Using Light to Control Protein-Protein Interactions:
Optogenetics in *Drosophila melanogaster*

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Abstract

Recent advancements in genetically encoded light-sensitive protein systems, also known as optogenetic systems, have stemmed from the many benefits of using blue light stimuli to selectively initiate protein-protein interactions. Such benefits include the non-invasive nature of light, the precision of the stimulus, and the reversibility of the protein-protein interactions in the dark. One specific optogenetic system from *Arabidopsis thaliana*, the CRY2/CIB module, offers a powerful genetically encoded mechanism by which to study the role of proteins in a tissue-specific manner during various stages of development. Using cloning techniques to generate CRY2 and CIB constructs in *Drosophila* specific vectors, we have attempted to adapt the CRY2/CIB system to *Drosophila*. We tested an oligomerizing version of the CRY2 component as a tool for the negative regulation of targeted proteins in *Drosophila*. Although we were unable to repeat the clustering results observed in yeast, we worked on modifying our light activation protocol and discovered the sensitivity of the system to inadvertent light stimulation during preparation for imaging. We also conducted cloning in order to perform a proof-of-concept experiment utilizing both cytoplasmically diffuse CRY2 and membrane-anchored CIBN. Thus far, germline transformants of the CIBN component have been generated, and work will continue to generate the CRY2 germline transformants. Additionally, we are also working on cloning variants of the small G protein Rho to form a fusion protein with the CRY2 component. At the plasma membrane, Rho proteins catalyze signaling cascades to affect actin and myosin formation and cytoskeletal changes. If *Drosophila* Rho1 proteins are successfully adapted to CRY2 components, upon blue light stimulation the recruitment of CRY2 to a CIB component anchored in the membrane could be spatially and temporally controlled to affect subsequent downstream events. The ability to drive Rho1 to the membrane at specific stages of development will generate a better understanding of the effects of altering cytoskeletal function during *Drosophila* morphogenesis and thereby give insight into wound healing and tissue regeneration processes in vertebrates.
Introduction

Many biological reactions utilize protein-protein interactions to drive cellular responses (Jones et al., 1996). Currently, a variety of methods exist for the external control of targeted biological reactions through catalyzing or terminating these protein-protein interactions. The activation or negative regulation of specific biological pathways can be manipulated through the exogenous control of protein-protein interactions such as dimerization, the joining of two proteins, and oligomerization, the joining of multiple proteins (Marianayagam et al., 2004). Chemical inducers have been used to control protein-protein interactions in a non-reversible manner (Spencer et al., 1993; Amara et al., 1997; Gestwicki et al., 2007); however, the use of chemical inducers can bear many challenges including the inability of the chemical inducers to be applied with spatial specificity and precision (Bishop et al., 2000), the difficulty in the removal of the stimulus (Padidam, 2003), and limited temporal resolution that is due to the time required for the diffusion of the chemical inducer (Bishop et al., 2000; Kennedy et al., 2010). Furthermore, the use of chemical inducers can be expensive and difficult to apply to real time, in vivo studies (Padidam, 2003).

As an alternative to using chemical inducers to control protein-protein interactions, recent advancements in the use of light to regulate specific interactions have generated a toolbox of reagents to selectively drive the localization of proteins and cellular components (Levskaya et al., 2009; Hughes et al., 2012; Tucker, 2012; Renicke et al., 2013; Taslimi et al., 2014; Duan et al., 2015; Guglielmi et al., 2015). The recent focus on these genetically encoded light-sensitive protein systems, also known as “optogenetic” systems (Miesenböck, 2009), has stemmed from the many benefits of using light as a stimulus for protein interactions. These advantages include the immediate application of the stimulus, the quick response of proteins to the light, the
reversibility and easy removal of the stimulus, and the ability to precisely apply the stimulus to just one targeted portion of a cell (Tucker, 2012). In addition, light is non-invasive to cells and is not susceptible to membrane pumps as chemical inducers may be (Higgins, 2007). As prolonged exposure to light can be phototoxic to cells, brief pulses of light are used for activation in optogenetic systems to preclude triggering a phototoxic response from the cells (Tucker et al., 2001; Bodvard et al., 2011; Hughes et al., 2012).

Given the numerous benefits of utilizing optogenetic systems to induce protein-protein interactions, recent research has focused on manipulating protein-protein interactions by attaching proteins of interest to these photosensitive proteins. By applying light of a particular wavelength to the isolated light-sensitive domains of the photosensitive proteins, researchers can control the interaction of the attached proteins to activate/terminate a reaction and study the effects of the targeted protein-protein interaction on cellular function (Yang et al., 2013; Kim et al., 2014; Lee et al., 2014; Taslimi et al., 2014). To date, numerous optogenetic systems have been utilized to control protein-protein interactions with light stimuli including LOV (light, oxygen, and voltage) domains (Renicke et al., 2013), PhyB domains (Shimizu-Sato et al., 2002; Levskaya et al., 2009), and UV-resistant locus 8 (Chen et al., 2013; Crefcoeur et al., 2013). These optogenetic systems have been successfully used to control protein degradation (Renicke et al., 2013), alter cell morphology in mammalian cells (Levskaya et al., 2009), induce the expression of marker genes (Shimizu-Sato et al., 2002), regulate organelle distribution in cells (Duan et al., 2015), and control protein secretions (Chen et al., 2013) with light stimulus.

One particular optogenetic system of interest is the CRY2/CIB light-sensitive system from Arbidopsis thaliana. CRY2 (cryptochrome 2) is a blue-light (460nm, range 390-530nm) absorbing photosensor protein that, when in a photoexcited state, binds to CIB1, a basic helix-
loop-helix transcription factor (see Figure 1; Liu et al., 2008). These proteins can bind to each other in a reversible protein-protein manner that can be blue-light activated in milliseconds and reversible with dissociation kinetics within minutes in the dark (Kennedy et al., 2010; Liu et al., 2011; Tucker, 2012).

Additionally, a truncated version of CIB1 that lacks the basic helix-loop-helix domain, CIBN (residues 1-170 of the full length CIB1; Liu et al., 2008), contains the functional light sensitive domain and can be used in lieu of the full length CIB protein (Kennedy

Figure 1: The concept behind the CRY2/CIB Reaction. With application of blue light stimuli, CRY2 and CIB interact and associated proteins are brought together to catalyze or regulate a reaction (ex. initiation of transcription, start of a signaling cascade). In the dark (removal of blue light stimulus), the interaction is terminated and the associated proteins are separated, terminating their reaction.
et al., 2010). Recently, researchers leveraged the CRY2/CIB1 interaction to reconstitute a bipartite Gal4 transcription factor that had been separated into the activating and binding domains (attached to a CRY2 protein and CIB1 protein respectively; Hughes et al., 2012). Upon blue-light stimulation (461nm, 1sec pulses every 2-3 minutes), the CRY2 construct was recruited to the CIB1 component, thus reconstituting the Gal4 transcription factor and driving Gal4-dependent transcription upon light activation (Hughes et al., 2012; Tucker, 2012). In a separate study, the interaction between CIB1 and CRY2 proteins was utilized to control the movement and dispersion of organelles in mammalian cells; organelles (anchored to CRY2 proteins) and truncated kinesin motor transport proteins (KIF5A, attached to CIB1 proteins) combined upon exposure to blue light pulses (200ms with 10sec intervals) thereby driving the movement of organelles to the plus end of microtubules as the KIF5A traveled along the microtubule (Duan et al., 2015).

