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Investigating the molecular function of Akirin during skeletal myogenesis in Drosophila melanogaster

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Abstract

The specification and differentiation of muscle precursor cells, or myoblasts, by the action of the mesodermal and muscle transcription regulator Twist is a key event in the formation of the *Drosophila* larval musculature. Akirin, a highly conserved nuclear protein, appears to play a critical role in the regulation of Twist-dependent gene expression during mesodermal specification and muscle development. Specifically, Akirin serves as a cofactor to promote interactions between regulatory transcription factors and multisubunit Brahma SWI/SNF-class chromatin remodeling complex to impact gene expression across varying targets. Using a genetic interaction screen in *Drosophila*, we have begun to identify other Akirin interacting proteins that participate in the process of muscle specification, patterning, and development. Our screening method has identified a number of proteins that genetically interact with Akirin during muscle patterning in the embryo, including *pannier* and *taf4* which both are involved in general transcription initiation. Importantly, we have identified a novel interaction between Akirin and the NuRD chromatin remodeling complex. These results also show context-dependent roles of Akirin at the transcriptional level, as interactions with SWI/SNF family function to facilitate gene expression, and interactions with NuRD family may function repress transcriptional activities. We have extended these studies to map the regions of the Akirin protein that are necessary for such interactions during embryonic muscle development. However, we tried and failed to demonstrate a physical link between Akirin and Twist using the methods in this thesis. By identifying new intermolecular partners for Akirin during myogenesis, we have shed new light on the molecular action of Akirin during embryonic development.

CHAPTER 1: INTRODUCTION

A fundamental question in developmental biology is how a single common progenitor gives rise to diverse cell lineages through distinct mechanisms of specification and organization. During embryonic development, these processes are governed by gene expression programs coordinated by tightly regulated transcription factor activities. At the level of gene expression, interactions of accessory proteins or cofactors contribute to the regulation of such developmentally critical transcription factors (Koster *et al.,* 2015). These interactions are controlled directly through binding and dimerization activity, or through indirect mechanisms with chromatin remodeling complexes to create an environment favorable for gene expression (Clapier and Cairns, 2009). Therefore, the identification of secondary cofactors is essential to further understand the regulation of transcription factors during development.

The work presented in this thesis aimed to further understand the molecular function of a novel protein during embryonic myogenesis. The formation of mesodermally derived tissues to form body walls or skeletal muscle is a requirement for developing *Drosophila* embryos. Within the mesoderm, myoblast populations arise and ultimately fuse to form a functional muscle fiber. Myoblast population dynamics are tightly controlled by gene expression programs that are moderated by myogenic transcription factors to determine muscle cell fates. Although many transcription factors oversee muscle development in mammals, the presence of a sole myogenic transcription factor, Twist, in *Drosophila melanogaster* makes it an ideal animal model for myogenesis (Baylies *et al.,* 1996). Despite the primary importance of myoblast mechanics for building and patterning the musculature, the regulatory signaling mechanisms that influence myogenic transcription at the level of gene expression remain to be elucidated.

Overview of Skeletal Myogenesis

During development of the animal embryo, cells are given regional and temporal cues from their surrounding environment to specify a particular cellular fate and ultimately differentiate. Diverse cell lineages are first defined by cell movements during a process called gastrulation that result in the formation of the three germ layers: ectoderm, mesoderm, and endoderm.

Following gastrulation, mesodermal cells give rise to somites that form bilaterally to the primitive spinal cord and run along its length from anterior to posterior. The somites then give rise to the sclerotome that forms the vertebrate and rib cartilage, the myotome that forms skeletal musculature, and the dermamyotome that forms dermis of the back and skeletal limb muscles. Within the myotome of maturing somites, a population of cells are induced by a family of myogenic regulatory factors (MRFs) to differentiate into a pool of myoblasts, which specify muscle cell fates (Weintraub *et al.,* 1989; Kopan *et al.,* 1994). Once myoblast pools are defined, the cell exits the cell cycle, aligns, and ultimately fuses into a functional, multinucleate muscle fiber (Figure 1).

In *Drosophila*, a muscle fiber arises from a pool of founder cells and fusion-competent myoblasts that remain after the formation of muscle (Baylies *et al.,* 1998). The specification and differentiation of myoblast populations result from a coordinated program of gene expression (Baylies and Bate 1996). Orthologs of genes that encode the regulators that guide the process of myoblast specification and differentiation are present across metazoans (Rochlin *et al.,* 2009). The function of myogenic transcription factors to commit mesodermal cells to a skeletal muscle cell fate during development is conserved in both vertebrates and invertebrates (Zhang *et al.,* 1999). Moreover, *Drosophila* represents an ideal animal model for studying myogenesis, as the identities and regulatory ability of the molecular players involved in muscle development are highly conserved from insects to mammals (Nowak *et al.,* 2009; Kim *et al.,* 2007; Richardson *et al.,* 2007).

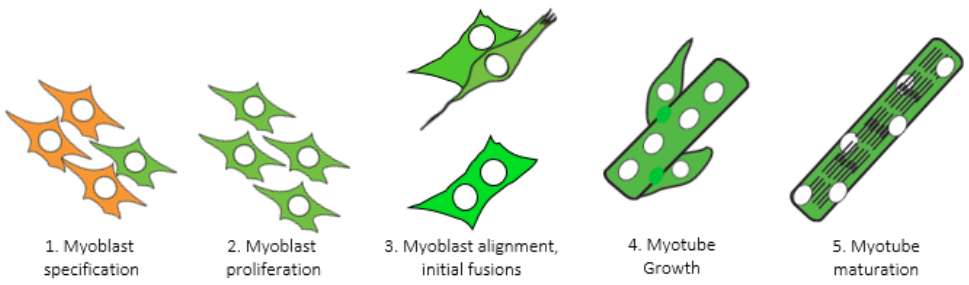

Figure 2: Overview of steps in myogenesis. During development of the animal embryo, the somites ultimately give rise to myogenic progenitors that form myoblasts upon determination. Given the appropriate environmental cues, the myoblasts will align and fuse into a multinucleate muscle fiber.

The Role of Twist in Patterning the Skeletal Musculature

The control of myoblast specification and differentiation is tightly controlled by gene expression mediated by myogenic transcription factors. A single transcription factor, Twist, is critical for somatic musculature patterning in *Drosophila melanogaster*, and is differentially expressed at different stages of embryonic development (Baylies and Bate 1996; Baylies *et al.,* 1998). Twist is a highly conserved basic helix-loop-helix (bHLH) transcription factor that is required for a diverse array of processes such as successful mesoderm formation in the fly embryo, as well as cancer metastasis in mammals (Yang *et al.,* 2004). During gastrulation, the mesoderm will ultimately give rise to the cardiac, visceral, somatic, and somatic gonad musculature. High levels of Twist expression drive mesodermal cells to a somatic muscle fate, whereas low levels of Twist expression result in the formation of other mesodermal derivatives (Baylies and Bate, 1996). Homologues of Twist have been identified in both invertebrates and vertebrates, and its expression pattern during mesodermal development is conserved in most species (Castanon and Baylies, 2002). Interestingly, Twist targets a diverse array of genes responsible for several steps in patterning the adult musculature, and is crucial for the formation of the mesoderm (Castanon *et al.,* 2001; Sandmann *et al.,* 2007).

Twist regulates myogenesis by directly controlling the expression of muscle-specific genes through binding of a conserved region within the bHLH domain (Castanon and Baylies, 2002). bHLH factors function by inducing transcription at E-box consensus sequences (CANNTG motif) found within regulatory regions of genes expressed in skeletal muscle cells (Ephrussi *et al,* 1985; Berkes *et al.,* 2005; Zhang *et al.,* 1999). DNA binding of bHLH factors is facilitated through dimerization activity with ubiquitous bHLH E proteins. During early development, Twist forms a homodimer to drive cells to a somatic muscle fate, whereas heterodimerization of Twist with other E protein partners such as Daughterless repress gene expression programs that are required for somatic myogenesis (Castanon *et al.,* 2001).

In addition to the bHLH region, *Drosophila* Twist contains a number of domains across its sequence that are critical for its function (Figure 2). GST-pull downs demonstrated that two glutamine rich regions of Twist, AD1 and AD2, are critical for its direct interaction with Dorsal to specify a mesodermal fate in the ventral region of *Drosophila* embryos (Shirokawa and Courey,

1997). Additionally, the first 330 amino acids of Twist directly interact with Taf4, the basal transcription factor TFIID subunit $TAF_{II}10$ (TBD-associated-factors), in order to facilitate Twist and Dorsal induced specification of mesodermal derivatives (Pham *et al.,* 1999). Interactions between Twist and components of the nuclear machinery support a general transcriptional role of Twist, as interactions between Twist and Taf4 can ultimately recruit RNA polymerase II to activate gene expression programs (Pham *et al.,* 1999). As discussed previously, Twist dimerizes with itself through the highly conserved bHLH region to promote DNA binding (Castanon *et al.,* 2001). However, when Twist is bound to Daughterless, another bHLH E protein, the DNA binding activity is reduced and gene transcription is repressed (Castanon *et al.,* 2001). Finally, the tryptophan-arginine (WR) motif is also highly conserved among Twist family members (Spring *et al.,* 2000). Although the exact function of the WR motif is unknown, it has been shown that it is critical for Twist interaction with Snail during cranial neural crest formation in *Xenopus* (Lander *et al.,* 2013), Twist-induced IL8 cytokine production in human breast epithelial cells (Li *et al.,* 2012), and repression of Runx2 by Twist during osteoblast differentiation in mice (Bialek *et al.,* 2004).

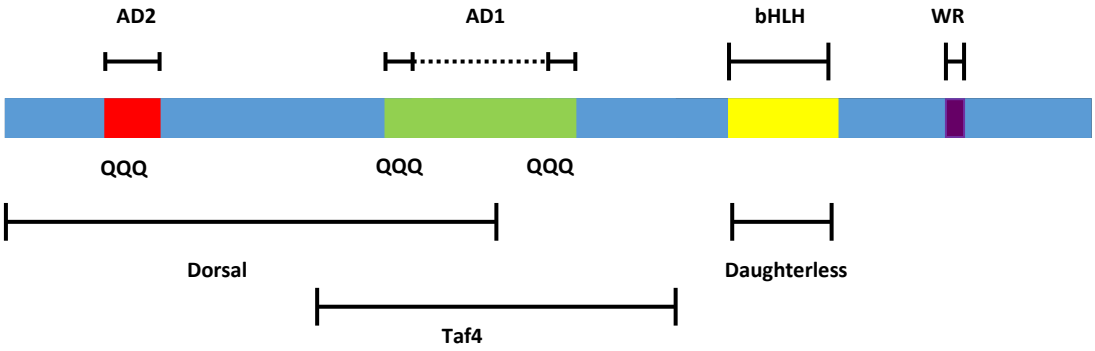

Figure 2: Critical domains of* Drosophila *Twist. Across its sequence, *Drosophila* Twist contains a number of differentially conserved domains that are critical for its function. Interactions with various regions of the two glutamine-rich motifs, AD1 (green) and AD2 (red), are important for Twist synergistic activation of Dorsal and Taf4. Protein dimerization and DNA binding activity is mediated through interactions with the bHLH motif (yellow). Finally, the WR motif (purple) is also highly conserved, but its exact function remains to be elucidated.

The modulation of Twist activity in the embryo and the wide-range of target genes suggests that Twist has a complex regulation whose current mechanism is still not well understood

(Baylies and Bate 1996; Baylies et al. 1998; Sandmann et al. 2007). Therefore, a key question in developmental biology is how a single transcription factor such as Twist can mediate an entire program of gene expression during2 muscle differentiation.

Chromatin Remodeling during Development

All living organisms undergo dynamic changes to gene expression programs in response to cellular and environmental signals. As genome size increased with the development of higher organisms in the Eukarya and Archaea domains of life, different mechanisms for chromatin remodeling among evolving species emerged to allow for accessibility of genomic sequences (Koster *et al.,* 2015). These mechanisms are in place to alter condensed DNA and allow access for regulatory proteins to recruit transcription factors that control gene activation or repression. This confers an evolutionary advantage, as a single regulatory protein can serve as a secondary cofactor for many different transcription factors, thereby reducing the number of regulatory proteins that need to be readily available at all times during the life of the cell.

The temporal regulation of the wide range of Twist targets during myogenesis suggests that some mechanism must exist at the DNA level that regulates their expression given the needs of the cell. As a transcription factor, one of the key functions of Twist is the recruitment of RNA polymerase II to muscle-specific genes. Given the high turnover of changes in gene expression programs during development, the chromatin environment may block the activation of target genes and may need to be remodeled to create an environment favorable for gene expression. Chromatin remodeling complexes confer an opportunity for regulation of transcriptional processes that ensure cellular homeostasis by binding with high affinity to covalent histone modifications to alter the chromatin environment (Clapier and Cairns, 2009).

It is important to understand how chromatin remodelers work to influence gene expression. The nucleosome is the structural unit of condensed DNA, consisting of 147 bp of DNA wrapped around four histone proteins forming a final octamer, thereby acting as a substrate for chromatin remodelers. This allows for extensive compacting of the genome in an orderly manner, but regulatory elements can often be packaged into inaccessible areas. During development, rapid cellular movements and specification requires global re-organization of

chromatin for subsequent changes in gene expression patterns (Wu, 2012). Mechanisms mediated by chromatin remodeling complexes confer an opportunity for nucleosomes and components of chromatin remodelers to directly regulate transcriptional processes that ensure cellular homeostasis (Tartey *et al,* 2015). Dynamic changes to the organization of chromatin is affected by DNA methylation, covalent modification of histones, and ATP-dependent alterations to chromatin that is influenced by other interacting partners such as transcription factors (Clapier and Cairns, 2009; Hota and Bruneau, 2016).

ATP-dependent chromatin remodeling complexes exist within Archaea and Eukarya domains and function by using energy derived from ATP-hydrolysis in order to restructure the nucleosome during the packaging of DNA or regulation of DNA accessibility (Clapier and Cairns, 2009). The catalytic activity of all chromatin remodeling complexes is carried out by an ATPase, a subunit of the SNF2 family of DNA helicases (Hota and Bruneau, 2016). These families are further defined into four distinct groups based on similarity of the ATPase domains and presence of particular domains across their sequence (Hota and Bruneau, 2016). The four major subfamilies include SWI/SNF (switching defective/sucrose non-fermenting), ISWI (imitation SWI), INO80 (SWI/SNF2 related inositol requiring 80), and CHD (chromodomain helicase DNA-binding) (Hota and Bruneau, 2016; Clapier and Cairns, 2009). Each subfamily contains a catalytic ATPase that uses ATP hydrolysis to alter nucleosome structure, and up to 16 other non-catalytic protein subunits that are responsible for specific genome binding activities (Hota and Bruneau, 2016). Although each chromatin remodeling family has a distinct structure, all are involved in developmental processes beyond transcription (Ho and Crabtree, 2010).

Akirin Serves as a Novel Regulator of Biological Processes

Akirin, a highly conserved nuclear cofactor, was first identified using RNA-mediated interference in *Drosophila melanogaster* (Goto *et al.,* 2008) and has been linked to regulation of gene expression by acting as a secondary cofactor to transcriptional activation in several biological signaling pathways, including innate immunity. Akirin is thought to function as a global regulator by mediating interactions between transcription factors and components of chromatin remodeling complexes to influence gene expression. Gene regulation is critical for

organismal development, so regulatory elements of transcriptional machinery are subject to constant adaption to contribute to its survival (Koster *et al.,* 2015). Understanding evolutionary conservation of Akirin and its diversity can unveil new knowledge about mechanisms of gene regulation during innate immune responses. Macqueen *et al.* (2009) provided a thorough overview of how the *akirin* gene family was established over evolutionary time in metazoans by using a comparative genomic approach. First, it was noted that *akirin* genes were not detectable in Bacteria and Archaea domains. However, an *akirin* protogene was identified in Placozoans, and the earliest detection of *akirin* was noted in two Protista taxa, Alveolata and Heterlobosea. Invertebrates have one isoform of *akirin*. However, examination of exon-exon boundaries revealed an exon duplication event in vertebrates, leading to the emergence of two homologues, *akirin1* and *akirin2*. Birds and reptiles have a single *akirin2* gene copy, and ray-finned fishes of the teleost class have a wide range of two to eight *akirin* family members (Chen *et al.,* 2013). Interestingly, *akirin* has not been detected in bacteria, yeast, or plants (Goto *et al.,* 2008). Multiple alignment sequences were used to construct a phylogenetic tree of invertebrate and vertebrate Akirin isoforms. High conservation of *akirin* genes across metazoans further support a long-standing role for Akirin in gene regulation, likely due to a decrease in genetic variability due to dynamic transcriptional needs of the organism to maintain cellular homeostasis and survival.

