in a manner reflecting the 'strong interaction' controls) were patched onto -Leu-Trp plates along with the controls and grown up overnight in the 30^{o}C incubator. The next day an autoclaved velvet was placed over a press and the –Leu-Trp plate with the colonies to be replicated was pressed agar-side down onto the velvet, and then the plate was finger-pressed gently but firmly to transfer the cells from the plate to the velvet. Then, one-by-one, additional plates were pressed agar-side down onto the velvet to transfer the cells to desired selective media. These plates were labeled in the same fashion as the original –Leu -Trp plate so that when colonies grew they could be properly identified. Colonies were also replicated to a new –Leu -Trp plate as a control. The same velvet was used for all replica plates of a particular series; when the transfers were complete the plates were then incubated overnight at 30^{o}C.

Plasmid Extraction

Potential interactor strains were streaked onto SC–Trp+cycloheximide plates, which were incubated at 30°C for three to four days. The isolated colonies were then transferred to –Trp+cycloheximide liquid medium for plasmid extraction.

3ml cultures of the cells from the SC-Trp+cycloheximide plates were grown overnight in –Trp+cyclo liquid medium. Two cultures were made for each strain, and the tubes were put in the 30°C roller/incubator for 3 days. When the cultures had grown, the cells were spun down in a microcentrifuge at 4000 rpm for one minute. 1.5ml of culture was placed into a microfuge tube, spun down, and the supernatant was poured off. This was done twice because the entire 3ml culture would not fit into one microfuge tube. Next 0.2mL of freshly prepared solution A (see appendix) was added to each tube, then 0.2mL of phenol chloroform (1:1) saturated with TE, and finally one scoop of glass beads (RLK lab patent #420). The tubes were then vortexed for one minute to break open the cells. Once this had been done, the tubes were centrifuged at top speed for five minutes so that the aqueous layer separated and rose above the phenol layer. The aqueous layer was then removed and transferred to a new tube. The isopropanol was added in a volume equal to 60% of the total remaining solution. The tube was then centrifuged at top speed for another five minutes in order to precipitate the DNA. When this was completed, the supernatant was carefully poured off, leaving the DNA pellet in the tube. 250 µl of 70% ethanol was then added to the DNA pellet and the tube was pulse vortexed. After this it was centrifuged for five minutes at top speed. Again the supernatant was carefully poured off so that only the DNA pellet remained in the tube. All tubes containing a pellet were laid over a paper towel to dry overnight. Once dry, the pellet was resuspended in 10µL of GDW (reagent grade distilled water) and referred to as the plasmid extraction solution and stored at 4° C.

Preparation of Electrocompetent Cells

DH5α cells were streaked onto LB medium and grown overnight at 37° C. The following day a fresh colony was used to inoculate 50 ml SOB (2% Bacto Tryptone, O.5% yeast extract, 10 mM NaCl, and 2.5 mM KCl) medium and then grown overnight at 37° C while being shaken vigorously. The next morning 0.5 ml from this culture was inoculated into 500 ml of SOB liquid medium. The culture was then incubated for approximately three hours at 37° C, while being shaken for aeration. The cells were removed from incubation when the culture yielded an OD_{550} reading of at least 0.8. The 500 ml culture was centrifuged at (2,600 x Gfor ten minutes. Following centrifugation the cell pellet was resuspended in

500 ml of cold wash buffer containing DI water and 10% glycerol. After resuspension the resulting solution was centrifuged again for 10 minutes at 2,600 x G (or until the solution was no longer turbid). After this step the supernatant was once again decanted and the cells were resuspended in another 500 ml of cold wash buffer. Following a third centrifugation the cell pellet was resuspended in two ml of cold wash buffer. Then 200 µl aliquots were made from the resuspensions. Subsequently the aliquots were flash frozen in liquid nitrogen and stored at -80^{o}C.

Electroporation of Electrocompetent Cells

2 µl of the plasmid extraction solution was combined with 23 µl of DH5α electrocompetent cells in a sterile, chilled micro-electroporation chamber, while in a cold room at 4° C. The micro-electroporation chamber was then transferred to an ice bath. Prior to preparation of the electroporation chambers the electroporator's compartment was filled with ice. Electroporation of the DH5α cells with the plasmid extract was carried out at 4 kV, 4 kΩ and 330 µF. The pulse duration was usually about 1.8 seconds. After the pulse the electroporated cells were transferred to 1 ml of warmed SOC media, containing 98% SOB (without magnesium), 20 mM of $MgCl_2$ + $MgSO_4$, and 20 mM Glucose. This solution was then incubated while shaking for one hour at 37^{o} C. The cells were then streaked onto LB agar plates (appendix A) with 100 µg/ml ampicillin, to select for the prey plasmid.

Minipreparation of DNA from *E.coli*

For all minipreps of plasmid DNA Invitrogen's QuiaPrep kit was used. First 5 ml LB cultures, containing antibiotics for plasmid selection, were inoculated with cells from an E. coli transformation or frozen strain and grown overnight in appropriate selection medium. In the morning 1 ml from each culture was transferred to a microfuge tube and spun at 13,000 RPM for one minute. The supernatant was decanted and another 1 ml was added to the microfuge tube. The tubes were then centrifuged again and the supernatant was decanted. The cells were resuspended in 250 µl of cold P1 buffer with RNAase added. Then 250 µl of P2 buffer was added to each microcentrifuge tube. The tubes were inverted several times. Following this step 350 µl of N3 buffer was added to each tube. Then the 1.5 ml eppendorf tubes were subjected to 10 minutes of centrifugation at 13,000 RPM. After centrifugation the supernatant from each eppendorf tube was added to a spin column. The spin columns were then centrifuged for one minute at 13,000 RPM. The flow-through was removed and 500 µl of PB buffer was added to each column. The tubes were then centrifuged again, the flow through was discarded, and 750 µl of PE buffer

was added. The spin columns were then subjected to two centrifugation cycles each followed by discard of the flow-through. 50 µl of elution buffer was added to the membrane of the spin column and the columns were transferred to new eppendorf tubes. The tubes sat for two minutes and were then centrifuged to elute the plasmid DNA from the spin column to the eppendorf tubes. All DNA preps were stored at -20^{o}C and a subsequent restriction digest followed by electrophoresis was used to confirm the identity of all minipreps.

Sequencing

In order to confirm the identity of bait and prey plasmids, discovered or used in the yeast two hybrid screen minipreps from bacterial cultures containing the plasmids were sent to the DNA Analysis Facility on Science Hill at Yale University.

Results

Mig-10, a gene involved in cell migration, is thought to code for an adapter molecule, which somehow mediates signal transduction between extra cellular sources and actin polymerization (or possibly depolymerization) machinery (Quinn et al,2006). In order to better understand the role that *mig-10* plays, a yeast two hybrid cDNA library screen was performed. The screen used Invitrogen's Proquest™ Two Hybrid System. While using MIG-10A(an isoform of MIG-10) as the bait, ten transformations were performed across two different screens using a cDNA *C. elegans* prey library (Invitrogen).

Screen 1 consisted of four transformations and screen 2 consisted of six transformations; each screen was performed independently but in parallel by two different researchers. These two screens combined resulted in a total of 900,000 clones being transformed. Each screen individually reached a benchmark of at least 400,000. Potential candidates were isolated by plating the transformations onto 65 mM 3AT selection plates. Relatively large colonies were streaked onto maintenance plates and subsequently replica-plated onto selective media. Candidates showing growth patterns indicative of a yeast two hybrid interaction were then isolated and sequenced. Subsequently, these prey plasmids were retransformed into another m*ig-10A* cell line to control for possible mutations and eliminate false positives.

