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Characterizing Cyclin J By Identifying Conserved Protein-Protein Interactions

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CHARACTERIZING CYCLIN J BY IDENTIFYING CONSERVED PROTEIN-PROTEIN INTERACTIONS

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DEDICATION

I want to thank God and my family for their support and dedicate this work and my upcoming career to them. By God's grace only am I even here to take advantage of this opportunity. For the support and patience of my family, I owe a debt that is incalculable. Through my career, I hope to show my gratitude by serving humanity through facilitating quality research that will improve the quality of life on Earth. Also through my career, I hope to earn the capacity to attempt to repay my parents by easing some of their financial burdens, so they can have more time to relax and enjoy life.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 Introduction

1.1 Cyclins are a superfamily of important regulators

The original Cyclin (Cyclin B) was first identified by Tim Hunt in 1982 by radiolabeling spawned sea urchin eggs to study protein production after fertilization [1]. The appearing and disappearing of this protein seemed to correspond to cycles of cell division [1]. Over the last 30 years, about 30 proteins have been discovered and categorized as members of the Cyclin superfamily. Cyclins are regulatory subunits of Cyclin-dependent kinases (Cdks). Although many members of the family share structural similarity, the one prerequisite feature is the presence of a Cyclin Box domain, a multi-helical motif also present in TFIIB and pRb [2]. The Cyclin Box imparts Cdk binding and activation [2]. A number of different Cdk proteins have been identified, and specific Cyclins form heterodimers with specific Cdks. The Cyclin is the regulatory subunit that activates kinase activity and directs substrate specificity, and the Cdk is the catalytic kinase subunit.

A subset of the Cyclins control the cell cycle [3]. Most of the Cyclins that regulate the cell cycle oscillate and are controlled by regulated degradation, to ensure that the cell cycle moves only in one direction [3]. The cell cycle consists of four stages: G1, which involves growth of the cell in preparation for DNA replication; S phase, which is replication of the DNA; G2, which involves growth of the cell in preparation for cell division; and M phase, the process in which the parent cell divides into two separate daughter cells [3]. The cell cycle Cyclin(s) dominating the landscape at a particular phase determine which Cdk is active and which proteins are phosphorylated [3].

The metazoan cell cycle Cyclins can be divided into four categories, based on the activity that they preside over. G1 Cyclins (Cyclin D), which maintain steady levels throughout the cell cycle, form complexes with Cdk4 that coordinate the cell cycle with extracellular signals and prepare the cell for S phase [4]. G1/S Cyclins (Cyclin E), which accumulate in late G1 and decline in S phase, form complexes with Cdk2 that work cooperatively with G1 Cyclin complexes to drive the cell cycle to enter S phase [4]. S phase Cyclins (Cyclin E and A), which have levels that remain high throughout S phase, form complexes with Cdk2 that promote the activities necessary for DNA replication and G2 [4]. Finally, M phase Cyclins (Cyclin A and B), which have high levels from late G2 through M phase, form complexes with Cdk1 that drive the transition from G2 to M phase and promote activities necessary for cell division [4].

As just stated, many highly studied Cyclins directly regulate the cell cycle, but other Cyclins have functions that are not directly related to the canonical cell cycle. Cyclin T, K, H, and C, for example, form complexes that have been identified as having a regulatory relationship to transcription and RNA Polymerase II function [5-8]. Cyclin F regulates Cyclin B nuclear localization [9]. Cyclin L has been implicated in mRNA splicing [10, 11]. Cyclin O has been shown to be required for the intrinsic apoptosis pathway in lymphoid cells [12]. Cyclin I has been shown to be required for Cdk5 activation in terminally differentiated kidney podocytes [13]. Also, a few Cyclins, for example Cyclin J and Y, are not well characterized.

1.2 Cyclin J is a protein of unknown function

1.2.1 Cyclin J has Cyclin Superfamily properties

Cyclin J was first identified in experiments that used *Drosophila* Cdk1 and Cdk2 as baits in a yeast two-hybrid (Y2H) library screen of a *Drosophila* embryonic cDNA library [14]. Possessing a Cyclin Box domain, Cyclin J is a member of the Cyclin superfamily [15]. Cyclin J is conserved among all metazoans [16]. All of the research done on Cyclin J to date has been done in *Drosophila*. Its mRNA and protein are present in the early embryo, then disappears, only to reappear in adult females [15, 17]. When probing protein extracts with affinity-purified rabbit polyclonal Cyclin J antibodies, Cyclin J was seen in unfertilized eggs, in embryos for the first few hours following fertilization, and in adult females [17].

As mentioned above, Cyclins function as part of a Cyclin/Cdk complex [2, 3]. The identity of the Cdk partner is key to its function and pathway placement. To identify the Cdk partner for Cyclin J, Kolonin et al. performed co-immunoprecipitation (CoIP) using an affinity-purified rabbit polyclonal Cyclin J antibody and lysate from different stages and tissues in *Drosophila* [17]. Probing lysate from unfertilized oocytes and early embryos, Cdk2 co-purified with Cyclin J [17]. In the same experiment, Cdk1 weakly co-purified with Cyclin J, but in lysate from unfertilized eggs only [17]. Again, the primary function of a Cyclin is to bind to and activate the kinase activity of a Cdk partner [3]. Consistent with this, Cyclin J immunoprecipitates made from unfertilized eggs and 0-4hr embryos were able to phosphorylate H1 proteins in an *in vitro* H1 kinase assay [17]. Surprisingly, experiments done by Lehner et al. suggested that the partner for Cyclin J

is Cdk1, and not Cdk2 [16]. The Lehner Lab experiments involved transgenic *Drosophila* lines that produced N and C-terminal GFP-Cyclin J fusion proteins. The method they used to precipitate and detect Cyclin J involved antibodies that bind to the GFP segment. They used Protein A-sepharose beads coated with affinity-purified rabbit GFP antibodies that bind to Cyclin J-EGFP and lysate made from whole ovaries. Here, Cdk1, and not Cdk2, co-purified with Cyclin J [16]. At present, we do not have a clear explanation for the different findings with respect to Cyclin J's Cdk partner. This investigation is designed to help address this question.

Cyclin J does appear to have Cyclin properties *in vivo*. Injecting affinity-purified rabbit polyclonal Cyclin J antibodies or Cyclin J-specific aptamers into embryos disrupted the cell cycle by causing telophase bridges and delayed progression through mitosis [17]. *Drosophila* Cyclin J is also capable of rescuing growth in Cyclin-deficient baker's yeast [15].

1.2.2 *CycJ*-null *Drosophila* mutants appear normal, but a genetic interaction occurs when the piRNA pathway is impaired

What happens when *CycJ* is deleted in flies? The Finley lab and the Lehner lab used the same method, which was deleting the space between two transposon insertion alleles, *XPd07385* and *RBe01160*, that each contain a FLP/FRT recombination site. This stretch of *chromosome 3L* contains *armi*, *CycJ*, and *CG14971*. To achieve single-null flies, all of the three genes were replaced in different combinations in the form of *P elements* [16, 18]. The Lehner lab determined that singular *CycJ*-null flies did not have diminished viability [16], while our lab observed decreased egg laying and decreased

egg hatching [18]. Aside from these differences, both groups concluded that the *CycJ*-null flies appeared normal [16, 18]. The singular *armi*-null mutant phenotype resulted in loss of germline cells and production of only 2 or 3 normal-looking egg chambers per ovariole [16, 18]. That is less than wild-type ovarioles, which average 3 to 5 [16, 18]. *armi* is a member of the transposon-silencing piRNA pathway [19]. The piRNA pathway's most important job is to protect the germline stem cells from transposon activity corrupting protein coding genes and cis elements [20]. It does this by processing transposon-complementary 26-31 nucleotide RNAs, called piRNAs, to direct pathway members to degrade transposon transcripts and silence their production through chromatin remodeling [20]. It was observed that the *armi, CycJ* double-null phenotype was different than the singular *CycJ*-null or *armi*-null, with production of 1 or 2 disorganized, overpopulated egg chambers, followed by the loss of germline cells [18]. This variation to the single-null phenotypes is a genetic interaction with the piRNA pathway that has been verified by ruling out any involvement with the other excised DNA and by reproducing a similar genetic interaction with other piRNA pathway members [18]. Based on this data, *CycJ* appears to be required for processes involved with proper egg chamber formation when the piRNA pathway is compromised.

1.3 Objectives and specific aims

Aside from the observations and facts discussed above, Cyclin J's function is poorly understood. Major unknowns include knowledge of the molecular pathway in which Cyclin J acts, exactly which Cdk is the true partner, the identity of upstream and downstream regulators, and how it interacts with the piRNA pathway. A broad tool that could start the process of elucidating many of these unknowns is studying Cyclin J's

protein-protein interactions (PPIs). Cyclin J's PPIs are likely to play important roles in each of these unknowns. To date, 36 PPIs have been identified using *Drosophila* Cyclin J as a bait in yeast two-hybrid (Y2H) library screens (Table 1) [21, 22]. One drawback of the Y2H assay is that it can detect false-positives [22]. Further testing of PPIs identified with this method is needed to increase confidence in the validity, and these 36 PPIs have yet to receive this scrutiny. Real *in vivo* physical interactions tell the story of the action of each protein, including Cyclin J.

Fly Protein	Fly Protein	Fly Protein	Fly Protein
cdk2	E5	SP2637	CG15043
cdk4	ena	Spn-A	CG15676
cdk5	gus	Stam	CG17508
cks30A	heph	Vm26ab	CG18273
cenG1A	His3.3A	fog	CG2865
Cks85A	MED15	CG11486	CG31204
Cpr64Aa	Pgam5	CG12723	CG5708
CycK	PHDP	CG13510	CG5731
dimm	RAF2	CG13900	CG7518

Table 1. Putative *Drosophila* Cyclin J protein interactions. Listed are 36 Drosophila proteins that were identified in yeast two-hybrid screens.

This project has one specific aim. It is to identify and test for biologically relevant Cyclin J PPIs. I am using approaches that involve two assays to test PPIs. The assays I am employing are the yeast two-hybrid assay and co-affinity purification (CoAP). When a PPI is detected using two different assays, for example Y2H and CoAP, it is more likely to be a true positive [23]. Orthologs of *Drosophila* Cyclin J's PPIs will also be tested. I am comparing *Drosophila*, mosquito, and human orthologs of PPIs. A PPI is also more likely to be a true positive when the PPI is conserved between more than one species.

CHAPTER 2 Materials and methods used to identify biologically relevant Cyclin J interacting proteins

2.1 Yeast two-hybrid

2.1.1 Overview of the yeast-two hybrid system

The yeast two-hybrid assay (Figure 1) is a molecular biology tool that employs fusion protein constructs produced in the nucleus of baker's yeast cells to identify binary PPIs through various reporter strategies [24]. I used a modified LexA system, based on the one described in Gyuris et al. [25]. Specifically, two haploid yeast clones that each produce a fusion protein to be tested are mated, then grown on media that allows two reporter genes to be tested. If the two fusion proteins interact, the reporters activate [14].

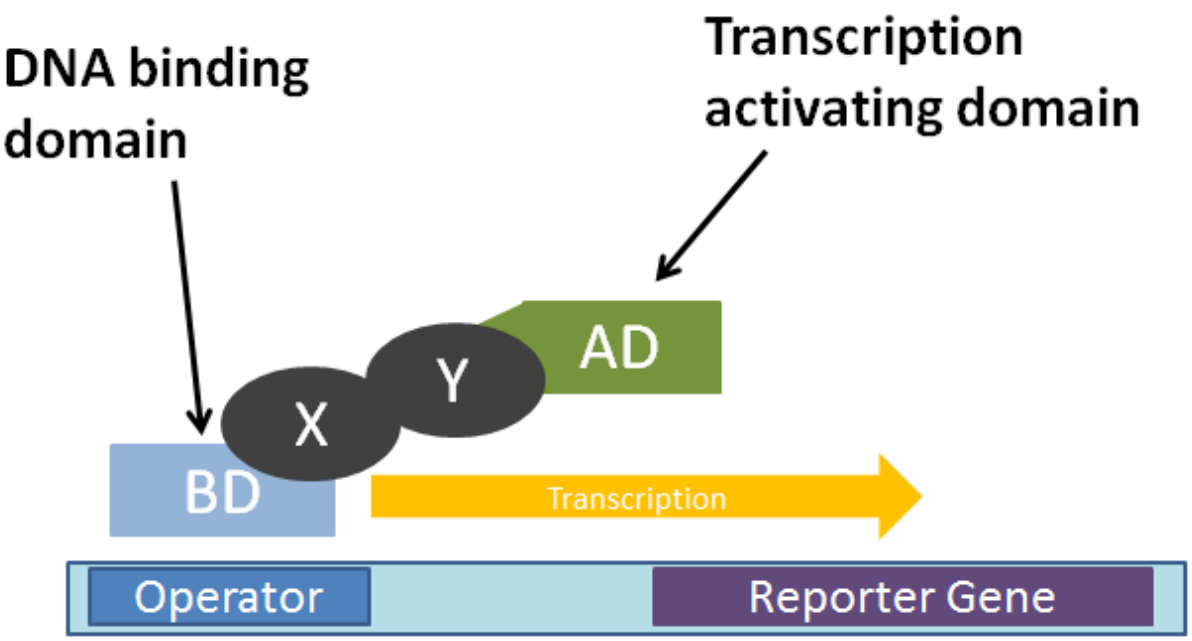


Figure 1. Yeast two-hybrid assay. The DNA binding (BD) and transcription activating (AD) domains of a transcription factor are used to create fusion proteins (BD-X & AD-Y) which activate transcription of reporter genes when they interact.

The test begins with creating yeast spots on solid media. The spots are made by the overlaying of two drops of the liquid haploid yeast cultures on agar plates of solid, rich media, where they will mate, and incubating the plates overnight. The two strains of yeast are called RFY309 and RFY231. Y2H-ready RFY309 has two key features. First, a bait plasmid, *pNLEX(NLS)*, which has a *HIS3* selection marker. This plasmid produces an N-terminal fusion protein (BD-X), made up of your protein of choice (X) and the LexA DNA-binding moiety (BD), whose production is constitutively induced by the *ADH1* promoter. It also has a second plasmid, *pSH18-34*, which contains the *LacZ* reporter. *pSH18-34* has a *URA3* selection marker and eight LexA operators

(*LexAopLacZ*) that control *LacZ* production. When LexA binds and recruits an activating domain, *LacZ* transcription is activated. The product enzyme, β-galactosidase, cleaves the X-gal substrate, that has been added to the media, into a blue product. Y2H-ready RFY231 also has two key features. The prey plasmid, *pJZ4*, has a *TRP1* selection marker that expresses AD-Y. AD-Y is an N-terminal fusion protein that involves your protein of choice (Y) and B42, an 89-amino acid domain that activates transcription in yeast (AD). *pJZ4* expresses AD-Y under control of the *GAL1* promoter, which is induced by galactose and repressed by dextrose. RFY231 also has an integrated DNA construct that consists of LexA operators (*LexAopLEU2*) that control *LEU2*. When transcription is activated, the host yeast cells are viable on leu^- media.

Following overnight growth, the rich media plates with the yeast spots are replica plated onto four indicator plates that select for diploid growth. Each reporter (*LacZ* and *LEU2*) involves a pair of plates. The X-gal indicator plates are $uracil^-$, $histidine^-$, $tryptophan^-$ (to select for diploid cells) plus the X-gal substrate, with one containing galactose, maltose, and raffinose (UHW-grmX) and the other containing dextrose (UHW-DX) as carbon sources. The leu^- indicator plates are $uracil^-$, $histidine^-$, $trypophan^-$, and $leucine^-$, with one containing galactose, maltose, and raffinose (UHWL-grm) and the other containing dextrose (UHWL-D) as carbon sources. Again, dextrose represses and galactose activates production of AD-Y.

After 3 days of growth, the spots on the indicator plates are scored based on their reporter activity. All reporter activity present on dextrose plates can only be an artifact of activation by BD-X itself. Thus, if yeast turns blue on UHW-DX plates and/or grows on UHWL-D, the BD-X activation will interfere with the interaction test. On

galactose^{+} media though, the prey is now potentially able to interact with the bait. If the BD-X has proven to not be self-activating, this reporter activity or growth is said to be galactose-dependent. If the AD-Y successfully pairs with the BD-X, growth on leu^{-} plates and/or blue color on X-gal plates are signals for an interaction. The overall score is the sum of the scores on *LacZ* and leu^{-} plates (see Figure 2). G-D scores are measured by the total score on the galactose plates minus the total score on the dextrose plates.