As Akirin contains no readily identifiable DNA-binding regions or functional domains, except for predicted nuclear localization sequences (NLS) (Macqueen *et al*., 2009), the exact mechanism by which Akirin interacts with transcription factors and components of the nuclear machinery is not completely understood. Even so, Akirin isoforms are expressed ubiquitously across a wide variety of cell types and tissues. Analysis of Akirin cellular localization revealed it is restricted to the nucleus (Goto *et al.,* 2008), likely due to putative but conserved nuclear localization sequences (NLS) found at the N-terminus in all metazoan isoforms of *akirin* (Macqueen *et al.,* 2009). However, after divergence from cartilaginous fish, teleost *akirin1* homologues lost their NLS for an as of yet unidentified reason (Liu *et al.,* 2015). Akirin isoforms are expressed at different levels in many tissue types, including the liver, kidney, skeletal muscle, heart and brain in fruit flies, fish, and mice (*Goto et al,.* 2008; Marshall *et al.,* 2008; Liu

et al., 2015). Moreover, mammalian *akirin2* is expressed in immune cells including macrophages and B cells (Tartey and Takeuchi, 2015) and demonstrated an increased expression in the liver, spleen, and kidney of miiuy croaker when challenged *in vivo* with infection by *Vibrio anguillarium* (Liu *et al.*, 2015). The cell types that respond are highly specific and subsequently remodel the chromatin environment to contribute to the demand for changes in gene expression in response to a cellular stimulus (Tartey *et al.,* 2015).

Several studies also indicate that Akirin interacts with a diverse array of transcription factors, subunits of chromatin remodeling complexes, and other regulatory elements to synergistically influence gene expression during other biological signaling pathways, such as innate immune responses. Through a yeast two-hybrid screen, Bonnay *et al.* (2014) found that Akirin interacts with a NF-κβ transcription factor, Relish, to facilitate the activation of Relish-dependent innate effector genes with H3K4ac epigenetic marks in *Drosophila melanogaster*. Immunoprecipitation assays demonstrated that Akirin mediates NF-κβ transcription via BAP60, a subunit of the Brahma (fly SWI/SNF homologue) chromatin remodeling complex. Interactions between Akirin, BAP60, and Relish also directly influenced the upregulation of Relish-dependent genes to stimulate the production of antimicrobial peptides (AMPs) or negative regulators of the Relish pathway (Tartey *et al.,* 2014). In shrimp, Akirin positively regulates IMD signaling and subsequent AMP expression through interactions with BAP60 and Relish, but also functions as a negative regulator of IMD signaling by interaction with 14-3-3 proteins (Hou *et al.,* 2013; Liu *et al.* 2016). In mammals, Akirin2 interacts with BAF60, a subunit of the mammalian SWI/SNF complex, and IκB-ζ, a subunit of the complex formed with NF-κβ to positively regulate pro-inflammatory gene cytokine production when the innate immune system was challenged (Tartey *et al.,* 2014). A potential role for Akirin as a global transcriptional regulator is supported by the fact that dynamic changes in gene expression patterns occur in mouse macrophages as a result of lipopolysaccharide (LPS) stimulation during gram-negative infection (Goto *et al.,* 2008). In sum, Akirin has demonstrated a conserved function among insects and mammals by physically linking NF-κβ/Relish signaling effector molecules with SWI/SNF chromatin remodeling complexes in order to create a transcriptionally active state at innate target genes. The regulators of these genes are evolutionarily malleable, as gene regulation programs need to be

adaptable to support environmental pressures (Koster 2015). Considering the role of Akirin in initiating innate immune responses by interacting with chromatin remodeling machinery, it is logical to suggest that it has evolved as an essential regulator of this biological process and can interact with other molecular players to regulate other biological processes, thereby reducing the number of regulatory proteins that need to be available during the life of a cell.

Akirin Interacts with Twist during Skeletal Myogenesis

Transcription factors exhibit regulatory properties through specific interactions directly with its target gene or indirectly with secondary cofactors. Akirin was identified as a potential cofactor for the wide range of Twist activity during somatic myogenesis in the fruit fly embryo (Nowak *et al.,* 2012). It is suggested that the interaction between Twist and Akirin is mediated through binding interactions with a subunit of the Brahma SWI/SNF (switching defective/sucrose nonfermenting) chromatin complex remodeler family in order to activate or repress target gene expression during embryonic development in *Drosophila* (Nowak *et al.,* 2012). Importantly, Twist and Akirin interaction was required for proper expression of *mef2*, an essential gene that initiates skeletal muscle formation during embryonic development and maintenance (Nowak *et al.,* 2012). There are two protein isoforms of Akirin in mice and humans (Tartey et al. 2014), but only one in *Drosophila*, again making it an ideal model for studying regulatory mechanisms of muscle development. A diverse array of skeletal muscle patterning defects were observed in *Drosophila* embryos lacking Akirin, including duplicated, missing, or mis-attached muscles as demonstrated in Figure 3 (Nowak *et al.,* 2012).

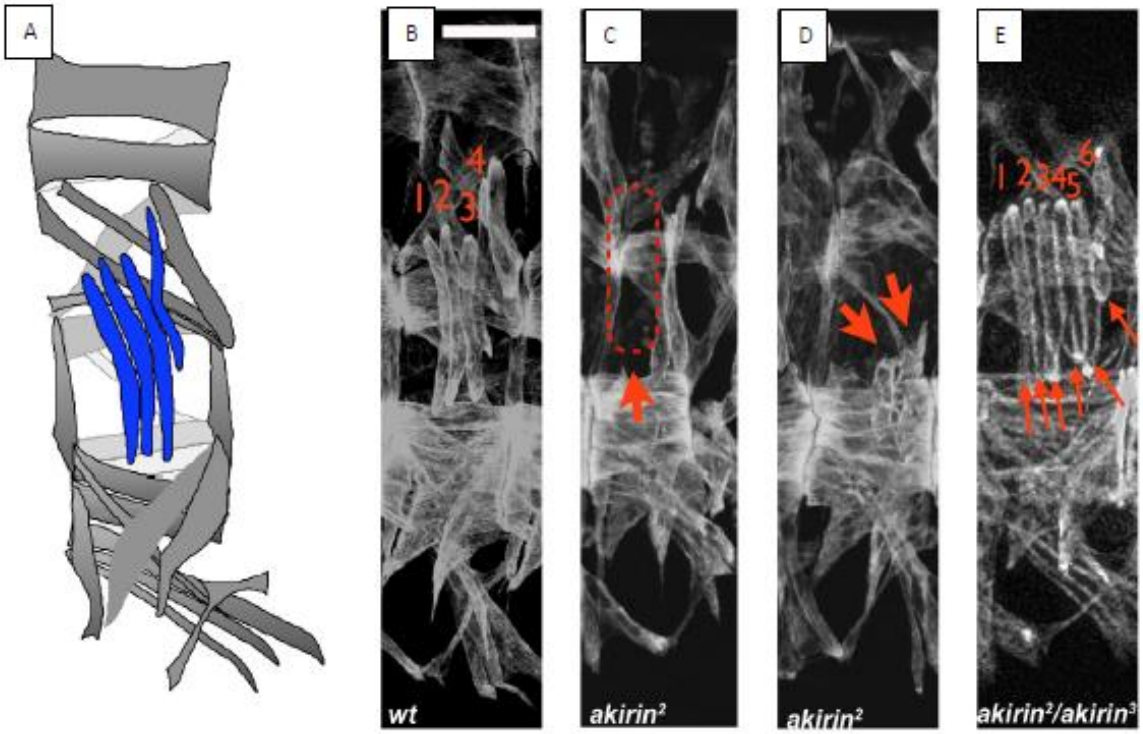

Figure 3: Muscle defects observed in* akirin *mutant embryos. *Drosophila* embryos at stage 16 in left lateral view, in which the images are oriented such that dorsal is up and anterior is left. Anti-tropomyosin antibodies are used to stain to visualize the musculature. A) Cartoon depiction of wild-type skeletal muscle anatomy of one abdominal hemisegment. B) Lateral transverse muscles labeled 1-4 in wild-type embryo. C-E) *akirin* mutant embryos with a host of deranged muscle phenotypes: C) missing muscles; D) mis-attached muscles; E) duplicated muscles. Red arrows directly indicate the affected lateral transverse muscles affected (Nowak *et al.,* 2012).

The broad scope of Twist and Akirin activities suggests that both function to mediate several regulatory processes, such as myogenesis and innate immunity, in animals via interactions with dynamic chromatin remodeling complexes. Gene regulation is critical for organismal development, so regulatory elements of transcriptional machinery are subject to constant adaption to contribute to its survival (Koster *et al.,* 2015). Akirin is thought to function as a global regulator by mediating interactions between transcription factors and other components of the nuclear machinery to synergistically influence gene expression in a variety of biological contexts. The work in this thesis is aimed to identify the domains of Akirin that are critical for interaction with the myogenic transcription factor Twist, and identify other proteins that interact with Akirin during muscle development. Chapter 2 outlines the first objective to map the domains of Akirin that physically interact with Twist, and identify domains of Twist

critical for interaction with Akirin. Methods to explore Akirin function were pursued using a candidate region approach, where specific and highly conserved regions of both Akirin and Twist were removed for downstream binding assays. In Chapter 3, the second objective of generating an interactome of Akirin is discussed. In this approach, interactions between *akirin* and other factors necessary for somatic muscle patterning were identified using double heterozygote *Drosophila* embryos as a rapid genetic screening technique.

By understanding gene regulation on the molecular level through proteins like Akirin, further insight is given into the control of critical transcription factors such as Twist. A more comprehensive understanding of these regulatory processes will also potentially reveal specific molecules that can be targeted for the development of better therapeutics for treating muscle wasting diseases, and chronic inflammatory states seen in diseases like cancer.

CHAPTER 2: MAPPING THE DOMAINS OF INTERACTION BETWEEN AKIRIN AND TWIST

The first objective of this thesis was to map the domains of Akirin that physically interact with Twist, and identify domains of Twist critical for interaction with Akirin. Methods to explore Akirin function were pursued using a candidate region approach, where specific and highly conserved regions across both Akirin and Twist were deleted (Figure 5A and 5B). Using this approach, it was hypothesized that *in vitro* binding of full-length constructs will verify that these two proteins interact, and binding of full-length Akirin or Twist with the various deletion constructs could reveal which regions are critical for binding to occur *in vivo,* thereby providing insight into the molecular function of Akirin. Materials and methods for this objective can be referenced in Chapter 5.

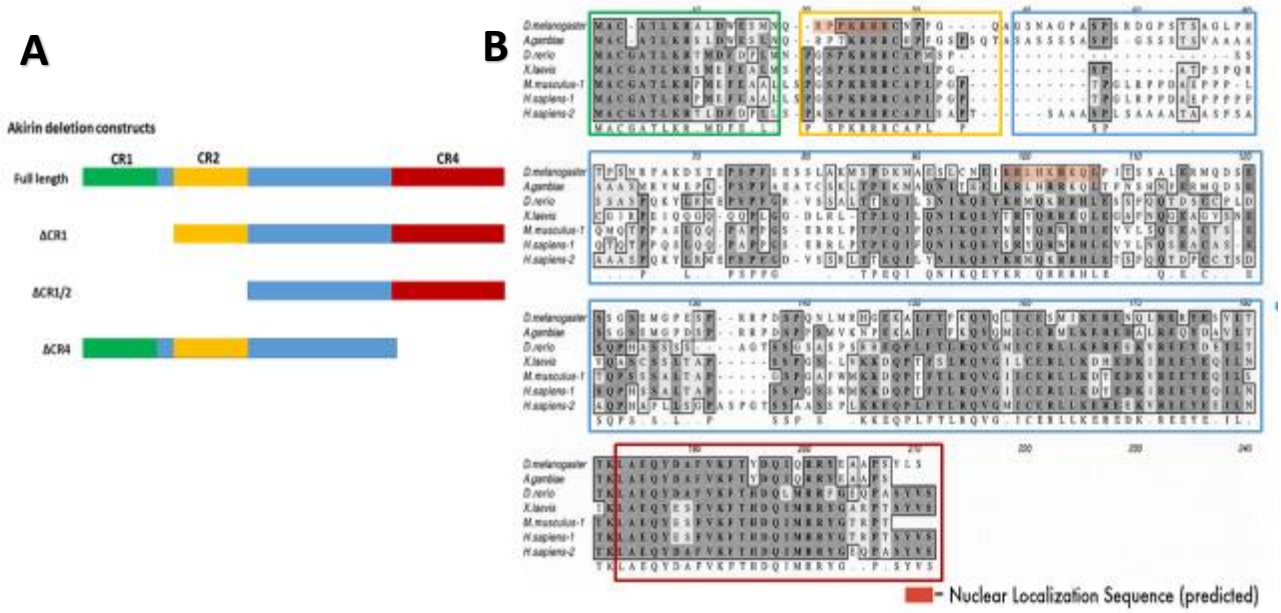

Figure 4: Akirin deletion constructs developed for this study. As Akirin has no readily identifiable functional domains, deletion constructs based off of the most highly conserved regions were constructed from sequence alignment (Macqueen and Johnson, 2009) using Gateway Technology (Invitrogen). ΔCR indicates which conserved region(s) have been removed from a particular construct. Full-length dAkirin includes all 201 amino acids, ΔCR1 (green) removes amino acids 1-14, ΔCR1/2 (yellow) removes amino acids 1-31, ΔCR4 (red) removes amino acids 174-201. Any binding interactions observed using these deletion constructs will give insight into the functional regions across its sequence and a better understanding of the molecular function of Akirin. Primers used to generate these constructs can be referenced in Chapter 5.

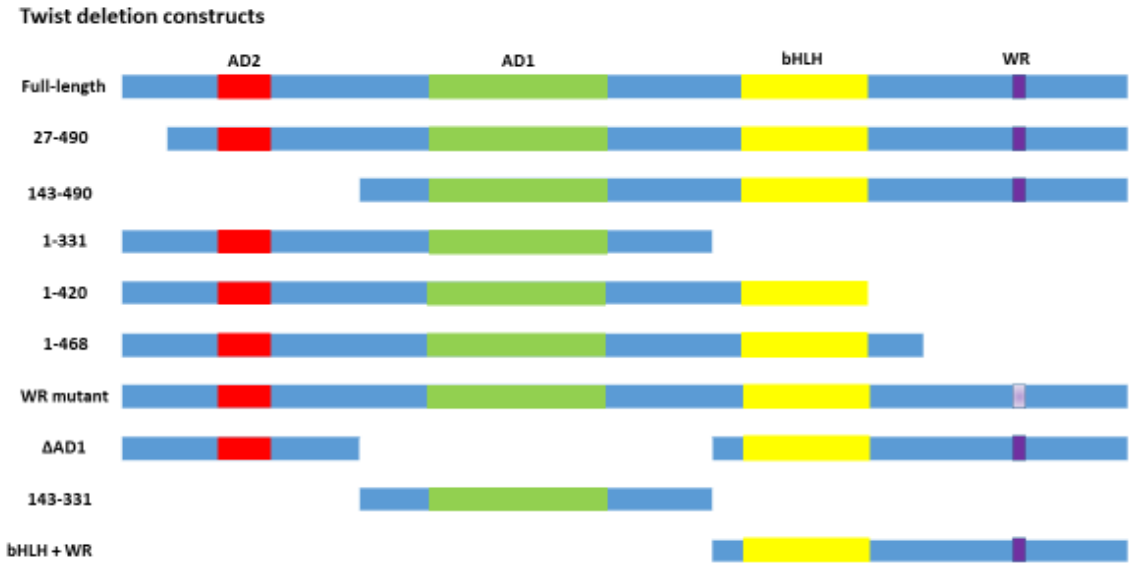

Figure 5: Structure of Twist and constructs developed for use in this study. This figure is a representation of all of the constructs built for observing the binding interaction between Twist and Akirin, specifically deleting known functional domains of Twist. Deletions are represented by missing links within each construct, in which each deletion construct name is listed on the left side of the figure. Across its sequence, Twist contains a number of domains: AD2 (red), AD1 (green), bHLH (yellow) and a WR motif (purple). ΔAD1 indicates that the AD1 region has been deleted, and the WR mutant is an alanine substitution for a conserved 6 amino acid sequence within the WR motif. Primers used to generate these constructs can be referenced in Chapter 5.

Results

Expression and Purification of full-length Twist from pDEST expression vectors

In order to confirm successful purification of GST-Twist, the protein was analyzed on a 15% SDS-PAGE gel and stained with Coomassie brilliant blue (Figure 6A). During the attempt to purify GST-Twist from BL21 (DE3) pLysS competent cells, the protein was present in the soluble supernatant. However, pure protein was not present in the eluted fractions after several attempts (Figure 6A).