The CRY2/CIB optogenetic system offers a unique genetically encoded light-regulated method to study morphogenesis in Drosophila melanogaster by controlling the activation and/or localization of cellular components. Drosophila is a genetically-tractable model organism that is ideal for studying cell and tissue morphogenesis. Drosophila development closely mirrors human development as several tissue movements mimic those found in mammalian developmental processes such as neural tube closure and palate formation (Martin et al., 2004). Additionally, by studying and gaining a better understanding of morphogenesis in Drosophila, researchers often gain insights into wound healing and tissue regeneration processes in vertebrates (Kiehart et al., 2000). In particular, the dorsal closure stage of Drosophila morphogenesis is of interest as the mechanics underlying the epithelial sheet movement that occurs during this process has been implicated in the epithelial sheet movement and wound healing processes of other organisms.
During dorsal closure, the lateral dorsal epidermal sheets migrate to cover a hole in the dorsal epidermis that consists of extraembryonic cells termed the amnioserosa (Kiehart et al., 2000; Harden, 2002). This movement is driven by actomyosin-rich “purse stings” that are present during developmental and wound healing processes across many species (Harden, 2002; Rodriguez-Diaz et al., 2008). The ability to regulate the activity in targeted regions of the actomyosin-rich purse strings using optogenetic systems and blue light stimulation serves as a promising method by which to study the purse string and actomyosin cytoskeleton dynamics during the dorsal closure stage of Drosophila morphogenesis.

The goal of our research is to modify the Arabidopsis system (CRY2/CIB) of light-induced interaction of proteins such that the system is functional in Drosophila to regulate proteins of interest during morphogenesis using blue-light stimulation. We formed a collaboration with Chandra Tucker (University of Colorado School of Medicine) who has successfully adapted these Arabidopsis components to regulate protein transcription in yeast and has provided us with CRY2/CIB constructs for our own experiments. We have taken several approaches to adapt this optogenetic system to Drosophila. We initially cloned and tested an oligomerizing version of the CRY2 protein alone (a mutated version of CRY2PHR with an E490G mutation, termed “Cry2Olig”, that clusters upon blue light stimulation; Taslimi et al., 2014) in Drosophila S2 cells to potentially use this construct to sequester, and thereby render inactive, proteins of interest (i.e. transcription factors, see Figure 2). We tested the efficacy of our light activation protocol with a positive control; a yeast-specific oligomerizing CRY2 construct was transformed into yeast to compare the published expression and localization of the
Cry2olig proteins in yeast (Taslimi et al., 2014) with the results we observed from the expression of the oligomerizing CRY2 construct in Drosophila S2 cells.

Second, to determine the functionality of this optogenetic system with both CRY2 and CIB components, we designed a proof-of-concept experiment to test our light activation protocols. We cloned CRY2PHR(dNLS)_mCherry (an mCherry fluorescent protein-tagged cytoplasmic variant of the CRY2 component that has the nuclear localization signal deleted) and CIBN(dNLS)_pmEGFP_CAAAX (an EGFP fluorescent protein-tagged version of the CIBN protein that has the nuclear localization signal deleted and an addition of a CAAX sequence which drives the CIBN construct to the membrane) from mammalian vectors into Drosophila-
specific vectors to test for expression of each component and the effects of light activation in the *Drosophila* system (see Figure 3). The deletion of the nuclear localization signal in the CRY2 and CIB constructs allows the CRY2 construct to remain diffuse in the cytoplasm prior to blue light stimulation, while the addition of the CAAX sequence (four C-terminal amino acids; [C] Cysteine- [AA] 2 Aliphatic Amino Acids – [X] Any Amino Acid), to the CIBN construct signals a post-translational modification that causes the CIBN to localize to the cellular membrane (Hancock *et al.*, 1991).

**Figure 3: CRY2PHR(dNLS)_mCherry and CibN(dNLS)_pmEGFP_CAAX constructs.** Schematic of the CRY2/CIB optogenetic system. The CRY2PHR(dNLS)_mCherry construct is cytoplasmically diffuse and the CibN(dNLS)_pmEGFP_CAAX construct (a version of the CIBN protein with the deletion of the nuclear localization signal, an added EGFP fluorescent protein, and an added CAAX sequence) is localized to the membrane. Similar to Figure 1, after the application of blue light stimulus, the CRY2 and CIB components interact. Since the CIBN component has a modification that makes it membrane localized, the CRY2 component is driven to the membrane after the application of the stimulus.
While our studies were in progress, Guglielmi et al. (2015) published a paper which reported the successful use of CRY2 and CIBN constructs to disrupt Drosophila morphogenesis by using the light-dimer pair to control Drosophila cell contractions. Based on our previous experiments and this initial successful adaptation of the CRY2/CIB optogenetic system in Drosophila, we will work on recapitulating their methodologies and repeating their experiments in the context of the dorsal closure stage of morphogenesis.

Guglielmi et al. (2015) used the CRY2/CIB system to control PI(4,5)P_2 depletion to study tissue mechanics and cell contractility during ventral furrow formation of Drosophila morphogenesis. Guglielmi et al. (2015) fused an inositol polyphosphate 5-phosphatase to CRY2 which was then recruited to a membrane-localized CIBN component upon blue light stimulation. This translocation of the inositol polyphosphate 5-phosphatase triggered PI(4,5)P_2 depletion, affecting cortical actin levels and cell contractions. We have received the flies used in their study (flies containing the CRY2 combined with Drosophila inositol polyphosphate 5-phosphatase OCRL and flies containing the CIBN with a plasma membrane-anchored EGFP protein) and will use these stocks to refine our methods of light activation and potentially use their method of PI(4,5)P_2 depletion to perturb actin function and tissue morphogenesis during dorsal closure.

