MOLECULAR DISSECTION OF PINCH:

INSIGHTS INTO INTEGRIN FUNCTION

IN *DROSOPHILA*

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Oncological Sciences

The University of Utah

August 2011

The University of Utah Graduate School

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ABSTRACT

Adhesion and migration are required for proper development and for the maintenance of adult tissues. Cellular adhesion is regulated by signaling, adaptor, and actin associated proteins that assemble at the cell cortex in response to integrin activation. PINCH, ILK, and RSU-1 are three such adaptor proteins that form a physical complex downstream of integrins and have evolutionarily conserved overlapping, yet not identical, roles in integrin-mediated adhesion. The 5 LIM domain scaffolding protein PINCH is the central molecule in this complex, making contacts with ILK via LIM1 and RSU-1 via LIM5. In *Drosophila*, PINCH and ILK are required for maintaining actin-membrane linkages in the embryonic muscle, and null mutant clones in the wing display a loss of adhesion resulting in blisters. RSU-1 is not required for viability, but like PINCH and ILK, is required for maintaining adhesion of the wing epithelia. To further understand the contributions of these proteins to integrin function *in vivo*, a molecular analysis of PINCH was conducted using mutations that disrupt the interaction with ILK ($PINCH^{Q38A}$ and $PINCH^{\Delta LIM1}$) or RSU-1 ($PINCH^{D303V}$ and $PINCH^{\Delta LIM5}$). We find that $PINCH^{Q38A}$ transgenes rescue the *stck* (PINCH) null mutant, and rescued flies display no defects in integrin function. However, a dramatic reduction in viability is observed in $PINCH^{Q38A}$ rescued flies upon loss of RSU-1. Disruption of the PINCH-ILK interaction does not affect the levels of either protein, in contrast to results observed with complete loss of either PINCH or ILK, suggesting that PINCH and ILK mediate other interactions

that contribute to their function and stability. PINCH$^{\Delta LIM1}$ transgenes rescue the late embryonic/early larval lethality of the *stck* null mutant, but rescued animals only survive to larval and pupal stages, indicating that LIM1 function is required later in development. PINCH^{D303V} transgenes rescue the *stck* null mutant, although rescued adult flies are not healthy. PINCH^{D303V} rescued flies exhibit reduced levels of RSU-1, which is mislocalized, indicating that the PINCH-RSU-1 interaction is required to maintain levels of RSU-1 and to localize RSU-1 to adhesion sites. PINCH$^{\Delta LIM5}$ transgenic flies were also generated, but the transgenes fail to rescue the *stck* null mutant due to instability of the transgenic protein. Taken together the data presented in this dissertation demonstrate functional consequences of disrupting the PINCH-ILK-RSU-1 complex, and suggest that multiple contacts made by each complex member contribute to protein localization, stability, and function downstream of integrin.

This dissertation is dedicated to my family.

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ACKNOWLEDGMENTS

I have many people to thank for their support and guidance over the last several years. First I must thank Mary Beckerle, my advisor. One reason I joined Mary's lab was because of my respect for her as a scientist and a leader. I feel that she has helped me to mature as a scientist, especially in helping me to express my thoughts clearly in my writing and in my public presentations. Even with her hectic schedule, she made time to meet and give advice during the most critical points for me in graduate school. This has made the biggest difference in the last nine months as I have prepared a manuscript to be published and for the preparation of this thesis.

I am fortunate to have a great committee. Anthea Letsou, Jody Rosenblatt, Mark Metzstein, and Kimble Frazer are all individuals whom I really like as people and respect as scientists. Each one of them brings a unique perspective to our meetings and I appreciate specific qualities in each of them. It has been a pleasure having their support and guidance over the years.

The members of the Beckerle lab have been excellent mentors and friends and I have enjoyed our conversations not only about science, but about our travels, and culinary adventures as well. I want to especially thank Julie Kadrmas and Kathleen Clark who played an especially important role in helping me learn to use *Drosophila* as a model system and have helped me develop as a scientist. I would also like to acknowledge my

fellow graduate students in the lab, Hsiao-Fen Han, Aashi Chaturvedi, and Laura Luettjohann. We have all experienced challenges as students, and knowing there are others around who understand has made a huge difference.