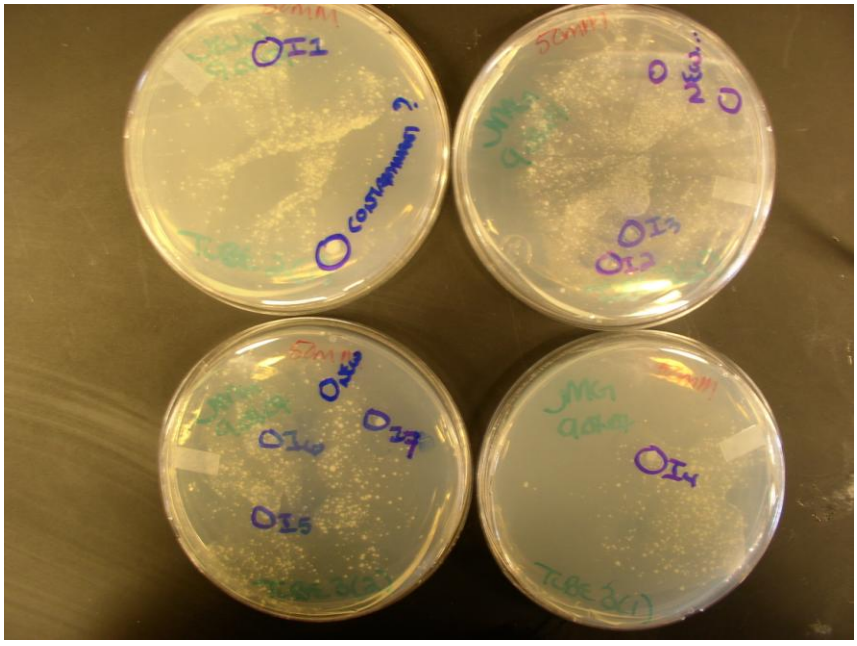


Figure 3: 50mM 3AT test transformation. Media is SC-Leu-Trp-His+50mM3AT. Possible interactors are circled in blue marker and labeled.

Determination of 3AT Concentration

To perform the library transformations, the correct 3AT concentration was needed. The desired 3AT concentration must prevent growth resulting from basal levels of transcription, while still allowing growth of potentially strong interactors. This required testing several different concentrations of 3AT.

Three sets of initial transformations were performed using concentrations of 25, 50 and 65 mM 3AT. The data for 25mM 3AT is not shown. The 50mM 3AT transformation is shown in Figure 3. Even though this was a concentration test transformation, several large colonies (colonies I1-I7) were isolated for characterization. Larger colonies indicate higher expression levels of the reporter genes, thus indicating a significant interaction.

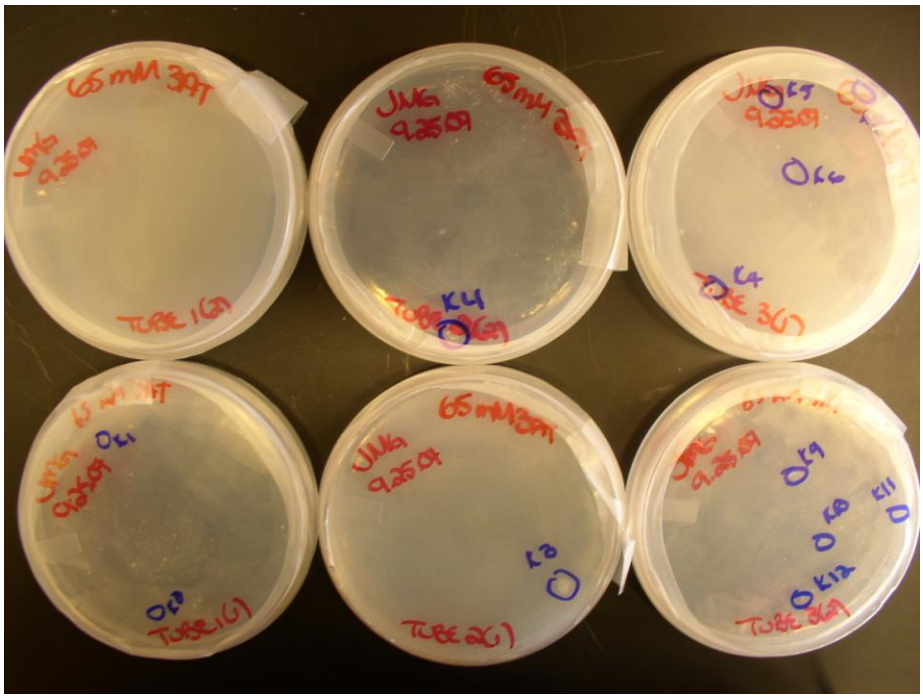


Figure 4: Fourth transformation from screen 1. Media is SC-Leu-Trp-His+65mM 3AT. Possible interactors are circled in blue marker and labeled K1-K12.

All transformations after the second were plated on –Leu-Trp–His medium containing a concentration of 65mM 3AT (Figure 4). Growth on 65mM 3AT showed a strong reduction in background colonies. Possible interactors were first circled and labeled, then picked to be plated on –Leu-Trp medium for replica cleaning/plating, as well as strain maintenance.

Plating Control Yeast Strains on Selective Media:

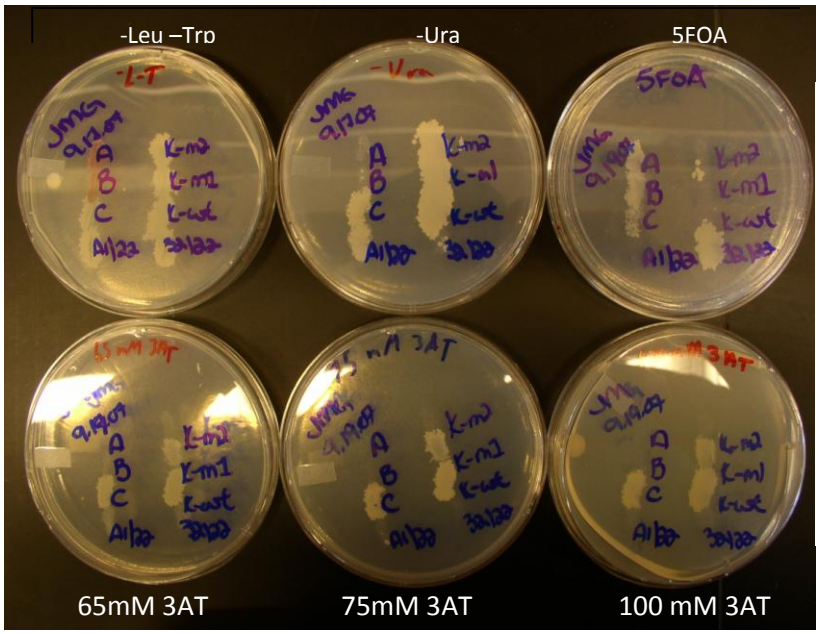
Control yeast strains were patched onto maintenance media (-Leu, -Trp), and then replica-plated onto various selective media (**Figure** ; Table 1). All plates were initially replica-cleaned after one day of growth, and all plates were transferred to a final –Leu-Trp plate as a control. If bait and prey interact *in vitro,* then they should activate the URA3 and HIS3 reporter genes. This interaction should manifest itself through growth of the respective strain on 65mM 3AT –His medium, as well as on -Ura medium.

The strain should not grow on 5FOA medium, which inhibits positive interactors that express the URA3 gene.

Table 1: Table showing Library Transformation controls, as given by Invitrogen. K-wt = strain 2; K-m1 = strain 3; K-m2 = strain 4; 32/22 = strain 5; A1/22 = strain 6. Not shown are the controls from the old Invitrogen kit, where C is a medium interactor, A is the weakest interactor, and B is somewhere in between. (Table taken from ProQuest™ Yeast Two Hyrbid manual.)