Since Guglielmi et al. (2015) have demonstrated the functionality of the CRY2/CIB system in Drosophila, we have also begun working on adapting and expanding the CRY2/CIB system to regulate targeted Drosophila proteins of interest during the process of dorsal closure. Specifically, we are interested in driving Drosophila Rho1 proteins to the membrane where they can regulate regionalized and targeted cytoskeletal processes during dorsal closure. As a small GTPase (G protein), Rho proteins regulate a variety of cellular processes including the regulation and rearrangement of the actomyosin cytoskeleton (Raftopoulou et al., 2004). Rho GTPases are a
large family of regulatory proteins found in eukaryotic organisms that, in addition to playing important roles in actomyosin cytoskeleton regulation and differentiation (Moon et al., 2003), can also regulate gene transcription (Hill et al., 1995), cell cycle progression (Olson et al., 1995), membrane trafficking (Ridley, 2001), apoptosis (Aznar et al., 2001), and neuronal development and synaptic functions (Stankiewicz et al., 2014). Although optogenetic systems have been used to recruit other small G proteins such as Rac (Yin et al., 2015) and Cdc42 (O’Neill et al., 2016) to the membrane, to our knowledge, optogenetic methods have not been adapted to control Drosophila Rho1 protein interactions. We particularly focused on Rho1 proteins, as Drosophila Rho1 is member of the Rho protein superfamily of GTPases and is a homolog of the mammalian RhoA (Magie et al., 1999), a protein that plays a role in human stem cell development (McBeath et al., 2004), cytokinesis (Kosako et al., 2000), actin polymerization (Watanabe et al., 1997) and myosin II contractility (Yamashiro et al., 2003). In Drosophila, Rho1 proteins play a role in cytoskeletal arrangements during Drosophila development through regulating cell cycle progression (Olson et al., 1995) as well as actin filament formation (Chrzanowska-Wodnicka et al., 1996) and contractility of myosin that binds to the actin filaments (Raftopoulou & Hall, 2004), thereby producing force and cell shape changes (Magie et al., 1999; Harden, 2002). Thus, with the successful adaptation of the CRY2/CIB system to Drosophila Rho1 proteins, blue light stimuli could induce actomyosin cytoskeletal changes during Drosophila morphogenesis to further our understanding of the roles of actomyosin cytoskeletal structure and function during the development of Drosophila. We are interested in targeting the wild type, constitutively active (Q63L), and dominant negative (T19N) versions of Drosophila Rho1 in addition to the equivalent mammalian RhoA homologs. These mutant versions of Rho will allow us to mimic both the activation (Q63L) and the inhibition (T19N) of Rho function during dorsal closure in a
tissue-specific manner to further explore the mechanics of *Drosophila* morphogenesis. In addition, we will be able to probe the potential functionality due to conservation of homology of the mammalian RhoA proteins in the *Drosophila* system.

As Guglielmi *et al.* (2015) have demonstrated the functionality of the CRY2/CIB optogenetic system in *Drosophila*, we can now adjust our light activation methodologies and target other proteins of interest to address the regulation of cell and tissue mechanics during *Drosophila* morphogenesis. In addition to testing an oligomerizing version of the CRY2 component and performing proof-of-concept experiments, we also have worked on adapting the light-sensitive system to specific *Drosophila* proteins to study morphogenesis and dorsal closure. By combining the various forms of small G protein Rho1 to CRY2 components and recruiting these constructs to a plasma-membrane localized CIBN component with blue light stimulation, Rho1 can interact with signaling cascades to affect actin and myosin formation and cytoskeleton changes. Thus, the CRY2/CIBN optogenetic system allows for a highly precise, non-invasive, and reversible method to better understand the effects of altering cytoskeleton and cell shape during *Drosophila* morphogenesis, thereby providing researchers with insights into wound healing and tissue regeneration processes in vertebrates.
Methods

DNA Constructs/Plasmids and Cloning Methods

General Overview of Cloning Methods

CRY2/CIB coding regions were cloned from mammalian and/or yeast vectors into *Drosophila*-specific pAW vectors (Thermo Fisher, San Jose, CA). These plasmids were constructed either via standard restriction enzyme cloning or through the Gateway cloning process using pCR™8/GW/TOPO® shuttle vectors (Thermo Fisher, San Jose, CA) following PCR amplification of the regions of interest. CRY2/CIB vectors obtained from the Tucker Lab (University of Colorado School of Medicine) were verified by restriction enzyme endonuclease mapping and sequencing. DNA was purified via the Wizard® Prep SV Miniprep DNA Purification System kit (Promega, Madison, WI) or through performing Boiling Mini-Preps using the protocol outlined in Harwood (1996).

*Cry2oligomerizing-mCherry_pAW*

To test the CRY2 oligomerizing construct in *Drosophila*, we cloned the Cry2oligomerizing-mCh-Clathrin (mCherry, N1 vector) construct sent by the Tucker Lab (Chandra Tucker, University of Colorado School of Medicine) into *Drosophila*-specific vectors. The coding region of the Cry2oligomerizing-mCherry component was cloned into *Drosophila*-compatible pAW vectors (P element Gateway vectors with Actin 5c promoter regions; Thermo Fisher, San Jose, CA) via restriction enzyme digest (utilizing XhoI and BsrGI (blunted) to digest the insert and XhoI and NheI (blunted) to digest the pAW vector) and in-gel ligation (EZclonesystems, New Orleans, LA) using T4 DNA Ligase (restriction enzymes and ligase provided by New England Biolabs® Inc., Ipswich, MA). The oligomerizing CRY2 component,
termed “Cry2Olig”, is a mutated version of CRY2PHR (a truncated version of CRY2, residues 1-498 of the full length CRY2 component) with an E490G mutation that clusters upon blue light stimulation (Taslimi et al., 2014).

**CRY2PHR(dNLS)_mCherry and CIBN(dNLS)_pmEGFP_CAAX**

CRY2PHR(dNLS)_mCherry and CIBN(dNLS)_pmEGFP_CAAX constructs for our proof-of-concept experiment were provided by the Tucker Lab (University of Colorado School of Medicine) and were cloned from mammalian vectors into pAW vectors via Gateway cloning (Thermo Fisher, San Jose, CA). CRY2PHR is a truncated (residues 1-498 of the full length CRY2; Taslimi et al., 2014) but fully functional version of the full length CRY2 component and the CRY2PHR(dNLS) is a cytoplasmic variant of the truncated CRY2PHR component with the deletion of the nuclear localization signal. The CIBN(dNLS)_pmEGFP_CAAX is a version of the CIBN protein with the deletion of the nuclear localization signal, an attached EGFP fluorescent protein, and an added CAAX sequence which drives the CIBN construct to the membrane.