In a similar fashion, successful purification of 6xHis-Twist was analyzed via SDS-PAGE gel analysis and Coomassie brilliant blue staining. Naturally expressed protein using this method was present in inclusion bodies, and pure protein was successfully isolated using gravity column

purification under denaturing conditions (Figure 6B). However, attempts to refold the protein using step-down dialysis were not successful, as the protein crashed out of solution upon introduction of elution buffer with no urea. Other attempts to rapidly exchange the elution buffer with urea into buffers supplemented with triton X-100, tween-20, surfactant P50, and octyl thioglucoside resulted in no recovery of the protein (data not shown). Another attempt to solubilize Twist was followed in a similar fashion as Shirokawa and Courey (1979), in which this group claims to have solubilized full-length Twist in buffer supplemented with NP-40. A chemical substitute of NP-40, IPEGAL, was used to reproduce these results with no success (Figure 6C). In addition, the same approach was taken with Twist 1-331, as this construct lacks the bHLH domain that is responsible for its dimerization and DNA-binding activity and was hypothesized to contribute to protein aggregation.

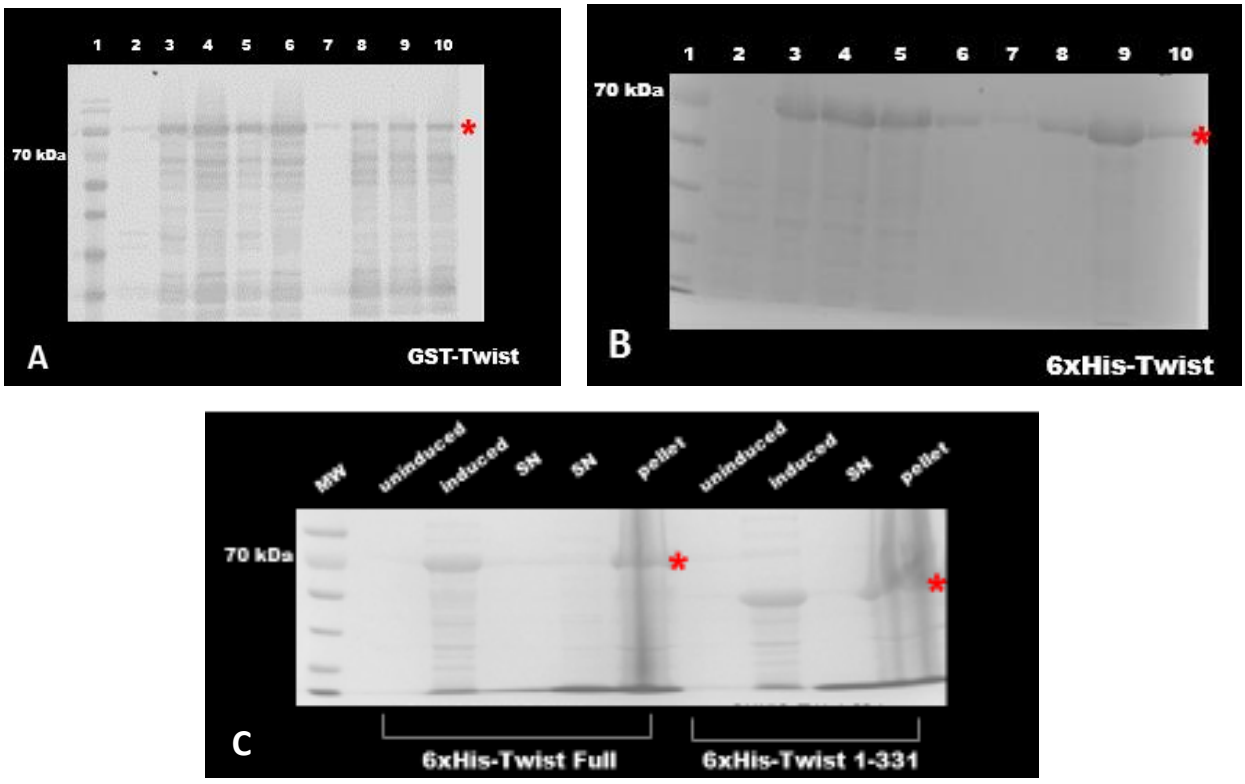

Figure 6: Initial attempts to purify Twist from pDEST vectors. Both protein purifications were analyzed by running purification samples on a 15% SDS-PAGE gel and Coomassie brilliant blue staining. Target protein is indicated by red asterisk. A) Purification of GST-Twist using Glutathione Sepharose did not yield pure protein products in the eluted fractions. B) Pure 6xHis-Twist was successfully isolated by gravity column purification under denaturing conditions. C) Solubilization of full-length Twist and Twist 1-331 in buffer supplemented with IPEGAL was attempted following protocols outlined by Shirokawa and Courey (2009); following harvest of clarified cell lysates, the target proteins remained in the pellet (SN = supernatant).

Expression and Purification of multi-tagged Twist and Akirin from pGEX-4t vector

Following the results of purification of full-length Twist constructs expressed from the pDEST vectors, a new approach to express soluble protein was undertaken. In this approach, the pGEX-4t vector was modified such that full-length Akirin and Twist would be tagged with GST on the N-terminus to facilitate solubility, and a C-terminal FLAG/6xHis tag to facilitate purification on 6xHis-tagged Talon metal affinity resin (Figure 7A). Upon expression of new constructs via IPTG induction, both proteins were present in the supernatant and were successfully eluted from the gravity column as pure protein under native conditions (Figure 7B,C).

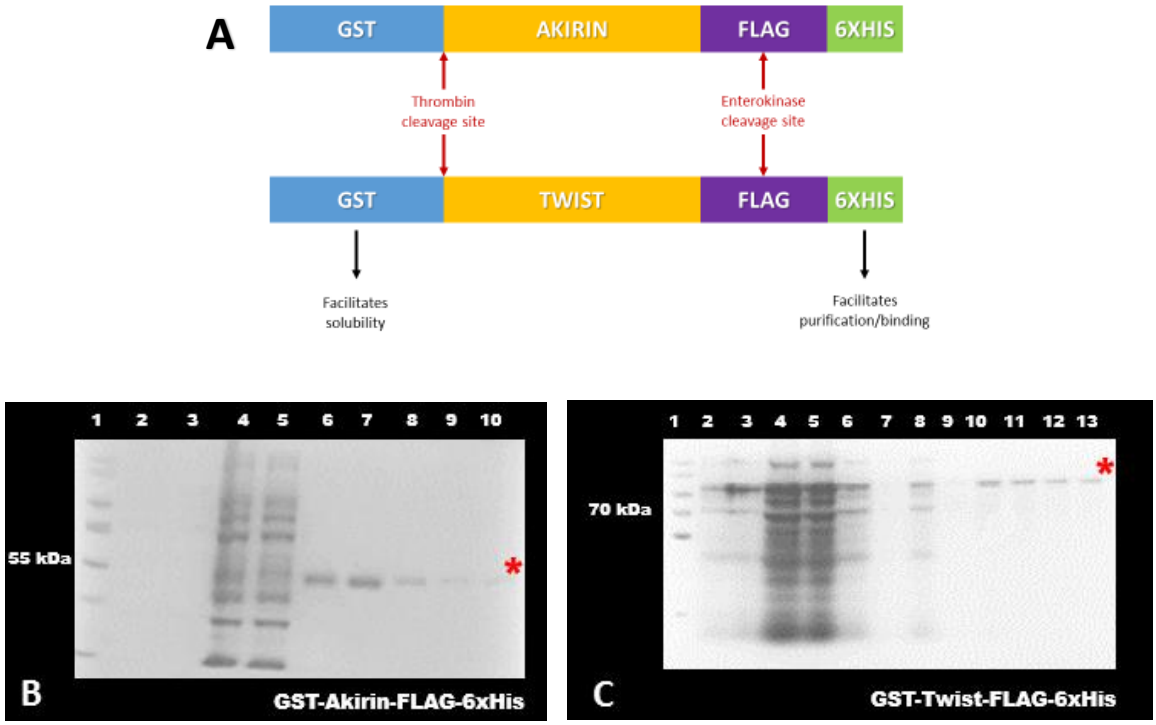

Figure 7: Multi-tagged Akirin and Twist for optimal expression and purification of target proteins under native conditions. A) Based on the results of initial purification attempts, each full-length construct was modified using the pGEX-4t vector to produce proteins with an N-terminal GST-tag to overcome solubility issues, and a C-terminal FLAG/6xHis-tag for optimal binding during purification. Each construct contained two proteolytic cleavage sites to reduce the number of tags present on the final protein products. B-C) Success of both purifications was analyzed by running purification samples on a 15% SDS-PAGE gel and Coomassie brillant blue staining. Both proteins were expressed in the supernatant and pure proteins were isolated by 6xHis-tagged Talon metal affinity resin. B) GST-Akirin-FLAG-6xHis (expected size 50 kDa; eluted proteins in lanes 6-10); C) GST-Twist-FLAG-6xHis (expected size 83 kDa; eluted proteins in lanes 10-13).

On-column Thrombin Cleavage of GST-tag

The new approach to purifying multi-tagged Akirin and Twist included a desire to remove the N-terminal GST-tag by thrombin cleavage for subsequent binding experiments. After unsuccessful attempts to cleave the GST-tag from purified proteins (data not shown), the proteins were purified again under the same conditions, with the exception of an overnight incubation of the protein-resin complex with thrombin. Pure protein was obtained using this approach, but the yield of both Akirin and Twist was much lower. In addition, the GST-tag on Akirin remained uncleaved (Figure 8A), and the GST-tag was only partially cleaved from Twist (Figure 8B). Based on these results, the pure protein products from the previous purification were biotinylated for use in BLI.

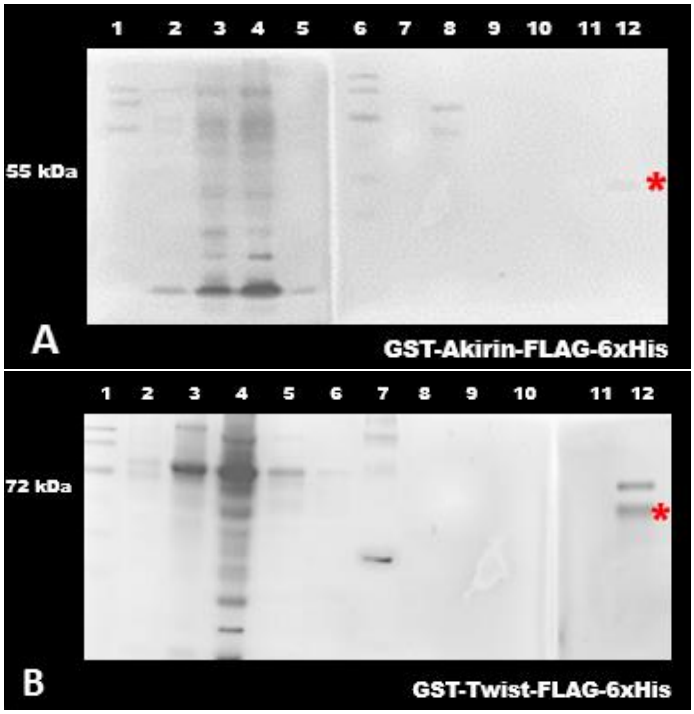

Figure 8: Expression and purification of multi-tagged Akirin and Twist with on-column thrombin cleavage. Success of both purifications was analyzed by running purification samples on a 15% SDS-PAGE gel and Coomassie brillant blue staining. Following an overnight incubation of the protein-resin complex with thrombin, Akirin did not demonstrate any loss of the GST-tag (A) and the GST-tag was only partially cleaved from Twist (B). Loss of GST from GST-Akirin-FLAG-6xHis was expected to result in fragment 24 kDa in size, and uncleaved eluted protein is in lane 12 (A). Loss of GST-Twist-FLAG-6xHis was expected to result in a fragment 57 kDa in size, and partially cleaved protein is found in lane 12 (B).

Biolayer interferometry of full-length Akrin and Twist

The first objective of this thesis was to observe the binding kinetics between Akirin and Twist. The potential binding interactions between Akirin and Twist was assessed by biotinylating either Akirin and Twist, binding the ligand to SA sensors, and observing their association. Although it initially appeared that the two proteins did interact, Twist non-specifically bound to the sensor with near 100% similiarity (Figure 9A,B).

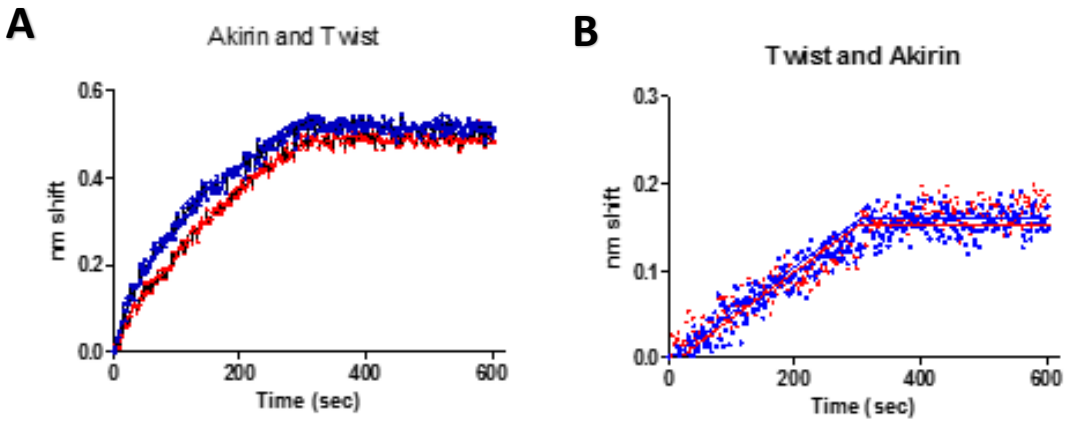

Figure 9: In vitro binding kinetics of Akirin and Twist. Binding kinetics of Akirin and Twist were performed by biolayer interferometry (BLI). Shown are association (0-300 sec) and dissociation (300-600 sec). Data are shown as colored points with lines showing fit to global single-state association-then-dissociation model. A) Streptavidin-coated sensors were loaded with (blue) or without (red) biotinylated Akirin then associated with Twist. (Akirin:Twist K_D = 4.820e-22; No ligand:Twist K_D = 2.481e-22). B) Reverse experiment with streptavidin-coated sensors were loaded with (blue) or without (red) biotinylated Twist then associated with Akirin. (Twist:Akirin K_D = 3.104e-20; No ligand:Akirin K_D = 6.167e-22).

Co-immunoprecipitation of Akirin and Twist in insect S2 cells

Following the results of protein binding assays from proteins purified in *E. coli,* the next approach was to assess the binding of Akirin and Twist in a eukaryotic system. Using this approach, Akirin constructs were built with an N-terminal HA-tag, and Twist constructs were built with a C-terminal FLAG-tag. Upon calcium phosphate transfection into insect S2 cells, expression of full-length constructs was successful and detected via Western blotting (Figure 10).

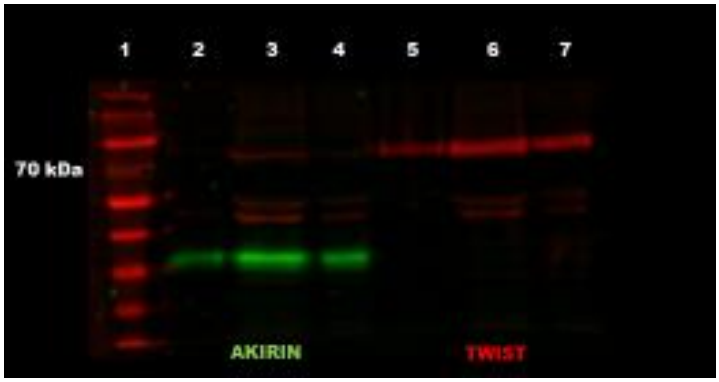

Figure 10: Verification of expression of full-length Akirin and Twist in insect S2 cells. Using calcium phosphate transfection, both HA-Akirin and FLAG-Twist were successfully expressed in insect S2 cells. Expression was verified in triplicate and detected by probing with rabbit anti-HA and mouse anti-FLAG. Akirin is labeled in green, and Twist is labeled in red. (Expected fragment size: HA-Akirin 25 kDA, FLAG-Twist 55 kDa).

Following successful expression of target proteins in insect S2 cells, the next step was to assess their binding capabilities *in vitro*. For this approach, FLAG-Twist was used as bait to pull-down the prey HA-Akirin (Figure 11). This method was similar to the experiments carried out by Liu *et al.* (2017) to demonstrate that Akirin interacts with a neuronal transcription factor, Geminin, during *Xenopus* neural development. The results show that FLAG-Twist was unable to pull-down HA-Akirin using this method. The Akirin construct was lost before harvesting the supernatant, perhaps due to degradation or non-specific binding to the resin.