	LEU2 Plasmid	TRP1 Plasmid	Purpose
1	none	none	Negative transformation control
2	pEXP™32/Krev1	pEXP™22/RalGDS-wt	Strong positive interaction control
3	pEXP™32/Krev1	pEXP™22/RalGDS-m1	Weak positive interaction control
4	pEXP™32/Krev1	pEXP™22/RalGDS-m2	Negative interaction control
5	pDEST™32	pDEST™22	Negative activation control
6	Bait plasmid	pDEST™22	Negative activation control; baseline
	If available:		
7	pDEST™32	Prey plasmid known to interact with bait	Negative activation control
8	Bait plasmid	Prey plasmid known to interact with bait	Bait-specific positive interaction control (if available)

Controls seemed to work inconsistantly (Table 1; **Figure** 5). At the highest level of 3AT concentration (100mM) K-wt, the strongest interacting control strain, showed the most growth, while A and B (weaker interacting controls) did not grow at all, showing that the interaction was too weak for expression to overcome resistance to 3AT. The empty vector strains, as expected, did not grow. The 5FOA plate had the inverse growth pattern of the –Ura plate, as it should (see Introduction). Unfortunately, in this figure A1/22 growth was inconsistent with the expected pattern. It should have grown on 5FOA and not grown on –Ura; **Figure** 5 shows that the opposite occurred. A1/22 did, however, perform as expected on the –His plates. Similar problems appeared with the K-m1 and K-m2 strains which are weak interactors yet they seem to grow just as well as K-wt on the –Ura and 100 mM 3AT plates. In later figures, these inconsistencies are not present, suggesting the results in **Figure** 5 were only due to insufficient replica cleaning.



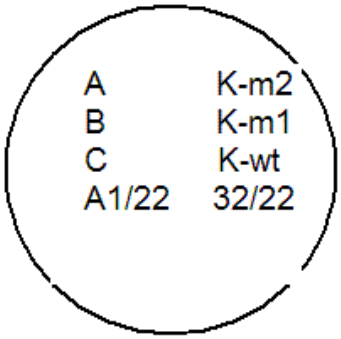


Figure 5: Replica plating with controls (left). The K-wt (wild-type) control is representative of a strong interactor, A is the weakest interactor, and A1/22 is meant to control for *mig-10*'s self activation. It contains a Mig-10A bait plasmid along with an empty pDEST22 prey vector. The 22/32 control is a strain containing both empty prey and bait vectors. Figure on right shows setup of control plates.

Screen 1 Candidate Characterization

The first screen resulted in 350,000 colonies being transformed. 31 colonies were isolated as potential interactors: of these, eleven colonies were picked as strong interactors, two as moderate interactors, and ten as weak interactors (see Appendix C: Table of Screen 1 Results). Strain nomenclature works as such: numbers indicated the order in which colonies in a given transformation were picked; letters correspond to the transformation. P denotes the first transformation, I the second, C the third, and K the fourth transformation. Replica plates displaying these isolated colonies are found in figures 6 through 8. Replica plating methods followed those of the control group mentioned in the previous section, and controls were plated separately from candidates for all but the third transformation. Although it is not shown, each replica set was plated onto an additional –Leu-Trp plate as a control for cell debris transfer.

For the first transformation, Figure 6 showed most of the possible interactors (that is, p3-p10) grew about the same on each plate. Even on 100mM 3AT, an extremely high concentration, all of the possible

interactors showed growth. For this reason, it was 5FOA that determined which colonies were picked as strong interactors. P6 and P9 did not grow on 5FOA media, indicating they were good interactors.

Colonies defined themselves better in the second replica plating series than in the first series, but there is still some ambiguity regarding which colonies are consistently strong interactors. 100mM 3AT and 5FOA plates played a large role in determining 'strong' interactors, because once again, on the other plates the growth of each possible interactor was about the same. However, on 100mM 3AT, I1 and I5 grew especially well, and on the 5FOA plate, these two did not grow well, indicating they were potentially strong interactors.

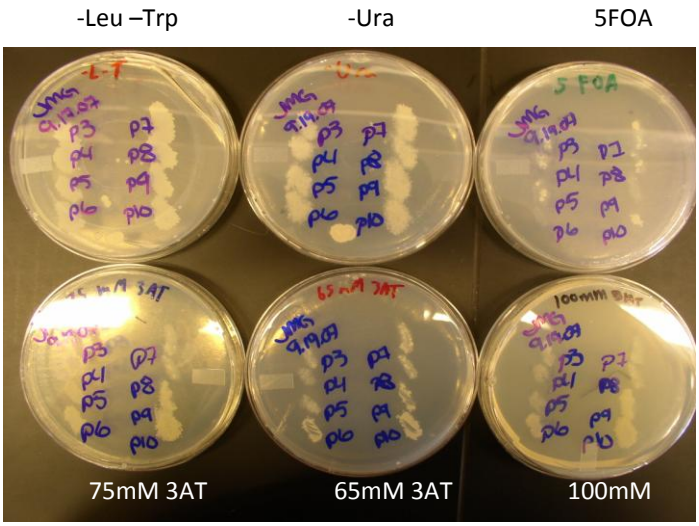
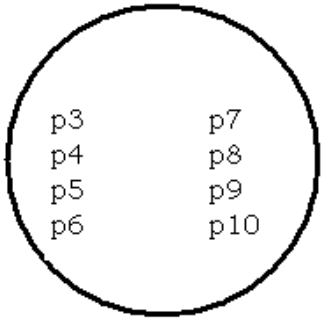


Figure 6: Replica plating for first transformation of screen 1. Media is once again written in marker on plates; potential interactors from the first transformation were labeled as p3-p10.



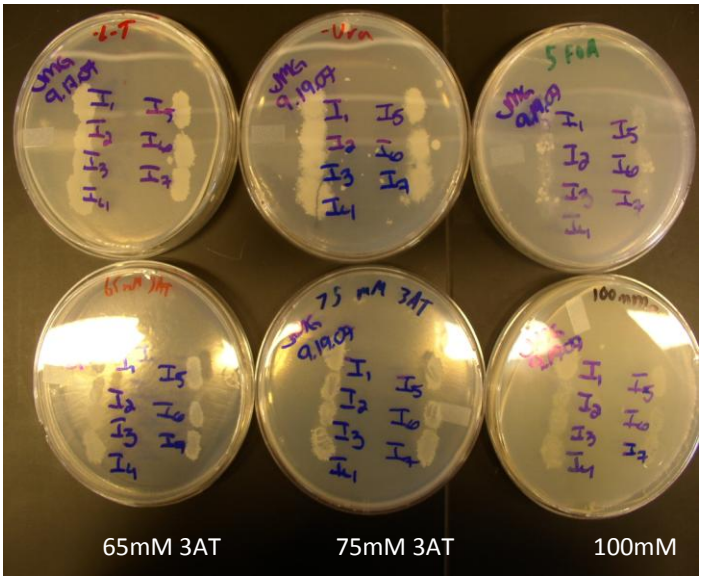
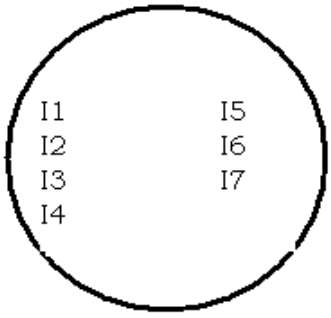
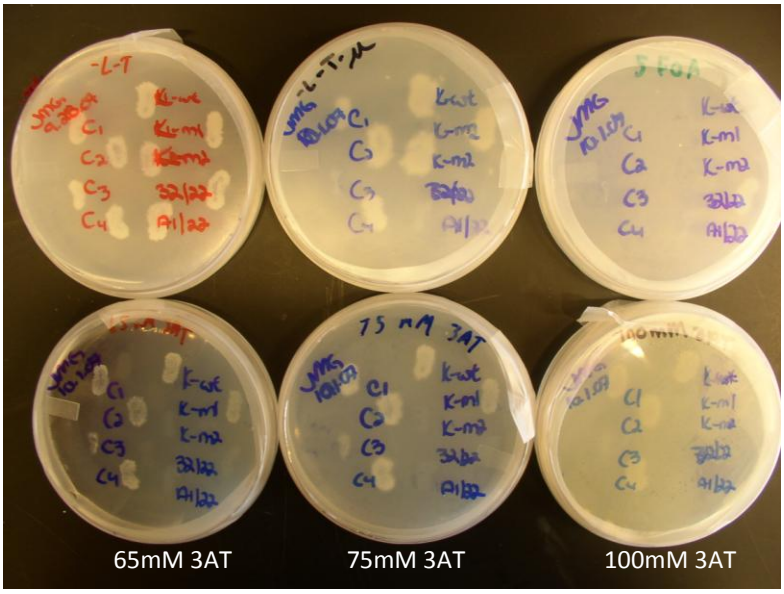


Figure 5: Second transformation from screen 1 replica plating series. Media is written with marker on plates, and candidates from the second transformation were labeled as I1-I7.