**Mammalian RhoA and Drosophila Rho1**

We received the wild type (WT), constitutively active (Q63L), and dominant negative (T19N) versions of the mammalian RhoA protein linked to CRY2 and mCherry from Chandra Tucker (Tucker Lab, University of Colorado School of Medicine). We also received the wild type (WT) and constitutively active (Q63L) versions of the *Drosophila* Rho1 protein from the *Drosophila* Genomics Resource Center (Bloomington, IN) and the dominant negative version (T19N) of the *Drosophila* Rho1 protein from the Strutt lab (The University of Sheffield,
Sheffield, England) to be cloned to replace RhoA variants in shuttle vectors via standard restriction enzyme cloning. Prior to recombination into pAW vectors via Gateway cloning (Thermo Fisher, San Jose, CA), these constructs were cloned into the pCR™8/GW/TOPO® shuttle vector (Thermo Fisher, San Jose, CA) as an intermediary step.

**Transformation and Transfection of DNA constructs into *Drosophila* Embryos/S2 Cells**

After cloning CRY2/CIB components into pAW vectors, the newly created *Drosophila* constructs were transfected into *Drosophila* S2 cells or were purified and injected into *Drosophila* embryos to generate germline transformants. Transfection of DNA into *Drosophila* S2 cells was performed as outlined in the protocol found in the FuGENE® HD Transfection Reagent Technical Manual (Promega, Madison, WI) and/or as stated in the protocol used by Cherbas *et al.* (1995) in Chapter 9 of “Methods in Cell Biology Vol. 44”. DNA constructs for injection into *Drosophila* embryos to generate germline transformants were prepared for injection using the protocol outlined in the Plasmid DNA purification User Manual in the NucleoBond® Xtra Midi section (NucleoBond® Xtra Midi prep by Macherey-Nagel, Bethlehem, PA). The purified pAW constructs were then injected by Model Systems Injection Service (Durham, NC) to generate *Drosophila* germline transformants.

**Microscopy: Imaging and Photo-Activation Methods (Drosophila S2 Cells)**

Transfected S2 cells were plated on dried Concanavalin A coated coverslips (1 mg/ml; Sigma-Aldrich, St. Louis, MO), allowed to spread for 15 minutes, and then imaged. Imaging of the S2 cells and blue light activation was performed as outlined in Tucker *et al.*, (2012) at approximately 23°C using a Zeiss Axiovert 200M confocal equipped with MetaMorph software (Molecular Devices, Sunnyvale, CA). A 100X 1.45 NA oil immersion objective was used. Z-
planes of 0.2µm per slice spacing were acquired and projected. S2 cells were exposed to blue light ($\lambda = 488$nm) under the Zeiss Axiovert 200M confocal for 6ms, 150ms or 800ms.

**Yeast-Positive Control**

The Cry2olig-mCherry construct, a yeast-specific oligomerizing version of the CRY2 construct, was received from the Tucker Lab (University of Colorado School of Medicine). Transformation of this yeast-specific DNA construct into yeast cells for imaging was kindly performed by Adam Leman (Haase Lab, Duke University) using standard protocols.

**Yeast Imaging and Photo-Activation Methods**

After selecting yeast colonies from the yeast transformation plate and culturing the cells overnight in a YEPD broth following the protocol outlined by Woods *et al.* (2001), the yeast cells were imaged using the Zeiss Axiovert 200M confocal inverted microscope. Extra caution was exercised to minimize the exposure of the yeast cell culture to white light (that includes blue wavelengths) by wrapping the culture tube in aluminum foil during the overnight growth and by covering the slides with foil. A droplet of the yeast liquid culture was imaged using the Zeiss Axiovert 200M confocal inverted microscope with a 100X 1.45 NA immersion oil objective, and the specimen was pulsed with light ($\lambda = 488$nm) for lengths of 0.25 seconds, 1 second, and 10 seconds to stimulate the yeast-specific CRY2 oligomerizing construct.
Results

Vector Verification

To test the functionality of the CRY2/CIB system in *Drosophila*, we first requested vectors containing variants of the CRY2 and CIB components from the Tucker Lab (University of Colorado School of Medicine) to adapt to the *Drosophila* system. All of the vectors were verified through restriction endonuclease mapping and complete sequencing of the region of interest (Eton Bioscience, Inc, Research Triangle Park, NC). After this initial vector verification, we cloned an oligomerizing version of the CRY2 component from the Cry2oligomerizing-mCherry-Clathrin construct sent by the Tucker lab for adaptation to the *Drosophila* system.

Re-creation of a CRY2oligomerizing construct and expression in *Drosophila* S2 cells

Our collaborators in the Tucker Lab (University of Colorado School of Medicine) have reported blue-light stimulated clustering and nuclear localization of a CRY2 oligomerizing construct tagged with mCherry fluorescent protein in yeast cells (Taslimi et al., 2014; Figure 2). To test if the same CRY oligomerizing construct was similarly nuclear-localized into puncta in *Drosophila* upon blue light stimulation, and therefore could be used as a negative regulator of protein interactions, the Cry2oligomerizing-mCherry was excised from a mammalian (N1) vector that also contained clathrin and was cloned into *Drosophila*-specific pAW vectors (P element Gateway vectors with Actin 5c promoter regions; Thermo Fisher, San Jose, CA). Sequencing of the Cry2oligomerizing-mCherry_pAW vector (Eton Bioscience, Inc., Research Triangle Park, NC) identified the addition of extra nucleotides (through nucleotide repair of blunt end cloning) encoding six amino acids (Tyr-Leu-Ala-Gly-Ser-Cys) that created a C-terminal end with both hydrophobic and hydrophilic properties.
The newly constructed Cry2oligomerizing-mCherry_pAW vector was then transfected into *Drosophila* S2 cells (Figure 4). Observation of the S2 cells co-transfected with a positive control (pA-GFP) confirmed a transfection rate of approximately 10%. The pA-GFP appeared diffuse in the cytoplasm and, as expected, did not localize to the nucleus after being exposed to blue light (488nm for 150ms, Tucker, 2012; Figure 4A). The CRY2oligomerizing_mCherry already had formed puncta but did not localize to the nucleus even after being exposed to blue light for 800ms (Figure 4B). A second transfection experiment demonstrated the CRY

![Figure 4: Cry2oligomerizing-mCherry_pAW transfection into S2 cells:](image)

The pA-GFP positive control demonstrated a more diffuse pattern with some puncta (a). The CRY2oligomerizing component of the Cry2oligomerizing-mCherry_pAW vector was clustered in various parts of the cytoplasm even before exposure of the cells to blue light (b). The punctate pattern was not nuclear-localized and the localization of the puncta did not change upon blue light stimulation (c, d).
component clustered in various parts of the cytoplasm even before exposure of the cells to blue light (Figure 4C). Observed on the fifth day after transfection, the punctate pattern was not nuclear localized and the localization of the puncta did not change upon blue light stimulation (Figure 4D). The pA-GFP positive control for the second transfection appeared diffuse but also contained some puncta (data not shown). A third transfection experiment was performed to visualize the formation of puncta at early stages in the transfection process. We found that on the third day following transfections, as the fluorescence began to be detected it was already localized in a punctate pattern.