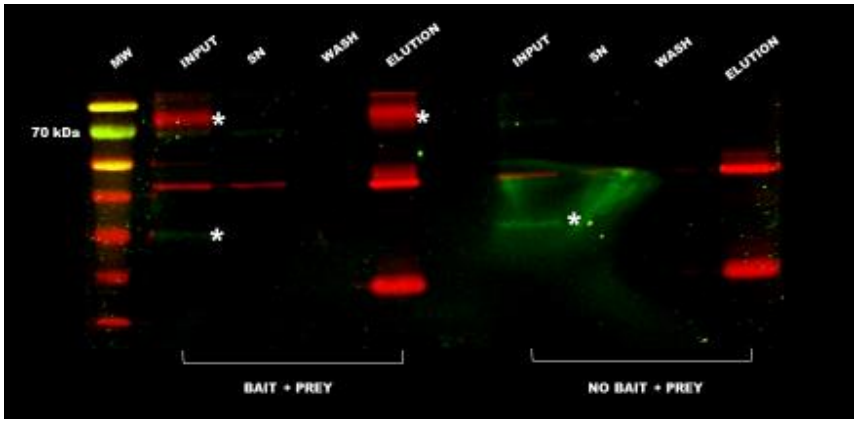

Figure 11: Co-immunoprecipitation of full-length Twist and Akirin in insect S2 cells. As a final approach to verifying interaction of Twist and Akirin, FLAG-Twist was used as bait to pull-down the HA-Akirin prey using FLAG-coupled agarose in a similar fashion as Liu *et al.* (2017). The experiment was duplicated without the bait to confirm that HA-Akirin is not non-specifically binding to the agarose. White stars indicate target proteins that were probed with rabbit anti-HA and mouse anti-FLAG, where FLAG-Twist labeled in green and HA-Akirin is labeled in red. (MW = molecular weight; SN = supernatant).

Discussion

The broad scope of Twist and Akirin activities suggests that both function as general transcription factors to mediate several regulatory processes in animals via chromatin remodeling complexes. As Akirin contains no readily identifiable DNA-binding regions or functional domains, except for predicted nuclear localization sequences (NLS) (Macqueen et al. 2009), the exact mechanism by which Akirin interacts with transcription factors and components of the nuclear machinery is not completely understood. Considering the lack of knowledge of the molecular function of Akirin, the first objective of this thesis was to map the domains of Akirin that physically interact with Twist, and vice versa. In this approach, pure protein was to be obtained for downstream functional assays. However, there were many technical challenges associated with attempts to purify full-length constructs of both proteins. Although some groups have claimed to have successfully purified Akirin and Twist, there is a lack of proof of purification for either protein, or the results were not reproducible (Liu *et al.,* 2016; Shirokawa and Courey, 1979). However, rabbit reticulocyte lysates were used to produce radiolabeled Twist to assess its dimerization activity (Shirokawa and Courey, 1979; Castanon *et al.,* 2001). In addition, the study that demonstrated a physical link between Akirin and Twist showed this by using *E. coli* cell lysates containing GST-Twist could pull-down radiolabeled Akirin produced by in vitro transcription/translation in rabbit reticulocyte lysates (Nowak *et al.,* 2012). It is possible that using in vitro translated proteins that were radiolabeled was a far more sensitive approach than used in this thesis to detect binding. Even so, this thesis presents novel purification methods for both Akirin and Twist. However, they were unable to bind using BLI. This may have been due to the presence of multiple tags on both ends of the recombinant proteins, which could have interfered with any possible interaction. Considering that Akirin is a nuclear protein, it also cannot be ruled out that there is another unknown intermediate player that allows for the previously reported physical association between Akirin and Twist.

Due to the challenges associated with proteins purified from *E. coli,* it was hypothesized that co-immunoprecipitation (CoIP) experiments using total transfected eukaryotic cell lysates may provide the additional co-factor or post-translation modification required for binding activities. In addition, Liu *et al.* (2017) demonstrated that Akirin interacts with a neurogenic

transcription factor, Geminin, along its C-terminus and the *Xenopus* SWI/SNF homolog, BAF53a, on its N-terminus by through CoIP experiments in transfected HEK cells. In a similar fashion, Akirin and Twist were expressed in insect S2 cells and assayed for binding using CoIP experiments. S2 cell expression was chosen for this objective to maintain any insect-specific protein modifications that may be critical for Akirin function in a muscle development context. Using this approach, Akirin and Twist were unable to bind. This may have been due to non-specific binding of Akirin to the resin, as Akirin was not present in any sample but the input. It should be noted that the S2 cell-based methods used in this thesis may not have been sensitive enough to detect binding or the tags may have interfered with binding.

It must be restated that the structure or folding of Akirin remains unknown. Given the lack of knowledge of predicted structure or molecular function of Akirin, there is no readily acceptable method to predict the domains that are responsible for its interaction with Twist. When this thesis project was undertaken, it was anticipated that the constructs may fail to interact using these approaches. One alternative approach may be to produce recombinant protein in a eukaryotic system, such as the baculovirus-based system, that will more likely yield properly folded Akirin and Twist that can be used for downstream binding assays such as CoIP or BLI. Another approach may be to use an *in vivo* cell-based reporter assay using a previously reported cassette that can ascertain the effects of Akirin-deletion constructs on Twist-dependent gene expression in cultured S2 cells (Castanon *et al.,* 2001; Wong *et al.,* 2008). Assaying for luciferase production in total transfected cells is a standard technique and can reveal which Akirin-deletion constructs are responsible for interacting with full-length Twist. Although these alternative approaches are viable, they are outside the scope of this thesis.

CHAPTER 3: GENERATION OF AN AKIRIN INTERACTOME

The second objective of this thesis was to identify other Akirin-interacting proteins during muscle development. Interactions between *akirin* and other factors necessary for somatic muscle patterning were identified using double heterozygote embryos as a rapid genetic screening technique (see below). A candidate approach was taken to generate an interactome of Akirin function during muscle patterning. The identities of factors that interact with Akirin during muscle development remain unknown, although repositories of protein-protein interaction data have identified a number of candidate proteins that are likely Akirin-interacting partners. The factors that were screened for this objective included a subunit of the NuRD chromatin remodeling complex (Mi-2), components of transcriptional machinery (Taf4), other transcription factors (Pannier), and novel genetic loci with predicted *akirin* interaction (*CG30497*). The NuRD complex in *Drosophila* is made up of 6 core subunits: Mi-2, MBD2/3, MTA, RPD3, p55, and p66/68 (Clapier and Cairns, 2009), and which there are mutant fly lines available for all but p55. Each one of these fly lines was obtained from the Bloomington Stock Center and balanced over appropriate balancer chromosomes during the course of this thesis project.

For a rapid screening method, possible genetic interactions between Akirin and these candidate proteins during embryonic development can be uncovered by examining double heterozygous embryos. The *akirin* mutant fly lines that will be used for all genetic interaction screens are either viable (referred to in this work as $akirin^{2}$) or homozygous lethal ($akirin^{3}$). $akirin^{2}$ is a P-element insertion into the first intron of *akirin,* and $akirin^{3}$ is an insertion into the first exon. Using this method, single heterozygous embryos demonstrate a wild-type muscle pattern. However, double heterozygous embryos exhibit a range of deranged muscle phenotypes when a genetic interaction required for the establishment of the final skeletal muscle pattern is disrupted. This forward genetic screen has been used by other research laboratories as a rapid technique to identify genes that are involved in embryonic patterning (Nowak *et al.,* 2012). The materials and methods for this objective are referenced in Chapter 5.

Results

Akirin Interacts with a Number of Candidate Genes during Myogenesis

The skeletal musculature in *Drosophila* embryos forms a stereotypic pattern in each abdominal hemisegment, which allows for rapid visual identification of disruptions in the muscle patterning (Bate 1990). Therefore, a double heterozygous genetic screen was undertaken to identify interactions between *akirin* and other factors that are necessary for proper muscle patterning. Single heterozygous embryos for the candidate genes do not show an abnormal muscle phenotype, but embryos double heterozygous for *akirin* and candidate genes for which there is a genetic interaction demonstrate a host of abnormal muscle patterns. Of the candidate genes for this screen, *pannier* and *taf4* appeared to genetically interact with *akirin* due to the significance of muscle defects observed in double heterozygous embryos (Figure 12 B-E, Table 1). The muscle defects observed included missing muscles, attachment defects, and duplicated muscles. Importantly, the observed defects in the muscle patterning were in accordance with those seen in *akirin* and *twist* mutant embryos (Nowak *et al.,* 2012). Although embryos double heterozygous for *CG30497* and *akirin* occasionally showed abnormal muscle patterning, the prevalence was not significant (Figure 12 F-G, Table 1) and was likely due to interference of balancer chromosome. These balancer chromosomes are highly altered and modified chromosomes made up of numerous inverted sections and marker genes, which are useful for maintaining a desired mutation in a heterozygous stock and prevent recombination. Considering that the observed muscle phenotypes may have been influenced by the respective balancer chromosomes for the candidate genes, wild-type embryo siblings were also assessed for the observed muscle defects. Although the abnormal muscle patterning occurred occasionally in the wild-type siblings, the prevalence was not significant, especially in comparison to the occurrence of errors in muscle patterning in double heterozygous embryos (Table 1). Taken together, these results suggest new modes of *akirin* interaction with predicted interacting partners during skeletal myogenesis.

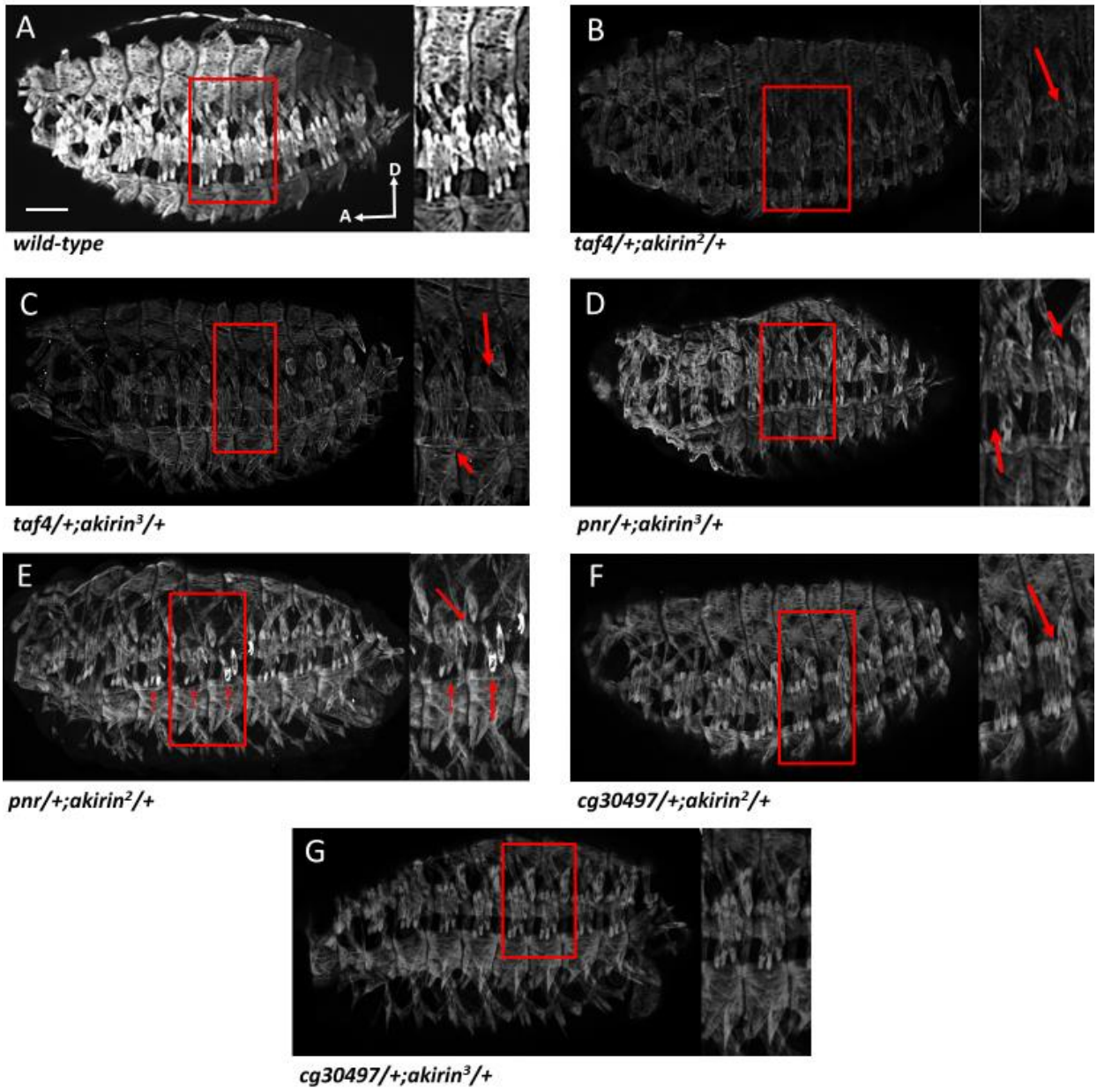

Figure 12: Akirin genetically interacts with components of the basal transcriptional machinery and other transcription factors during skeletal muscle development. Double heterozygous have a wide range of defects in the muscle patterning including missing muscles, attachment defects, and duplicated muscles. The double heterozygous embryos that exhibit muscle defects suggest that there is a genetic interaction between *akirin* and the predicted partner (quantification of muscle phenotypes can be found in Table 1). (A-G) depict lateral views of stage 16 wild-type and embryos heterozygous for the indicated partner and *akirin*. All embryos are stained with anti-myosin heavy chain to reveal the skeletal musculature. Scale bar = 50 µm.

Table 4. Penetrance of muscle phenotypes in mutant embryos double heterozygous for* akirin *and the indicated partner.

Genotype	Embryos examined	Penetrance[1]	Missing muscles[2]	Attachment defects[2]	Duplicated muscles[2]
taf4/+ x akirin^2/+	37	45.9%	35.3%	0%	64.7%
taf4/+ x akirin^3/+	18	55.6%	50%	20%	30%
[a]wild-type	56	5.4%	33.3%	0%	66.7%
pnr/+ x akirin^2/+	29	55.2%	25%	12.5%	62.5%
pnr/+ x akirin^3/+	11	27.8%	66.7%	33.3%	0%
[b]wild-type	36	8.3%	100%	0%	0%
CG30497/+ x akirin^2/+	25	4.0%	100%	0%	0%
CG30497/+ x akirin^3/+	42	9.5%	75%	0%	25%
[c]wild-type	36	5.6%	100%	0%	0%

[1] Penetrance represents the percentage of mutant embryos that exhibit a muscle defect in at least 2 hemisegments.
[2] The observed defect in mutant embryos is given as a percentage to represent prevalence of missing, attachment, or duplication events.
[a-c] Wild-type siblings carrying the respective balancer chromosome for each mutation; [a]*TM3,DfdGMRYFP,* [b]*TM6B,DfdGMRYFP,* [c]*TM3,sqhmCherry*

Akirin Interacts with the NuRD Chromatin Remodeling Complex during Myogenesis

Akirin was previously identified as physical and genetic link between the myogenic transcription factor Twist and the Brahma subunit of the SWI/SNF-class BRM chromatin remodeling complex (Nowak *et al.,* 2012). To further supplement the development of an Akirin interactome during muscle development, the double heterozygous genetic screen was used to identify whether *akirin* also interacts genetically with other chromatin remodeling complex families. Using this approach, *akirin* appears to interact genetically with *mi-2* and *simjang* (p66), both of which are subunits of the nucleosome remodeling and deacetylase (NuRD) complex (Figure 13, Table 2). For further confirmation of an interaction between *akirin* and *mi-2,* polytene chromosomes were used to assess whether they colocalize across the genome. Polytene chromosomes played a role in early attempts to isolate and map the *Drosophila* genome. They can readily be isolated from the salivary glands of wandering third instar larvae, in which the cells are undergoing rapid DNA replication to increase transcriptional activities, but not cell division, thereby leading to giant chromosomes. Regions of active gene transcription

can readily be identified across the polytene chromosomes, and can be analyzed for potential colocalization at these regions. Considering this technique, further verification of the data obtained from the double heterozygote genetic screen was undertaken by demonstrating that *akirin* significantly colocalizes with *mi-2* in polytene chromosomes, both of which individually are broadly distributed throughout the genome (Figure 14A; Nowak *et al.,* 2012; Murawska *et al.,* 2008). Taken together, these results present a new mechanism of transcriptional control mediated by Akirin interactions during muscle development in the fruit fly.