For the third series of replica plating, only the largest colonies were picked from the original plates. This is different from the first transformation, in which several colonies were chosen regardless of size. This change in experimental practice was due to an increase in skill and discrimination on the researcher's part. Controls were placed on the same plate as the four candidate strains. The A1/22 strain did not grow on the 3AT or –Ura plates. However, it also did not grow on the 5FOA plate. Given the fact that A1/22 didn't grow on 5FOA, it may be that not enough cell debris was transferred from the master plate to stimulate growth. Most of the strains on these replica plates suggest some level of interaction; only the results for C3 did not indicate a strong or moderate interaction. C1, C2, and C4 all grew well on the positive selection plates, and did not grow on 5FOA.



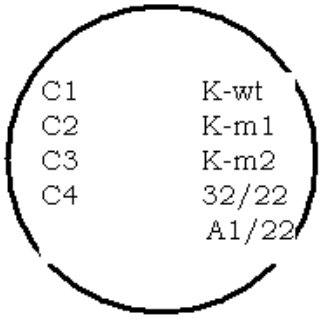
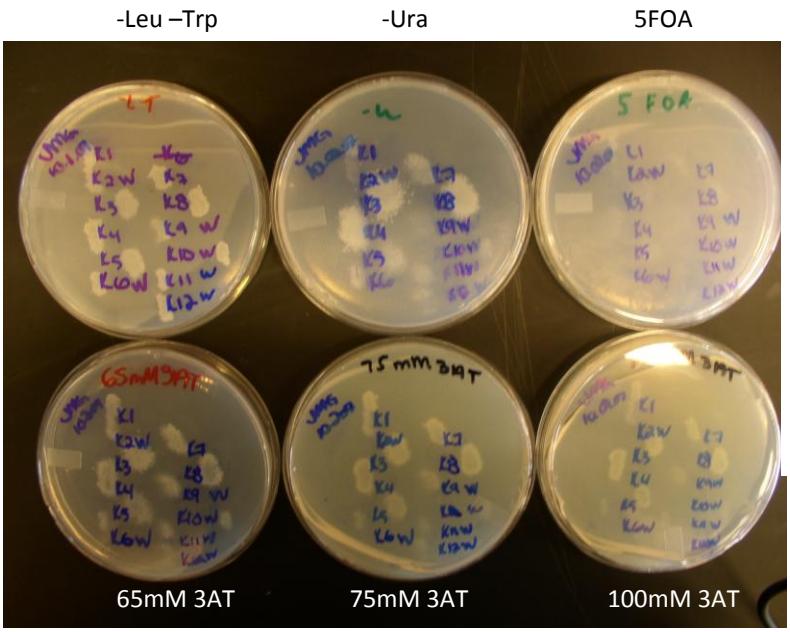


Figure 6: Replica plating for third transformation from screen 1. Media is written on plates in marker, and candidates from the third transformation were labeled as C1-C4. Selected controls are also included.



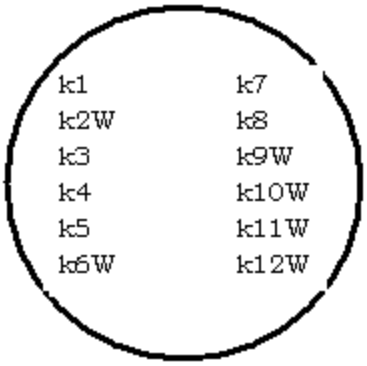


Figure 7: Replica plating for fourth transformation from screen 1. Media is written in marker on plates, and candidates from the fourth transformation were labeled K1-K12. A 'W' next to a colony indicates comparatively weak interaction.

The fourth transformation of screen 1 produced a lot of candidates for interaction. Compared to previous transformations, several of the picked colonies were noticeably smaller, so these were labeled with a 'W' (for 'weak') prior to incubation. The 5FOA plate showed that most of the colonies were interacting, so it was ultimately the 75mM and 100mM concentrations of 3AT that determined the strongest interactors. Note that K3, K4, and K8 grew well, even with a concentration of 100mM 3AT.

DNA from six of the seven candidates that were identified as strong interactors was isolated and sent off for sequencing (Table 2). Five sequenced prey plasmids were retransformed into the *mig-10A2* strain, a separately matained Mig-10A cell line, and growth patterns still indicated strong interaction.

Screen 2 Candidate Characterization

More than 400,000 clones were screened in screen 2. Fifteen independent candidate interactors were isolated using 65 mM 3AT selection plates. The nomenclature is based on growth strength during the initial selection. "V" stands for a moderate interactor (medium colony) while "S" stands for a strong interactor (large colony). The numbers correspond to the order in which the colonies were isolated. Each candidate was initially transformed into the Mig-10A1 strain; some of these candidates were later

retransformed into the Mig-10A2 strain. The 15 putative interactors were patched onto –Leu-Trp medium and then replica plated onto various selective media. The results of the replica plating are shown(Figure 10). All of the strains shown in this figure had been transformed into both Mig-10A1 and Mig10A2 (the separately maintained Mig-10A cell line).

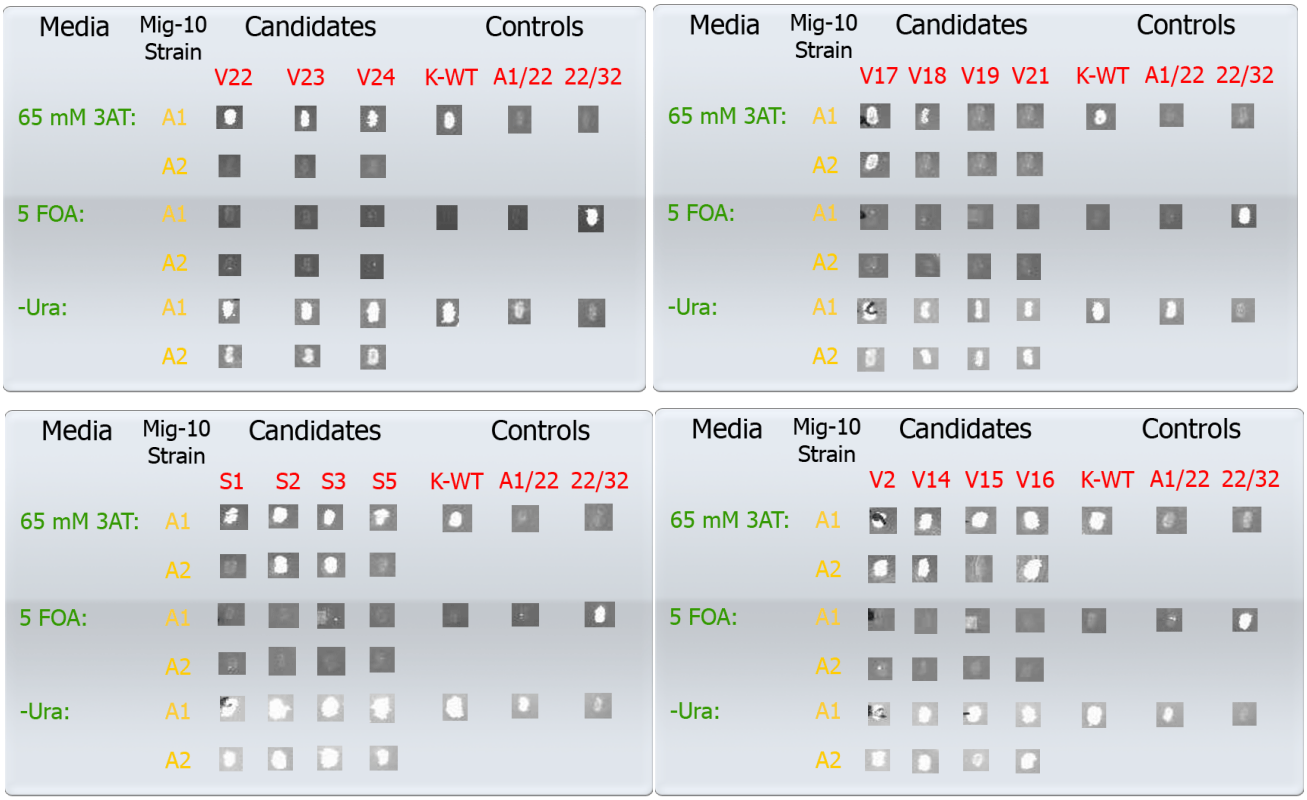


Figure 8: The screen 2 replica plating results, showing strength of interaction colonies versus controls. Candidates are shown interacting with both MIG-10A1 and MIG-10A2 strains. Each individual square corresponds to a single replica set. Each square also has its own set of controls to account for and normalize the differences across individual replica sets. It should be noted that there was some variability in the A1/22 strains growth on the –Ura plates.