I am fortunate to have made great friends in my time in Utah. I would like to thank my classmates for their wonderful support in all matters relating to science, grad school and life in general. Through them I have built a great network of friends who have participated in great camping trips, have challenged me on the trails and mountainsides, and most importantly we have shared many great meals together. Through my great friends I also met Paul, who has made the last two years of my life so special. I've enjoyed our adventures and time together in Moab and Salt Lake City immensely.

Finally I have to thank my family. My parents, Ziad and Ralda, have always been supportive of all my endeavors. They have taught me to be generous and to work hard and to put care and effort into everything I do. My younger siblings, Laila and Paul, both finished their PhDs before I did. They are models of hard work, focus, and dedication and I have so much respect for them. I am so proud of them and all of their accomplishments.

CHAPTER 1

INTRODUCTION

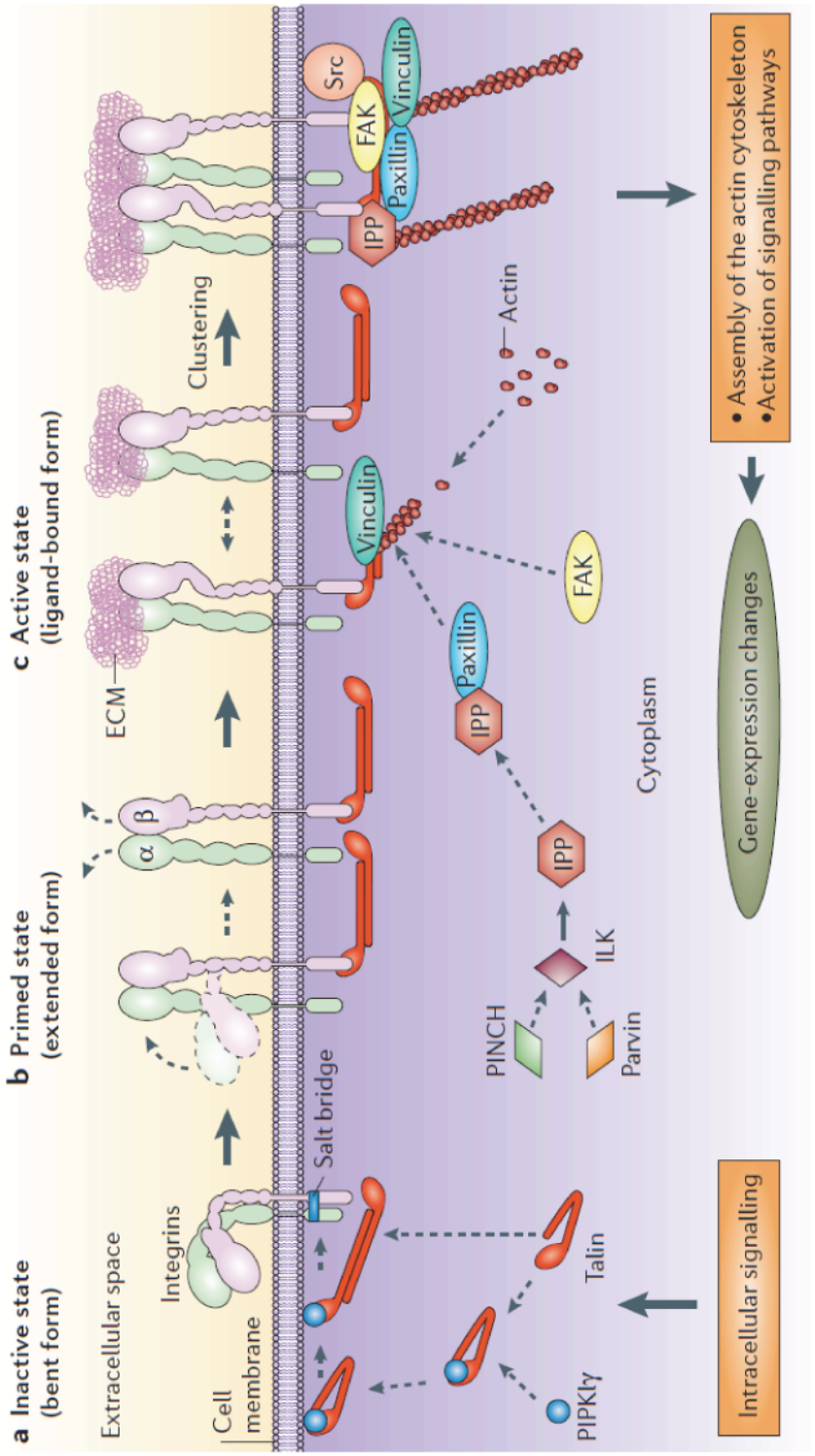
Overview of cell adhesion

Cellular responses to signals from the environment that affect cell shape change, adhesion and motility have important implications during development and in the maintenance of adult tissues. During development, gastrulation and neural tube formation are both achieved by epithelial sheet morphogenesis. In adults, cells interact with extracellular matrix (ECM) proteins to maintain tissue integrity in locations such as the myotendinous junction. Cell adhesion and migration are also important properties of cells in the immune system and at the site of injury during the process of wound healing. Finally, understanding how cells interact with the ECM has important implications for understanding tumor metastasis (Legate et al., 2006; Xia and Karin, 2004). Understanding how adhesion is regulated at the cellular level will broaden our knowledge of these important biological processes

Integrin structure and function

Integrins are the primary transmembrane receptors responsible for transducing signals between the ECM and the cytoplasm to cause changes in adhesion and cell shape. Integrins consist of α and β heterodimers that, upon receiving signals from within the cell, undergo a conformational change to become activated and interact with the ECM. Activated integrins are then able to cluster and assemble different protein complexes at the cytoplasmic face of the cell, including signaling proteins and proteins that either directly or indirectly stabilize the actin cytoskeleton (Bokel and Brown, 2002; Brown et al., 2000; Legate et al., 2006) (Fig 1.1). Integrin adhesion complexes can contain approximately 150 proteins, whose specific regulation is responsible for carrying out downstream functions (Zaidel-Bar et al., 2007).