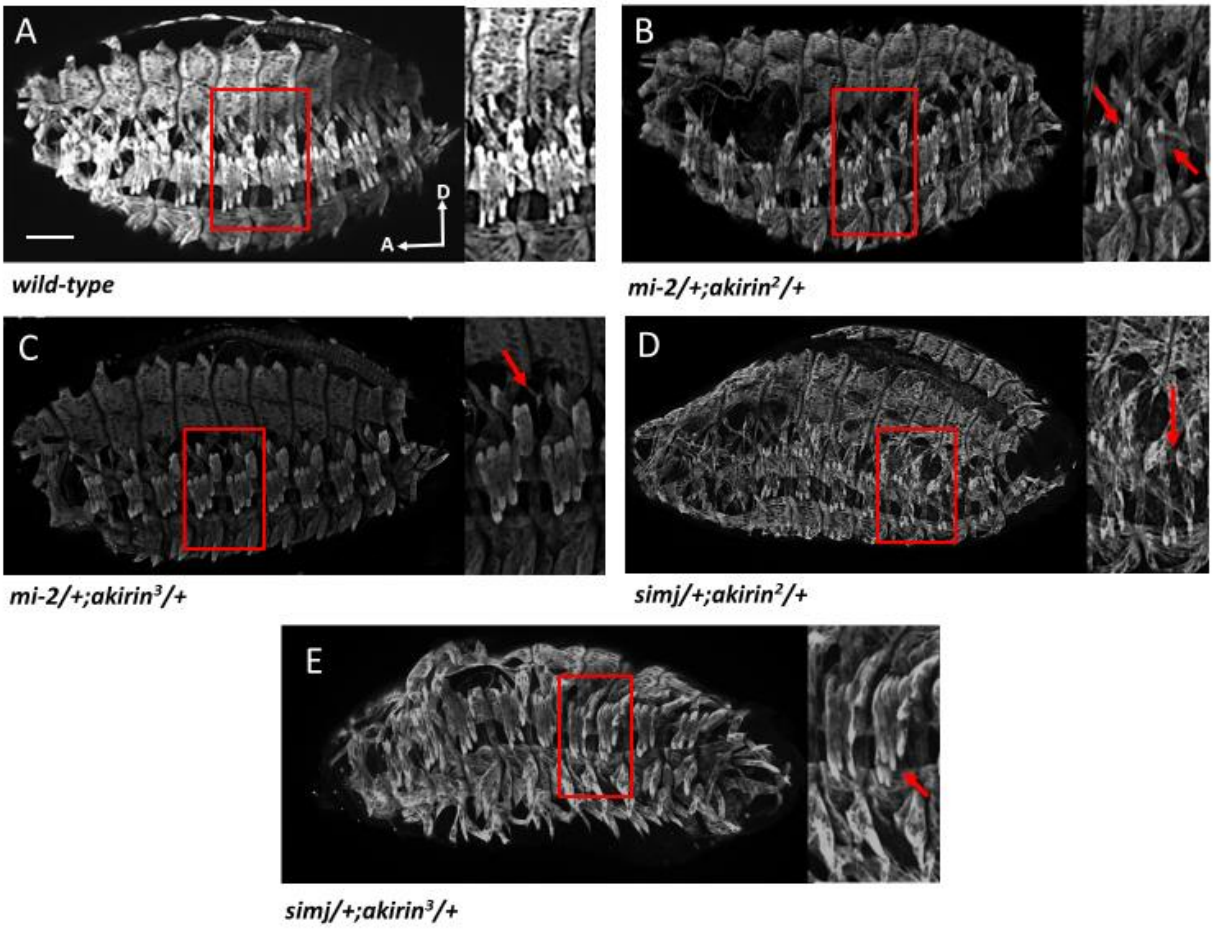

Figure 13: Akirin genetically interacts with Mi2 and simjang subunits of the NuRD chromatin remodeling complex during skeletal muscle development. Double heterozygous have a wide range of defects in the muscle patterning including missing muscles, attachment defects, and duplicated muscles. The double heterozygous embryos that exhibit muscle defects suggest that there is a genetic interaction between *akirin* and indicated subunits of the NuRD complex (quantification of muscle phenotypes can be found in Table 2). (A-G) depict lateral views of stage 16 wild-type and embryos heterozygous for either *mi2* or *simjang (simj)* and *akirin*. All embryos are stained with anti-myosin heavy chain to reveal the skeletal musculature. Scale bar = 50 µm.

Table 5: Quantification of genetic interaction between akirin and NuRD complex subunit genes

Genotype	Embryos examined	Penetrance[1]	Missing muscles[2]	Attachment defects[2]	Duplicated muscles[2]	Stubby muscles[2]
mi2/+ x akirin^2/+	36	50.0%	44.4%%	5.6%	50%	0%
mi2/+ x akirin^3/+	17	58.8%	40.0%	20.0%	20.0%	20.0%
simj/+ x akirin^2/+	24	50.0%	25.0%	0%	75.0%	0%
simj/+ x akirin^3/+	12	41.7%	20.0%	40.0%	40.0%	0%
[a]wild-type	72	5.6%	50.0%	0%	50.0%	0%

[1]Penetrance represents the percentage of mutant embryos that exhibit a muscle defect in at least 2 hemisegments.

[2]The observed defect in mutant embryos is given as a percentage to represent prevalence of missing, attachment, or duplication events.

[a] Wild-type siblings carrying the respective balancer chromosome for the indicated mutations; [a]*TM3,DfdGMRYFP*

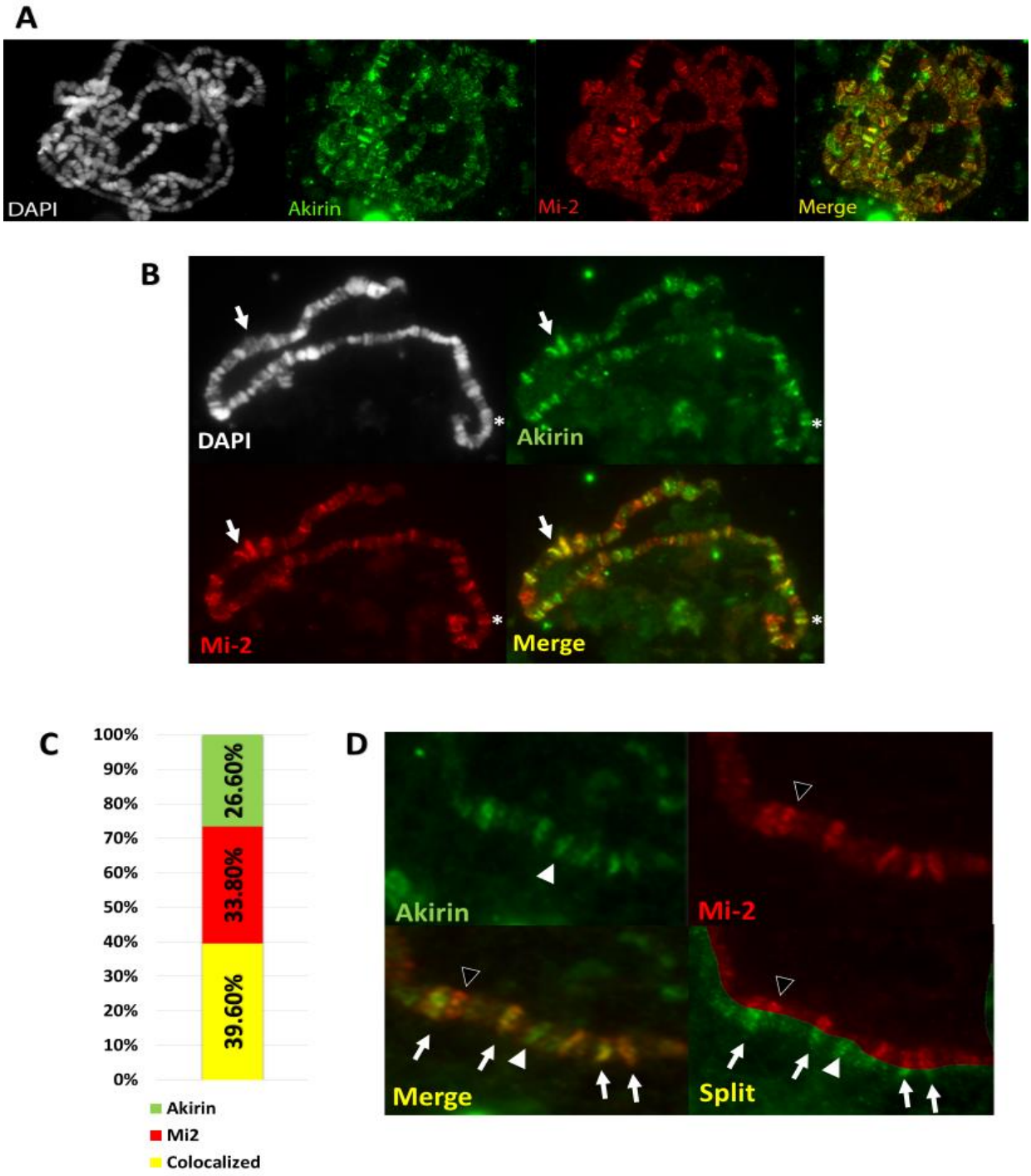

Figure 14: Akirin colocalizes with Mi-2, a subunit of the NuRD chromatin remodeling complex. A) Wild-type polytene chromosomes were stained with antibodies against Akirin (green) and Mi-2 (red), and counterstained with DAPI to visualize DNA. Akirin and Mi-2 colocalize extensively, and individually are broadly distributed throughout the genome. B) Akirin and Mi-2 colocalize in regions of puffed (white arrow) and non-puffed (white asterisk) regions. C) Quantitation of both colocalization and regions of non-overlap observed between Akirin and Mi-2. D) Demonstration of colocalization through detail of polytene chromosomes squashes. Regions of colocalization are indicated by white arrows, where Akirin signal without Mi-2 is represented by black arrowhead and Mi-2 signal without Akirin is represented by white arrowhead.

Discussion

One of the principal questions addressed by this thesis project was: through which mechanisms does Akirin influence gene expression programs, despite the lack of key DNA-binding domains or identifiable catalytic activity? Based on previously published data, the hypothesis is that Akirin serves as a regulator of gene expression by mediating interactions between nuclear machinery and transcription factors that together influence gene expression programs. The results of this thesis suggest novel interactions between *akirin* and a number of candidate molecular partners during skeletal myogenesis.

There are four primary families of ATP-dependent chromatin remodeling complexes: SWI/SNF, ISWI (imitation switch), INO80 (inositol required 80), and CHD (chromodomain, helicase, DNA binding). Several studies have demonstrated interactions between Akirin and subunits of the SWI/SNF class chromatin remodelers in various biological signaling pathways (Bonnay et al. 2014; Liu et al. 2017; Nowak et al. 2012, Tartey et al. 2014). However, Akirin has not been shown to genetically interact with ISWI or INO80 (Nowak *et al.,* 2012; unpublished data). As interactions with Akirin and CHD family remodelers has not yet been assessed, one of the candidate proteins for double heterozygous genetic screen in this thesis included a mutant Mi-2/NuRD (nucleosome remodeling and deacetylase) catalytic subunit of the CHD family. Embryos double heterozygous for *akirin* and *mi-2* demonstrated a host of abnormal muscle phenotypes with a high degree of significance in comparison to wild-type embryos carrying a balancer chromosome (Figure 13, Table 2). Although embryos singly heterozygous for these mutations do not show a muscle phenotype (indicated by wild-type), disrupted muscle patterning was observed in *akirin*^{2}*/+,mi-2/+* (50%, n=36, Figure 13B) and *akirin3/+,mi-2/+* (58.8%, n=17, Figure 13C) embryos. Initially, it was not expected that a genetic interaction would be seen between *akirin* and *mi-2,* as no interactions were previously identified using the double heterozygote genetic screen. The NuRD complex has repressive roles, as it contains methyl CpG-binding domain (MBD) proteins that recognize methylated DNA in order to stop gene expression (Clapier and Cairns, 2009). In mammals, the complex also associates with histone deacetylases (HDAC1/2), which together work to compact histones and thereby

prevent transcription (Clapier and Cairns, 2009). Another repressive function of NuRD comes from its ability to recruit DNA transcriptional repressors (Murawska *et al.,* 2008). These results also show context-dependent roles of Akirin at the transcriptional level, as interactions with SWI/SNF function facilitate gene expression (Nowak *et al.,* 2012) and interactions with NuRD may function repress transcriptional activities.

To further examine the role of Akirin and Mi-2 during muscle development in *Drosophila*, we have started to assess somatic muscle phenotypes of other NuRD core subunit mutant embryos. During the course of this thesis project, only the p66/68 (*simjang; simj)* line was able to be characterized using the double heterozygote genetic screen. An abnormal muscle phenotype was observed in *akirin*^{2}*/+;simj/+* (50%, n=24, Figure 13D) and *akirin*^{3}*/+;simj/+* (41.7%, n=17, Figure 13E) mutant embryos. Interactions between *akirin* and other core NuRD complex subunits will be carried out by undergraduate researchers as this project continues on. In accordance with the scope of this thesis, further observation of the interaction between *akirin* and *mi-2* was carried out by assessing the degree of colocalization in polytene chromosomes. Immunostaining of polytene chromosomes with antibodies against Akirin and Mi-2 (Figure 14) revealed that Akirin colocalized with Mi-2 throughout the genome in both areas of active and non-active gene transcription (39.6% colocalization). Taken together, these data support a role for Akirin as a general regulator of gene expression by mediating interactions with multiple families of chromatin remodeling complexes. Future directions of this project will include co-immunoprecipitation studies with whole embryonic extracts to see if tagged Akirin protein associates with Mi-2 *in vivo*.

Another candidate interacting protein for the double heterozygote genetic screen was Taf4, a subunit of TFIID complex that recruits the entire RNA polymerase II holoenzyme to the site of active transcription mediated by Twist (Pham et al. 1999). The glutamine-rich motif AD1, and to a lesser extent AD2, of *Drosophila* Twist is responsible for its transcriptional activity. *akirin2/+;taf4/+* (45.9%, n=37, Figure 12B) and *akirin3/+;taf4/+* (55.6%, n=18, Figure 12C) demonstrated a host of deranged muscle phenotypes, suggesting that *akirin* interacts with *taf4* genetically during muscle development. Furthermore, the GATA transcription factor fly homolog, Pannier (Pnr), is involved in development and contains a glutamine-rich region within

its C-terminus. A previous study identified that *akirin* interacts genetically with *pannier* (Pena-Rangel et al. 2002), therefore making it an ideal candidate to confirm a potential interaction using this double heterozygote genetic screen. Again, double heterozygous mutant embryos displayed disrupted muscle phenotypes including *akirin^{2}/+;pnr/+* (55.2%, n=29, Figure12D) and *akirin^{3}/+;pnr/+* (27.8%, n=11, Figure 12E) embryos. Although three of the four candidate genes for this double heterozygous genetic screen appeared to interact genetically with *akirin, CG30497*, a candidate gene that was predicted to interact with *akirin* in a yeast two-hybrid screen (Giot *et al.,* 2003) failed to demonstrate an interaction with *akirin* during embryonic muscle development (*akirin^{2}/+,CG30497/+,* 4.0%*,* n=25, Figure 12F; *akirin^{3}/+,CG30497/+,* 9.5%, n=42, Figure 12G). Although *akirin* and *CG30497* do not appear to interact genetically during muscle development, they may interact in another biological process. Taken together, these results further exemplify a context-dependent role for Akirin in a diverse array of developmental processes.

CHAPTER 4: INTRACELLULAR DELIVERY OF BIOLOGICALLY ACTIVE PROTEIN CARGO IN *DROSOPHILA* S2 CELLS

Introduction

Cell penetrating peptides (CPPs) are groups of short peptides that have been shown to overcome challenges of delivering bioactive macromolecular cargo to the interior of the cell, and are collectively considered as strong candidates for mediating therapeutic delivery of user-defined cargo into mammalian cells (Chen *et al.,* 2012; LeCher *et al.,* 2017). There are more than 1,600 CPPs depicted in a recent database collection (Agrawal *et al.,* 2016), with a diverse array of biochemical natures including hydrophilic, hydrophobic, amphiphilic, anionic, and cationic properties (LeCher *et al.,* 2017). CPPs were originally thought to enter cells through direct penetration, but that has since been debunked as CPPs are brought into the cell through endocytic mechanisms. There remains much controversy over the practical use of CPPs as therapeutic agents, with most of the challenges being related to endosomal escape of cargo and requirements for cellular entry (LeCher *et. al,* 2017). Recently, a novel CPP-adaptor method was developed from a twelve amino acid sequence of the HIV protein transactivator of transcription (TAT) that is capable of translocating across the plasma membrane of mammalian cells (Salerno *et al.,* 2016; LeCher *et al.,* 2017). In this method, TAT fused to calmodulin was able to associate to user-defined cargos containing a calmodulin (CaM) binding site (CBS) with high affinity in the presence of calcium, and that affinity decreases once the concentration of calcium becomes negligible (Salerno *et al.,* 2016). Importantly, baseline ionic gradients present in eukaryotic cells is taken advantage of in order to release cargo taken up in endosomes in a high-affinity, non-covalent manner (LeCher *et al.,* 2017). Taken together, this novel CPP-adaptor system overcomes issues associated with endosomal entrapment through the use of a non-covalent Ca^{+}-dependent linker model.