These results indicate that S2, S3, V14, V16 and V2 all had a strong interaction with MIG-10. All of these candidates had growth comparable to the K-WT positive control. Additionally, they all grew well upon the retransformation. However, it should be noted that V2 has not yet been sequenced so its identity is not yet known. As a consequence there will be no discussion on V2 in later sections. V17 also appeared to show a moderate interaction. It had identical results in both the A1 and A2 strains, but did not grow as well as the positive control. However, it was well above the growth rates of the negative controls. S1,

S5, V15, V18, V22, V23 and V24 all seemed promising for the initial transformation into MIG-10A1. However, the retransformation into MIG-10A2 seemed to eliminate these candidates as potential interactors. Also, V19 and V21 did not appear to interact with MIG-10 in either the A1 or A2 strain. This occurred despite the fact that previous replica plates with the MIG-10A1 strain suggested they were interactors (data not shown). It should be noted that there appear to be some problems with the –Ura selection plates. Most strains showed some form of growth on this media. This is probably due to the fact that these plates were allowed to incubate a day longer than normal after replica cleaning. Finally, it was concluded that S2, S3, V14, V16, V2 and V17 interact with MIG-10 in the yeast two hybrid system.

Sequence Results

Table 2: Sequencing results providing a summary of sequence information obtained from screens 1 and 2. For an explanation of candidate nomenclature see opening paragraphs of both Screen 1 and Screen 2 candidate characterization sections. The "Matches Up Upon Double Transformation" column tells whether or not the growth patterns were similar for that candidate when it is transformed into either MIG-10A1 or the MIG-10A2 strain. It should be noted that S6 and V13, although shown in table 2, was not a part of figure 12. This is because they had not yet been retransformed into Mig-10A2.

Candidate	Matches Up upon Double Transformation	Do Results Indicate Interaction?	Gene Accession Number	Protein Identity
S1	no	possibly weak	NM_066550.1	A Novel Protein
S2	yes	yes	NM_065823.3	Abi-1
S3	yes	yes	NM_065823.3	Abi-1
S5	No	possibly weak	NM_060409.4	Ribosomal
S6	not retransformed	possibly	NM_065823.3	Abi-1
V2	not Sequenced			
V13	not retransformed	possibly	NM_067169.3	Arx-7, an ARp2/3 complex component
V14	yes	yes	NM_065823.3	Abi-1
V15	no	possibly weak	NM_066119.4	Translation Elongation Factor
V16	yes	yes	NM_065823.3	Abi-1
V17	yes	yes	NM_068440.3	COP-9 SigNalosome subunit
V18	no	possibly weak	NM_060151.5	lin-53 nucleosome remodeling factor
V19	yes	no	NM_073883.3	Collagen
V21	yes	no	poor sequence data	Unavailable
V22	no	possibly weak	NM_059015.2	Ribosomal Protein, Large subunit (rpl-4)
V23	no	possibly weak	NM_171488.3	Translation Initiation Factor
V24	no	possibly weak	NM_059579.2	A Novel Protein
P6	yes	yes	NM_001083203.1	Mitochondrial phosphate carrier protein
P9	yes	yes	NM_060151.5	lin-53 nucleosome remodeling factor
P10	not retransformed	possibly	not sequenced	Unavailable
I1	not retransfomed	possibly	not sequenced	Unavailable
I5	yes	yes	NM_058687.2	Collagen
C1	no	no	NM_171472.2	Aldehyde dehydrogenase
C2	not retransformed	possibly	not sequenced	Unavailable
C4	yes	yes	NM_076382.3	ammonium transporter homolog
K3	not retransformed	possibly	not sequenced	Unvailable
K4	not retransformed	possibly	not sequenced	Unavailable
K8	yes	yes	NM_065823.3	Abi-1

If the aforementioned candidates of S2, S3, V14, V16, V17, p6, p9, I5, C4, and K8 do interact with MIG-10 *in vivo,* then the sequencing results would have to support this. Prey vectors from potential interactor strains, which had been found in screens 1 and 2, were isolated and sequenced (see Methods). The sequencing results were then analyzed using NCBI's nucleotide blast. The sequences were aligned with the Reference mRNA Sequence Database. One of the sequences found in both screen was lin-53, however, in the second screen it did not match up upon double transformation.

More interesting is that the sequencing results (Table 2) show that S2, S3, S6, V14, V16 and K8 are all the same protein, ABI-1. The fact that *abi-1* was sequenced multiple times suggests a real interaction with MIG-10. Table 2 also shows that V17 is a Cop-9 signalasome subunit. It should be noted that V13 was not retransformed. Still, sequencing of V13 raises an interesting possibility, if interaction is confirmed after retransformation. V13 is an Arp2/3 complex component, ARX-3. Both ARX-3 and ABI-1 are components of actin polymerization machinery. Even more interesting, it has been suggested that these two proteins, along with their respective complexes, directly cooperate during the polymerization process (Disanza et al, 2005).

Discussion

Mig-10 and *abi-1*

The results in this paper provide an important step to identifying how MIG-10 functions in a pathway that relates extra-cellular cues to cytoskeletal dynamics. These important clues may contribute to our understanding of how guidance cues drive axonal outgrowth and cell body migration during development.

According to the yeast two hybrid library screens described in this paper, MIG-10A interacts with ABI-1, which is an Abl interactor ortholog. Orthologs of *abi-1* are associated with growth cone particles, synaptosomes, and may also be involved with cytoskeletal reorganization (Wormbase). The domain structure of ABI-1 can be seen in Figure 9. The fact that *abi-1* was isolated six times (and confirmed upon retransformation five times) eliminates the possibility of a false positive.

ABI-1 is a protein which, in vertebrate systems, may be linked to actin-related cytoskeletal dynamics. It is thought to be involved with two different protein complexes, each pertaining to actin organization (Disanza et. al, 2005). The first complex in which ABI-1 can be found in is known as the WAVE-Abi1-Nap1-PIR121 complex. This set of proteins may orient globular actin for polymerization at barbed ends by the Arp2/3 complex, essentially acting as an activator of the Arp2/3 complex. Interestingly enough, screen 2 also revealed that MIG-10 potentially interacts with an Arp2/3 complex member, ARX-3. The second ABI-1 containing complex involves Eps8 and is responsible for Rac activated capping assemblies. Capping assemblies attach to the barbed ends of filamentous actin (Disanza et. al, 2005). This molecular capping prevents further polymerization and helps to prune growing actin branches. The fact that ABI-1 is involved with both capping and polymerization suggests that it is an integral player in the balancing act, which must be performed during site directed actin polymerization. Perhaps, MIG-10 enables the polymerization activity while silencing its capping effects, thus allowing for site directed actin filaments and axonal outgrowth.