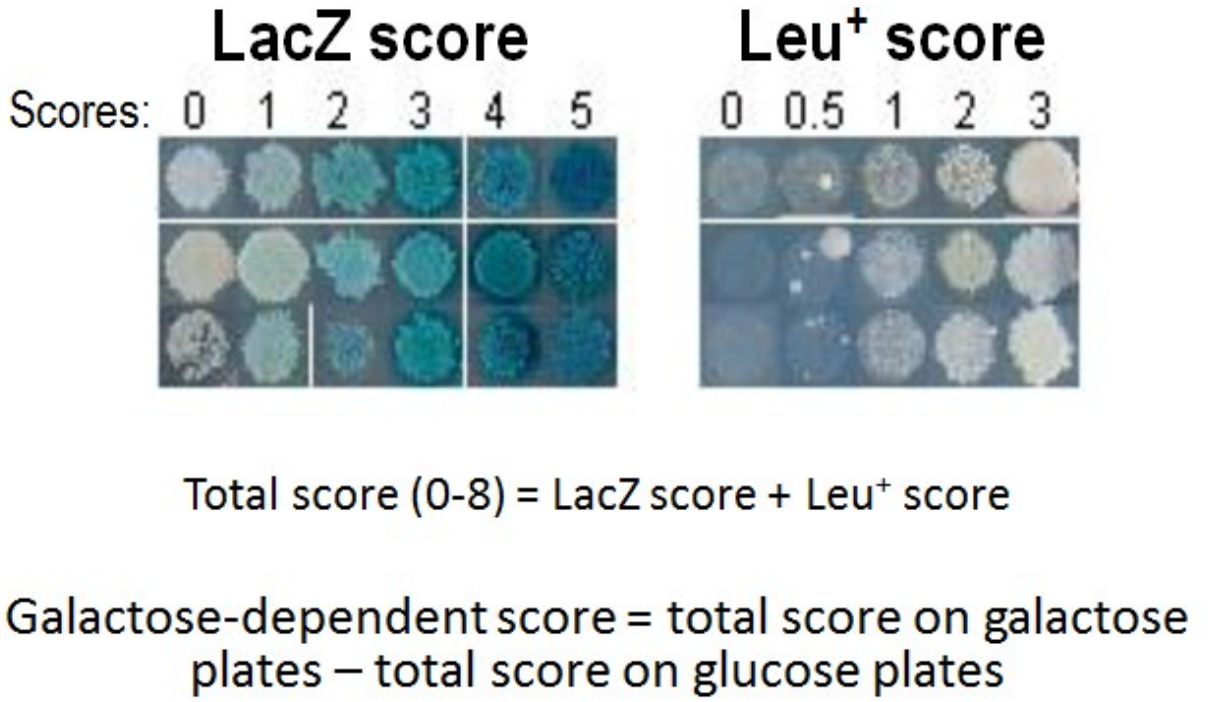


Figure 2. Assessing and calculating yeast two-hybrid reporter scores. Protein interactions are assessed based on the reporter activity on the indicator plates (UHW/grmX, UHW/DX, UHWL/grm, & UHWL/D). U=uracil$^-$, H=histidine$^-$, W=tryptophan$^-$, L=leucine$^-$, g=galactose carbon source, r=raffinose carbon source, m=maltose carbon source, D=dextrose/glucose carbon source, X=addition of the X-gal substrate to the solid media. The *LacZ* reporter is measured by the darkness of the blue color created by the cleavage of X-gal into a blue product by β-galactosidase. Production of β-galactosidase is initiated by interaction of BD-X and AD-Y. The stronger the interaction, the darker the color produced. The LacZ score (numbered 0-5) = the UHW/grmX score (galactose-associated activation) – UHW/DX (background activation). The leucine reporter is measured by the growth of yeast on leucine$^-$ solid media. Production of leucine is initiated by interaction of BD-X and AD-Y. The stronger the interaction, the greater the growth. The Leu$^+$ score (numbered 0-3) = the UHWL/grm score (galactose-associated activation) – UHWL/D (background activation).

This framework lends itself to many applications, so Y2H assays range from individual pair screenings to high-throughput proteome mapping [21, 22]. Its versatility is an asset, but Y2H is subject to the limitations that come with testing PPIs within the microenvironment of the yeast nucleus. Besides instances where BDs cause high background reporter activation, certain ADs can activate reporters regardless of the BD, which may occur when AD-Y contains a prey protein able to interact with many proteins, due to the nature of its chemical makeup. PPIs involving these “sticky” proteins are comprised of mostly false positives.

2.1.2 Yeast two-hybrid library screens

Pre-mating library tests

I conducted yeast two-hybrid library screens to identify human and mosquito Cyclin J interactors using the protocol described in, “Interaction mating methods in two-hybrid systems,” as detailed below [26]. Before the assay begins, each bait must be tested against the library being screened, plus a control library (consisting of just the empty AD vector *pJZ4* with no cDNA inserts), to determine whether a library screen is feasible and how many clones must be tested to achieve significant results. The steps are below.

The first step is to prepare bait/library mixtures. I grew a 30mL culture of the bait yeast strain in a 250mL flask in liquid uracil$^-$, histidine$^-$, dextrose yeast media overnight shaking at 30˚C. In the morning, I measured the OD_{600} and took a portion of the culture to inoculate another 30mL liquid culture in a 250mL flask, to grow under similar conditions until mid to late log phase. An OD_{600} of 1.0 = 2 x 10^7 cells/mL. The human

bait culture OD_{600} was 0.980, which equals 1.96 x 10^7 cells/mL. The mosquito bait culture OD_{600} was 0.975, which equals 1.95 x 10^7 cells/mL. For each mixture, the bait strain must contain double the cells of the library strain to ensure that every library clone is able to mate with an available bait yeast clone. The HeLa cDNA library contains 4.0 x 10^7 colony forming units (cfu)/100µL. I mixed aliquots of 200µL of HeLa cDNA library yeast cells and 8.2mL of human Cyclin J bait yeast culture in 50mL Falcon tubes. For each tube, I spun the contents down at 1000 x g at room temperature and poured out the supernatant. I twice added 10mL of water and spun down at the same rate to wash the cells. Following the second wash and spin down, I resuspended the pellets in 2.5mL of liquid yeast extract, peptone, dextrose (YPD) media and 2.5mL of freezing solution, made 1 mL aliquots of the mixture in Eppendorf tubes, and froze all but one of the aliquots at -80˚C. The control library contains 2.55 x 10^7 cfu/125µL. I mixed 250µL of control library yeast cells and 2.6mL of human Cyclin J bait yeast culture in 50mL Falcon tubes. I spun down, washed, resuspended, and froze all but one of the aliquots in the same manner as done previously. I prepared the mixture of mosquito Cyclin J and the mosquito and control libraries in a similar manner as done with the human Cyclin J mixtures. The mosquito cDNA library contains 3.5 x 10^7 cfu/100µL. I mixed aliquots of 200µL of mosquito cDNA library yeast cells and 7.2mL of mosquito Cyclin J bait yeast culture in 50mL Falcon tubes, spun down, washed, resuspended, and froze all but one of the aliquots in the manner I have described. I mixed 250µL of control library yeast cells and 2.6mL of mosquito Cyclin J bait yeast culture in 50mL and again performed the same steps that ended in freezing all but one of the aliquots. Following the procedure described above, I was ready to do the pre-mating library tests. Through this,

I also created the frozen mixtures of human and mosquito Cyclin J bait yeast cells, in mixtures with the HeLa and mosquito libraries respectively, for the actual screen at the ratios necessary for proper mating.

With the bait/library mixtures prepared and ready, the next step was to test their effectiveness in this Y2H format. I used the bait/control library mixtures to grow colonies on solid UHWL/grm plates to determine leu^+ diploids and UHW/grm plates to determine the number of diploids (diploid forming units or dfu) in each aliquot. With this information, I could calculate the number of leu^+ colonies that arise by transactivation by the bait alone per 100µL versus the number of diploids formed per 100µL. This is the transactivation potential. A transactivation potential of less than 1×10^{-5} leu^+ colonies/dfu is ideal for conducting a Y2H library screen. When factoring in the number of individual clones contained in the test library, the number of colonies needed to saturate the library can be calculated. I used the bait/HeLa and mosquito cDNA library mixtures to grow colonies on solid UHWL/grm and UHW/grm plates so that I could calculate the number of leu^+ colonies produced per 100µL versus the number of diploids formed per 100µL. Analysis of this result tells us how much of the bait/HeLa or mosquito cDNA library mixture is needed to produce the desired number of leu^+ colonies.

I made 300µL aliquots of bait/control library and bait/HeLa and mosquito cDNA library mixtures at 10^{-4}, 10^{-5}, and10^{-6} concentrations in sterile water and plated 100µL of the contents onto 128x86mm yeast peptone dextrose (YPD) plates, in which the haploid yeast mate more effectively. I also made 300µL aliquots of bait/control library and bait/HeLa and mosquito cDNA library mixtures at undiluted, 10^{-1}, 10^{-2}, and 10^{-3} concentrations in sterile water and plated 100µL of the contents onto 128x86mm YPD

plates for mating. I incubated all these plates at 30˚C overnight. The next day, I used sterile technique with a glass scraper to remove all yeast that grew on each plate into 50mL Falcon tubes. I washed the yeast in the tubes with 10mL of sterile water, spun down the tubes at 1000 x g for 3 minutes, and poured out the supernatants. I then added 500µL of liquid UHWL/grm media to all the tubes with 10^{-4}, 10^{-5}, and10^{-6} concentations, mixed the contents, and plated them onto 128x86mm petri plates containing solid UHWL/grm media. I also poured 500µL of liquid UHW/grm media into all the tubes with the undiluted, 10^{-1}, 10^{-2}, and 10^{-3} concentrations, mixed the contents, and plated them onto 128x86mm petri plates containing solid UHW/grm media. I incubated all these plates at 30˚C for 3 days. Following that, I counted the colonies produced, multiplied the numbers by the dilution factor, and calculated the average colonies produced by 100µL for each combination.

The average number of human Cyclin J bait/control library colonies that grew on UHWL/grm plates was 1 per 100µL. The average number of human Cyclin J bait/control library colonies that grew on UHW/grm plates was 9.3 x 10^5 per 100µL. The average number of human Cyclin J bait/HeLa cDNA library colonies that grew on UHWL/grm plates was 7 per 100µL. The average number of human Cyclin J bait/HeLa cDNA library colonies that grew on UHW/grm plates was 2.9 x 10^6 per 100µL.

The average number of mosquito Cyclin J bait/control library colonies that grew on UHWL/grm plates was 785 per 100µL. The average number of mosquito Cyclin J bait/control library colonies that grew on UHW/grm plates was 1.9 x 10^7 per 100µL. The average number of mosquito Cyclin J bait/mosquito cDNA library colonies that grew on UHWL/grm plates was 4.6 x 10^3 per 100µL. The average number of mosquito Cyclin J

bait/mosquito cDNA library colonies that grew on UHW/grm plates was 1.4 x 10^7 per 100µL.

The transactivation potential of the human Cyclin J bait is 1.0 x 10^{-6} leu^+ colonies/dfu. To screen all of the 1 x 10^7 individual clones contained within the HeLa cDNA library, I needed to plate at least 10^7 dfu and could expect 10 leu^+ colonies to form from the bait alone. The human Cyclin J/HeLa cDNA library mixture gave rise to 2.0 x 10^{-6} leu^+ colonies/dfu. 1 leu^+ colony is formed per 100µL. I attempted to produce 100-150 colonies, I used 1.4mL of the human Cyclin J/HeLa cDNA library mixture to perform the screen.

The transactivation potential of the mosquito Cyclin J bait is 4.2 x 10^{-5} leu^+ colonies/dfu. To screen all of the 1 x 10^7 individual clones contained within the mosquito cDNA library, I needed to plate at least 10^7 dfu and to expect 430 leu^+ colonies to form just from the bait alone. The mosquito Cyclin J/mosquito cDNA library mixture gave rise to 3.3 x 10^{-4} leu^+ colonies/dfu. 815 leu^+ colonies are formed per 100µL. I chose to attempt to produce 576 colonies, so I used 50µL of the mosquito Cyclin J/mosquito cDNA library mixture to perform the screen.

Mating the bait and library, and selection of positives

I thawed one aliquot of the human Cyclin J/HeLa cDNA library mixture and prepared to plate 1mL, and I also thawed one aliquot of the mosquito Cyclin J/mosquito cDNA library, removed 50µL, and added 950µL of sterile water to prepare to plate a final volume of 1mL. I plated the two 1mL preparations onto separate 128x86mm YPD plates and incubated them at 30˚C overnight. The next day, I used sterile technique with

a glass scraper to remove all yeast that grew on the two plates into two 50mL Falcon tubes. I washed the yeast in the tubes with 10mL of sterile water, spun down the tubes at 1000 x g for 3 minutes, and poured out the supernatants. I then added 3mL of liquid UHWL/grm to the tubes, mixed the contents, and plated them onto large 22cmx22cm square petri containing solid UHWL/grm media. I incubated the plates at 30˚C for three days. I picked 77 yeast colonies from the human Cyclin J/HeLa cDNA library plate, 576 yeast colonies from the mosquito Cyclin J/mosquito cDNA library plate, and placed the colonies into wells of separate 96-well plates with 200µL of liquid UHW/D media to turn off production of the AD fusion protein. I incubated the cultures while shaking at 30˚C for 3 days. I used the Biomek NX-MC robot (Beckman Coulter) to transfer 3µL of each well to YPD plates to form circular colony spots and incubated the spots at 30˚C overnight. The next day, I replica plated the spots onto the four indicator plates using a sterile velvet cloth and incubated them at 30˚C for 3 days. I took photos and scored the reporter activity using the G-D method.

Human Cyclin J / HeLa cDNA library mating

The human Cyclin J/HeLa cDNA library screen plates resulted in strong growth of all spots on the UHWL/D plates, most likely due to activation by the BD alone. The combination of that result plus almost no difference in activation of the X-gal reporters between the spots on the UHW-Dx and UHW/grmx plates is a scenario where interactions between human Cyclin J and HeLa cDNA library may not be able to be screened using this format. To rule out errors in performing the mating, I performed a second mating. That mating resulted in a similar outcome, so I discontinued the human Cyclin J/HeLa cDNA library screen at that point.

Mosquito Cyclin J / mosquito cDNA library mating

Initially, I tested 576 diploid clones that resulted from the mating of mosquito Cyclin J and the mosquito cDNA library. Following initial scoring the interactions, I chose a wide variety of scores to continue testing, because biologically relevant PPIs may or may not be strong interactions *in vivo*. Also, only choosing to continue testing a small subset of reporter scores may result in choosing many of the same clones. A broad base of reporter scores insures against that outcome.

Selecting and preparing clones for re-testing

I set out to reduce the number of plates being handled from 6 to 4. I wrote a program that was used by the Biomek NX-S8 (Beckman Coulter) to transfer 30µL of each of the clones from the original liquid cultures to new 96-well plates with fresh liquid UHW/D media.

I re-tested the 378 prospective mosquito Cyclin J interactors by sub-cloning them into fresh yeast and mating these yeast with the Cyclin J bait. This process begins with colony PCR of the diploids that have been re-arrayed for further testing. To make solid cultures to colony PCR, I used the Biomek NX-MC to transfer 3µL of each well to solid W/D plates and incubated the spots at 30˚C overnight. Next, I performed a colony PCR of each spot to create a reservoir of ORF sequence to use for further study. I used GoTaq Polymerase (Promega), the primers *BCO1* and *BCO2*, which amplify the library inserts, and a small bit of cells from the colony spots in a 30µL total volume to perform the reaction. I tested for the presence of a PCR product by electrophoresing 5µL from each reaction on a 0.9% agarose gel. About 70% of reactions appeared successful.

In an effort to reduce the potentially high number of duplicate clones, I restriction digested each colony PCR reaction and electrophoresed the reactions on 4% agarose gels to compare the banding patterns of the digests. Duplicate clones will produce identical digested patterns. Each reaction involved Alu I (NEB), buffer 2 (NEB), and an aliquot from the colony PCR reaction. Following the analysis of the gels, I concluded that no duplicate clones were obvious among the clones contained in the four plates being tested. This result was surprising and is unlikely to be a reflection of the true composition. I concluded that the restriction digests contained many incomplete digests, where the presence of intermediate products confounded the process of analyzing the gel for duplicates. With the exorbitant amount of time to produce and analyze the colony PCR products and the amount of materials needed to conduct the restriction digest tests, I decided to move to the next step without eliminating any clones from contention.