Although this CPP-adaptor system shows much promise for developing therapeutics that can be readily delivered to cells, more studies need to be carried out to validate its capability of delivering biologically active protein cargo. It was hypothesized that the TAT-CaM system could be combined with a binary conditional mutagenesis system to further support its use to deliver

active biomolecules. The conditional mutagenesis system exploited in this approach was the UAS-GAL4 system, which is commonly used as a genetic tool in *Drosophila melanogaster* for targeted gene expression in a tissue-specific manner (Duffy 2002). GAL4 is a galactose-inducible yeast transcriptional activator first identified in *Saccharomyces cerevisiae* (Laughon *et al.,* 1984; Duffy 2002). Specifically, GAL4 targets a 17 base-pair sequence dubbed as an upstream activating sequence (UAS) that is similar to multicellular eukaryotic enhancer elements (Duffy, 2002). Exploiting this binary method as a genetic tool, a tissue-specific promoter is fused to GAL4, which is then capable of binding to a UAS sequence that is fused to a gene of interest (Figure 15A). Without GAL4 induction, the gene of interest is maintained in a transcriptionally inactive state. When two flies carrying theses constructs are mated, the resulting progeny express the gene of interest in a GAL4-induced pattern in the desired tissue. Taking advantage of this system, it was hypothesized that CBS-fused GAL4 could be introduced to S2 insect cells transfected with a UAS-eGFP plasmid construct via Tat-CAM. Upon introduction of the cargo, it would be free to enter the nucleus due to its internal nuclear localization sequence (Chan *et al.,* 1998) and activate transcription of the extracellular plasmid, thereby expressing eGFP in the nucleus (Figure 15B). If this approach was successful, it would be another validation of delivery of biologically active cargo using the novel CPP technology. In addition, proof of concept in insect cells could prove useful for delivering Akirin deletion constructs to see if they were capable of outcompeting endogenous Akirin. Moreover, UAS-GAL4 coupled CPP-adaptor system could be applied to adjust transcript levels on demand, and allow for observations on how dynamic changes to that transcript could affect developmental processes, such as the formation of myotubes in culture.

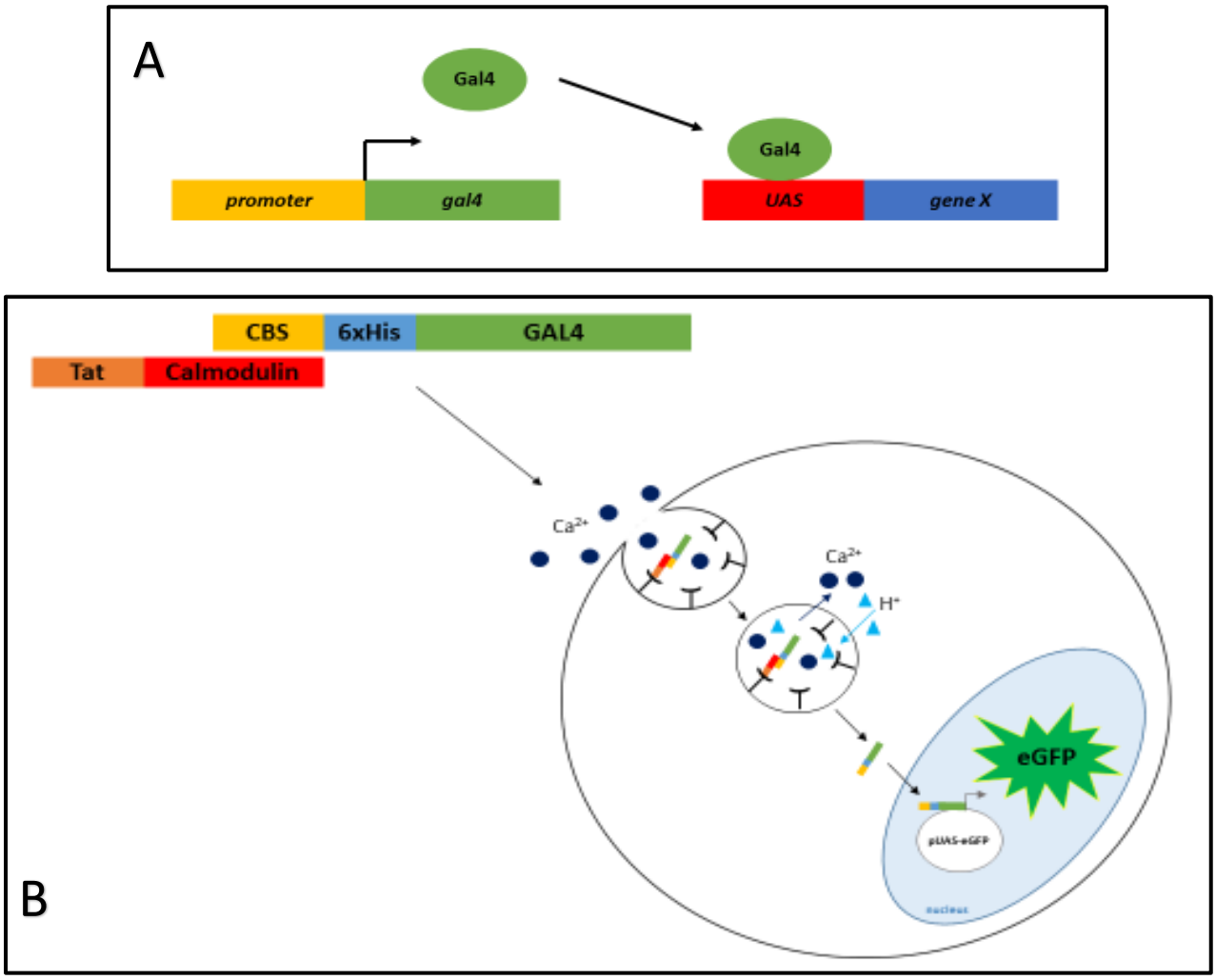

Figure 15: Experimental approach for coupling the binary UAS-GAL4 system to TAT-CaM adaptor system. A) General overview of UAS-GAL4 transgenic system used in *Drosophila.* This system is composed of two independent lines, in which the *Gal4* gene (green) is under the control of a tissue-specific promoter (yellow), and the responder line in which a gene of interest (*gene X,* blue) is under the control of UAS (red). Combining these two lines by mating results in an expression of the UAS-transgene in the cells or tissues in which GAL4 (green circle) is expressed. B) Cartoon depiction of experimental approach, adapted from LeCher *et al.* (2017). Using this experimental approach, purified CBS-GAL4 is coupled to TAT-CaM and allowed to enter insect S2 cells transfected with pUAS-eGFP through endocytic mechanisms. After the endosome travels into the cell, acidification of the endosome results in an exchange of hydrogen ions to the interior of the endosome, and a release of calcium ions taken up from outside the cell. The decreased concentration of calcium ions inside of the endosome results in a decreased affinity of TAT-CaM to CBS-GAL4, thereby releasing the cargo. The cargo is thought to be sorted and released by the formation of multiple smaller intracellular vesicles, and the later processing of TAT-CaM remains a guess (LeCher *et al.,* 2017). The cargo is released into the cytosol, where it is redirected to the nucleus due to its internal NLS. Once inside the nucleus, the cargo activates transcription from pUAS-eGFP, thereby producing functional eGFP that can be viewed under a fluorescent microscope.

Results

CBS-6xHis-GAL4 was cloned into pET19-b (Genewiz) and was successfully purified from *E. coli* by gravity chromatography with Calmodulin 4B-Sepharose (Figure 16A-B). There were challenges with purification, as the target protein was unable to bind to His-tagged metal affinity resin as indicated by the presence of the target protein in the flow-through fraction. Fractions containing the target protein were then applied to gravity chromatography with Calmodulin 4B-Sepharose and recovered in the eluted fractions. As the final product did have contaminating proteins present, presence of the target protein was confirmed by Western blotting (Figure 16B). Before delivering this construct to transfected S2 cells, the purified protein construct was validated through successful binding and dissociation from biotinylated TAT-CaM via BLI (Figure 16C,D).

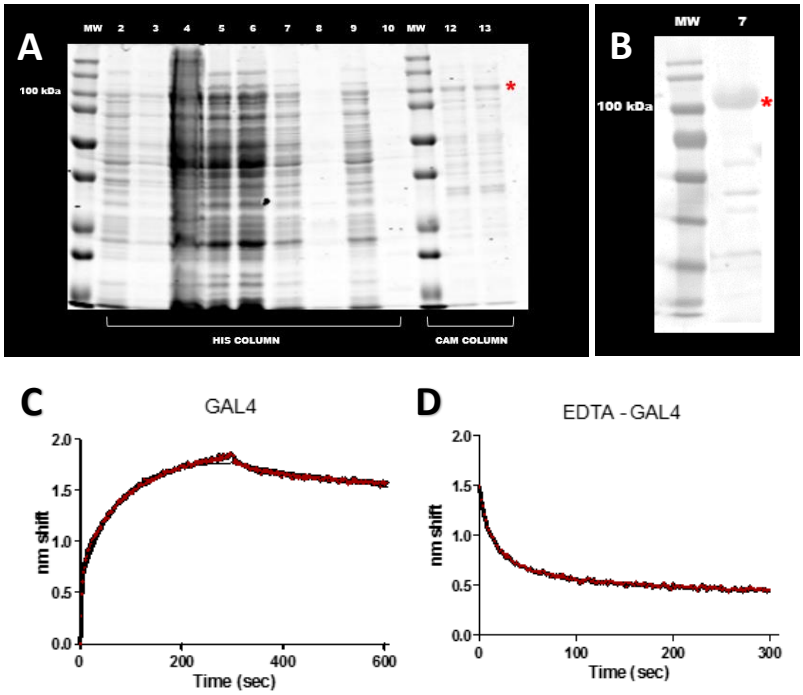

Figure 16: Purification and validation of CBS-6xHis-GAL4. A) Success of purification was assessed by Coomassie brilliant blue staining. In the first attempts to purify CBS-6xHis-GAL4 on His-tagged metal affinity resin, the target protein was largely present in the flow-through and washes (lanes 6, 7, 9). These fractions were then applied to gravity chromatography using Calmodulin 4B-Sepharose, in which the desired protein construct was present in the eluted fractions (lanes 12, 13; target protein indicated by red star). B) As the final elution fractions had contaminating proteins present, the concentrated protein fraction was analyzed by Western blotting and probed with mouse anti-6xHis (target protein indicated by red star). Note that (B) is a composite figure, lanes were omitted from the same blot for sake of clarity/direct comparison to the ladder. C-D) Association and Dissociation of GAL4 to TAT-CaM as measured by BLI. C) Association occurred between 0 and 300 seconds (K_D = 4.934e-11). D) dissociation in EDTA occurred beyond 300 seconds.

In the first attempts to deliver CBS-GAL4 in S2 cells, the protein was found to be biologically active due to its ability to activate UAS-eGFP (Figure 17). However, this was found to be true in both the presence and absence of TAT-CaM (Figure 17B,C). The non-treated cells did not show leaky expression of the promoter, as eGFP was not detected cells that were transfected but not exposed to TAT-CaM or GAL4 (Figure 17A).

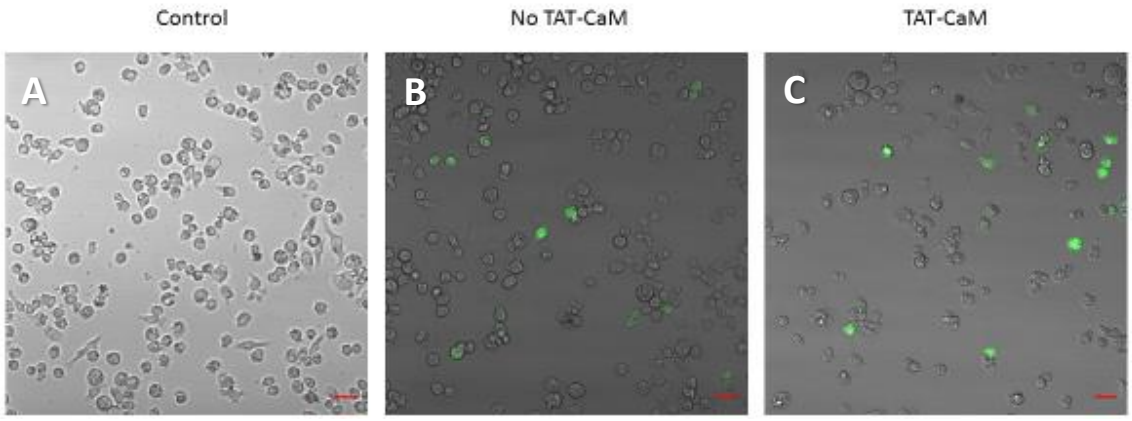

Figure 17: TAT-CaM mediated delivery of CBS-GAL4 into insect cells for activation of eGFP. S2 insect cells were transfected with pUAS-eGFP and successful delivery of CBS-GAL4 was assessed by activation of functional eGFP as detected by fluorescent microscopy. A) Transfected cells with no treatment; B) Transfected cells treated with GAL4, but not TAT-CaM; C) Transfected cells treated with both GAL4 and TAT-CaM. Although eGFP activation was successful upon GAL4 treatment, GAL4 was able to enter the cells in both the presence and absence of TAT-CaM. Scale bars = 20 µm.

In the first set of experiments, cellular uptake of TAT-CaM was carried out in media supplemented with serum. Considering that the media supplemented with serum may have influenced the cells to take up solutes from its environment, serum-free media was used for penetration assays in addition to fluorescent labeling of CBS-GAL4 to confirm intracellular localization of the protein. Using this approach, CBS-GAL4 was successfully delivered into the cell, again both in the presence and absence of TAT-CaM (Figure 18 A,B).

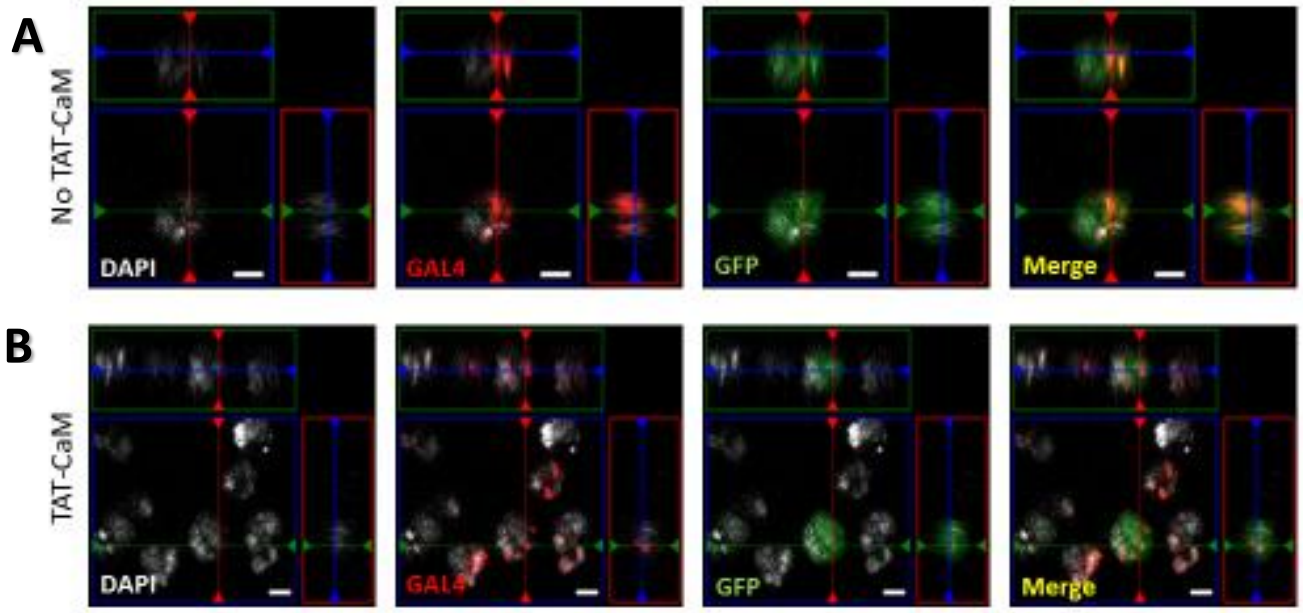

Figure 18: TAT-CaM mediated delivery of labeled CBS-GAL4 into insect cells for activation of eGFP. S2 insect cells were transfected with pUAS-eGFP and successful delivery of CBS-GAL4 was assessed by activation of functional eGFP (green) as detected by fluorescent microscopy. A) Transfected cells treated with GAL4 (red), but not TAT-CaM; B) Transfected cells treated with both GAL4 and TAT-CaM. Although eGFP activation was successful in some cells upon GAL4 treatment, GAL4 was able to enter the cells in both the presence and absence of TAT-CaM. Scale bars = 5 µm.