Comparison of CRY2oligomerizing_mCherry to a Positive Yeast Control (Cry2olig-mCherry)

Given the formation of Cry2olig puncta in Drosophila S2 cells prior to light activation, we attempted to verify our methods of light activation. We used a yeast-specific Cry2olig-mCherry (a Cry2Oligomerizing construct functional in yeast) sent by the Tucker lab and transformed into yeast as our positive control. This positive control has been demonstrated by Taslimi et al. (2014) to oligomerize in yeast and HEK293 cells upon blue light stimulation. In collaboration with the Haase Lab (Duke University), the Cry2olig-mCherry construct was transformed into yeast and colonies were selected, cultured, and imaged. On first observation (Figure 5), the Cry2olig-mCherry construct appeared to be clustered in puncta located outside of

![Figure 5: CRY2olig_mCherry response to light exposure in Yeast](image)

**Figure 5: CRY2olig_mCherry response to light exposure in Yeast:** Before light activation, the Cry2olig-mCherry was clustered in puncta in the cytoplasm (a). After 1 second of blue light exposure ($\lambda = 488$nm), no change was observed in the cell (b). After 10 seconds of blue light exposure ($\lambda = 488$nm), the Cry2olig_mCherry remained stationary and no change was observed in the cell (c).
the nucleus. After pulsing the yeast cells with blue light ($\lambda = 488$nm for 0.25 seconds, 1 second, and 10 seconds), the clusters remained stationary and no nuclear localization was observed.

Due to the inability to repeat the results of Taslimi *et al.* (2014), a second transformation into yeast was performed. The second transformation was kept in the dark in all stages of the growth process to reduce the opportunity for accidental light activation prior to observation. In the second transformation, the Cry2Olig-mCherry was observed with a similar punctate pattern and no additional aggregation or nuclear localization was observed after activation with light ($\lambda = 488$nm for 0.25 seconds, 1 second, and 10 seconds; Figure 6).

![Figure 6: CRY2olig-mCherry response to light exposure in Yeast (Second Transformation, three yeast cells clumped together).](image)

Given the lack of success in reproducing the results, and presumably the light activation protocols, we decided that it would be beneficial to conduct a proof-of-concept experiment to refocus our research from using CRY2 to terminate transcription to adapting both the CRY2 and CIB components to recruit proteins to the cell membrane. To aid in this shift in focus, our collaborators in the Tucker lab sent two new DNA constructs to help us conduct this proof-of
concept experiment: the expression and light activation of CRY2PHR(dNLS)_mCherry and CIBN(dNLS)_pmEGFP_CAAX.

**Proof-of-concept experiment: Recruiting CRY2 to a membrane-anchored CIBN**

The CRY2PHR(dNLS)_mCherry and CIBN(dNLS)_pmEGFP_CAAX components were cloned into *Drosophila*-specific pAW vectors via Gateway cloning (Thermo Fisher, San Jose, CA). The inserts were verified to be in the pCR™8/GW/TOPO® vector through sequencing (Eton Bioscience, Inc., Research Triangle Park, NC). We then performed a LR Recombination Reaction into *Drosophila*-specific pAW vectors (ThermoFisher, San Jose, CA) and again used sequence verification (Eton Bioscience, Inc., Research Triangle Park, NC) to ensure that the inserts were in pAW vectors with no unexpected point mutations.

Upon verifying and purifying the two constructs, the CRY2PHR(dNLS)_mCherry_pAW and the CIBN(dNLS)_pmEGFP_CAAX_pAW constructs were given to the Model Systems Injection Service (Durham, NC) for the injection and germline transformation into *Drosophila* embryos. Transgenic flies containing the CIBN(dNLS)_pmEGFP_CAAX_pAW construct were generated, however the generation of germline transformants of the CRY2PHR(dNLS)_mCherry_pAW construct were not obtained after two separate injection runs.

**Successful use of the CRY2/CIB optogenetic system in Drosophila**

As we were generating constructs for our proof-of-concept experiment, the De Renzis group (European Molecular Biology Laboratory, Heidelberg, Germany) published a paper reporting their successful use of the CRY2/CIB optogenetic system in *Drosophila* to disrupt morphogenesis (Guglielmi *et al.*, 2015). With this report of the successful use of CRY2 and
CIBN components in *Drosophila*, in addition to continuing our cloning for our proof-of-concept experiment, we also proceeded to work on adapting the De Renzis group’s methods of light activation to our constructs. Recently, the De Renzis group have sent us the flies used in their study (flies containing the CRY2 combined with *Drosophila* inositol polyphosphate 5-phosphatase OCRL and flies containing the CIBN with a plasma membrane-anchored EGFP protein) to refine our methods of light activation and potentially use light activation to perturb actin function and tissue changes during dorsal closure. Since we were sent the original *Drosophila* lines used in their study, we are currently working on crossing the lines to generate flies that express both constructs. Given the successful demonstration of the CRY2/CIB optogenetic system by Guglielmi *et al.* (2015), we also moved forward to build both mammalian RhoA CRY2 and *Drosophila* Rho1 CRY2 fusion constructs.

**Generation of CRY2_mCherry- Rho protein fusion constructs**

The mammalian RhoA variants (wild type, constitutively active, and dominant negative) were sent to us by our collaborators in the Tucker lab. Cloning of the mammalian RhoA proteins into *Drosophila*-specific pAW vectors will allow us to test the conservation of Rho functionality across phylogeny. Thus far, the dominant negative (T19N) and constitutively active (Q63L) cytoplasmic versions of the mammalian RhoA construct with a mCherry-tagged CRY2 component have been successfully cloned (via Gateway cloning; Thermo Fisher, San Jose, CA) into shuttle vectors and sequence verified (Eton Bioscience, Inc., Research Triangle Park, NC). Additionally, the dominant negative version (T19N) of the mammalian RhoA linked to a CRY2 component without an mCherry tag has also been successfully cloned into a shuttle vector (via Gateway cloning; Thermo Fisher, San Jose, CA) and sequence verified (Eton Bioscience, Inc.,
Research Triangle Park, NC). Work is still progressing to generate the constitutively active (Q63L) version of the mammalian RhoA linked to a CRY2 component without an mCherry tag in a shuttle vector. Once the five variations of the CRY2_RhoA constructs (CRY2_mCherry_WTRhoA, CRY2_mCherry_T19NRhoA, CRY2_mCherry_Q63LRhoA, CRY2_T19NRhoA, and CRY2_Q63LRhoA) are cloned into shuttle vectors and sequence verified, we will perform a LR Recombination Reaction (Thermo Fisher, San Jose, CA) to clone the variations of the CRY2_mammalianRhoA components into Drosophila-specific pAW vectors for injection into Drosophila embryos, the subsequent generation of Drosophila germline transformants, and light activation tests in cells of Drosophila embryos that are also expressing CIBN(dNLS)_pmEGFP_CAAX.