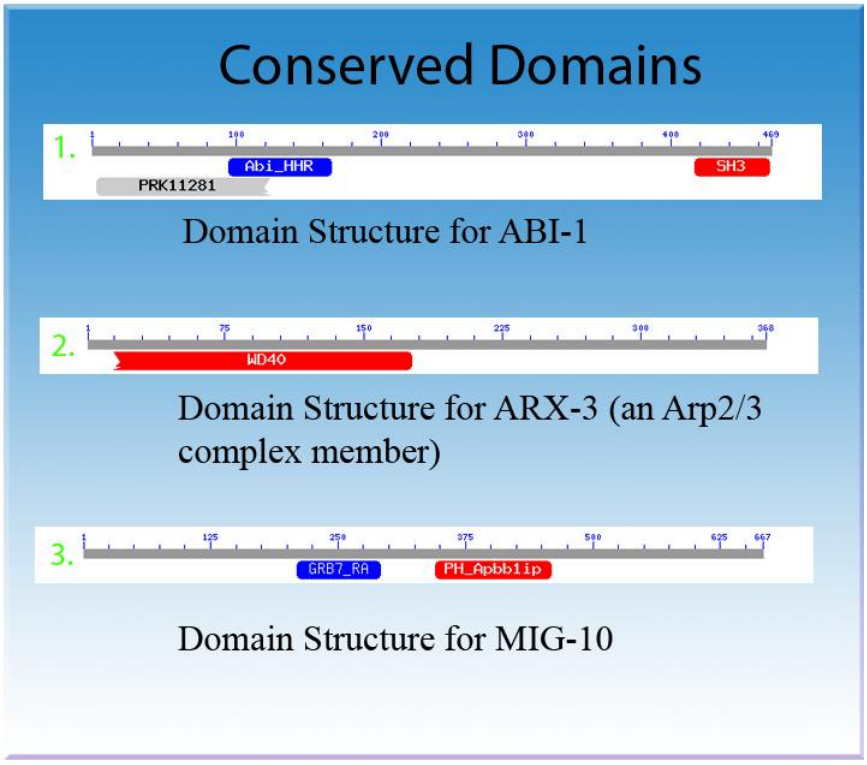


Figure 9: Conserved Domain Structures of MIG-10 and two interactors identified by the screen

The domain structure of ABI-1 provides several important clues (Figure 11). ABI-1 contains an SH3 domain which is known to interact with proline-rich regions. MIG-10A actually has several proline-rich regions. ABI-1 also contains an Abi_HHR domain and interacts with tyrosine kinases of the Abl-kinase family; this is indicative of proteins involved with intracellular signaling cascades.

Aside from evidence obtained through identifiable/functional domains, there are other reasons why the interaction between MIG-10A and ABI-1 is most likely real. As explained in the introduction, MIG-10 is probably involved with recruiting proteins involved in actin polymerization machinery. In the case of axon guidance, we know that the direction of this polymerization is then determined by cellular polarity created by extracellular cues in the form of netrin and slit. Interaction with ABI-1, a protein directly involved with actin polymerization, fits well with our model of MIG-10 function. Interaction with the ARP 2/3 complex member, ARX-3, also fits the model; however, this interaction has not yet been confirmed by retransformation.

In addition to ABI-1 and Arx2/3, a couple of other proteins worth mentioning were found to interact with MIG-10. One was the COP9 signalsome (candidate V17), which is conserved between plants and

mammals, including humans. It is also thought to be conserved among most multi-cellular eukaryotes as well, but is not thought to be present in many, if any, single-cell eukaryotes, as COP9 is not found in *Saccharomyces cerevisiae* (a single-cell eukayote) (Wei et al., 1998). The COP9 gene encodes a protein that is involved in regulating multiple cell-signaling pathways. It is associated with a kinase activity that phosphorylates the regulators involved in signal transduction (Wei and Deng, 1999).

The other protein worth mentioning is LIN-53 (candidate p9), which is a nucleosome remodeling factor (Wormbase). LIN-53 works in opposition to the Ras signaling pathway (Xiaowei and Horvitz, 1998). If MIG-10 does interact with a nucleosome remodeling factor (such as LIN-53) *in vivo*, it might influence gene expression. Like COP9, LIN-53 is further down the pathway than what interaction with ABI-1 and Arx2/3 suggest, but it still suggests involvement in signaling cascades.

Drawbacks of the Yeast Two Hybrid System

There are several points one must remember when using the two-hybrid system to screen for protein-protein interactions. Just because proteins interact in this system, it does not necessarily mean that they interact *in vivo*; i.e. interaction in yeast nuclei may not mimic the intracellular conditions of a *C. elegans* neuron or excretory cell. Yeast is also a very simple eukaryote compared to other eukaryotic organisms, such as *C. elegans*. In many multi-cellular eukaryotes, posttranslational modification plays a large role in protein expression; while still present in yeast cells, the process is less elaborate.

Another point to remember is that the entire system is based on the two hybrid proteins: bait and prey. While very practical and functional, the hybrid proteins may not necessarily reflect the natural proteins, which could be another cause for false interaction, or lack of interaction altogether. Also, with the cDNA library, some of the natural proteins are so large, that in the system, only part of the protein (and therefore only some of the domains) are included as prey. This could result in some domains that are normally unexposed to become exposed to interaction, or conversely domains that are normally exposed to interaction with other proteins' domains to become be unavailable for interaction.

Folding mechanics may play a role as well. Whenever a protein folds *in vivo*, the folding pattern for any given region is dependent on the sequence which comes before and after it, i.e. there are spatial and temporal aspects of folding. Either the activation or DNA binding domains could theoretically alter folding. However, these domains have been chosen for their ability to fold independently.

An additional point to take into consideration is that because these interactions are not taking place *in vivo* in the normal timeline of the organism, the interactions that are found by the two-hybrid system may in fact be biologically irrelevant in the natural setting. It is possible, for example, that even though the two proteins interact in the yeast nucleus, *in vivo* they do not even exist simultaneously during the cell cycle, or perhaps they are located in different compartments, or cells entirely. While this does not technically negate the interaction, it does make it irrelevant in terms of using the interaction to further characterize the gene of interest.

A final point on the caveats of yeast two hybrid, is that proteins with large hydrophobic regions will often interact promiscuously. This is a characteristic artifact of trans-membrane proteins placed in the yeast two hybrid system (Invitrogen, 2005). To show that both ABI-1 and MIG-10 do not have large trans-membrane segments, the sequence for each was analyzed using the "DAS" TM-Segment Prediction program (Figure 10). The results of this analysis suggest that neither protein has a large trans-membrane span.

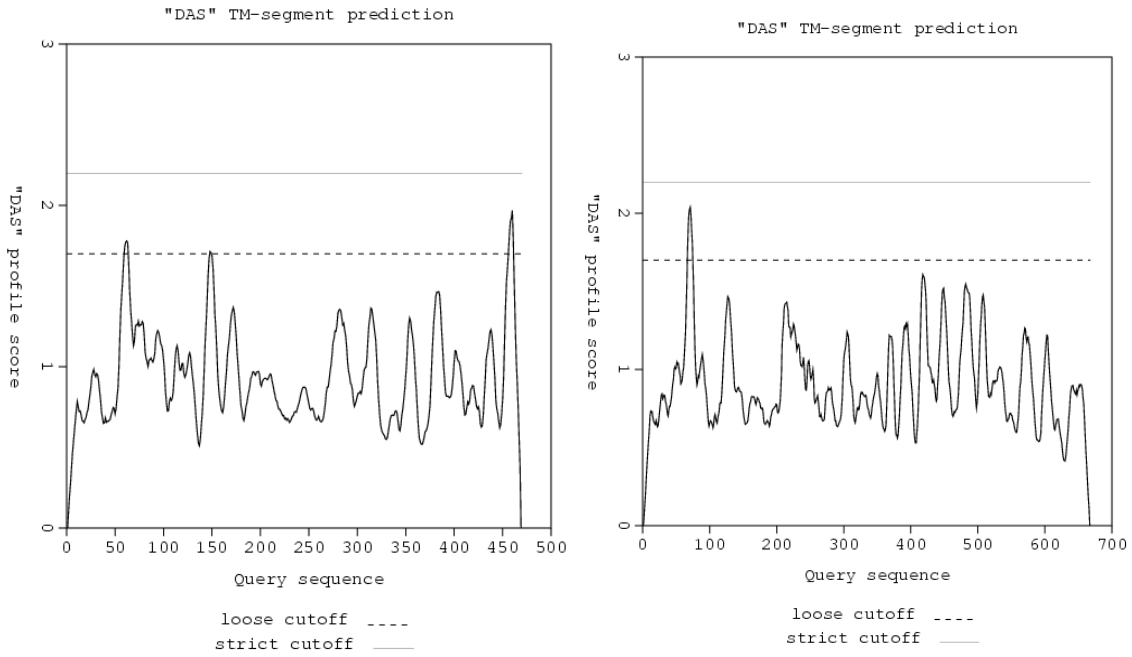


Figure 10: TM Segment Prediction Plots for *mig-10*(right) and *abi-1*(left). Scores above line marks correspond the trans-membrane segments.