The next steps involved harnessing the yeast gap repair mechanism to produce yeast with newly-made AD plasmids that contain copies of the inserts made from the colony PCR of the re-arrayed clones. Gap repair uses the process by which the yeast cells repair double-strand breaks by inserting a PCR product into a linearized plasmid where each has homologous ends that the yeast machinery uses to join together. I used linearized *pRF4-5*$_o$, an AD plasmid similar to the original AD plasmid used in the mosquito cDNA library, and the PCR products, which contained the cDNA library ORF DNA and the necessary flanking DNA that is homologous to the ends of the linear *pRF4-5*$_o$.

Before performing the reaction, I made competent yeast that readily were able to execute the gap repair reaction. I grew RFY231 cells in liquid YPD until late log phase,

then centrifuged them at 1000 x g at 4˚C for 15 minutes, washed them with sterile water, and centrifuged them again under the same conditions. Then, I suspended them in TrisHCl/LiAc/EDTA (TLE), DMSO, salmon sperm DNA, and Eco RI and Xho I linearized *pRF4-5*$_o$. Following that, I distributed the cell suspension into 1.5 mL tubes and froze them at -80˚C.

With the competent cells prepared, I began the gap repair procedure. It first involved mixing together 5μL of each of the colony PCR reactions, with 10μL of competent RFY231 cells, and 30μL of PEG/TrisHCl/LiAc/EDTA (PTLE) in each well of a 96-well PCR plate at room temperature. Next, I incubated the plate on a heating block at 30˚C for 30 minutes and 42˚C for 15 minutes. After allowing it to cool to room temperature, I added 70μL of liquid W/D+15% glycerol media to each well. Following that step, the reaction was ready to plate onto solid media. I used the Biomek NX-S8 to distribute 30μL of each reaction onto a large, 46 partition 22cmx22cm square petri containing solid W/D media. With four 96-well plates to process, I performed the above steps for each and ended up with 8 of the large partitioned plates with a gap repair reaction in each partition. I incubated each of these at 30˚C for approximately 3 days. Following that incubation, many of the partitions contained yeast colonies. I used a sterile toothpick to transfer a colony from each partition that contained one to a well on four new 96-well plates that contained 120μL of liquid W/D+15% glycerol media and incubated the plates at 30˚C for 3 days while shaking.

Re-testing the Cyclin J interactions

The yeast in the four 96-well plates contained inserts that were created by colony PCR of the inserts from the initial prospective mosquito Cyclin J interactors. The next step was to mate these freshly created yeast clones with the mosquito Cyclin J-BD yeast to test the reproducibility of the interactions. Following that, I also mated all the prospective interactors with several control BDs. Some AD fusion constructs produce positive interaction scores with many BDs, likely having more to do with the nature of these "sticky" AD proteins rather than any type of biologically relevant PPI. By testing these prospective interactors in this manner, the reporter scores that resulted from matings with control BDs provided information to help determine which of these clones in the four plates were high-confidence interactions.

I grew a fresh batch of RFY309/*pNLEXattR-aaecycj* in liquid UH/D media at 30˚C while shaking overnight. I aliquoted about 120µL of the culture into each well of a 96-well PCR plate to aid in the robot work. I used the Biomek NX-MC to distribute 3µL drops to make spots of the liquid culture onto 4 solid YPD plates. I also took the 4 PCR plates of the newly-made gap repaired-yeast cultures that contained the prospective interactors and used the robot to distribute 3µL drops of each culture on top of those spots on the YPD plates. I incubated the plates at 30˚C overnight and then replica plated each plate onto the 4 indicator plates using a sterile velvet cloth. I incubated the indicator plates at 30˚C for 3 days, took photos, and scored the reporter activity. Many of the spots from the YPD plates failed to grow on the indicator plates. This indicates that the spots did not have diploid yeast. Many of the colony PCR reactions appeared to have failed, suggesting that the gap repair reaction would not have been successful

either. The empty spots corresponded to these. Of the 384 clones I attempted to reproduce through gap repair, 271 were successfully sub-cloned and re-tested. Of those 271, 239 had positive interaction scores.

Specificity test

I also assayed five control BDs with the prospective ADs to best anticipate which interactions had the highest confidence. The control BD plasmids were already constructed and were members of the lab's plasmid bank. I obtained samples of *pRFHM202-cdi5* (*Drosophila* Cyclin J), *pRFHM12-cdc2* (*Drosophila* Cdk1), *pRFHM13-cdc2c* (*Drosophila* Cdk2), *pRFHM1-Bicoid*, and *pNLEXattR-aaeCyclin Y*, all various forms of N-terminal DNA-binding fusion proteins. I used the LiAoc yeast transformation protocol to transform RFY309 with each of these BD plasmids. Following this, I was ready to test the control BDs against the prospective ADs in Y2H assays. I mated the BD and AD yeast in the same format as previously described. I cataloged the reporter scores of each BD with each AD and used the scores in my analysis of the prospective PPI's confidence.

2.1.3 Directed yeast two-hybrid assays

I used the yeast two-hybrid assay to test for interactions involving the human or mosquito orthologs of *Drosophila* Cyclin J interactors. The constructs I used in this screen are listed in Figure 3. I used the LiAoc yeast transformation protocol to transform RFY309 yeast with the BD vectors and RFY231 yeast with the AD vectors. I transferred single yeast colonies from these reactions into wells of 96-well plates with the appropriate selective liquid media and incubated them at 30˚C for 3 days while shaking.

I performed the matrix screen by using a multichannel pipettor and a 96-well placement guide to manually overlay 5µL of liquid cultures to make yeast spots on YPD plates. I incubated the plates at 30˚C overnight and then replica plated each plate onto the 4 indicator plates using a sterile velvet cloth. I incubated the indicator plates at 30˚C for 3 days, took photos, and scored the reporter activity.

BD Domains	AD Domains	AD Domains
dmCyclin J	aaeSpndle-A	hsSpndle-A
aaeCyclin J	aaeCdk1	hsCdk1
hsCyclin J	aaeCdk2	hsCdk2
aaeCdk1	aaeCyclin J	hsCyclin B
hsCdk1	aaeCyclin B	hsCyclin J
aaeCdk2	aaeCks85A	hsCks1B
hsCdk2	aaeCks30A	hsCks2
dmBicoid	aaeGus	hsGus
aaeCyclin Y		hsCdk5
hsCks2		AD only
hsSpindle-A		
hsCdk5		
hsCyclin Y		
hsGus		
aaeCks30A		
aaeGus		

Figure 3. Directed yeast two-hybrid assays design. Listed are the constructs used in the directed yeast two-hybrid assays. There are 16 BDs, 17 ADs, plus 1 AD only control. Each BD is tested against each AD.

2.2 Co-affinity purification to test protein interactions

2.2.1 Overview of the co-affinity purification system

Co-affinity purification (CoAP) is another common tool of choice in testing PPIs (Figure 4). Our lab uses a Cu^{2+}-induced Gal4 expression system to make NTAP-X and Myc-Y N-terminal fusion proteins in *Drosophila* S2 or S2R+ cells. If X and Y interact, Myc will CoAP with NTAP. Each culture is co-transfected with plasmids expressing the

pair of putative interactors, or one with an empty NTAP vector control, and *pMT-Gal4* (plasmid bank #966). Expression of the NTAP and Myc fusion proteins is controlled by a *UAS* promoter responsive to Gal4, which itself is induced by $CuSO_4$ action on the *metallothionein* promoter of *pMT-Gal4*. The CoAP is performed by incubating protein lysate with agarose beads coated with rabbit IgG antibodies. The NTAP tag stably binds to the IgG beads. The reaction is washed repeatedly and then Western blot is performed for NTAP and Myc. The Myc-tagged protein will only be present if it was "pulled down" by the NTAP-X construct. All "pull downs" must be compared to an NTAP-vector-only control to prove that what is being "pulled down" is the result of the protein in question and not the NTAP itself. When *Drosophila* cells are used to test *Drosophila* proteins, the cellular environment may be well-suited to provide the mechanisms to bestow the native post-translational modifications. This ability makes CoAP superior to Y2H when it comes to testing PPIs of proteins that exhibit certain requisite post-translational modifications, but it is still fallible. Many proteins fail to be produced effectively for CoAP. Whether it be the wrong post-translational modification, the lack of a necessary stabilizing partner molecule, or a total incompatibility with the cell type, some protein combinations cannot effectively be tested by CoAP.

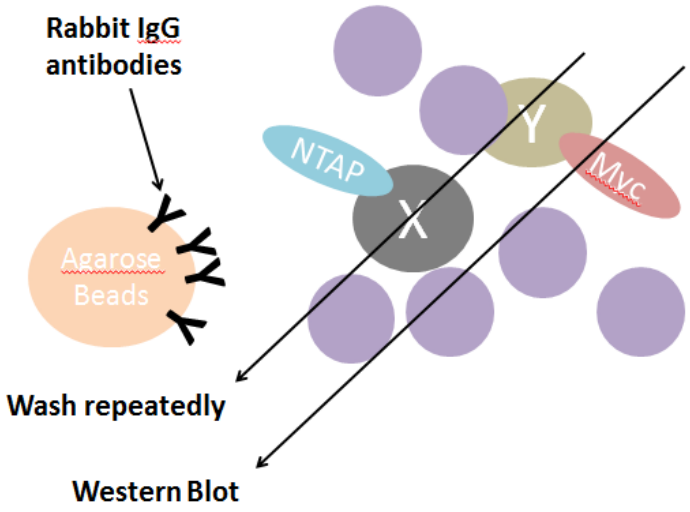


Figure 4. Co-affinity purification. X and Y are proteins being tested for interaction, and N-terminal fusion proteins are made to simplify binding and detection. NTAP-X and Myc-Y are co-expressed in S2R+ or S2 cells and harvested. The lysate is incubated with rabbit IgG antibodies that bind NTAP constructs. The mixture is incubated on ice and washed with cold lysis buffer 5 to 10 times. Following washing, the mixture is Western blot for NTAP and Myc. If Myc-Y is co-detected with NTAP-X and not with an NTAP-only control, the test is positive for a PPI between X and Y.

Y2H and CoAP techniques are complementary to each other in the information they yield. Y2H technologies make the screening of whole cDNA libraries a straightforward endeavor. The troublesome effects of the sometimes high false-positive rate in Y2H PPIs are mitigated by further testing. CoAP is a very powerful tool for detecting PPIs that fills this need. CoAP has two main advantages that complement the simple, yet limited, ability of Y2H assays. Growing properly folded, functional fusion proteins is best done in a system that matches the native conditions as closely as possible. Growing the fusion constructs in conditions similar to the native cell environment, as opposed to the nucleus of yeast as in Y2H, promotes the addition of

post-translational modifications that may be key in functional proteins. This is one advantage. As all proteins that associate with the bound NTAP-X are “pulled down,” distant, yet biologically relevant relationships can be demonstrated. While this can be considered an advantage, it can complicate the picture of what is interacting with what. The ability for Y2H to discriminate between the binary and complex interactions supports the case for these two techniques being an effective combination, when used in tandem.

To carry out CoAP, 2 to 3 million S2R+ cells are seeded in approximately 2mL of Schneider’s liquid media in a cell culture dish. After 24 hours, Effectine Transfection Reagent (Qiagen) is used to transfect the cultures with the desired combinations of the 3 necessary plasmids (the NTAP-X plasmid, the Myc-Y plasmid, and *pMT-Gal4*). About 18 hours later, $CuSO_4$ is used to induce plasmid expression of NTAP-X and Myc-Y. Approximately 72 hours after that, the cells are harvested, protein extracts are made, and protein concentration is tested using a Bradford assay (Biorad). Following both an NTAP and Myc Western to verify that the proper size protein constructs were produced, the CoAP is conducted. An aliquot of Rabbit IgG coated agarose bead solution is placed in a 1.5mL tube. 250-500µg of protein extract is added to the tube and the 5-10 rounds of washing with fresh lysis buffer, centrifugation, and removal of the supernatant is performed. Through this process, all contaminants that do not bind the NTAP construct are removed. The purified protein extract is analyzed by Western blot again for NTAP and Myc. The verified presence of the Myc-tagged protein and the NTAP-tagged protein in the same CoAP sample is a positive PPI result if the empty vector control fails to retain the Myc-tagged protein.

2.2.2 Co-affinity purification of orthologs of *Drosophila* Cyclin J protein interactions

The results of the CoAP experiments are not known at the time of this report, but the design of the first set of CoAPs is in Figure 5. I used the aaeCyclin J, aaeCyclin B, aaeCdk1, aaeCdk2, and aaeCks85A entry vectors and the *pHZ13attR* (NTAP fusion) and *pHZ12attR* (Myc fusion) destination vectors in an LR reaction to create the plasmids for the CoAP test.

NTAP fusion	Myc fusion
NTAP-aaeCyclin J	Myc-aaeCks85A
NTAP-aaeCyclin J	Myc-aaeCdk1
NTAP-aaeCyclin J	Myc-aaeCdk2
NTAP-aaeCyclin B	Myc-aaeCdk1
NTAP-aaeCyclin B	Myc-aaeCdk2
vector only	Myc-aaeCks85A
vector only	Myc-aaeCdk1
vector only	Myc-aaeCdk2

Figure 5. Co-affinity purification design. Listed are the constructs used in the co-affinity purification (CoAP) test of previously identified Cyclin J PPIs. NTAP-X fusion constructs or vector only controls are co-expressed with the adjacent Myc-Y fusion constructs and are tested for CoAP.

2.3 Plasmids and strains

Entry vectors (Appendix B)

Destination vectors (Appendix B)

Yeast strains (Appendix B)

Constructing the Cyclin J baits (human)

I began the construction of the human Cyclin J bait by withdrawing the *pDONR223-hscycj* Gateway (Invitrogen) entry plasmid from the hORF cDNA library [27]. I used *M13F* and *M13R* to sequence the ORF and flanking regions of *pDONR223-hscycj*. The sequence file names are hsCycJF-M13F(-21) and hsCycJR-M13R. These sequence results encompassed the whole human *CycJ* ORF. *pDONR223-hscycj* corresponds to human Cyclin J isoform 2 (GenBank accession: NP_061957.2), is a perfect match to it, and is in the expected reading frame. I was now ready to create the bait plasmid. I used *pDONR223-hscycj* and the destination plasmid *pNLEXattR* in an LR reaction (Invitrogen) to create *pNLEXattR-hscycj*. The final step in creating the bait was transforming the proper yeast with the bait plasmid. I used an LiAoc yeast transformation protocol to transform RFY309 with *pNLEXattR-hscycj*. The bait yeast was selected on solid uracil$^-$, histidine$^-$, dextrose yeast plates.

Constructing the Cyclin J baits (mosquito)

I used Herculase (Stratagene) high-fidelity polymerase and *aeCycA3F* & *R* primers to PCR-amplify the mosquito Cyclin J ORF from the mosquito cDNA library [28]. Next, I used Herculase, *DM1* & *DM2* primers, and the previous PCR product to PCR-

amplify a new product that is the mosquito Cyclin J ORF flanked by full *attB1* & *attB2* sites. With these sites, the PCR product was ready to be cloned into the Gateway entry plasmid. I used this PCR product and *pDONR221* (Invitrogen) in a BP reaction (Invitrogen) to create *pDONR221-aaecycj*. I used *M13F* and *M13R* to sequence the ORF and flanking regions of *pDONR221-aaecycj*. The sequence file names are aaeCycJF-M13F(-21) and aaeCycJR-M13R. These sequence results encompassed the whole mosquito *CycJ* ORF. *pDONR221-aaecycj* corresponds to the putative *Aedes aegypti* Cyclin A3 (NCBI Reference Sequence: XP_001653129.1), which is considered *Aedes aegypti* Cyclin J. This construct does have an N294H amino acid inconsistency with that sequence and is in the expected reading frame. I was now ready to create the bait plasmid. I used *pDONR221-aaecycj* and the destination plasmid *pNLEXattR* in an LR reaction (Invitrogen) to create *pNLEXattR-aaecycj*. The final step in creating the bait was transforming the proper yeast with the bait plasmid. I used an LiAoc yeast transformation protocol to transform RFY309 with *pNLEXattR-aaecycj*. The bait yeast was selected on solid uracil$^-$, histidine$^-$, dextrose yeast plates.