In addition to working on mammalian RhoA cloning, we are also currently working on using standard restriction enzyme cloning to replace the mammalian RhoA variants in the CRY2_mCherry shuttle vector with the Drosophila Rho1 variants (wild type, constitutively active Q63L, and dominant negative T19N). Once the Drosophila Rho1 variants have been verified to have replaced the mammalian RhoA in the shuttle vectors through sequence verification, we will perform a LR Recombination Reaction (Thermo Fisher, San Jose, CA) to clone these variants of the CRY2_mCherry_DrosophilaRho1 components into Drosophila-specific pAW vectors to be injected into Drosophila embryos for the generation of germline transformants and testing of light activation protocol.
Discussion

As we undertook a multi-pronged approach to reconstituting the CRY2/CIB system in *Drosophila*, we first focused on testing oligomerizing CRY2 components before working on adapting both the CRY2 and CIB components to the *Drosophila* system and using their light-activated interaction to regulate the translocation of Rho proteins.

We began our exploration of adapting the *Arabidopsis thaliana* CRY2/CIB optogenetic system to *Drosophila* by focusing on CRY2/CIB constructs that had already been adapted to yeast. In yeast, the CRY2/CIB1 light dimerization system has had success in inducing transcription and protein production from the application of blue light to the light-sensitive system (Hughes *et al.*, 2012). Hughes *et al.* (2012) were able to reconstitute a bipartite Gal4 transcription factor through blue light activation after separating and attaching the Gal4 activating and binding domains to CRY2 and CIB components, respectively. When blue light stimulation catalyzed the interaction of the CRY2 and CIB constructs and thus reconstituted the Gal4 transcription factor, the Gal4 was then able to bind to a UAS enhancer region which then initiated the transcription of a targeted genomic sequence. In order to study this system in *Drosophila*, we requested and received constructs from the Tucker lab (University of Colorado School of Medicine) that contained either variants of the *Arabidopsis* photoreceptor cryptochrome 2 (CRY2) or variants of the transcription factor CIB1. Ultimately, all of the vectors were verified through restriction endonuclease mapping and sequencing, allowing us to proceed with utilizing the constructs to explore CRY2/CIB functionality in *Drosophila*.

One of the vectors received from the Tucker Lab was an oligomerizing construct, Cry2oligomerizing-mCherry-Clathrin, which contains a mutated (E490G) version of the truncated (residues 1-498 of the full length CRY2; Taslimi *et al.*, 2014) CRY2PHR. Prior
research has highlighted the oligomerization of this CRY component in the nucleus in yeast cells upon blue light stimulation (Taslimi et al., 2014). We decided to test this oligomerizing CRY2 construct in *Drosophila*; if the oligomerizing CRY2_mCherry construct was nuclear localized upon blue light activation in *Drosophila*, it may be possible to take advantage of this localization to sequester and inactivate proteins of interest that we fuse to the CRY2. Sequencing of the Cry2oligomerizing-mCherry_pAW construct revealed the addition of extra nucleotides (the result of unintended nucleotide repair) that encoded six amino acids (Tyr-Leu-Ala-Gly-Ser-Cys) and created a C-terminal end with both hydrophobic and hydrophilic properties. As these extra amino acids are not neutral, it was possible that this addition would affect our results, however we proceeded with transfecting the construct into *Drosophila* S2 cells. Observation of the S2 cells with the positive transfection control of cytoplasmic GFP (pA-GFP) confirmed a low transfection rate of approximately 10% (Figure 4A). After the first transfection attempt (Figure 4B), the CRY2 oligomerizing component appeared diffuse in the cytoplasm and did not localize to the nucleus after being exposed to blue light (488nm for 150ms, based off the protocol presented in Tucker, 2012). This result was different than the clustering behavior observed in other preliminary research which reports that the “CRY2olig” protein oligomerization in yeast occurs when CRY is exposed to blue light (488nm) for 6ms, an exposure time significantly less than the amount of light to which the tested S2 cells were exposed (Taslimi et al., 2014). Even when exposed to blue light stimuli for longer times (800ms), the CRY component did not localize to the nucleus, prompting us to re-transfect a new batch of S2 cells to discount the possibility of the low transfection rate and/or a sub-optimal batch of S2 cells affecting our initial result. However, the second transfection experiment also demonstrated the CRY component clustered in various parts of the cytoplasm prior to exposure of the cells to blue light (Figure 4C).
Observed on the fifth day after transfection, the punctate pattern was not nuclear-localized and the localization of the puncta did not change upon blue light stimulation (Figure 4D). The pA-GFP positive control for the second transfection also contained some puncta. Although the exact reason for the puncta pattern could not be discerned, it is possible that since the Drosophila cells were expressing a foreign and unrecognized protein, the CRY protein was sequestered to lysosomes (producing a punctate pattern) and therefore could not oligomerize upon light activation. Another possibility is that the extra hydrophobic and hydrophilic amino acids added to the C-terminal end during the cloning process may have also affected our results. Given that the puncta did not appear to be nuclear-localized, it was unlikely that the observed puncta were derived from the oligomerizing nature of the CRY component. If the puncta were a result of the oligomerizing nature of the CRY component, we would have expected them to be nuclear localized as reported in yeast (Taslimi et al., 2014) even prior to light stimulation, thereby indicating our protocol did not have the proper light-activation protection. We believe that the punctate pattern in both the control and the experimental S2 cells is related to the overall health of the cells and the inadvertent exposure to white light that begins the aggregation process. If the S2 cells were in a robust state, it is possible our inadvertent aggregation of Cry2olig via light contamination would have resulted in the visualization of the translocation of the construct to the nucleus. Another possibility for the premature aggregation of the Cry2olig is the potential inability of the Cry2olig protein to fold properly in a Drosophila S2 cell.

Due to the abnormal response of the CRY2 oligomerizing construct in S2 cells to light exposure, we decided to conduct a positive control test with a yeast-specific oligomerizing version of the CRY2 construct, Cry2Olig-mCherry, that has reported functionality in yeast (Taslimi et al., 2014). By imaging and light-activating Cry2Olig-mCherry transformed into yeast
cells, we could test our light activation protocol which would give us a better indication if the lack of light-activated nuclear clustering of the modified oligomerizing CRY construct in S2 cells was due to the incompatibility of the protein with *Drosophila* systems, the health of the S2 cells, or due specifically to issues with our light activation protocol.