In the next set of experiments, the ability of S2 cells to uptake myoglobin was used to ascertain if transfection was directly related to the ability of S2 cells to uptake the CBS-cargo in serum-free media, as myoglobin is a well vetted control with the TAT-CaM system. In this approach, fluorescently labeled CBS-myoglobin demonstrated an ability to enter non-transfected S2 cells in both the presence and absence of TAT-CaM (Figure 19A,B).

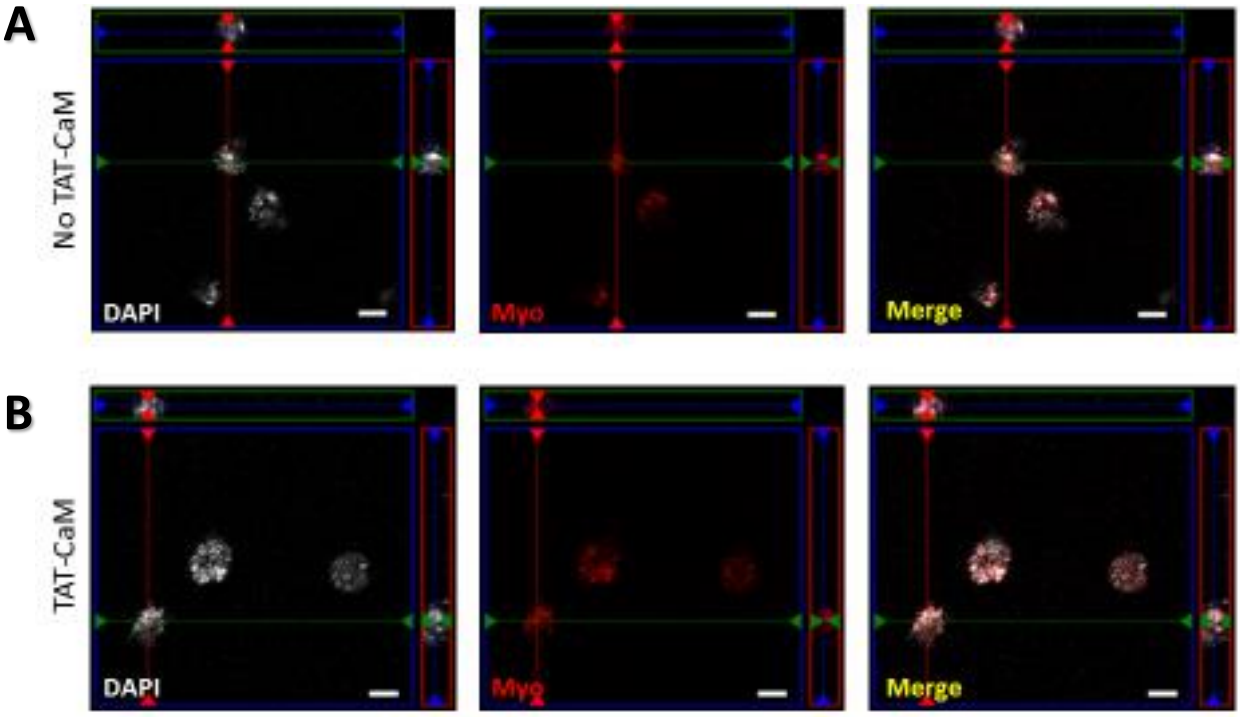

Figure 19: TAT-CaM mediated delivery of labeled CBS-myoglobin into insect cells. S2 insect cells were treated with a well-vetted control, CBS-Myoglobin (Myo). A) Transfected cells treated with Myo (red), but not TAT-CaM; C) Transfected cells treated with both Myo and TAT-CaM. In accordance with the previous results, Myo was able to enter the cells in both the presence and absence of TAT-CaM. Scale bars = 10 µm.

In accordance with the previous results, CBS-myoglobin was able to penetrate the cell in both the presence and absence of TAT-CaM. However, the nuclei appeared very fragmented, a phenotype that is associated with apoptotic or dying cells. Given the concern for the health of the cells, cellular uptake of myoglobin was repeated in addition to propidium iodine (PI) staining. PI staining revealed that all cells in both the experimental and control groups for myoglobin uptake were undergoing early apoptosis (Figure 20).

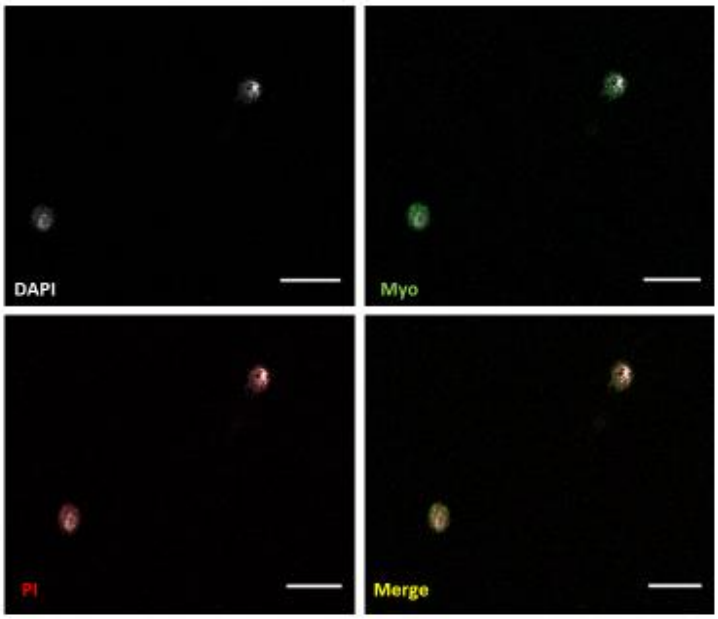

Figure 20: Propidium iodine staining of TAT-CaM mediated delivery of CBS-Myoglobin into insect cells. Based on previous observations and concerns about overall cell health, cells were exposed to propidium iodine (PI; red) staining to ascertain cell viability upon TAT-CaM mediated delivery of CBS-Myoglobin (green). Cells that demonstrated successful uptake of CBS-Myoglobin in the presence or absence or TAT-CaM also tested positive for PI stain, thereby indicating that the cells are necrotic or apoptotic as PI cannot cross the membrane of healthy cells. Only cells from the experimental group that were treated with both CBS-Myoglobin and TAT-CaM are shown for sake of clarity, as both groups demonstrated similar results. Scale bars = 20 µm.

Discussion

The goal of these experiments was to show successful delivery of biologically active cargo into insect S2 cells through the use of the TAT-CaM adaptor system. In these experiments, CBS-GAL4 was able to activate the transfected plasmid and resulted in the expression of functional eGFP in all experiments. However in the initial experiments, CBS-GAL4 was able to activate eGFP in both the presence and absence of TAT-CaM. The expression of eGFP was not due a leaky reporter, as its expression was not observed in non-treated transfected cells. Passive uptake of CBS-GAL4 was observed after serum was omitted. Next, a well vetted control, CBS-myoglobin was used as the protein cargo to assess the permeability of

the insect cell membranes. Again, passive uptake was observed as cells readily took up the cargo in both the presence and absence of TAT-CaM. Importantly, it was noted that the nuclei appeared fragmented, a phenotype that is associated with apoptotic cells. The experiment was repeated in addition to propidium iodine (PI) staining, which revealed that cells that had taken up the protein cargo were necrotic and/or apoptotic. S2 cells normally exist as semi-adherent cells in culture, but become more adherent upon calcium phosphate transfection. Even so, the cells can easily be resuspended by pipetting. Given that there are stringent washing steps during the penetration assay, it is possible that any cells that remain after cellular uptake are no longer healthy and have dropped out of solution. Taken together, the results suggest that insect S2 cells are not a good cell culture system to be applied to the TAT-CaM technology.

Using the novel CPP-adaptor method, several versatile cargo proteins have been shown to be delivered to multiple live cell types with little to no cytotoxic effects (Salerno *et al.,* 2016). However, there has been limited research on cellular uptake of CPPs in insect cells. One study has shown that CPP-TAT fusion with enhanced green fluorescent protein (eGFP) allowed for an improved ability of cellular uptake of eGFP in larval midgut cells of silkworms (Cermenati *et al.,* 2011). Another group demonstrated the ability of three distinct arginine-rich CPPs to deliver DNA cargo encoding eGFP and RFP (red fluorescent protein) into insect Sf9 (*Spodoptera frugiperda*) cells with functional expression of both cargos at the protein level with little cytotoxicity (Chen *et al.,* 2011). If this project were to be continued, a comparative experiment could be carried out in Sf9 cells, as they are the only insect cells that have been successfully applied in published CPP-adaptor studies.

CHAPTER 5: MATERIALS AND METHODS

Part 1: Generation and Purification of Akirin and Twist Constructs

1.1 Plasmid generation

To generate purified proteins of candidate regions for interaction between Akirin and Twist, protein expression vectors were constructed using Gateway Technology (Invitrogen). Candidate regions of Twist and Akirin are represented in Figures 4 and 5, respectively. PCR constructs were generated using the primers listed in Table 1. For Twist constructs, the PCR reaction protocol was 5 minutes at 95^{o}C, 34 cycles of 95^{o}C for 1 minute, 58^{o}C for 1 minute, 68^{o}C for 2 minutes, and a final extension at 68^{o}C for 15 minutes. PCR constructs were then inserted into pENTR (Invitrogen) by SD-TOPO cloning. Akirin constructs were propagated into pENTR by a previous lab member, Crystal Chiu. Following generation of pENTR plasmids, each construct was propagated into two separate protein expression vectors, pDEST15 (GST-tag, Invitrogen) and pDEST17 (6xHis-tag, Invitrogen), using LR recombination. The presence of each candidate region in the destination vectors was verified by restriction digestion with *AscI* and *NotI-HF*, as well as sequencing (primers listed in Table 3).

Sequence	Purpose
5’- CACCATGGCCTGTGCAACCCTG -3’	Akirin N-terminal forward
5’- TTACGACAGGTAGCTAGGCGCTG -3’	Akirin C-terminal reverse
5’- CACCATGAACCAGCGGCCTCCGAAG -3’	AkirinΔCR1 forward
5’- CACCATGGCTAGCAATGCAGGTCCAGCGTC	AkirinΔCR1 reverse
5’- TTACTTGGTGGTCAGCACGGACTCG -3’	AkirinΔCR4 reverse
5’- CACCATGATGAGCGCTCGCTCGGT -3’	Twist N-terminal forward
5’- CTATGCCTTCTGGTGCTGCG -3’	Twist C-terminal reverse
5’- CTAGTCCAGCAAACTGCCGG -3’	Twist 1-331 C-terminal reverse
5’- CTAACTCGAGCTGAGCATGC -3’	Twist 1-420 C-terminal reverse
5’- CTACGGGATAATGGGTGCTC -3’	Twist 1-468 C-terminal reverse
5’- CACCATGCAGAACAATGTGATCAA -3’	Twist 27-490 N-terminal forward
5’- CACCATGTCTTGGAACGAGCACG -3’	Twist 143-330 N-terminal forward
5’- CACCAACCAGCGGGTCAT -3’	Twist bHLH+WR N-terminal forward
5’- GTTGTATGACGCTCTTGATGTT -3’	pDEST15 sequencing forward
5’- CTTCCTTTCGGGCTTTGTTAG -3’	pDEST15 sequencing reverse
5’- AGATCTCGATCCCGCGAAAT -3’	pDEST17 sequencing forward
5’- CAGCCAACTCAGCTTCCTTTC -3’	pDEST17 sequencing reverse

Table 6: Primers used in this study for protein expression plasmid propagation using Gateway Technology (Invitrogen). *Generation of PCR constructs with the N-terminal leader sequence “CACC” allowed for rapid cloning in the correct orientation for protein expression. The primers used for verification of successful cloning by Sanger sequencing are also listed.*

1.2 Protein Purification of Full-length Twist from pDEST vectors

The full-length Twist expression vectors were transformed into BL21 (DE3) pLysS competent cells (New England Bio Labs) and clones were cultured in 1 L of LB medium containing ampicillin at 30^{o}C to an OD_{600} of approximately 0.4. Proteins were expressed following isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma-Aldrich) induction at a final concentration of 0.2 mM. Cells were harvested by centrifugation at 7,000 rpm at 4^{o}C for 10 minutes. GST-Twist containing cells were homogenized in lysis buffer (50 mM sodium phosphate pH 7.4, 150 mM NaCl) supplemented with lysozyme and DNase. Following homogenization, cells were lysed using a

French press. Lysates were then clarified by centrifugation at 18,000 rpm for 1 hour. GST-Twist present in the soluble supernatant was purified using Glutathione Sepharose 4B resin (bioWORLD). The resin was equilibrated with lysis buffer, and the clarified lysate was incubated with the equilibrated resin for 1 hour at 4^{o}C with gentle rotation. The protein-resin complex was added to a gravity column, and the mixture was washed twice with wash buffer (50 mM sodium phosphate pH 7.4, 200 mM NaCl) and protein was eluted with elution buffer (50 mM dibasic sodium phosphate pH 8.0, 150 mM NaCl + 20 mM fresh reduced glutathione). Success of purification was analyzed by SDS-PAGE and Coomassie brilliant blue staining.

Following the results of attempting to purify GST-Twist, 6xHis-Twist was expressed and collected in a similar fashion. Harvested BL21 (DE3) pLysS competent cells containing 6xHis-Twist were resuspended in lysis buffer (20 mM Tris base pH 7.4, 500 mM NaCl, 10 mM imidazole, 8M urea, 10 % glycerol) containing 8M urea, as the protein was sequestered into inclusion bodies. After lysate clarification, 6xHis-Twist in the supernatant was purified using His-tagged Talon metal affinity resin (ClonTech laboratories). Following equilibration of the resin with lysis buffer, the clarified lysate was incubated with the resin for 1 hour at 4^{o}C with gentle rocking. The resulting protein-resin complex was washed with was buffer (20 mM Tris base pH 7.4, 500 mM NaCl, 25 mM imidazole, 8M urea, 10 % glycerol), and the target protein was eluted with elution buffer (20 mM Tris base pH 7.4, 500 mM NaCl, 250 mM imidazole, 8 M urea, 10 % glycerol). Buffer exchange was carried out by step-down dialysis with a Slide-A-Lyzer^{TM} dialysis cassette (ThermoFisher Scientific). The first step into lysis buffer supplemented with 6 M urea was allowed to go overnight at room temperature with gentle stirring, followed by exchange into lysis buffers with containing 6 M and 4 M urea for 2 hours at room temperature for each step. Buffer exchange into 2 M urea was carried out at 4^{o}C for one hour, followed by a final exchange into HBSTG (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% Tween-20, 10% glycerol). The success of purification was analyzed by SDS-PAGE and Coomassie brilliant blue staining.

1.3 Protein Purification of Multi-tagged Akirin and Twist Constructs

The pGEX-4T1 vector was modified such that both Twist and Akirin were tagged with GST on the N-termini, and FLAG/6xHis on the C-termini. Upon receipt of lyophilized plasmid containing the desired constructs from Genewiz, the plasmid was transformed into Library Efficiency DH5α competent cells (ThermoFisher Scientific). Single colonies that resulted from cells grown overnight on ampicillin and chloramphenicol resistant LB plates at 37^{o}C were inoculated into 5ml of fresh LB supplemented with ampicillin and chloramphenicol. Overnight cultures were then mini-prepped using a QIAprep spin miniprep kit to create working DNA stocks.

After confirming that both of the target proteins were present in the supernatant upon IPTG induction, proteins were purified using His-tagged Talon metal affinity resin (ClonTech laboratories) using the same approach as 6xHis-Twist. Since the new constructs were not sequestered into inclusion bodies upon expression, urea was omitted from all buffers used for purification. After obtaining pure protein in the eluted fractions from the resin, 3ml of the best eluted fractions were injected into Slide-A-Lyzer^{TM} dialysis cassettes (ThermoFisher Scientific) for overnight buffer exchange into HBSTG. Success of purification was analyzed by SDS-PAGE and Coomassie brilliant blue staining. Pure proteins were quantified using Pierce^{TM} BCA Protein Assay (ThermoFisher Scientific) and stored at -80^{o}C.

1.4 Thrombin cleavage of GST-tag

In order to remove the GST-tag from the multi-tagged constructs for use in biolayer interferometry (BLI) binding assays, site-specific proteolytic cleavage was performed using thrombin while carrying out purification. Clarified lysates were prepared in a similar fashion as previously discussed for 6xHis-tagged Talon metal affinity gravity column purification. After clarified lysates were combined with equilibrated resin, 10 units of thrombin (GE Healthcare) dissolved in 1xPBS was added to the mixture and incubated for 16 hours at 4^{o}C. The mixture was added to a gravity column, washed with wash buffer twice, and cleaved proteins were eluted with elution buffer. Analysis of successful cleavage and purification was assessed by SDS-PAGE and Coomassie brilliant blue staining.