Constructing the directed assays baits and preys

I cloned the other ORFs for the screen from either the HeLa or mosquito cDNA library, or I obtained entry clones through the hORF cDNA library [27]. For a list of the constructs used in the directed assays, see Figure 3. I used Herculase high-fidelity polymerase and the appropriate primers (see Appendix A) to PCR-amplify the selected human and mosquito ORFs flanked by the *attB1* & *attB2* sites necessary for the BP reaction, and then I performed the BP reaction to create Gateway entry vectors (see Appendix B). After sequencing and analyzing these entry vectors I made or obtained

from the hORF library in a manner similar to the human and mosquito Cyclin J baits, I was able to verify that all the entry vectors matched the desired ORFs. Details of these constructs are in the Finley Lab plasmid bank. I used the constructs to perform several LR reactions to create the destination vectors, then used an LiAoc yeast transformation protocol to transform the appropriate yeast strain in order to conduct the directed assays.

Hela cDNA library

Made by Jeno Gyuris [25], the library is comprised of cDNA from HeLa cells grown in 5% serum on plates to 70% confluence. cDNAs are inserted into *pJG4-5*, a Y2H vector used to make B42 activating domain N-terminal fusion proteins. The vector is *TRP1*$^{+}$, and a *GAL1* promoter is used to drive fusion protein production. The cDNA library contains 9.8 x 10^{6} individual members, >90% of which contain a cDNA insert whose average size ranges between 1 kb and 2 kb.

Mosquito cDNA library

Made by Dumrong Mariang [28], the library is comprised of pooled cDNA from ten stages of Aedes aegypti development. The cDNA is inserted into *pRF4-5*$_{o}$, a Y2H vector used to make B42 activating domain N-terminal fusion proteins. The vector is *TRP1*$^{+}$, and a *GAL1* promoter is used to drive fusion protein production. The cDNA library includes more than 10^{7} individual members, 64% of which contain a cDNA insert whose average size ranges between 0.3 kb and 4 kb. The average size of the inserts is 1.4 kb.

CHAPTER 3 Identification of Cyclin J interacting proteins in mosquito and human

3.1 Introduction

To identify biologically relevant Cyclin J PPIs, I performed two yeast two-hybrid (Y2H) library screens and a Y2H directed assay. More information about the Cyclin J PPI network would be a useful resource in the quest to shed light on the pathways in which Cyclin J acts. Thirty-six putative PPIs were previously identified for *Drosophila* Cyclin J, but these were detected by Y2H, a technique that can result in false positives [21, 22]. Since all the putative PPIs involve *Drosophila* Cyclin J, probing other species for conserved PPIs could increase confidence in PPIs that span more than one species. Of specific interest was the identification the Cdk partner for Cyclin J. The preparations for conducting CoAP to test for PPIs involving Cyclin J are complete, but the results of the CoAP assays cannot be included in this report, due to time constraints.

3.2 A screen for human proteins that interact with Cyclin J

To identify human Cyclin J interacting proteins, I conducted a Y2H screen of a HeLa cell cDNA library (see section 2.3 for details). First, I created a human Cyclin J bait by sub-cloning the human Cyclin J ORF from the human ORF cDNA library [27] into the Y2H bait vector. Before conducting the screen, I tested the human Cyclin J bait and determined that it did not activate the Y2H reporters appreciably on its own. To begin the screen, I mated the human Cyclin J bait yeast with the HeLa cDNA library yeast and selected colonies in which the reporters were active. I picked 77 positive colonies of various sizes and tested them on Y2H indicator plates. Pictures of these indicator plates are presented in Figure 6a. I scored the reporter activity of the yeast spots and found

that only 2 exhibited weak total scores (see Figure 2 for scoring calculations). The leucine reporter appears to be activated by the bait alone for all 77. This is seen when looking at Figures 6a and comparing the UHWL/D plates (bait-only active) and UHWL/grm plates (bait and prey active). Growth is observed equally on both plate types. This is interpreted as the bait activating the leucine reporter. The 2 positive *LacZ* scores are very weak, and I decided not to continue the screen for the sake of 2 questionable prospective interactions. To be sure that this result was not due to an error on my part, I re-performed the mating, this time selecting 138 positive diploid colonies of various sizes and testing them on Y2H indicator plates (Figure 6b-c). On this attempt, I observed the same leucine activation and 5 positive *LacZ* scores that were very weak. This confirmed that a Y2H screen using this bait/library combination is not a worthwhile undertaking to find biologically relevant Cyclin J PPIs.

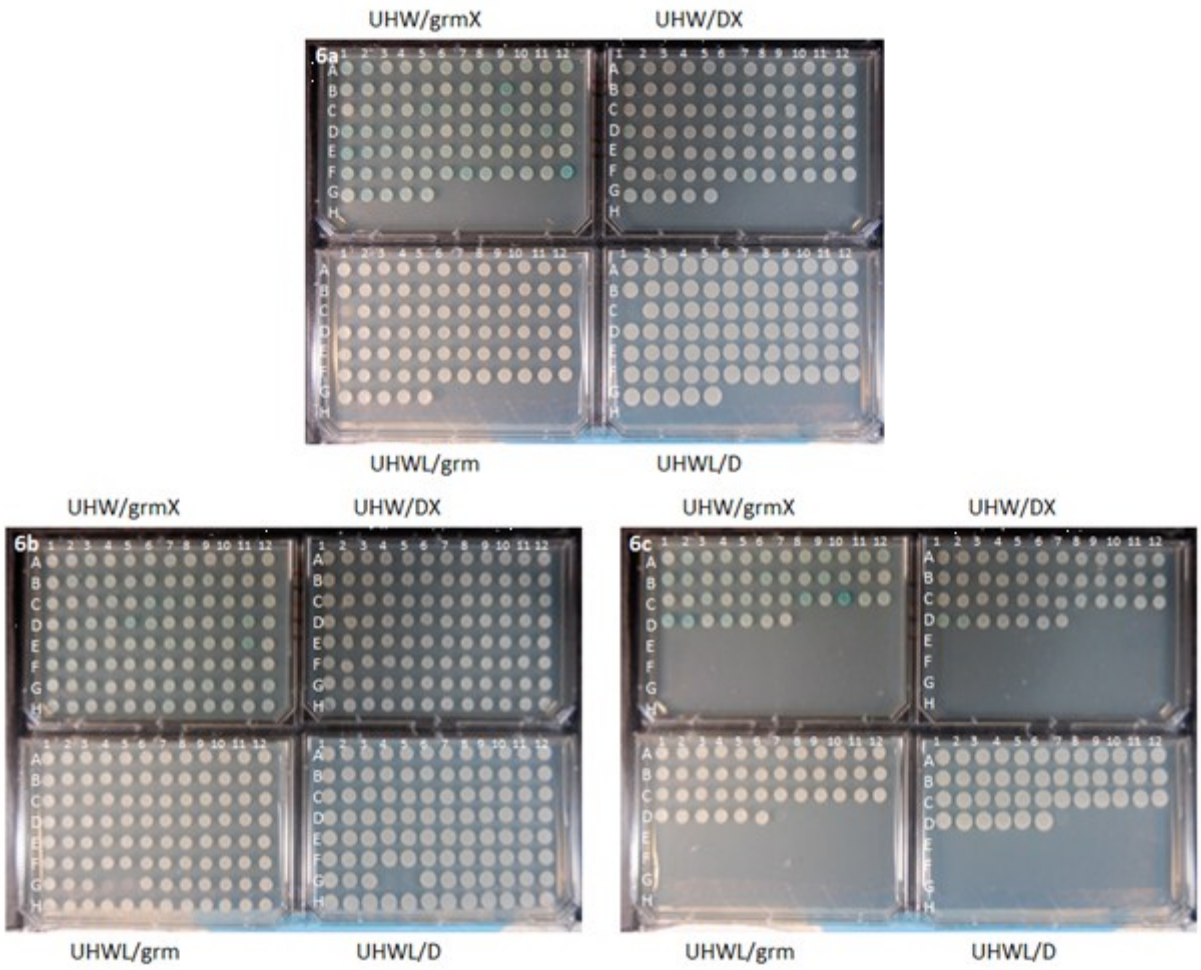
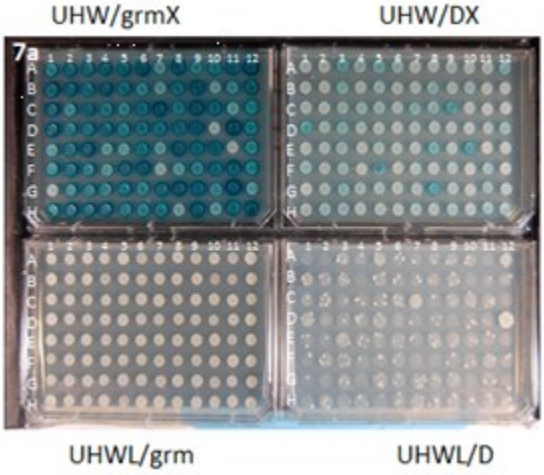
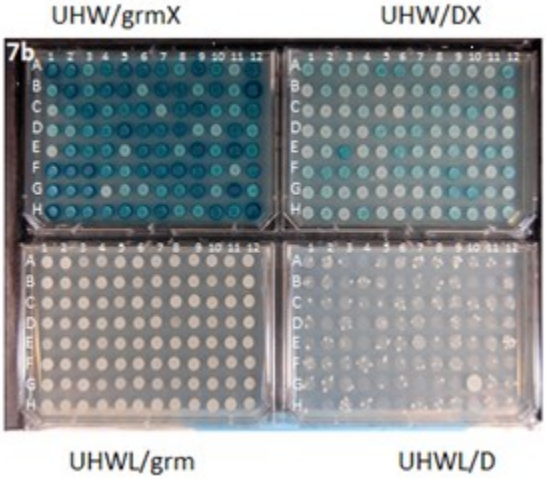


Figure 6a-c. Human Cyclin J library screen indicator plates. Indicator plates for the human Cyclin J interaction screen of the HeLa cDNA library. (a) Plate 1 (b) Plate 2 (c) Plate 3. All plates lack uracil, histidine, and tryptophan (UHW) to select for diploids. The bottom two plates in each figure lack leucine (L); colonies that grow on these have the leucine reporter activated. The top two plates in each figure contain the X-gal substrate (X); colonies that are blue on these plates have an active *LacZ* reporter. The right two plates in each figure have glucose (D), which represses production of the library protein. The left two plates have galactose (plus raffinose and maltose, grm) which activates production of the library protein.

3.3 A screen for mosquito proteins that interact with Cyclin J

To identify mosquito Cyclin J interacting proteins, I conducted a Y2H screen of a mosquito cDNA library [28]. First, I created a mosquito Cyclin J bait by sub-cloning the *Aedes aegypti* Cyclin J ORF, that I PCR amplified from the mosquito cDNA library, into the Y2H bait vector. Before conducting the screen, I tested the mosquito Cyclin J bait and determined that it did not activate the Y2H reporters appreciably on its own. To begin the screen, I mated the mosquito Cyclin J bait yeast with the mosquito cDNA library yeast and selected colonies in which the reporters were active. I picked 576 positive colonies of various sizes and tested them on Y2H indicator plates. Pictures of these indicator plates are presented in Figure 7a-f. I scored the reporter activity of the yeast spots, 574 had galactose-dependent reporter activity, suggesting an interaction between Cyclin J and those library proteins. I chose 378 clones of various positive reporter scores to be re-arrayed into four 96-well plates for further study. I performed colony PCR to amplify the prospective interacting cDNA inserts with flanking regions used to conduct gap repair cloning. I performed restriction digests of the PCR reactions with Alu I to identify duplicate clones (Figure 8a-d). The digests were not useful because the reactions appeared incomplete. I then decided to move forward with the gap repair cloning. I used the gap repair technique to sub-clone all of the 378 initial positive ORFs into Y2H prey vectors in fresh yeast to re-test the interactions.


UHW/grmX
UHW/DX
7a
UHWL/grm
UHWL/D


UHW/grmX
UHW/DX
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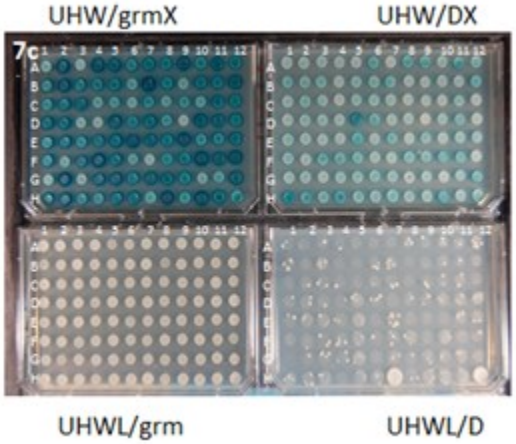
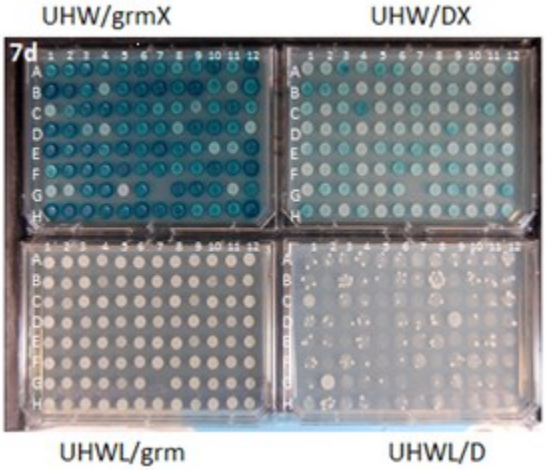
UHWL/grm
UHWL/D


UHW/grmX
UHW/DX
7c
UHWL/grm
UHWL/D


UHW/grmX
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7d
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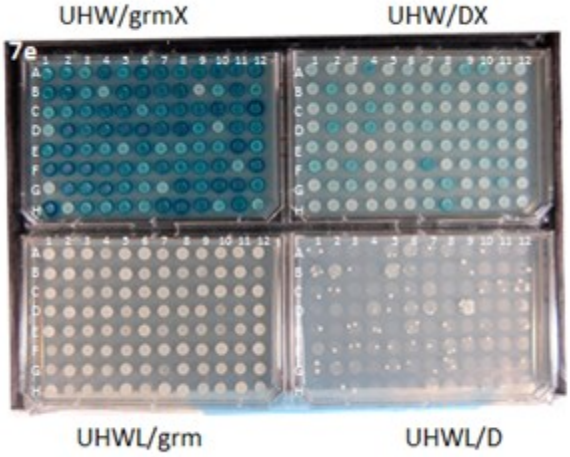
UHWL/D


UHW/grmX
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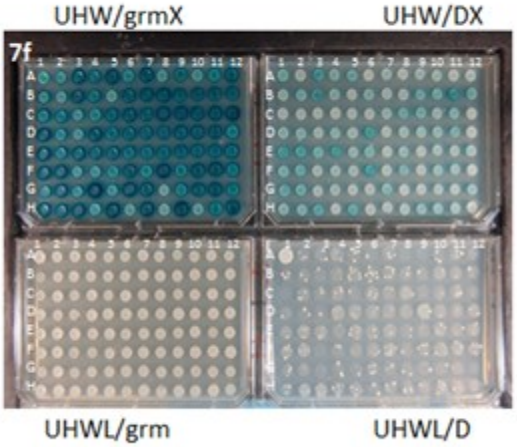
UHW/grmX
UHW/DX
7f
UHWL/grm
UHWL/D

Figure 7a-f. Mosquito Cyclin J library screen initial clones. Indicator plates for the mosquito Cyclin J interaction screen of the mosquito cDNA library. There were 576 clones tested. 574 had positive galactose-dependent scores. (a) Plate 1 (b) Plate 2 (c) Plate 3 (d) Plate 4 (e) Plate 5 (f) Plate 6. Plate labels are as in Figure 6.