We received the Cry2olig-mCherry construct in a yeast vector from the Tucker lab to be transformed into yeast. The first transformation into yeast and observation of the cells revealed the Cry2olig-mCherry construct clustered in puncta outside of the nucleus. After pulsing the yeast cells with light (\(\lambda=488\text{nm}\) for 0.25sec, 1sec, 10sec), the clusters remained stationary and no change was observed (Figure 5). A second transformation into yeast was kept in the dark in all stages of the culture process. In these cells, Cry2olig-mCherry was slightly more diffuse in the cytoplasm but with puncta already formed, and no clustering was observed after activation with light (\(\lambda=488\text{nm}\) for 0.25sec, 1sec, 10sec; Figure 6). It is possible that we could not successfully reconstitute this positive control experiment due to a combination of unintended light stimulation of the cells before they were observed under the microscope and/or overgrowing the yeast cells before imaging them. However, as with the S2 cell transfection, if unintended light stimulation occurred, we would still expect the puncta to be nuclear-localized as demonstrated by Taslimi *et al.*, (2014). Future work should focus on imaging during the yeast growth cycle and not during overgrown conditions. In addition, samples for imaging should be completely covered during growth and preparation for imaging by taking precautions such as covering growing samples, using a dark room to plate cells, and using yellow wavelength light to focus the microscope and to prepare samples for imaging (Guglielmi *et al.*, 2015). Additionally, the observed yeast cells from the second transformation appeared smaller and a green fluorescence was detected from the cells. Due to the size difference and unexpected green fluorescence, it was determined that the
observed yeast may have been contaminated during the transformation process by other samples from our collaborator which may have affected our results.

Given our inability to recapitulate the Cry2 oligomerizing results of Taslimi et al., (2014) in both *Drosophila* S2 cells and yeast cells, and upon reflection of the overall project goal of adapting this optogenetic system to *Drosophila*, we decided that the focus of the research may be better if shifted from using the oligomerizing CRY2 component to inhibit protein function via nuclear localized oligomerization to adapting both the CRY2 and CIB components to *Drosophila* to utilize the light-sensitive system to recruit proteins to the cell membrane in the *Drosophila* embryo. Additionally, due to inconsistencies and challenges when working with the transient transfection of *Drosophila* S2 cells, we decided to proceed with generating germline transformants of our constructs so that our initial proof-of-concept experiments will be conducted in *Drosophila* embryos, thereby reducing artifacts due to the health of the S2 cells and inconsistencies in the transfection protocol. Working with germline transformants will also allow us to generate permanent fly stocks of these constructs.

To aid in this shift in focus to a proof-of-concept experiment, our collaborators in the Tucker lab sent two new DNA constructs: CRY2PHR(dNLS)_mCherry and CIBN(dNLS)_pmEGFP_CAAX. Both constructs contain the deletion of the nuclear localization signal (dNLS), and the CRY2PHR version is a truncated (residues 1-498 of the full length CRY2; Taslimi et al., 2014) yet fully functional version of the CRY2 component.Additionally, the CIBN construct is modified with a “CAAX box” sequence that results in prenylation of the CIBN and insertion into the plasma membrane (pm), thereby causing the CIBN construct to anchor in the membrane. Ideally, upon blue light stimulation, diffuse, cytoplasmic CRY2PHR(dNLS)_mCherry would be recruited to the membrane where the CIBN construct is
located. If the system is shown to be functional in *Drosophila*, the CRY2PHR(dNLS)_mCherry construct could then be fused with proteins of interest to catalyze signaling cascades and subsequent cellular changes such as cytoskeleton rearrangements when recruited to the CIBN in the cellular membrane.

After Gateway cloning (Thermo Fisher, San Jose, CA) the CRY2PHR(dNLS)_mCherry and CIBN(dNLS)_pmEGFP_CAAX components into *Drosophila*-specific pAW vectors, both constructs were given to the Model Systems Injection Service (Durham, NC) for the injection and germline transformation into *Drosophila* embryos. Although transgenic flies containing the CIBN(dNLS)_pmEGFP_CAAX_pAW construct have been generated, after two injections, we still lack any germline transformants for the CRY2PHR(dNLS)_mCherry_pAW construct and will need to discern the reason for the lack of transformants through construct verification and reinjection into *Drosophila* embryos. There are many potential reasons for the lack of transgenic flies including the toxicity of the construct to *Drosophila* development or other complications with the vector. As a first step, we will re-sequence the CRY2PHR(dNLS)_mCherry_pAW construct prior to another germline injection attempt. We are now imaging our CIBN(dNLS)_pmEGFP_CAAX transgenic lines to ensure that the construct fluoresces. We are also establishing crosses to map the genomic location of the transgenes that integrated into the genome.

If the germline transformants of CRY2 component can be generated, we will then cross these lines together and image the resulting progeny during embryogenesis to determine if both components are expressing (i.e. if the red CRY2PHR(dNLS)_mCherry_pAW construct is found in the cytoplasm and the green CIBN(dNLS)_pmEGFP_CAAX_pAW is found in the membrane). Once we confirm both lines are expressing, we will test the light activation protocol
to determine if the CRY2 component is recruited to the CIBN component in the membrane in individual cells during *Drosophila* embryogenesis.

While we were generating constructs for our proof-of-concept experiment, Guglielmi *et al.* (2015) published a paper reporting their successful use of the CRY2/CIB optogenetic system in *Drosophila* to disrupt morphogenesis by using the light-dimer pair to control *Drosophila* cell contractions. By combining the CRY2 with *Drosophila* inositol polyphosphate 5-phosphatase OCRL (tagged with mCherry florescent protein) and the CIBN with a plasma membrane-anchored EGFP protein, Guglielmi *et al.* (2015) were able to cause defects in morphogenesis with the application of blue light (488nm, 1sec). Upon blue light activation, the CRY2 construct was recruited to the plasma membrane-anchored CIBN, thereby recruiting the inositol polyphosphate 5-phosphatase to the plasma membrane where the phosphatase would dephosphorylate PI(4,5)P$_2$ to PI(4)P (Guglielmi *et al.*, 2015). The depletion of PI(4,5)P$_2$ subsequently depleted the depolymerization of cortical actin, thereby affecting cell contractility during morphogenesis and allowing the researchers to analyze the role of cortical actin in *Drosophila* morphogenesis. Guglielmi and colleagues in the De Renzis group (European Molecular Biology Laboratory, Heidelberg, Germany) also noted that the rapid activation of the system with blue light without requiring the addition of an exogenous chromophore allowed the CRY2/CIBN system to be an effective mechanism to disrupt *Drosophila* morphogenetic processes with high spatiotemporal precision and reversibility at room temperature with a half-life of approximately 8.9 minutes. With this report of the successful use of CRY2 and CIBN components in *Drosophila*, in addition to continuing our progress on our proof-of-concept experiment, we have begun work with the fly lines used by the De Renzis group to refine our methods of light activation. Additionally, working with their flies may allow us to use their
method of light activation and PI(4,5)P₂ depletion to perturb actin function and tissue mechanics during *Drosophila* morphogenesis to address our own questions about the process of dorsal closure. Since we received the original *Drosophila* lines used in their study, we are currently working on crossing the lines to generate flies that express both CRY2 and CIB constructs to conduct these experiments.