Suggestions for Future Research

As with most areas of science, the more we discover, the more questions we raise. The results presented in this paper are good starting points for future research, but there is still so much more that needs to be explored. Accordingly this paper has several recommendations for future groups who may continue with this work.

First of all, to determine if the interaction between MIG-10 and ABI-1 occurs in vivo, genetic evidence is needed. It would be interesting to look at *abi-1* and *mig-10* double mutants, as well as each individually to see if they produce similar phenotypes. Studies involving RNAi might also provide useful information. If MIG-10 and ABI-1 are a part of the same pathway, then they should both affect developmental axon guidance in a similar manner. A series of genetic crosses between Mig-10 and Abi-1 mutants could provide evidence for this. If the Mig-10 and Abi-1 mutant phenotypes are identical, then this would suggest that they operate in the same pathway. However, only a double mutant can provide conclusive evidence. If the double mutant's phenotype is identical to both single mutants, then MIG-10 and ABI-1 are most likely operating in the same pathway. Conversely if the double mutant amplifies and/or creates additional phenotypes, then these two proteins are most likely operating in multiples pathways, some of which do not overlap.

It should be noted that even if genetic evidence shows that these two proteins operate in the same pathway, it does not necessarily imply a physical interaction between them. A more convincing genetic assay for the purpose of elucidating an in vivo interaction could be done through the creation of a trans heterozygous worm for *abi-1* and *mig-10* mutations. If a trans heterozygote worm leads to an amplification of mutant phenotypes such as axon guidance, while allowing the animal to escape the more adverse or lethal phenotypes associated with double mutants then in vivo physical interaction between *mig-10* and *abi-1* becomes a stronger possibility.

Obtaining genetic evidence through *abi-1* mutants may prove to be difficult. The ABI-1 protein is important to actin polymerization. This could create problems when examining the phenotype and, more importantly, creating viable worms. Instead, one could create a transgenic worm with an inducible siRNA corresponding to the *abi-1* gene, which would only be activated in the nervous system. Perhaps a specific metabolic product or promoter could switch on *abi-1* silencing during embryonic development when cell migration occurs.

Potential interactors discovered in screens 1 and 2 were only retransformed into a separately maintained *mig-10A* cell line. It would be interesting to retransform these candidates into other isoforms of *mig-10*, such as *mig-10B* or *mig-10C*, though the latter may show a level of self-activation too high to make this valuable. If a candidate interacts with the *mig-10A* isoform, but not *mig-10B*, this could lead to analysis on which domains of *mig-10* are interacting. Another interesting experiment would be to test ABI-1's interaction with only the RAPH region of MIG-10.

Following this line of thought, another set of experiments to consider would be a reverse hybrid screen. This screen involves mutating various regions of either *mig-10A, abi-1,* or *arx-3,* then plating strains containing various bait and prey combinations on 5FOA medium to see which mutations negate interaction. This experiment could be done to determine the regions of interaction on these respective proteins. If a mutant does grow on 5FOA (meaning it no longer interacts), it would be informative to see what genetic change caused it to do so. Mutations could be single base pair changes or whole domain deletions.

Our candidates were all discovered by screening the cDNA library for interaction with *mig-10A*. If this MIG-10A interaction proves relevant in vivo, it is highly recommended that future groups continue to screen the cDNA library for interactors, using at least the *mig-10B* isoform. A mini-screen using Mig-10B (about 70,000 colonies were transformed, data not shown) produced a couple of possible interactors, though time constraints did not allow for further analysis of these colonies. Given the strong results of the *mig-10A* screen, it is quite plausible that another screen of the library (using a different isoform) could also provide valuable clues towards MIG-10's function. Additionally, it would be interesting to see if the results of these screens were similar or dissimilar to the *mig-10A* screen. If they are new, they should then be retransformed with *mig-10A* to see if they interact with both isoforms. If not, this can again lead to analysis on which domains of the *mig-10* gene are interacting.

The evidence provided in this paper raises interesting questions regarding the legitimacy of a MIG-10/ABI-1 interaction, MIG-10's role in actin polymerization, and the MIG-10 signaling cascade in general. Future experiments will need to substantiate the evidence using biochemical and genetic means. If successful, further steps will have been taken toward understanding MIG-10's functional pathway. This will help to complete our understanding of axonal and cell body migration during development.

References

Bear, M. F., Connors, B. W., Paradiso, M. A. *Neuroscience Exploring the Brain*, third edition, Lippincott Williams and Wilkins

Chang, C., Adler, C. E., Krause, M., Clark, S. G., Gertler, F. B., Tessier-Lavigne, M., Bargmann, C. I., MIG-10/Lamellipodin and AGE-1/PI3K Promote Axon Guidance and Outgrowth in Response to Slit and Netrin**,** (2006) *Current Biology* **16**, 854-862

Disanza, A., Steffen, M., Hertzog, E., Rottner, K., Scita, G., Abil regulates the activity of N-WASP and WAVE in distinct actin-based processes, (2005) Cellular and Molecular Life Sciences **62**, 955-970

Gomperts, B. D., Kramer, I. M., Tatham, P. E. R. *Signal Transduction*, first edition, Elsevier

http://www.wormbase.org/db/gene/gene?name=WBGene00003243;class=Gene

Krause, M., Dent, E. W., Bear, J. E., Louriero, J. J., Gertler, F. B., ENA/VASP PROTEINS: Regulators of the Actin Cytoskeleton and Cell Migration, (2003) *Annu. Rev. Cell Dev. Biol.* **19**, 541-561

Krause, M., Leslie, J. D., Stewart, M., Lafuente, E. M., Valderrama, F., Jagannathan, N., Strasser, G. A., Rubinson, D. A., Liu, H., Way, M., et al., Lamellipodin, an Ena/VASP Ligand, Is Implicated in the Regulation of Lamellipodial Dynamics, (2004) *Dev. Cell* **7**, 571-583

Invitrogen, 2005

Lafuente, E. M., van Puijenbroek, A. A., Krause, M, Carman, C. V., Freeman, G. J., Berezovskaya, A., Constantine, E., Springer, T. A., Gertler, F. B., Boussiotis, V. A., RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion, (2004) *Dev. Cell* **4**, 585-595

Manser, J., Wood, W. B., Mutations affecting embryonic cell migrations *C. elegans* (1990) *Developmental Genetics* **11**, 49-64

Manser, J., Rooprapunt, C., Margolis, B., *C. elegans* Cell Migration Gene*mig-10*Shares Similarities with a Family of SH2 Domain Proteins and Acts Cell Nonautonomously in Excretory Canal Development (1997) *Developmental Biology* **184**, 150-164

Quinn, C., Pfeil, D. S., Chen, E., Stovall, E. L., Harden, M. V., Gavin, M. K., Forrester, W. C., Ryder, E. F., Soto, M. C., Wadsworth, W. G., UNC-6/Netrin and SLT-1/Slit Guidance Cues Orient Axon Outgrowth Mediated by MIG-10/RIAM/Lamellipodin, (2006) *Current Biology* **16**, 845-853

Wei, N.; Tsuge, T.; Serino, G.; Dohmae, N.; Takio, K.; Matsui, M.; Deng, XW. „The COP9 Complex is Conserved Between Plants and Mammals and is Related to the 26S Proteasome Regulatory Complex." *Current Biology*. 1998.

Wei, Ning; Deng, Xing-Wang. „Making Sense of the COP9 Singalsome: a Regulatory Protein Complex Conserved from *Arabidopsis* to Human." *ScienceDirect*. 1999.