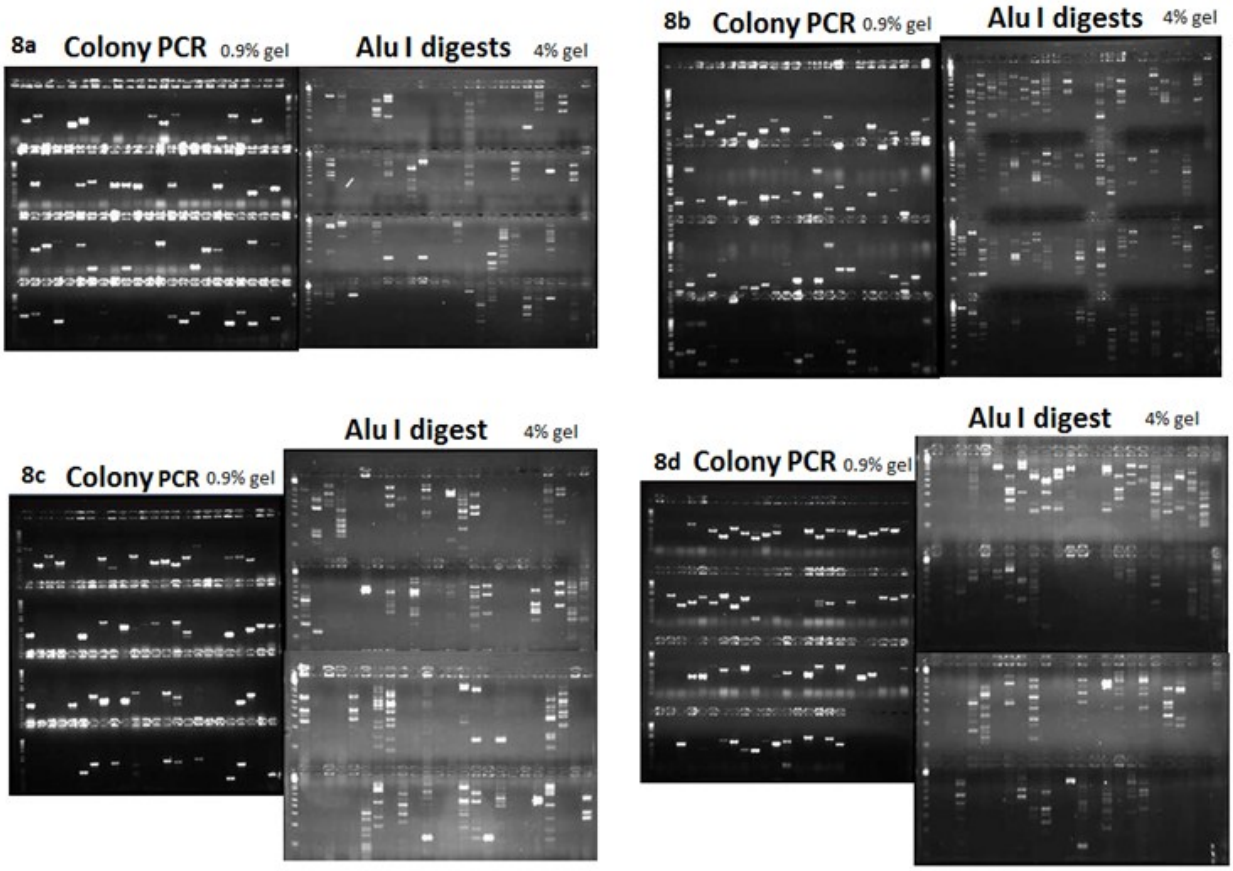

Figure 8a-d. Colony PCR and Alu I restriction digests. I restriction digested the colony PCR reactions of the 378 prospective mosquito Cyclin J interactors, ran them on agarose gels, and compared the band pattern of the digests to identify identicals. Left side – Colony PCR using GoTaq (Promega) and the primers *BCO1* & *BCO2* of the 378 prospective mosquito Cyclin J interactors. Reactions are electrophoresed at 100 volts for 30 minutes on 0.9% agarose gels. Right side – Alu I restriction digests using the colony PCR reaction, the NEB buffer 2, and Alu I restriction digest. Reactions are electrophoresed at 100 volts for 45 minutes on 4% agarose gels. (a) Plate 1 (b) Plate 2 (c) Plate 3 (d) Plate 4.

I re-tested the 378 reconstituted initial positives by mating them to the mosquito Cyclin J bait again. Pictures of these indicator plates are presented in Figure 9a-d. I scored the reporter activity of the yeast spots and found that 271 of the 384 were successfully re-tested. Growth of yeast spots on both of the UHW plates (which select for diploid yeast; meaning that both the bait and library protein are present) signified a successful re-test. For example, Plate 1, spots A1 was not a successful re-test and spots A2 was a successful re-test. A positive reporter score is constituted by an increase in reporter score of the grm plate (where the bait and library protein are expressed) over the D plate (where only the bait is expressed). Of the 271, positive reporter scores were found in 239. Plate 2, spots A3 exemplifies positive reporter scores with both reporters active, and F2 exemplifies a positive reporter score with only one. I then tested the prospective interactors by mating them with 5 control baits to assess specificity. Taking specificity into account, I chose 94 yeast clones of various positive reporter scores and sequenced their inserts. Out of the 94, 71 were unique mosquito transcripts. Table 2 includes the mosquito protein ID, the reporter score from the Cyclin J test, and the *Drosophila* ortholog, if one is known.

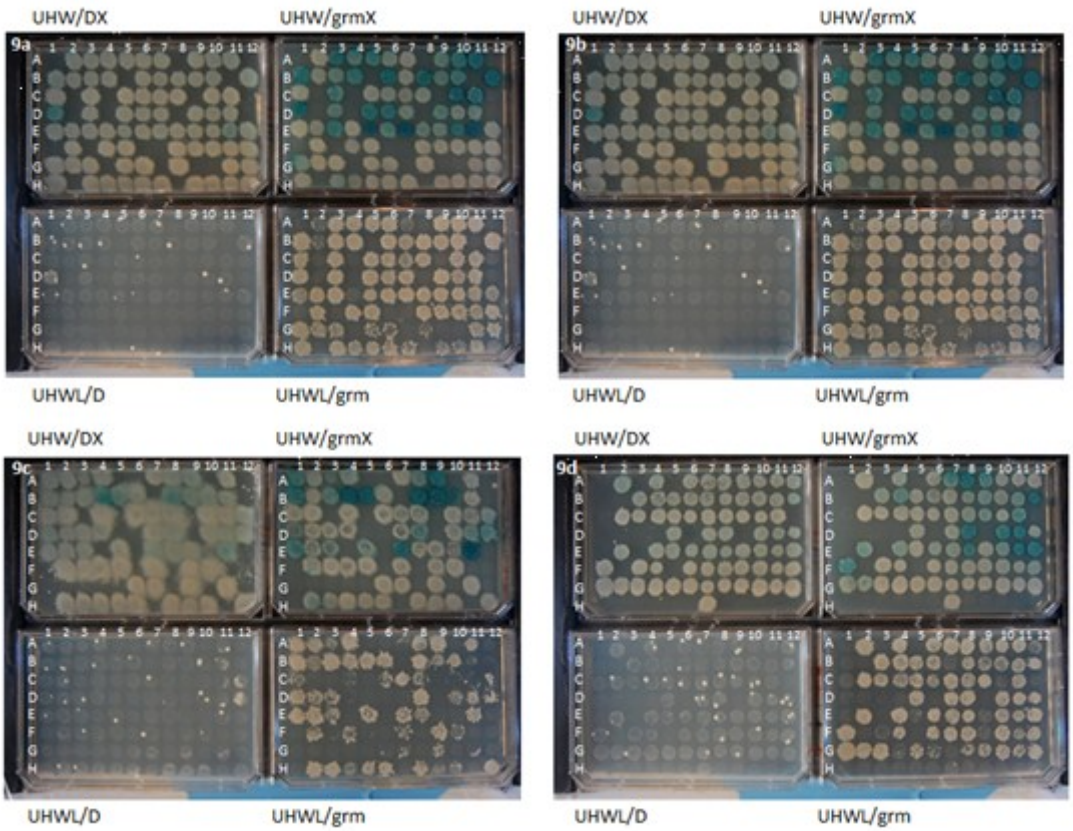

Figure 9a-d. Mosquito Cyclin J library screen clones re-test. I successfully sub-cloned the inserts of 271 of the 378 initial positive mosquito Cyclin J interactors and created fresh yeast to re-test the interactions with the mosquito Cyclin J bait. 239 of the 271 had positive galactose-dependent scores. (a) Plate 1 (b) Plate 2 (c) Plate 3 (d) Plate 4.

Protein ID	X detected	Total score	Fly Ortholog	Protein ID	X detected	Total score	Fly Ortholog	Protein ID	X detected	Total score	Fly Ortholog
AAEL005868-PA	2	6.5 & 7	N/A	AAEL001791-PA	1	3	CG10283	AAEL011881-PA	1	5	HIP-replacement
AAEL007948-PA	2	2 & 4	N/A	AAEL004588-PA	2	3 & 3	Cpr67B	AAEL010368-PA	1	6	Pex14
AAEL011554-PB	1	3	CG13349	AAEL010821-PA	1	3	RpLP0	AAEL011255-PA	1	3	ns1
AAEL008605-PA	2	5 & 3	CG8891	AAEL005451-PA	3	4, 5 & 5	RpL38	AAEL012585-PA	1	2.5	RpL7
AAEL000978-PA	1	3	CG11417	AAEL007595-PA	1	7	N/A	AAEL000269-PA	1	6.5	CG15019
AAEL003005-PA	2	3 & 3	N/A	AAEL008309-PA	1	4	CG33713	AAEL010302-PA	1	6	N/A
AAEL014583-PA	2	3 & 3	RpLP2	AAEL001099-PA	1	4	IntS6	AAEL005819-PA	1	5	CG10184
AAEL008735-PA	2	2 & 2.5	CG6739	AAEL008542-PA	1	7	Khc	AAEL004059-PB	1	2	Eip55E
AAEL015143-PB	1	2	Pabp2	AAEL012156-PA	1	4	Ime4	AAEL008736-PB	1	3	Ssb-c31a
AAEL009991-PA	1	2	N/A	AAEL004313-PA	1	3	Fkbp13	AAEL003324-PA	1	4	RpLP1
AAEL010821-PA	2	3 & 2	RpLP0	AAEL007306-PA	1	3	Actn	AAEL012359-PA	1	3	awd
AAEL010850-PA	1	6	wupA	AAEL003750-PB	1	3	Nlp	AAEL002572-PA	1	3	Mlc2
AAEL003715-PA	2	6 & 7	N/A	AAEL000854-PA	1	3	CG6028	AAEL006129-PA	1	3	Nero
AAEL003750-PB	2	3 & 3	Nlp	AAEL003303-PA	1	3	N/A	AAEL014899-PA	1	7	CG3603
AAEL002040-PA	2	6 & 4	N/A	AAEL014694-PA	1	4	qin	AAEL007135-PA	1	2	Prx5
AAEL013766-PA	1	6	Gasp	AAEL014702-PA	1	3	Aats-lys	AAEL011704-PA	1	3	Hsp83
AAEL003872-PA	1	2	Tctp	AAEL012807-PA	1	2	Uch	AAEL014583-PA	1	3	RpLP2
AAEL002675-PA	1	5	arg	AAEL011904-PC	1	2	N/A	AAEL004500-PB	1	2.5	Ef2b
AAEL002542-PA	1	4	Tpi	AAEL005712-PA	1	5	msps	AAEL011854-PA	1	4	N/A
AAEL002040-PA	1	4	PebIII	AAEL002464-PA	1	3	CG8210	AAEL007884-PA	1	3	stmA
AAEL002759-PD	1	8	Tm2	AAEL003126-PA	1	3	CG8290	AAEL011711-PA	1	2	Nacalpha
AAEL002761-PC	1	7	N/A	AAEL003095-PA	1	5	N/A	AAEL007699-PB	4	5,5,4 & 2	RpL9
AAEL005733-PB	1	4	Mhc	AAEL009906-PB	1	2	N/A	AAEL002764-PA	1	5	CG5214
AAEL002833-PA	1	3	Cp1	AAEL008852-PA	1	5	Mys45A				

Table 2. Mosquito library screen results. I identified 71 unique mosquito proteins in the yeast two-hybrid screen of the mosquito cDNA library. This table illustrates the mosquito protein ID, the total reporter score, and the *Drosophila* ortholog, if one exists (N/A if no ortholog exists). The "X detected" column shows the number of instances that gene was detected out of the 94 sequenced.

None of the sequenced library proteins matched orthologs of the putative *Drosophila* Cyclin J interactors. Seventy-one new putative Cyclin J PPIs were identified and are valuable, but no overlap with previous results indicates that no conserved PPIs were detected.

3.4 Directed assays for potentially conserved Cyclin J interactions

I set out to answer three questions in this set of experiments. (1) Does human Cyclin J interact with human Cdk1 and/or Cdk2? (2) Does mosquito Cyclin J interact with mosquito Cdk1 and/or Cdk2? (3) Are other candidate Cyclin J interactions conserved between human, mosquito, and *Drosophila*? I created Y2H constructs expressing activation domains (AD) or DNA-binding domains (BD) fusion proteins for

several human and mosquito orthologs of previously identified *Drosophila* Cyclin J interactors to address these questions. I used the format used in the re-testing of Cyclin J interactors to mate each AD with each BD (see Figure 3).

The most important PPI for a Cyclin is its Cdk partner. Previously, *Drosophila* Cyclin J was shown to interact with Cdk2, but not Cdk1, in a Y2H assay [15]. Also, research done in two labs conflict with each other over the identity of Cyclin J's *in vivo* Cdk partner. The Finley lab used CoIP to demonstrate that Cyclin J co-purifies with Cdk1 and Cdk2 [17]. The Lehner lab reported that Cyclin J co-purifies with Cdk1 only [16]. I tested two other proteins for conservation of a PPI with *Drosophila* Cyclin J, Gus and Spindle-A. According to Flybase, a *Drosphila* database, Gus is a protein that has been linked to the processes of oocyte anterior/posterior axis specification and dorsal appendage formation [29]. Spindle-A is a protein that has been linked to the processes of germarium-derived oocyte fate determination, female meiosis, double-strand break repair, polarity specification of dorsal/ventral axis, intracellular mRNA localization, cell cycle checkpoint involvement, oogenesis, polarity specification of anterior/posterior axis, and karyosome formation in *Drosophila*, also according to Flybase [30].

3.4.1 Testing for a human Cyclin J interaction with human Cdk1 and Cdk2

I set out to obtain evidence of the identity of the Cyclin J/Cdk partner by looking for potentially conserved interactions in other species. To test this, with respect to human (hs) Cyclin J, Cdk1, and Cdk2, I performed Y2H assays with each of the three as ADs and BDs. Previous Y2H results have shown that *Drosophila* (dm) Cyclin J, Cdk1, and Cdk2 all interact with Cks30A and Cks85A [22]. In my assays, I used the

human orthologs of Cks30A and Cks85A, respectively Cks2 and Cks1B, as positive controls to validate proper construct function, since these two proteins are well-known Cdk interactors. I was unable to produce an hsCks1-BD, so the hsCks2-BD, hsCks1-AD, and hsCks2-AD constructs were the controls I had at my disposal.

I was unable to test human Cyclin J interactions because the Cyclin J constructs that I used appeared to be non-functional in the two-hybrid assays. The hsCyclin J-BD did not produce a positive interaction score with the hsCdk1-AD (Figure 10a; spots A10) or the hsCdk2-AD (Figure 10a; spots A11), both of which were able to interact with mosquito Cyclin J-BD.

The hsCyclin J-BD does not appear to be a reliable construct, because it does not produce a positive interaction score with the hsCks1B-AD (Figure 10a; spots B2) or the hsCks2-AD (Figure 10a; spots B3). Both of the human Cks AD constructs appear to be working in this assay, since they show interactions with the mosquito Cyclin J-BD (see Figure 10d; spots B2 & B3). The failure of the human Cyclin J-BD to successfully screen the HeLa cDNA library also points to the ineffectiveness of this construct. The hsCdk1-AD's reliability cannot be reliably tested by hsCks2-BD, because hsCks2-BD does not appear to be a reliable construct itself. The hsCdk1-AD does produce a positive interaction score with the mosquito Cyclin J-BD (Figure 10d; spots A10), so that does indicate that it is valid to some degree. The hsCdk2-AD's reliability also cannot be reliably tested by hsCks2-BD, because hsCks2-BD does not appear to be a reliable construct itself. Like hsCdk1-AD, hsCdk2-AD produces a positive interaction score with the mosquito Cyclin J-BD (Figure 11d; spots A11), so that indicates that it is also valid to some degree.