Given that Guglielmi et al. (2015) demonstrated the functionality of the CRY2/CIB system in *Drosophila*, we are proceeding to clone variants of Rho proteins with the CRY2/CIB system to expand and advance the use of this system to disrupt *Drosophila* morphogenesis. Since Rho proteins play a role in cytoskeletal arrangements during early *Drosophila* development (Harden, 2002), we will attempt to adapt the CRY2/CIB system to Rho proteins and use blue light stimuli to recruit Rho proteins to the cellular membrane and induce cytoskeletal changes during *Drosophila* morphogenesis. The ability to alter cytoskeletal function in a developmental stage and in a tissue-specific manner will help us understand the role of the actomyosin cytoskeletal structure during morphogenesis. As the tissue mechanics of *Drosophila* morphogenesis are similar to the tissue mechanics of wound healing and tissue regeneration processes in other organisms (Martin & Parkhurst, 2004), a better understanding of actomyosin cytoskeletal dynamics could provide insight into wound healing and tissue regeneration processes in vertebrates.

To study the potential use of a CRY2-Rho construct to disrupt *Drosophila* morphogenesis, we are working on cloning wild type, constitutively active, and dominant negative versions of both mammalian RhoA and *Drosophila* Rho1 into *Drosophila*-specific vectors containing CRY2 and mCherry components. Our collaborators (Chandra Tucker, University of Colorado School of Medicine) sent us the wild type (WT), constitutively active
(Q63L), and dominant negative (T19N) versions of the mammalian RhoA linked to CRY2 with and without and mCherry. We have cloned the CRY2_mCherry_RhoA components into a shuttle vector and are currently working on utilizing Gateway cloning (Thermo Fisher, San Jose, CA) to recombine the components into Drosophila-specific pAW vectors to co-express with the CIBN(dNLS)_pmEGFP_CAAAX construct in germline transformants. The expression of mammalian Rho variants in the CRY2/CIB system will help decipher the conservation of Rho function across phylogeny in a temporally controlled manner. Thus, if mammalian RhoA is not able to function as a small GTP protein throughout the completion of Drosophila development, perhaps switching it on and off through the use of optogenetics at a specific developmental stage and in limited tissues will allow us to test for any conserved functionality. If the mammalian RhoA is shown to generate a phenotype in Drosophila development, this would indicate the conservation of Rho functionality across phylogeny.

Concurrently, we are working on cloning Drosophila Rho1 variants to replace the mammalian RhoA variations in the shuttle vectors containing CRY2 and mCherry for subsequent cloning into Drosophila-specific pAW vectors (via Gateway cloning; Thermo Fisher, San Jose, CA). We received the DNA of the wild type and constitutively active (Q63L) versions of Drosophila Rho1 from the Drosophila Genomics Resource Center (Bloomington, IN) and the dominant negative version (T19N) of Drosophila Rho1 from the Strutt lab (The University of Sheffield, Sheffield, England). After the successful cloning to replace the mammalian RhoA in the shuttle vector, we plan to perform a LR recombination of these Rho constructs into pAW vectors via the Gateway cloning protocol (Thermo Fisher, San Jose, CA) for injection into Drosophila embryos to generate germline transformants. Expression of the mCherry tagged CRY2-Rho1 variants and the CIBN(dNLS)_pmEGFP_CAAAX_pAW construct will be tested and
upon blue light stimulation, it is expected that we will observe the recruitment of the CRY2 component to the CIBN component at the membrane of cells in the Drosophila embryos. This would indicate that the Drosophila Rho1 constructs are at the membrane and if functional, we may see a net effect on the actomyosin cytoskeleton as a result of the Rho1 signaling pathway being disrupted or activated due to the dominant negative (T19N) Rho1, wild-type, or constitutively active (Q63L) Rho1. With the successful adaptation of the CRY2/CIB system to Drosophila Rho1 proteins, we can potentially use blue light activation to induce actomyosin cytoskeletal changes during Drosophila morphogenesis to further our understanding of the role of actomyosin cytoskeletal structure during early development of Drosophila.

Overall, although our attempts thus far have not yet optimized the adaptation of the CRY2/CIB system to Drosophila, progress has been made in better understanding the challenges that arise with using optogenetic systems to regulate protein-protein interactions in Drosophila and the adaptation of the CRY2/CIB system to specific Drosophila proteins. Our exploration with the oligomerizing CRY2 constructs indicates the sensitivity of the optogenetic system to inadvertent and unintentional light stimulation. Additionally, with our difficulties in successfully transfecting CRY constructs into S2 cells with transfection rates no greater than 10% and the questionable health of the S2 cells prior to transfection, expression of the CRY2 and CIB components in an S2 cell culture system are not likely to yield functional data. As a result, we will proceed directly to the generation of germline transformants to eliminate errors that are a result of tissue culture artifacts. Future work will focus on working with the fly lines sent from the De Renzis group and the exploration of both repeating their light activation protocol and optimizing these light activation protocols to our lab. Furthermore, the use of the fly lines generated by the De Renzis group will serve as another method to disrupt Drosophila
morphogenesis to study the mechanisms behind actomyosin regulation during dorsal closure. While future work should continue on cloning CRY2_mCherry_mammalian RhoA components into Drosophila-specific vectors to test for the conservation of Rho functionality across phylogeny, it is unclear if the expression of mammalian Rho variants in a Drosophila system will generate phenotypes. Therefore, we will continue with the cloning of Drosophila Rho1 proteins into CRY2_mCherry constructs, as altering Drosophila Rho1 functionality is an exciting potential mechanism for regulating actin and myosin function during Drosophila morphogenesis. As Guglielmi et al. (2015) have now demonstrated a successful adaptation of the CRY2/CIB system to Drosophila, moving forward and adapting this optogenetic system to the study of Drosophila proteins such as Rho1 advances and expands the potential use of this non-invasive and reversible mechanism as a new genetic tool to better understand dorsal closure and Drosophila morphogenesis.
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Works Cited