Part 2: Biolayer Interferometry of Full-Length Akirin and Twist

2.1 BLI

Target ligands were biotinylated with 2.5 µl of NHS-LC-LC-biotin (succinimidyl-6-[biotinamido]-6-hexanamidohexanoate) (ThermoFisher Scientific) dissolved in DMSO and 100 µl of target ligand for 30 minutes at room temperature. Excess biotin was removed from each sample by rapid buffer exchange into fresh HBSTG using Zeba^{TM} Spin desalting columns (ThermoFisher Scientific. BLI kinetic assays were carried out on a ForteBio Octet QK biosensor equipped with streptavidin (SA) sensors. All assays were performed in a black 96-well plate at 25^{o}C. Both ligand and analyte were diluted to 1 µM in a final volume of 200 µl per well. After loading biotinylated target ligands to the SA sensor, a baseline was established in HBSTG before allowing ligand to interact with the desired analyte.

Part 3: Protein Expression in *Drosophila* S2 Cells

3.1 Plasmid generation using Gateway Technology (Invitrogen)

In accordance with previous results, the approach to observing the binding interactions between Twist and Akirin was taken in a new direction to express proteins in a eukaryotic system and assess their binding *in vivo.* Akirin was cloned into pAHW (*Drosophila* Genomics Research Center), a plasmid driven by an act5c promoter to produce target protein with an N-terminal 3xHA tag when expressed in *Drosophila* S2 cells. Similarly, Twist was cloned into pAFW (*Drosophila* Genomics Research Center) for expression of target protein with an N-terminal FLAG tag. The new constructs were propagated using the Gateway Technology system (Invitrogen) in the same manner as the pDEST vectors.

3.2 Transient transfection of S2 cells using calcium phosphate method

To express target proteins using the newly constructed plasmids, the DNA was transfected into *Drosophila* Schneider Line 2 (S2) cells using calcium phosphate method. S2 cells were maintained and transfected as previously described (Castanon *et al.,* 2001). $3x10^6$ cells were seeded into a 6-well plate in 3 ml Schneider's complete *Drosophila* medium with L-glutamine (ThermoFisher Scientific) and grown for 12 hours at 29^{o}C. The transfection mix was prepared by slowly adding solution A (36µl of 2 M calcium chloride, 19µg DNA, sterile water to 300µl) to solution B (300µl HEPES buffered saline; 50 mM HEPES pH 7.1 , 1.5 mM sodium phosphate, 280 mM NaCl) with gentle vortexing. The transfection mix was allowed to sit at room temperature for 40 minutes until a fine precipitate formed. The transfection mix was then added dropwise to the cells with gentle swirling, and the cells were incubated at 29^{o}C for 16 hours. The calcium phosphate solution was removed and the cells were washed twice with PBS, then resuspended in 3ml of fresh medium.

3.3 Co-immunoprecipitation

To analyze protein interactions using the co-immunoprecipitation (CoIP) method, 3 ml of transfected cells were harvested by centrifugation at 1000 x g for 3 minutes and resuspended in 1 ml PBS. The cells were pelleted again, resuspended in 50µl lysis buffer (60 mM Tris-HCl pH 7.8, 150 mM NaCl, 1mM EDTA, 1% Triton-X100; supplemented with 1x fresh Halt^{TM} protease inhibitor cocktail) and incubated on ice for 20 minutes. Following lysis, the nuclei and cell debris were pelleted by centrifugation for 10 minutes at 12,000 x g, and the supernatant was transferred to a new sterile tube. 40 µl of Anti-FLAG M2 affinity gel agarose suspension was equilibrated with TBS (Tris-buffered saline; 50 mM Tris-HCl pH 7.5, 150 mM NaCl) by washing the packed gel with 0.5 ml TBS three times. 500 µl each of cell lysates containing 3xHA-Akirin and FLAG-Twist were added together with equilibrated agarose and incubated overnight at 4^{o}C. The reaction was washed two times with TBS and the interacting products were eluted by adding SDS-PAGE sample buffer (0.125M Tris-HCl pH 6.8, 2% SDS, 0.2% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol) to a final concentration of 1X, and heating the samples to 60^{o}C for 5 minutes. The CoIP reactions were analyzed by SDS-PAGE and western blot.

For western blotting, the samples were transferred to a PVDF membrane at 2.5 A for 10 minutes using Trans-Blot Turbo Transfer system (BIORAD). The membrane was blocked with Odyssey blocking buffer and probed with 1:10,000 rabbit anti-HA (Sigma-Aldrich) and 1:10,000 mouse anti-FLAG (Sigma-Aldrich). Appropriate fluorescently-labeled secondary antibodies (Licor) were used at a concentration of 1:15,000.

Part 4: Generation of Akirin Interactome

4.1 *Drosophila* stocks and genetic crosses

Drosophila melanogaster stocks were maintained on Nutri-Fly^{TM} Molasses Formulation at 27^{o}C. The *Drosophila* strains used in this study were *w;akirin^{2}, w;akirin^{3},e/TM3,sqhmCherry, w;ly,hs-hid/TM3,sqhmCherry*, *w;Dr/TM3,DfdGMRYFP, Mi-2^{4}red^{1}e^{4}/TM6B,Sb^{1}Tb^{1}ca^{1}, ru^{1}h^{1}Diap^{1}st^{1}pnr^{1}sr^{1}es,ca/ TM3,Sb^{1}Tb^{1}, P{EP}CG30497^{EP2618}, Taf4^{1}/TM6C,cu^{1}Sb^{1}ca^{1}, w^{1118};P{w[mGT]=GT1}simj^{BG00404}/TM6B,P{w[+mC]=35UZ]DB1,Tb^{1}.* Strains used in this study were either kindly provided by the Bloomington *Drosophila* Stock Center (Indiana University), previously published (Nowak *et al.*, 2012), or generated in our laboratory.

4.2 Double heterozygote genetic screen

To observe interactions between *akirin* and other factors necessary for somatic muscle patterning, double heterozygote embryos were examined as a rapid genetic screening technique (Figure 21).

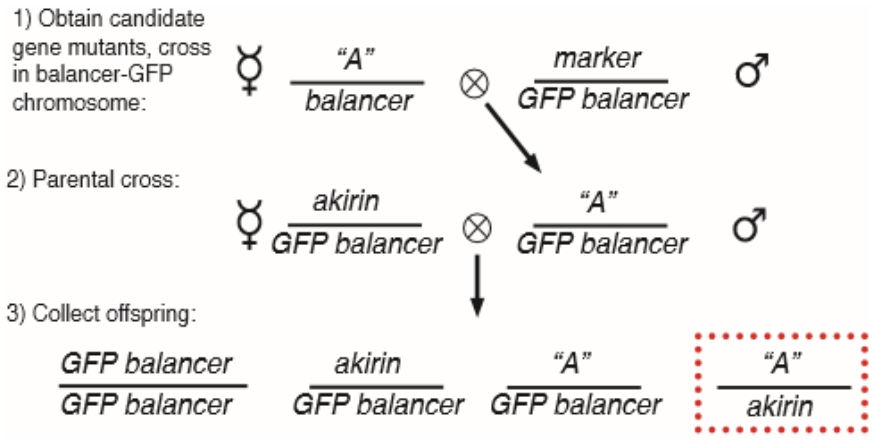

Figure 21: Scheme for collecting double heterozygous mutants for genetic screen. *akirin* heterozygous mutants are crossed with flies carrying the GFP marker. F1 embryos are collected and fixed with paraformaldehyde and stained with antibodies to reveal their musculature under a fluorescent microscope. Both of the parental lines carry the GFP marker in order to easily visualize double heterozygous mutants.

4.2.1 Embryo collection

To prepare embryos for immunohistochemistry, embryos were collected and stained following previously established protocols (Nowak *et al.*, 2012; Beckett and Baylies, 2006; Artero *et al.*, 2003). Embryos were collected on 60mm grape agar plates at stage 16 (~14 hours), followed by dechorionation in 50% bleach and fixation in 4% paraformaldehyde. Embryos were devitellinized in 50% heptane/methanol and stored at -20^{o}C.

4.2.2 Embryo immunohistochemistry

Antibodies and dilutions used for embryo immunostaining were as follows: anti-Myosin monoclonal mouse 1^{o} antibody (gift of S. Abmayr; 1:400) to stain *Drosophila* musculature, anti-GFP monoclonal rabbit 1^{o} antibody (abcam, 1:800) to stain for wild-type markers, ThermoFisher Scientific Alexa-fluor^{TM} 488 anti-mouse (1:200), ThermoFisher Scientific Alexa-fluor^{TM} 555 anti-rabbit (1:200), Jackson Immunoresearch biotin SP conjugated affine pure donkey anti-rabbit (1:200), Jackson Immunoresearch biotin SP conjugated affine pure donkey anti-mouse (1:200).

Stored embryos were washed in PBT (Phosphate buffered saline + 0.03% Triton X-100), blocked with PBT + 2% bovine serum albumin (BSA), and incubated at 4^{o}C overnight with the

appropriate primary antibody. Following the addition of the primary antibody, embryos were rinsed and washed with PBT-BSA. The appropriate secondary antibody was added to the embryos and allowed to incubate for 1 hour at room temperature. Fluorescently-labeled stained embryos were rinsed and washed with PBT-BSA. Embryos were allowed to sink in Vectashield overnight and were subsequently mounted for microscopy.

For diaminobenzidine (DAB) colorimetric staining, embryos with the appropriate antibodies were rinsed and washed in PBT-BSA, followed by incubation with Vectastain A+B kit (Vector Labs) for 30 minutes at room temperature. Embryos were again rinsed and washed in PBT-BSA, and resuspended in 600µl PBT-BSA, 20µl nickel chloride, and 30µl DAB. The embryos were placed onto a watch glass and a hydrogen peroxide solution was added to the mixture for color development. The reaction was stopped by rinsing the embryos several times in fresh PBT-BSA. Following the first color development, embryos were incubated with the next appropriate secondary antibody for 1 hour at room temperature. The secondary color development was repeated without nickel chloride in the DAB mixture. Following the last PBT-BSA wash, embryos were dehydrated in washes with: 70% ethanol, 90% ethanol, 100% ethanol, 100% dry ethanol, and acetone. The stained embryos were allowed to sink in a 1:1 araldite:acetone mixture at 4^{o}C and mounted in 100% araldite for microscopy.

4.2.3 Microscopy

A Zeiss LSM confocal 700 microscope was used for immunofluorescent imaging, and a Zeiss Axio Imager.M2 microscope was used for bright field imaging to quantify muscle phenotypes. A Plan-Apochromat20x/0.8 M27 objective was equipped on both microscopes. Zeiss Zen Black was used to produce maximum intensity projections, and Zeiss Zen Blue software was used for all final image processing.

4.3 Polytene chromosome immunohistochemistry

Antibodies and dilutions used for polytene chromosome immunostaining were as follows: rat anti-Mi2 (Active motif; 1:10), rabbit anti-Akirin (Nowak *et al.,* 2012; 1:50), ThermoFisher Scientific Alexa-fluor™ 488 anti-rabbit or anti-rat (1:250), ThermoFisher Scientific Alexa-fluor™ 555 anti-rabbit or anti-rat (1:250).

Preparation of polytene squash chromosomes and immunostaining was performed as previously described (Karam *et al.,* 2010). Salivary glands were dissected from y^1w^{67c23} (wild-type) 3rd instar larvae in 1xPBS+0.1% Triton X-100. The salivary glands were then placed in an acetic acid fixative (50% glacial acetic acid, 0.1% Triton X-100, 3.7% formaldehyde) for 2 minutes. Following fixation, glands were transferred to 45% acetic acid. 1-2 glands were placed in a drop of 45% acetic acid on a subbed microscope slide (slides dipped in 10% poly-L-lysine and allowed to drip dry) and covered with a siliconized coverslip (coverslips dipped in Sigmacote). Using a hammer (Carolina Biological Supply), the polytene chromosomes were squashed by repeatedly tapping in a spiraling motion. The slide was flash frozen in liquid nitrogen and stored at -80% until ready for immunostaining.

For immunostaining, slides were placed in a Coplin jar with antibody dilution buffer (1xPBS, 0.1% Triton X-100, 1% BSA) and washed for 30 minutes three times. 35µL of primary antibody diluted in antibody dilution buffer was added to the slide, sealed with parafilm and incubated overnight at 4°C in a humidified chamber. Following overnight incubation, the slides were washed with antibody dilution buffer three times for 15 minutes each wash. 50µL of secondary antibodies diluted 1:250 in antibody dilution buffer was added to each slide, covered with sealed parafilm, and incubated at 37°C for 30 minutes in a humidified chamber. The slides were washed with antibody dilution buffer three times for 15 minutes each wash and rinsed with 1xPBS. Slides were stained with DAPI for 1 minute and rinsed in 1xPBS. One drop of Vectashield was added to the specimen and covered with a coverslip until ready for microscopy. Adobe Photoshop was used to globally adjust brightness and contrast over processed images.

Part 5: Intracellular Delivery of Purified GAL4 into *Drosophila* S2 Cells

5.1 CBS-GAL4 Expression and Purification

5.1.1 Construction of vector encoding CBS-GAL4

A protein expression vector was constructed to generate pure protein for intracellular delivery. The pET19-b vector was modified with an N-terminal 6xHis-tag for purification, followed by a sequence encoding CBS and GAL4. Plasmids were generated in a similar fashion as described in section 1.1.

5.1.2 CBS-GAL4 Purification

For generation of pure CBS-GAL4 for intracellular delivery, protein expression and 6xHis purification was carried out as described in section 1.2. Subsequent Calmodulin (CAM) purification was carried out by equilibrating Calmodulin 4B Sepharose with equilibration buffer (50 mM Tris pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$, 10 % glycerol). Flow-through and lysate samples harvested during His-purification were incubated with equilibrated resin for one hour at 4C. The protein-resin complex was added to a gravity column, washed twice with equilibration buffer, and protein was eluted with elution buffer (50 mM Tris pH 8.0, 150 mM NaCl, 4 mM EDTA, 10% glycerol). A buffer exchange into NEB Buffer and concentration of eluted products was performed at the same time by using a Sartorius Vivaspin 20 spin column (10,000 MWCO, PES membrane filter). Protein fractions from both purifications were analyzed via SDS-PAGE and Coomassie brilliant blue staining. Final eluted product was verified by Western blotting by probing with mouse anti-6xHis (BioLegend).

5.2.1 Verification of CBS-GAL4 functionality with TAT-CaM system

BLI measurements were performed as previously described for Akirin and Twist in section 2.1 above. Baseline data was obtained in NEB buffer, and dissociation was measured in buffer with 10 mM EDTA.

5.3 Calcium phosphate transfection of insect S2 cells

Transfection of insect S2 cells using calcium phosphate method was performed as described in section 3.2. For these experiments, cells were transfected with pUAS-eGFP (gift of M. Baylies). Following transfection, cells were transferred to a 4-well or 8-well chambered coverglass and incubated for 16 hours at 29C.

5.4 Penetration assay using TAT-CaM

5.4.1 Uptake

CBS cargo proteins were labeled in given experiments with DyLight 550 or 488 NHS-Ester (ThermoFisher Scientific). For uptake experiments, a final concentration of 1 uM of CBS cargo protein (GAL4 or myoglobin) and 1 uM of TAT-CaM were mixed and incubated at room temperature for 5 minutes. Protein mix was added to media without serum to a final volume of 250 µl, then added to the cells in chambered coverglass following filter sterilization. Cells were incubated at room temperature for one hour with intermittent, gentle rocking. Cells were washed with PBS three times, and the nuclei were stained with 1 drop of NucBlue for 10 minutes at room temperature. Cells were washed five times with PBS and subjected to confocal microscopy to visualize uptake.

5.4.1 Microscopy

Confocal imaging of cellular uptake experiments were performed as previously described in section 4.2.3. Zeiss Zen Blue used to produce orthogonal projections of regions of interest and process all final images presented in this thesis.

Integration of Thesis Research

The work presented in this thesis encompassed several facets of biology ranging from genetics to biochemistry. Although some avenues of the thesis research were not as fruitful as hypothesized, this research uncovered a novel interaction between Akirin and the NuRD chromatin remodeling complex during skeletal muscle development in *Drosophila melanogaster.* The use of a diverse array of disciplines were used during this thesis project: biology, biochemistry, biotechnology, cell biology, chemistry, developmental biology, genetics, immunohistochemistry, and organismal biology. The application of several techniques from a variety of disciplines provides a new context for mechanisms leading to gene expression changes during muscle development in the fruit fly, which can potentially allow for a greater understanding of transcriptional control during mammalian development. Hopefully, the research in this thesis will allow for the identification of molecular players involved in building and maintaining the somatic musculature, thereby providing more specific targets for the therapeutic intervention in diseases such as muscular dystrophies and cancer cachexia.

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to keep moving forward when I wanted to give up. I would also like to thank him for all of his conversations about video games and music when I needed a distraction from lab work. Without his support and guidance, I would not be the scientist or person that I am today and I will definitely miss working in his lab.

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