The hsCdk1-BD does not appear to be a reliable construct, because it does not produce a positive interaction score with the hsCks1B-AD (Figure 10b; spots B2) or the hsCks2-AD (Figure 10b; spots B3). The hsCdk2-BD appears to mildly activate the reporters on its own, but it produces a positive interaction score with the hsCks2-AD (Figure 10b; spots D3) and appears to be a reliable construct. The hsCyclin J-AD does not appear to be a reliable construct, because it does not produce a positive interaction score with the hsCks2-BD (Figure 10c; spots B1).

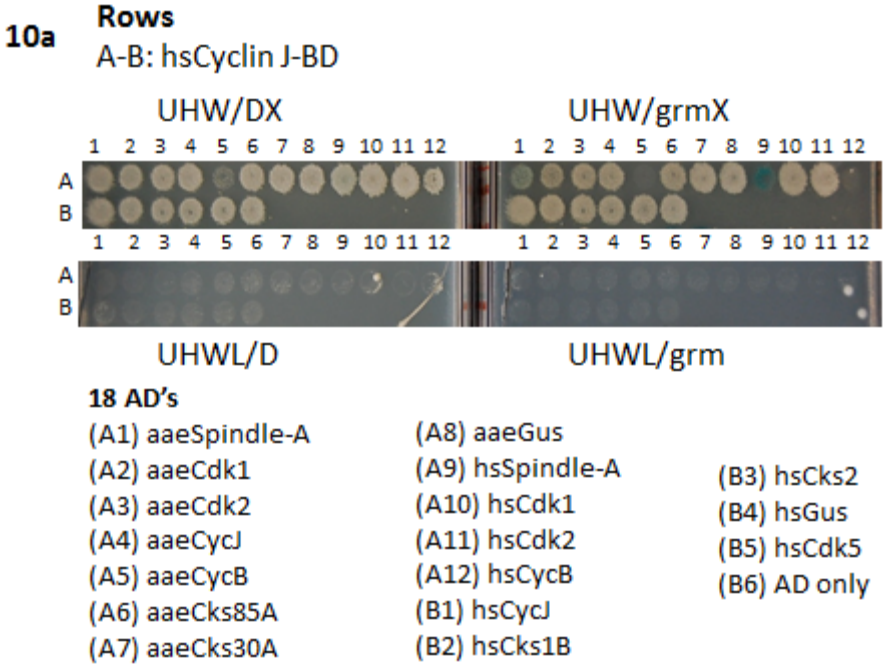

Figure 10a. Directed yeast two-hybrid assays with hsCyclin J. All spots have hsCyclin J-BD. They also have the indicated AD proteins. Plate labels are as in Figure 6.

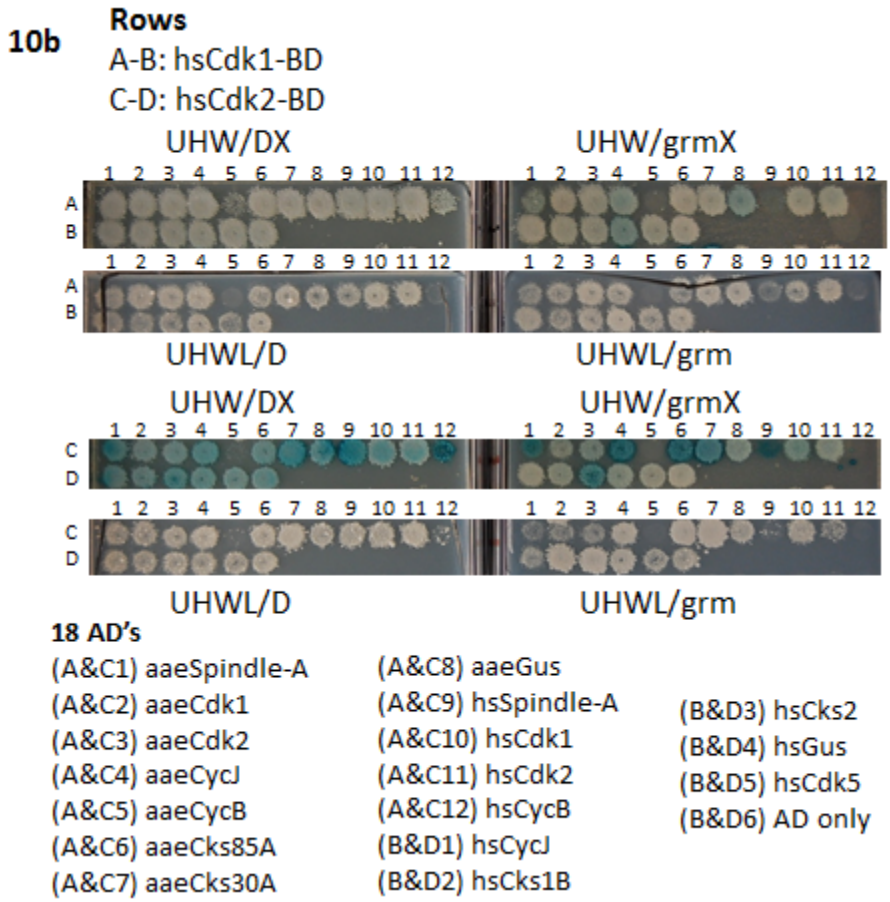

Figure 10b. Directed yeast two-hybrid assays with hsCdk1 and hsCdk2. All spots in A & B have hsCdk1–BD and all spots in C & D have hsCdk2–BD. They also have the indicated AD proteins. Plate labels are as in Figure 6.

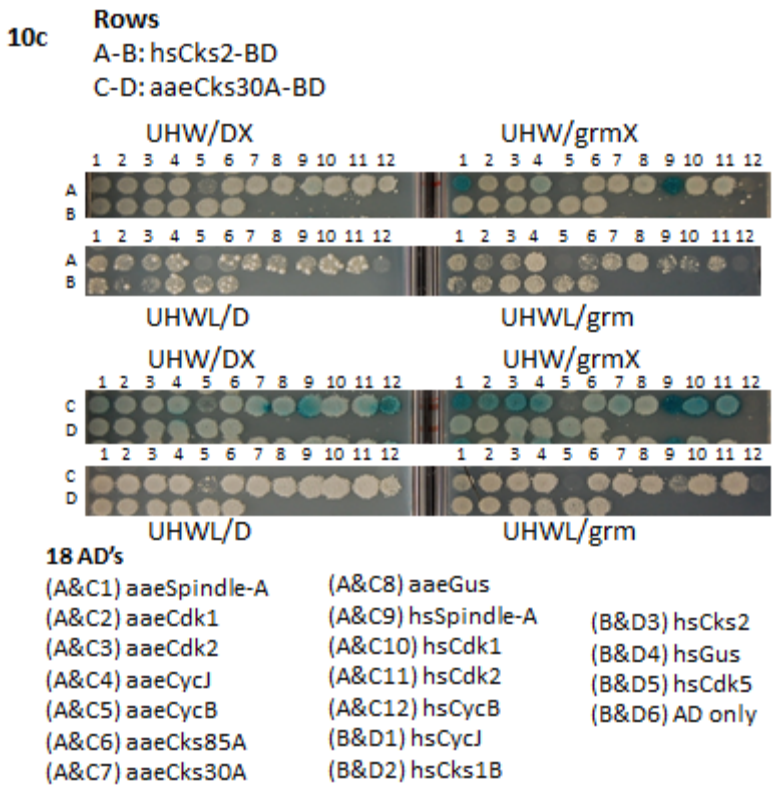

Figure 10c. Directed yeast two-hybrid assays with hsCks2 and aaeCks30A. All spots in A & B have hsCks2–BD and all spots in C & D have aaeCks30A–BD. They also have the indicated AD proteins. Plate labels are as in Figure 6.

The unreliability of the hsCyclin J-BD and AD constructs and the hsCdk1-BD construct makes the direct testing of this partnership impossible in my assays. Therefore, I will not reference any further testing that involves human Cyclin J constructs. There are caveats involving the interactions detected between human Cdk1 and Cdk2 and mosquito Cyclin J that will be detailed in the section that involves the mosquito tests.

3.4.2 Testing for a mosquito Cyclin J interaction with mosquito Cdk1 and Cdk2

I conducted the testing that involved the mosquito constructs similarly to the testing of the human counterparts. To test the potential Cyclin J/Cdk1 and 2 partnerships, I performed Y2H assays with the BD and AD versions of mosquito (aae) Cyclin J, Cdk1, and Cdk2. Again, previous Y2H results have shown that *Drosophila* Cyclin J, Cdk1, and Cdk2 all interact with Cks30A and Cks85A. In these assays, I used the mosquito orthologs of Cks30A and Cks85A, that in this case share the same name, as positive controls. I was unable to produce an aaeCks85A-BD, so I had the aaeCks30A-BD, aaeCks30A-AD, and aaeCks85A-AD constructs as the controls at my disposal.

Mosquito Cyclin J and Cdk2 interact, suggesting that their interaction may be conserved. The aaeCyclin J-BD produced a positive interaction score with the aaeCdk2-AD (Figure 10d; spots A3). Also, the hsCdk2 J-BD did produce a clear positive interaction score with the aaeCyclin J-AD (Figure 10b; spots C4). Here, I was finally able to produce some convincing data concerning the identity of a Cdk partner for Cyclin J. The aaeCyclin J-BD did not produce a positive interaction score with the aaeCdk1-AD (Figure 10d; spots A2), even though the Cdk1-AD was capable of interacting with aaeCks30A-BD (Figure 10c; spots C2). The aaeCdk1-BD produced an ambiguous result with the aaeCyclin J-AD (Figure 10e; spots A4). The faded, dark color of the yeast spot on the UHW/grmX plate could indicate that Cyclin J and Cdk1 is a toxic combination to yeast cells. This result could indicate an interaction, but the lack of a clear indicator suggests that the interaction cannot be determined with this BD/AD combination.

The aaeCyclin J-BD is a reliable construct, because it produces a positive interaction score with the aaeCks85A-AD (Figure 10d; spots A6) and the aaeCks30A (Figure 10d; spots A7). The aaeCyclin J-AD also appears to be a reliable construct, because it produces a positive interaction score with the aaeCks30A-BD (Figure 10c; spots A4).

The aaeCdk1-BD is a reliable construct, because it produces a positive interaction score with the aaeCks85A-AD (Figure 10e; spots A6) and the aaeCks30A (Figure 10e; spots A7). The aaeCdk1-AD also appears to be a reliable construct, because it produces a positive interaction score with the aaeCks30A-BD (Figure 10c; spots C2).

The aaeCdk2-BD is a reliable construct, because it also produces a positive interaction score with the aaeCks85A-AD (Figure 10e; spots C6) and the aaeCks30A (Figure 10e; spots C7). The aaeCdk2-AD also appears to be a reliable construct, because it produces a positive interaction score with the aaeCks30A-BD (Figure 10c; spots C3).

Based on these results, I conclude that mosquito Cyclin J interacts with mosquito Cdk2, but most likely, not Cdk1.

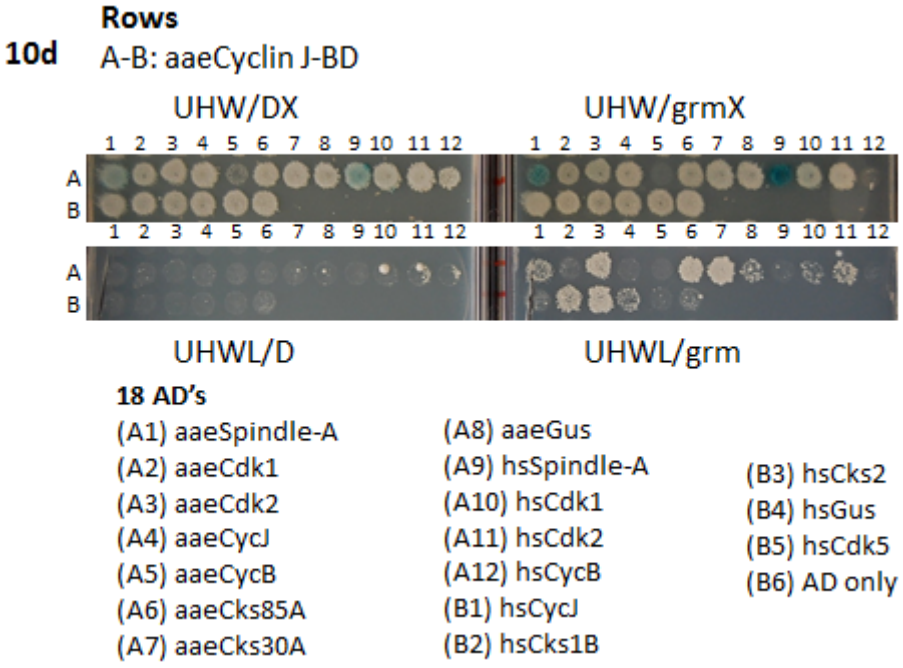

Figure 10d. Directed yeast two-hybrid assays with aaeCyclin J. All spots have aaeCyclin J-BD. They also have the indicated AD proteins. Plate labels are as in Figure 6.

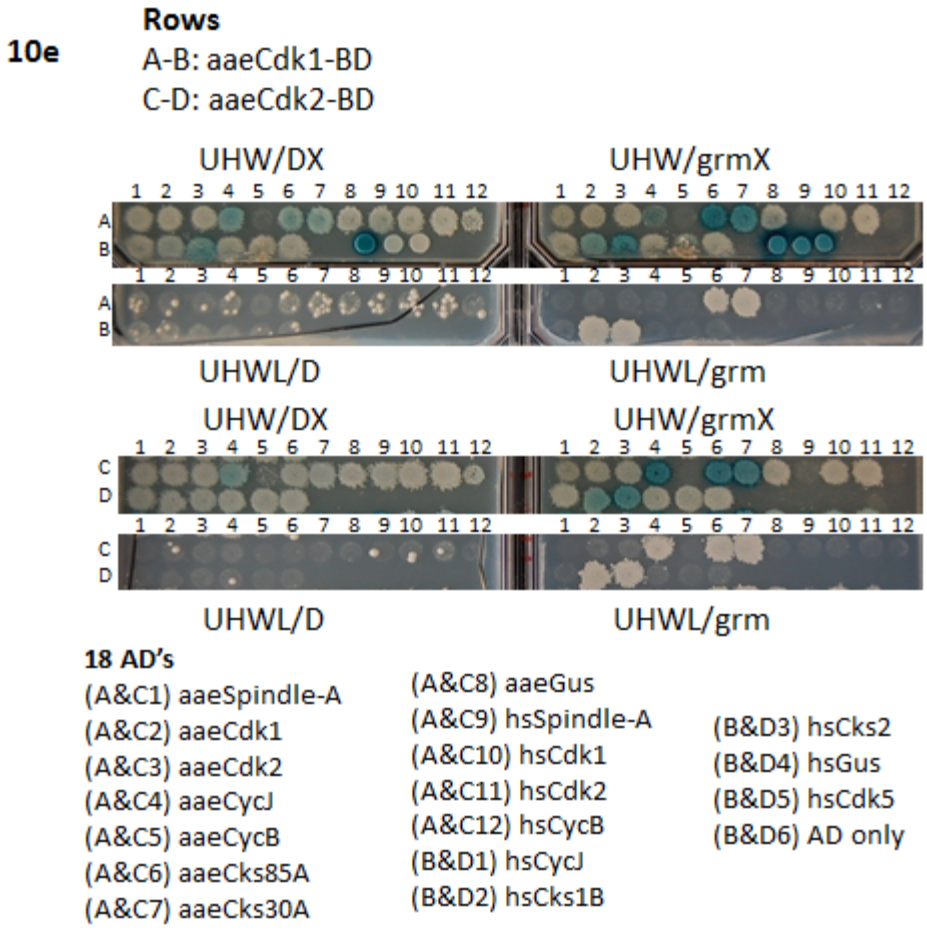

Figure 10e. Directed yeast two-hybrid assays with aaeCdk1 and aaeCdk2. All spots in A & B have aaeCdk1–BD and all spots in C & D have aaeCdk2–BD. They also have the indicated AD proteins. Plate labels are as in Figure 6.

I conclude that the Cyclin J/Cdk2 partnership is supported by my results. The interaction test was unambiguously positive in both BD and AD orientations for both constructs. The Cyclin J/Cdk1 interaction cannot be excluded, however. This conclusion is based on two results. The apparent toxic product that results from the aaeCdk1-BD/aaeCyclin J-AD test and the positive score of the aaeCyclin J-BD/hsCdk1-AD test warrants further investigation of this partnership.