Xiaowei, Lu; Horvitz, Robert. "*lin-35* and *lin-53*, Two Genes that Antagonize a *C. elegans* Ras Pathway, Encode Proteins Similar to Rb and Its Binding Protein RbAp48." (1998)Cell **95**, 981–991

Yu, T. W., Bargmann, C. I, Dynamic regulation of axon guidance, (2001) *Nature Neuroscience* **4**, 1169-1176

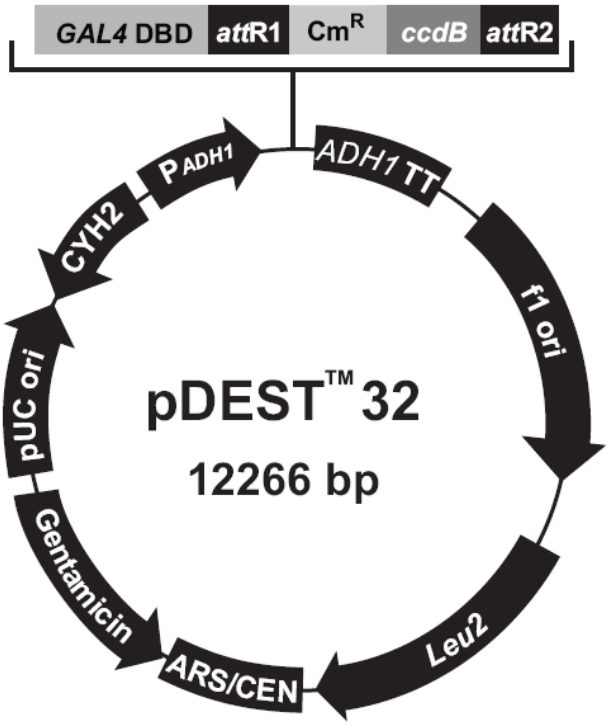
Appendices

Appendix A: Media Recipes

Medium	Recipe (for 1 Liter)
YPAD	Bacto-yeast extract (10 G), Bacto-peptone (20 g), Dextrose (20 g), Adenine sulfate (100 mg)
SC	Yeast nitrogen base without amino acids (6.7 g), Amino acid powder mix (1.4 g) not containing leucine, tryptophan, uracil or histidine, Bacto agar (20 g), After autoclaving add 20 ml 50% glucose
-Leu	To Autoclaved SC Medium add 20 mM (stock conc.) uracil (8 ml), 100 mM (stock conc.) histidine-HCL (8 ml) and 40 mM (stock conc.) tryptophan (8 ml)
-Leu-Trp	To Autoclaved SC Medium add 20 mM(stock conc.) uracil (8 ml), 100 mM(stock conc.) histidine-HCL (8 ml)
-Ura-Leu-Trp	To Autoclaved SC Medium add 100 mM(stock conc.) histidine-HCL (8 ml)
-His-Leu-Trp +65mM 3AT	To Autoclaved SC Medium add 20 mM(stock conc.) uracil (8 ml) and 3AT equivalent to 65 mM 3AT (final concentration)
5 FOA Plates	To Autoclaved SC Medium add 5FOA powder (2 grams) and 20 mM(stock conc.) uracil (8 ml)
Cyclohexamide	To Autoclaved SD medium add Cyclohexamide, final concentration should be 10 ug/ml along with 20 mM (stock conc.) uracil (8 ml), 100 mM (stock conc.) histidine-HCL (8 ml) and 20 mM (stock conc.) leucine (8 ml)
LB	1.0 % Tryptone, 0.5% Yeast Extract, 1% NaCl

Appendix A: Media Recipes for Yeast Two Hyrbrid Experiments, for plated media add 20 g of Bactoagar per liter. Also, for liquid media glucose can be added to media in powder form prior to autoclaving.

Appendix B: Yeast Two Hybrid Vectors



Comments for pDEST™ 32
12266 nucleotides

ADH1 promoter: bases 103-1557
GAL4 DNA binding domain: bases 1581-2024
*att*R1 site: bases 2037-2161
Chloramphenicol resistance (Cm^R) gene: bases 2411-3070
*ccd*B gene: bases 3411-3716
*att*R2 site: bases 3757-3881
ADH1 transcription termination region: bases 4119-4276
f1 origin: bases 4603-5058
Leu2 gene: bases 5767-6861
ARS4/CEN6 origin: bases 7589-8107
Gentamicin resistance gene: bases 8452-8985 (c)
pUC origin: bases 9833-10506
Cycloheximide sensitivity (CYH2): bases 11445-11894 (c)

(c) = complementary strand

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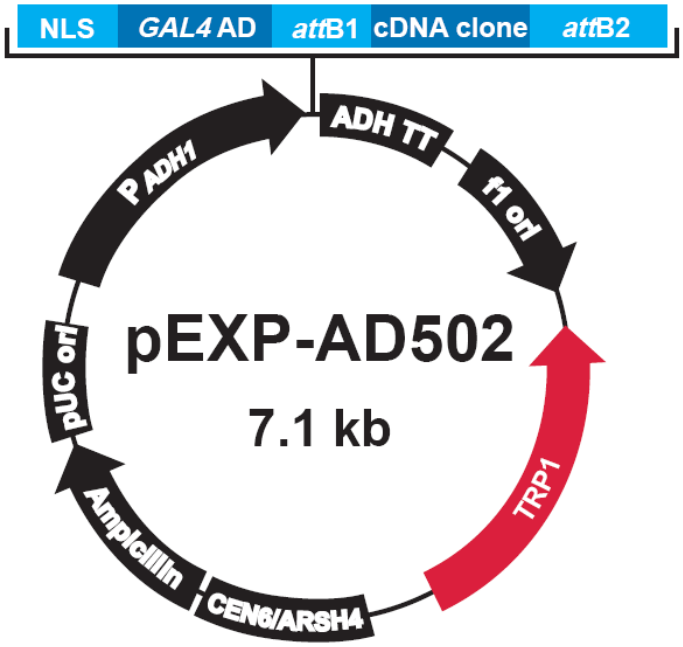
Figure 11:pDEST32 Map (provided by Invitrogen)



Comments for pEXP-AD502 (no insert)
7146 nucleotides

ADH1 promoter: bases 64-1517
Initiation ATG: bases 1523-1525
SV40 nuclear localization signal (NLS): bases 1526-1548
GAL4 activation domain: bases 1556-1903
*att*B1: bases 1910-1934
*att*B2: bases 1979-2003
ADH1 transcription termination region: bases 2045-2511
f1 origin: bases 2703-3158
TRP1 gene (c): bases 3255-3929
TRP1 promoter (c): bases 3930-4031
CEN6/ARSH4 (c): bases 4461-4979
bla promoter: bases 5012-5116
Ampicillin (*bla*) resistance gene: bases 5111-5971
pUC origin: bases 6116-6789
(c) = complementary strand

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Figure 12:pEXP-AD502 Map (provided by Invitrogen)

Appendix C: Table of Screen 1 Results

Table 3: Screen 1 interactor results. Strong interaction means the colony displayed growth well above control, while a weak interactor looked to be only slightly above the level of the control.

Candidate	Interaction Strength	Identity	Transformed with Mig-10A2
p6	Strong	NM_001083203.1	successful
p9	Strong	NM_060151.5	successful
p10	Strong	n/a	n/a
I1	Strong	n/a	n/a
I5	Strong	NM_058687.2	successful
C1	Strong	NM_171472.2	failed
C2	Strong	n/a	n/a
C4	Strong	NM_076382.3	successful
K3	Strong	n/a	n/a
K4	Strong	n/a	n/a
K8	Strong	NM_065823.3	successful
p3	Weak	n/a	n/a
p4	Weak	n/a	n/a
p5	Weak	n/a	n/a
p7	Weak	n/a	n/a
p8	Weak	n/a	n/a
I2	Weak	n/a	n/a
I3	Weak	n/a	n/a
I4	Weak	n/a	n/a
I6	Weak	n/a	n/a
I7	Weak	n/a	n/a
K1	Moderate	n/a	n/a
K7	Moderate	n/a	n/a