3.4.3 Testing for a mosquito Cyclin J interaction with mosquito Gus

Gus has been shown to interact with Cyclin J in a previous Y2H assay [22]. I set out to test the conservation of this interaction. I was able to create BD and AD human and mosquito Gus constructs, and I tested their interaction with the mosquito Cyclin J BD and AD.

Mosquito Cyclin J and Gus interact, suggesting that this interaction may be conserved. The aaeGus-BD produced a positive interaction score with the aaeCyclin J-AD (Figure 10f; spots C4), which was validated in an earlier section. The aaeCyclin J-BD also produced a very weak positive interaction score with the aaeGus-AD (Figure 10d; spots A8) and the hsGus-AD (Figure 10d; spots B4). I did not create positive controls for the Gus constructs, so their validity cannot be unquestionably determined. The hsGus-BD did not produce a positive interaction score with the aaeCyclin J-AD (Figure 10f; spots A4).

Based on these results, I conclude that mosquito Cyclin J interacts with mosquito Gus.

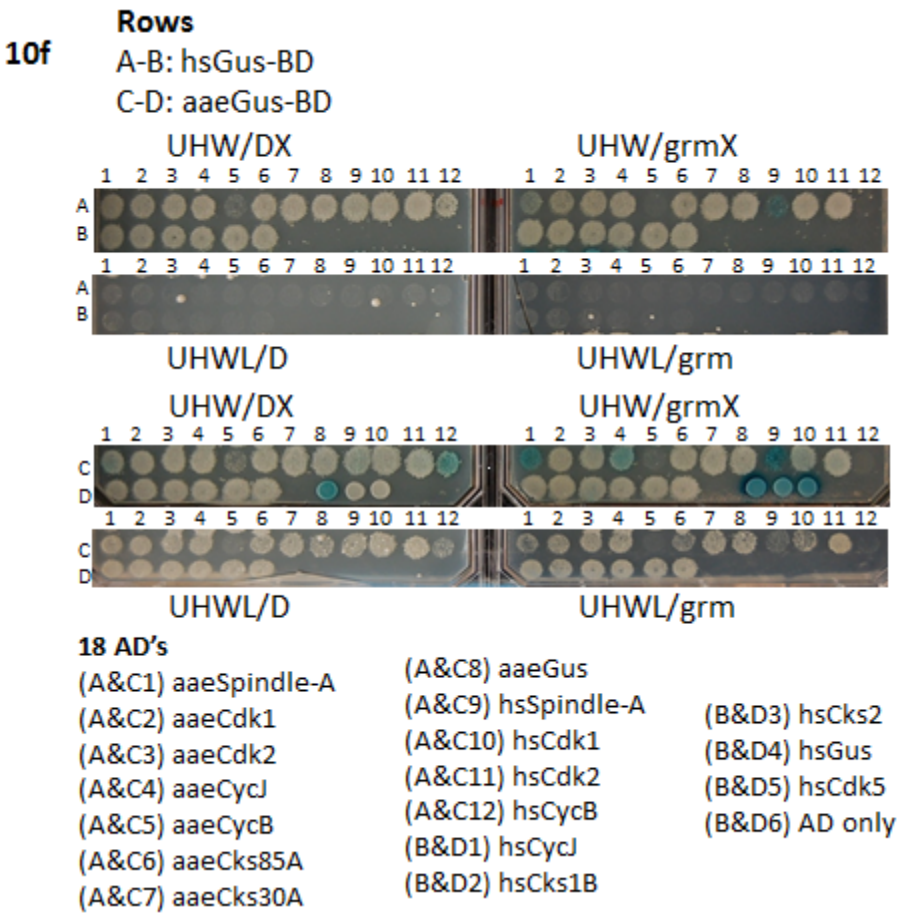

Figure 10f. Directed yeast two-hybrid assays with hsGus and aaeGus. All spots in A & B have hsGus–BD and all spots in C & D have aaeGus–BD. They also have the indicated AD proteins. Plate labels are as in Figure 6.

I conclude that the Cyclin J/Gus PPI is supported by my results. All of the interaction tests that involved a mosquito Cyclin J construct, which has been shown to be valid, consistently showed a positive test result for both human and mosquito Gus. While a positive test result across species is encouraging to have, the test result between same-species constructs is more valuable and relevant. That is why the mosquito Cyclin J/mosquito Gus reporter scores lead me to believe that the interaction is supported, even though the mosquito Cyclin J/human Gus reporters were not active.

3.4.4 Testing for a mosquito Cyclin J interaction with mosquito Spindle-A

Spindle-A has been shown to interact with Cyclin J in a previous Y2H assay [22]. I set out to test the conservation of this interaction in mosquito and human orthologs. I was able to create an hsSpindle-A-BD construct and a human and mosquito AD construct. I tested for their interaction with the mosquito Cyclin J BD and AD.

Mosquito Cyclin J and Spindle-A interact, but the conservation of the PPI is questionable, due to the conflicting results of Spindle-A BD and AD tests I will soon discuss. The hsSpindle-A-BD did not produce a positive interaction score with the aaeCyclin J-AD (Figure 10g; spots A4), which was validated in an earlier section. I did not create positive controls for the Spindle-A constructs, so their validity cannot be unquestionably determined.

The aaeCyclin J-BD produced a positive interaction score with the aaeSpindle-A-AD (Figure 10d; spots A1) and the hsSpindle-A-AD (Figure 10d; spots A9), but both of these ADs may be “sticky” constructs since they both appear to interact positively with almost every BD I tested.

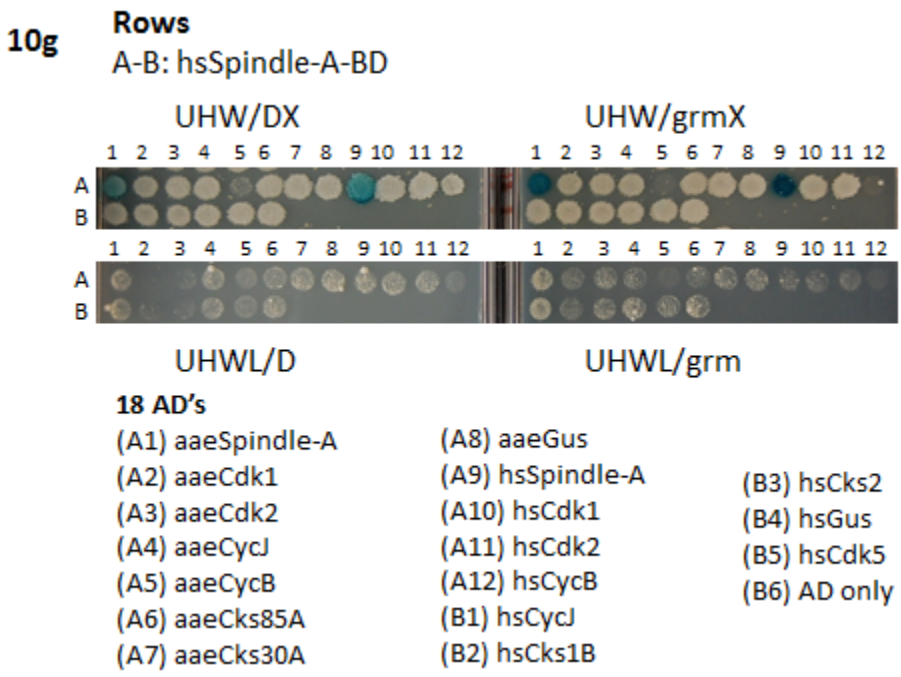

Figure 10g. Directed yeast two-hybrid assays with hsSpindle-A. All spots have hsSpindle-A-BD. They also have the indicated AD proteins. Plate labels are as in Figure 6.

I conclude that the Cyclin J/Spindle-A PPI is not supported by my results. This is for two reasons. First, not only did the human and mosquito Cyclin J-BDs detect interactions with human and mosquito Spindle-A-ADs, but several other BDs detected interaction with both Spindle-A ADs. These include hsCdk2, dmBicoid, aaeCyclin Y, hsCks2, hsSpindle-A, and aaeCks30A (results not shown). Second, the hsSpindle-A-BD does not detect an interaction with aaeCyclin J-BD or any of these other constructs in the AD orientation. It only detects interactions with human and mosquito Spindle-A-ADs. I believe that both of the Spindle-A-AD constructs are "sticky," and therefore activate reporters in many cases, regardless of any true PPI. I also believe that the result

demonstrated by the hsSpindle-A-BD of only interacting with itself and its mosquito ortholog is more likely a valid set of results.

11a

Interacting BD	Interacting AD	G-D score
hsCyclin J	hsSpindle-A	5
aaeCyclin J	aaeSpindle-A	4
aaeCyclin J	aaeCdk2	3
aaeCyclin J	aaeCks85A	3
aaeCyclin J	aaeCks30A	3
aaeCyclin J	aaeGus	0.5
aaeCyclin J	hsSpindle-A	5
aaeCyclin J	hsCdk1	0.5
aaeCyclin J	hsCdk2	1
aaeCyclin J	hsCks1B	2
aaeCyclin J	hsCks2	3
aaeCyclin J	hsGus	0.5
dmCyclin J	aaeCdk2	3
dmCyclin J	aaeCks85A	0.5
dmCyclin J	aaeCks30A	2

11b

Interacting BD	Interacting AD	G-D score
hsCdk1	aaeCyclin J	1
hsCdk1	aaeGus	1
hsCdk1	hsGus	1
aaeCdk1	aaeCks85A	8
aaeCdk1	aaeCks30A	6
aaeCdk1	hsCks1B	5
aaeCdk1	hsCks2	6
hsCdk2	aaeSpindle-A	2
hsCdk2	aaeCyclin J	3
hsCdk2	aaeCks85A	3
hsCdk2	hsSpindle-A	1
hsCdk2	hsCks2	2
aaeCdk2	aaeCyclin J	8
aaeCdk2	aaeCks85A	8
aaeCdk2	aaeCks30A	7
aaeCdk2	hsCks1B	5
aaeCdk2	hsCks2	6

11c

Interacting BD	Interacting AD	G-D score
hsGus	aaeSpindle-A	1
hsGus	hsSpindle-A	4
aaeCks30A	aaeSpindle-A	4
aaeCks30A	aaeCdk1	2
aaeCks30A	aaeCdk2	3
aaeCks30A	aaeCyclin J	2
aaeCks30A	aaeCks30A	1
aaeCks30A	hsSpindle-A	2
aaeCks30A	hsCdk1	3
aaeCks30A	hsCdk2	3
aaeGus	aaeSpindle-A	4
aaeGus	aaeCyclin J	2
aaeGus	hsSpindle-A	4

Figure 11a-c. Directed yeast two-hybrid assays reporter scores. A summary of all interactions observed in the directed assays together with reporter scores (11a) Directed yeast two-hybrid assays interactions detected 1 (11b) Directed yeast two-hybrid assays interactions detected 2 (11c) Directed yeast two-hybrid assays interactions detected 3.

CHAPTER 4 Conclusions and discussion

4.1 Significance of the results to date

The present total of 107 putative Cyclin J PPIs are valuable resources to continue the process of characterizing Cyclin J with more information. I chose to focus on the *Drosophila* orthologs of the mosquito Cyclin J interactors detected in the screen of the mosquito library, because information about Drosophila proteins is more readily available than mosquito proteins. Two of the great advantages of the *Drosophila* model organism are the number of techniques have been developed to manipulate the genome and there are many resources available to look up findings, such as the Flybase online database [30]. Most of the following information was located through the Flybase database. Many of the *Drosophila* orthologs of the newly identified putative interactors are known to be highly expressed in ovaries and early embryos and are genetically linked to sterility defects. As C*ycJ* and *armi* were shown to genetically interact, *CycJ* could also modulate a number of these phenotypes. I have chosen to highlight several of these newly-identified PPIs to discuss some notable connections to oogenesis.

For proteins to be directly functionally related, they need to occur in the same space at the same time. So, for the PPIs identified in this screen to be biologically relevant, they need to share these characteristics. Many of the PPIs detected in this screen have been documented to occur in the cell types essential to oogenesis. Since Cyclin J only occurs in ovaries and early embryos, co-expression of its putative interactors in these specialized cell types is a critical attribute.

The fusome is a highly important structure in oogenesis that connects the cytosol of the 16 germline cells that are derived from a germline stem cell to become the 15 nurse cells and the 1 ooctye [31]. Cysteine proteinase-1 (Cp1) is an endopeptidase linked to autophagic cell death that was identified by screening a library of proteins known to be present in fusomes using a protein trap assay [31, 32]. Ubiquitin carboxy-terminal hydrolase (Uch) is a ubiquitin thiolesterase linked to protein deubiquitination that was identified by *in situ* hybridization to be present in nurse cells and oocytes [33].

Another important cell type in oogenesis is the follicle cell. They line the outside of the germarium, which is the vessel-like organ that transitions into the egg chamber and is the structure in which the process of oogenesis occurs [34]. Stambha A (Stm A), a protein of unknown function, and Kinesin heavy chain (Khc), a motor protein that has been linked to regulation of pole plasm specificity, are proteins that were identified by *in situ* hybridization to be present in germline stem cells, follicle cells, nurse cells, oocytes, and embryos [34-38]. These documented localizations and links to oogenesis make these PPIs biologically relevant, and worthy targets to pursue in the further study of Cyclin J function.

Mutant phenotype analysis provides clues to the function of proteins. Several of the PPIs detected in this screen have previously documented mutant phenotypes involving oogenesis defects. Any involvement with oogenesis makes a PPI identified in this screen a good candidate to pursue further connections to Cyclin J. Nascent polypeptide associated complex protein alpha subunit (Nac α) is annotated to have a function of protein binding and is linked to regulation of pole plasm specificity, a critical process in anterior-posterior axis specification [39]. There is a mutant fly line created by

P element mutation (Nac α^{04329}), which has a documented oogenesis defect that manifests as fewer than normal, tiny collapsed eggs [40]. Cp1, which was mentioned above to localize to fusomes, has a mutant phenotype, created by deletions within the ORF, in which the flies produce a normal amount of eggs that never hatch [41]. Qin is a fairly newly characterized piRNA pathway member linked to post-transcriptional gene silencing by RNA that has a FLPase-mutated allele (Qin^{M41-13}), which has a mutant phenotype that manifests as egg chambers with abnormal numbers of oocytes and germline stem cell maintenance defects [42, 43]. Inducer of meiosis 4 (Ime4) is a protein that has methyltransferase activity and is linked to egg chamber encapsulation of the oocyte [44]. There is a mutant fly line created by a P element mutation ($Ime4^{c1}$), which has a documented oogenesis defect that manifests as compound egg chambers with supernumerary nurse cells [44]. This phenotype seems to exactly match the *armi*, *CycJ* double-null phenotype [18]. Heat shock protein 83 (Hsp83) has ATPase-coupled protein folding activity and is also linked to regulation of pole plasm specificity [45, 46]. $Hsp83^{e6A}$ and $Hsp83^{19F2}$ are mutations caused by ethyl methanesulfonate exposure, and the fly lines with these mutant alleles manifest with approximately 75% of the egg chambers arresting at different developmental stages, not later than stage 9, and the remaining egg chambers show a prominent defect in the transfer of nurse cell cytoplasm to the oocyte, a necessary step in oocyte maturation [47].

Another common factor among several PPIs detected is a connection to Notch signaling. This signaling pathway is well-established for the roles it has in many processes during egg chamber development [48]. A common theme seen in the processes that involve the Notch signaling pathway is cell fate specification [48]. It is a

key factor in maintaining the germline stem cell niche, the differentiation of polar cells and stalk cells, the establishment of the anterior-posterior axis, and the differentiation of the follicle cells into their specialized parts of the egg chamber [48]. Two proteins that were identified in the screen have features that are instantly recognizable within the known framework of the Notch signaling pathway. CG33713 is a protein of unknown function that has an ankyrin repeat-containing domain and a FERM/acyl-CoA-binding, 3-helical bundle domain. Domains with ankyrin repeats are known to facilitate binding between proteins, and they are known to be necessary for the Notch intracellular domain (NICD) to bind downstream targets [48]. FERM/acyl-CoA-binding, 3-helical bundle domains are known to localize a protein to the cell membrane [49]. This combination of features mirrors Notch itself, which is located at the cell membrane and has an ankyrin repeat domain. Another protein detected in the screen was AAEL009906-PB. No orthologous *Drosophila* protein has been identified to date. This protein also has no identified function, but it has a PDZ domain, which facilitates binding between proteins [48]. Several known Notch ligands have PDZ domains [48]. These domains are important for the organizing of cell-cell junctions [48].

Aside from these proteins with common Notch-associated domains, six other proteins identified in the screen have been directly linked to the Notch signaling pathway. This has been done through RNA interference (RNAi) assays. RNAi assays hijack the innate post-transcriptional gene silencing machinery to target and “knock down” one given gene at a time, in order to study the resulting phenotype [50]. Three proteins, already mentioned earlier for having mutant phenotypes that involve oogenesis, Nac α, Khc, and Ime4, have also been linked to Notch signaling through

specific tests that identify Notch signaling defects by the phenotype produced. Nac α scored hits on two assays that tested for Notch signaling defects by causing defects in bristle morphology and a fatal malformation of the notum when “knocked-down” [51]. Khc also scored hits on the same two assays [51]. Ime4 scored hits on four assays that tested for Notch signaling defects by causing defects in bristle morphology, loss of bristles, increased lateral inhibition, and a fatal malformation of the notum when “knocked-down” [51]. CG10492, which has a CCHC-type zinc finger domain but an unknown function, scored one hit on an assay that tested for Notch signaling defects by causing a fatal malformation of the notum when “knocked-down” [51]. Translationally-controlled tumor protein (Tctp), which has Ras guanyl-nucleotide exchange factor activity and is annotated to be a positive regulator of cell size, scored two hits on assays that tested for Notch signaling defects by causing a non-fatal malformation of the notum and a defect in planar polarity when “knocked-down” [51, 52]. Finally, Nero, which has deoxyhypusine monooxygenase activity and is annotated as being a positive regulator of autophagy, scored hits on two assays that tested for Notch signaling defects by causing defects in bristle morphology and loss of bristles when “knocked-down” [51, 53].

The connections between what is theorized about Cyclin J and the experimental knowledge regarding the newly-identified PPIs are numerous. Like Cyclin J, many of these putative PPIs are known to localize to cell types within the ovary. Mutating *CycJ* has been shown to produce mutant phenotypes that involve egg laying and hatching. When deleted with *armi*, severe egg chamber abnormalities are seen. Several of the newly-identified PPIs have documented mutant phenotypes that involve eggs and oogenesis. Also, the newly-identified PPIs are enriched with RNAi phenotypes linked to

the Notch signaling pathway. A couple have domains that are common features of proteins of the Notch pathway, which is well-understood to be indispensable for normal oogenesis. With the unearthing of all these details, many of the above proteins are likely related to Cyclin J function.

4.2 Future directions

To create a thorough profiling of Cyclin J's PPI activity, 3 immediate tasks are necessary. The first is the completion of the CoAP tests. Further testing of the mosquito Cyclin J with the two Cdks and an attempt to conduct a successful PPI test with human Cyclin J with the two Cdks will likely yield valuable data involving the identity of a Cyclin J, Cdk partnership. Next, the mosquito library screen identified 71 new putative Cyclin J PPIs. Similar to the process of the directed assays utilized here, testing for conservation of these PPIs with their human and *Drosophila* orthologs would increase confidence in them in a way similar to the aim of this project. In addition, manipulation of the ORF of Cyclin J, followed by repetition of the PPI tests previously completed will work to map the domains essential to Cyclin J PPIs. Further down the road, following confirmation of the PPIs, creating double-mutant *Drosophila* strains may confirm or deny observable phenotypic relationships controlled by the PPIs, similar to the tests involving the piRNA pathway proteins.

APPENDIX A

(1) List oligonucleotides, the Finley Lab bank #, and sequence. (2) List oligonucleotides, the Finley Lab bank #, and general target.

Oligonucleotide list 1

- aeCycA3F (bank#1195)
  - 5' AAAAGCAGGCTG CATGGAAATCTTCAG AAAT AACCCG
- aeCycA3R (bank#1196)
  - 5' AGAAAGCTGGGTTTCATTC CAGTTTGGCG CGT
- aeCdk1F (bank#958)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTT CATGGAG GATTT CC A A AAG AT AG AAAAG A
- aeCdk1R (bank#959)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTCT CA CTCG ATTGGGG CCG C
- aeCdk2F (bank#960)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTT C ATGC AC A AAAT AGG CGA CT ACC
- aeCdk2R (bank#961)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTC CTA CTGT CCGTT AGTTTTT CC ATT C
- aeGusF (bank#1197)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTG C ATGGA ATT AG CA CGT AAA AG ATA C AAA AGT
- aeGusR (bank#1198)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTGTT ATCTT CG ATC CCG AT AC AGC AG AT AGGTTTTC
- aeSpnAF (bank#1199)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTG C ATGGCT CA A CAG ATGG AG A AAA ACGTG
- aeSpnAR (bank#1200)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTGCT ATT CTTTC A CAT CTC CG ATTC CGTC CG
- aeCks30AF (bank#1203)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTG C ATGAGT GTTA A AG AC ATCT ATT ATT CGG AC AA AT ATT ACG ACG
- aeCks30AR (bank#1204)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTGTT ATGC CGTCGT CTTTGGC CG
- aeCks85AF (bank#1205)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTG C ATGC CGTC CGAT C AGA TCC A AT ACT C
- aeCks85AR (bank#1206)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTGTT AAG CCG CC A AA CGGG ACG
- aeCycBF (bank#1207)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTG C ATGGC CA C AC ACTT CCG C ATTG
- aeCycBR (bank#1208)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTGCT AC AGGTT C AGTTC AC CGTC TAT C AGC
- hsSpnAF (bank#1201)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTG C ATGGC A ATGC AG ATGC AGC TTG AAG
- hsSpnAR (bank#1202)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTGTC AGT CTTTGGC AT CTC CC ACT CC
- hsCdk5F (bank#1191)
  - 5' AAAGCAGGCTGGATGC AG AAAT ACG AG A AA CTGG AA AAG
- hsCdk5R (bank#1192)
  - 5' GAAAGCTGGGTGCTAGGGCGGA C AGA AGT
- hsCks2F (bank#1193)
  - 5' AAAGCAGGCTGGATGGC CC AC A AGC AG AT
- hsCks2R (bank#1194)
  - 5' GAAAGCTGGGTGTCATTTTTGTTGAT CTTTTGG A AGAGG
- hsCycBF (bank#1209)
  - 5' GGGGACAAGTTTGTACAAAAAAGC AGGCTG C ATGGCG CTC CGAG TC ACC
- hsCycBR (bank#1210)
  - 5' GGGGACCACTTTGTACAAG AAAG CTGGGTGTT AC AC CTTTGC C AC AGC CTTGG

Oligonucleotide list 2

- M13F & M13R (Invitrogen)
  - amplifies insert in pDONR221
- DM1 (bank# 988) & DM2 (bank# 989)
  - Extends Gateway attB1 and attB2 sites
- 5'RT (bank# 891) & 3'RT (bank# 892)
  - amplifies region between homologous recombination sites used in gap repair
- BCO1 (bank# misc) & BCO2 (bank# misc)
  - amplifies insert in pJG4-5
- HZ12_myc_seq (bank# 881) & HZ12_rev_seq (bank# 882)
  - amplifies insert in pHZ12
- DL41 (bank# 545) & HZ12_rev_seq (bank# 882)
  - amplifies insert in pHZ13

APPENDIX B

(Entry vectors) List entry vectors, origin, primers used, reference ID, and the Finley Lab bank #. (Destination vectors) List destination vectors, the assay involved, cell type transfected, and the Finley Lab bank #.

Entry vectors

Entry Vectors	Source	F Primer	R primer	Reference ID	Plasmid Bank#
pDONR221-aaecycj	mosq. cDNA lib.	aeCycA3F	aeCycA3R	XP_001653129.1	1128
pDONR221-aaecdk1	mosq. cDNA lib.	aeCdk1F	aeCdk1R	XP_001653367.1	2056
pDONR221-aaecdk2	mosq. cDNA lib.	aeCdk2F	aeCdk2R	XP_001662490.1	2057
pDONR221-aaecks30A	mosq. cDNA lib.	aeCks30AF	aeCks30AR	XP_001649286.1	2058
pDONR221-aaecks85A	mosq. cDNA lib.	aeCks85AF	aeCks85AR	XP_001650539.1	2059
pDONR221-aaecycb	mosq. cDNA lib.	aeCycBF	aeCycBR	XP_001660628.1	2060
pDONR221-aaegus	mosq. cDNA lib.	aeGusF	aeGusR	XP_001655083.1	2061
pDONR221-aaespn-A	mosq. cDNA lib.	aeSpnAF	aeSpnAR	XP_001657421.1	2062
pDONR223-hscycj	hORF library	N/A	N/A	NP_061957.2	2038
pDONR223-hscdk1	hORF library	N/A	N/A	NP_001777.1	2064
pDONR223-hscdk2	hORF library	N/A	N/A	NP_001789.2	2065
pDONR221-hscdk5	Hela cDNA lib.	hsCdk5F	hsCdk5R	NP_004926.1	2063
pDONR223-hscks1B	hORF library	N/A	N/A	NP_001817.1	2066
pDONR221-hscks2	Hela cDNA lib.	hsCks2F	hsCks2R	NP_001818.1	2067
pDONR221-hscycb	Hela cDNA lib.	hsCycBF	hsCycBR	NP_114172.1	2068
pDONR221-hsgus	Hela cDNA lib.	hsGusF	hsGusR	NP_079382.2	2069
pDONR221-hsspn-A	Hela cDNA lib.	hsSpnAF	hsSpnAR	NP_002866.2	2070

Destination vectors

Destination Vectors	Fusion Construct	Assay	Cell type	Plasmid bank#
pNLEXattR-aaecycj	BD-aaeCycJ	Y2H	RFY309	1129
pNLEXattR-aaecdk1	BD-aaeCdk1	Y2H	RFY309	2071
pNLEXattR-aaecdk2	BD-aaeCdk2	Y2H	RFY309	2072
pNLEXattR-aaecks30A	BD-aaeCks30A	Y2H	RFY309	2073
pNLEXattR-aaecks85A	BD-aaeCks85A	Y2H	RFY309	2074
pNLEXattR-aaecycb	BD-aaeCycB	Y2H	RFY309	2075
pNLEXattR-aaegus	BD-aaeGus	Y2H	RFY309	2076
pNLEXattR-aaespn-A	BD-aaeSpn-A	Y2H	RFY309	2077
pNLEXattR-aaecycy	BD-aaeCycY	Y2H	RFY309	2083
pNLEXattR-hscycj	BD-hsCycJ	Y2H	RFY309	1130
pNLEXattR-hscdk1	BD-hsCdk1	Y2H	RFY309	2078
pNLEXattR-hscdk2	BD-hsCdk2	Y2H	RFY309	2080
pNLEXattR-hscks2	BD-hsCks2	Y2H	RFY309	2079
pNLEXattR-hsgus	BD-hsGus	Y2H	RFY309	2081
pNLEXattR-hsspn-A	BD-hsSpn-A	Y2H	RFY309	2082
pNLEXattR-hscycy	BD-hsCycY	Y2H	RFY309	2084
pHM202-CDI5	BD-dmCycJ	Y2H	RFY309	499
pRFHM1	BD-dmBicoid	Y2H	RFY309	488

Destination Vectors	Fusion Construct	Assay	Cell type	Plasmid bank#
pJZ4attR-aaecycj	AD-aaeCycJ	Y2H	RFY231	2085
pJZ4attR-aaecdk1	AD-aaeCdk1	Y2H	RFY231	2086
pJZ4attR-aaecdk2	AD-aaeCdk2	Y2H	RFY231	2087
pJZ4attR-aaecks30A	AD-aaeCks30A	Y2H	RFY231	2088
pJZ4attR-aaecks85A	AD-aaeCks85A	Y2H	RFY231	2089
pJZ4attR-aaecycb	AD-aaeCycB	Y2H	RFY231	2090
pJZ4attR-aaegus	AD-aaeGus	Y2H	RFY231	2091
pJZ4attR-aaespn-A	AD-aaeSpn-A	Y2H	RFY231	2093
pJZ4attR-hscycj	AD-hsCycJ	Y2H	RFY231	2092
pJZ4attR-hscdk1	AD-hsCdk1	Y2H	RFY231	2094
pJZ4attR-hscdk2	AD-hsCdk2	Y2H	RFY231	2095
pJZ4attR-hscdk5	AD-hsCdk5	Y2H	RFY231	2096
pJZ4attR-hscks1B	AD-hsCks1B	Y2H	RFY231	2097
pJZ4attR-hscks2	AD-hsCks2	Y2H	RFY231	2098
pJZ4attR-hscycb	AD-hsCycB	Y2H	RFY231	2099
pJZ4attR-hsgus	AD-hsGus	Y2H	RFY231	2100
pJZ4attR-hsspn-A	AD-hsSpn-A	Y2H	RFY231	2102
pHZ13attR-aaecycj	NTAP-aaeCycJ	CoAP	S2R+	2101
pHZ13attR-aaecycb	NTAP-aaeCycB	CoAP	S2R+	2103
pHZ12attR-aaecdk1	Myc-aaeCdk1	CoAP	S2R+	2104
pHZ12attR-aaecdk2	Myc-aaeCdk2	CoAP	S2R+	2105
pHZ12attR-aaecks85A	Myc-aaeCks85A	CoAP	S2R+	2106

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ABSTRACT

CHARACTERIZING CYCLIN J BY IDENTIFYING CONSERVED PROTEIN-PROTEIN INTERACTIONS

by

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August 2013

Advisor: Russell L. Finley

Major: Biochemistry and Molecular Biology

Degree: Master of Science

Cyclins are proteins that bind to Cyclin-dependent kinases, or Cdks, through a conserved domain called the Cyclin Box. Many Cyclins regulate the cell cycle. A few Cyclins impact cellular processes outside of the cell cycle. Also, a few Cyclins have poorly understood functions.

Cyclin J is a member of the Cyclin superfamily of proteins. Cyclin J is conserved among all metazoans, but is presently not well understood. All the research done on Cyclin J has been done in *Drosophila*.

Its mRNA is present in the early embryo, then disappears, only to reappear in adult females. When probing protein extracts with antibodies, Cyclin J can be seen in unfertilized oocytes and embryos for the first few hours following fertilization. Immunoprecipitating Cyclin J from unfertilized oocytes and early embryos, Cdk2 co-immunoprecipitates. The same assay co-immunoprecipitates Cdk1 in unfertilized eggs only. Another group has observed very different

results in regard to Cdk interaction. They observe Cyclin J to co-immunoprecipitate with Cdk1 and not to interact with Cdk2 in whole ovaries.

This project has one specific aim. It is to identify and test for biologically relevant Cyclin J protein-protein interactions (PPIs). I am using approaches that involve two assays to test PPIs. The assays I am employing are the yeast two-hybrid assay (Y2H) and co-affinity purification (CoAP). When a PPI is detected using two different assays, for example Y2H and CoAP, it is more likely to be a true positive. Orthologs of *Drosophila* Cyclin J's PPIs will also be tested. I am comparing Drosophila, mosquito, and human orthologs of PPIs. A PPI is also more likely to be a true positive when the PPI is conserved between more than one species.

AUTOBIOGRAPHICAL STATEMENT

Ever since I can remember, I have been interested in and amazed by how things work. Learning about the nature and history of the universe is a craving that I have never tired of from an early age. To me, all areas of nature are based on and contain layers of miracles upon miracles. Life is no exception. The evolution of life on Earth is so filled with stunning expressions of the capacity of life that I am flabbergasted by every time I gain new insight into it. Once I accepted that my destiny is to be a part of studying life, my life has felt right in a profound way. I feel that my experiences in life have made me uniquely qualified to be a valuable part of a research team. Having worked many years in the restaurant industry, I have gained a strong appreciation for the power of a good balance between teamwork and independence. As in the restaurant industry, there is a lot of value to be gained through utilizing the strengths of your coworkers in performing your work. Also, the care in which you perform your work directly influences the quality of the product you produce. Through my experiences as an undergraduate, the most important lesson I learned was that the power of hard work and attention to detail can exceed sheer talent and pedigree. Through my experiences as a graduate student in the Finley Lab, the most important things I learned were a higher level of the execution of, and a deep appreciation for, the design and documentation of experiments and the presentation of findings. Also, I have a richer appreciation for the power of protein-protein interactions. Going forward as a professional research technician, I plan to continue learning while being a part of research that produces new-found knowledge that contributes to improving health.