Figure 1.1: Integrin activation and assembly of downstream proteins. (A, B, C) Upon receiving signals from inside the cell, integrin receptors undergo a conformational change that allows them to engage the extracellular matrix. This is classically described as inside-out signaling. (C) Once integrins are activated, they cluster and relay signals back into the cytoplasm allowing the assembly of downstream proteins such as adaptor molecules, signaling molecules and cytoskeletal regulators that affect cell shape changes and downstream signaling. This is classically described as outside-in signaling. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology 7, 20-31 (January 2006) | doi:10.1038/nrm1789.


a Inactive state
(bent form)
b Primed state
(extended form)
c Active state
(ligand-bound form)
Extracellular space
Integrins
Cell membrane
Salt bridge
α
β
ECM
Clustering
Src
FAK
Paxillin
Vinculin
IPP
Actin
PINCH
Parvin
ILK
PIPKIγ
Talin
Cytoplasm
Gene-expression changes
• Assembly of the actin cytoskeleton
• Activation of signalling pathways
Intracellular signalling

In *Drosophila* there are two β integrins (PS and ν) and five α integrins (PS1-5), making the study of integrins much less complicated than in vertebrates where there are eight β and 18 α subunits which can assemble into 24 distinct heterodimers (Brower, 2003; Hynes, 2002). *Drosophila* βPS integrin, encoded by the *mysospheroid* (*mys*) locus, is the most ubiquitous β-integrin as it is expressed in all tissues and has been shown to interact with at least three of the α subunits. *mys* mutants in *Drosophila* exhibit some defects in embryonic germ band retraction and dorsal closure, but show 100% penetrance in muscle detachment phenotypes in stage 16-17 embryos indicating the importance of integrins in adhesion and migratory processes (Leptin et al., 1989; Newman and Wright, 1981; Pines et al., 2011; Wright, 1960). Furthermore, *mys* null clones in the wing result in blisters, indicating a loss of adhesion between the two sheets of epithelia that make up the wing (Fig 1.2 A,B,D) (Brabant et al., 1996; Brower and Jaffe, 1989). Indeed, the *Drosophila* embryonic muscle and the developing and adult wing have proven to be excellent models for studying integrin-mediated adhesion and have provided the means to identify downstream genes involved in integrin function (Loer et al., 2008; Prout et al., 1997; Walsh and Brown, 1998).

Molecular scaffolds are required for the assembly and function of integrin adhesion complexes

One critical event required for maintaining integrin activation is the binding of Talin to the cytoplasmic tail of β-integrin (Calderwood, 2004). Talin functions as a platform to assemble many signaling proteins and adaptor proteins that regulate the actin cytoskeleton (Fig 1.1) (Calderwood and Ginsberg, 2003). Furthermore Talin has the capacity to directly bind Actin, which together with its other roles, demonstrates the

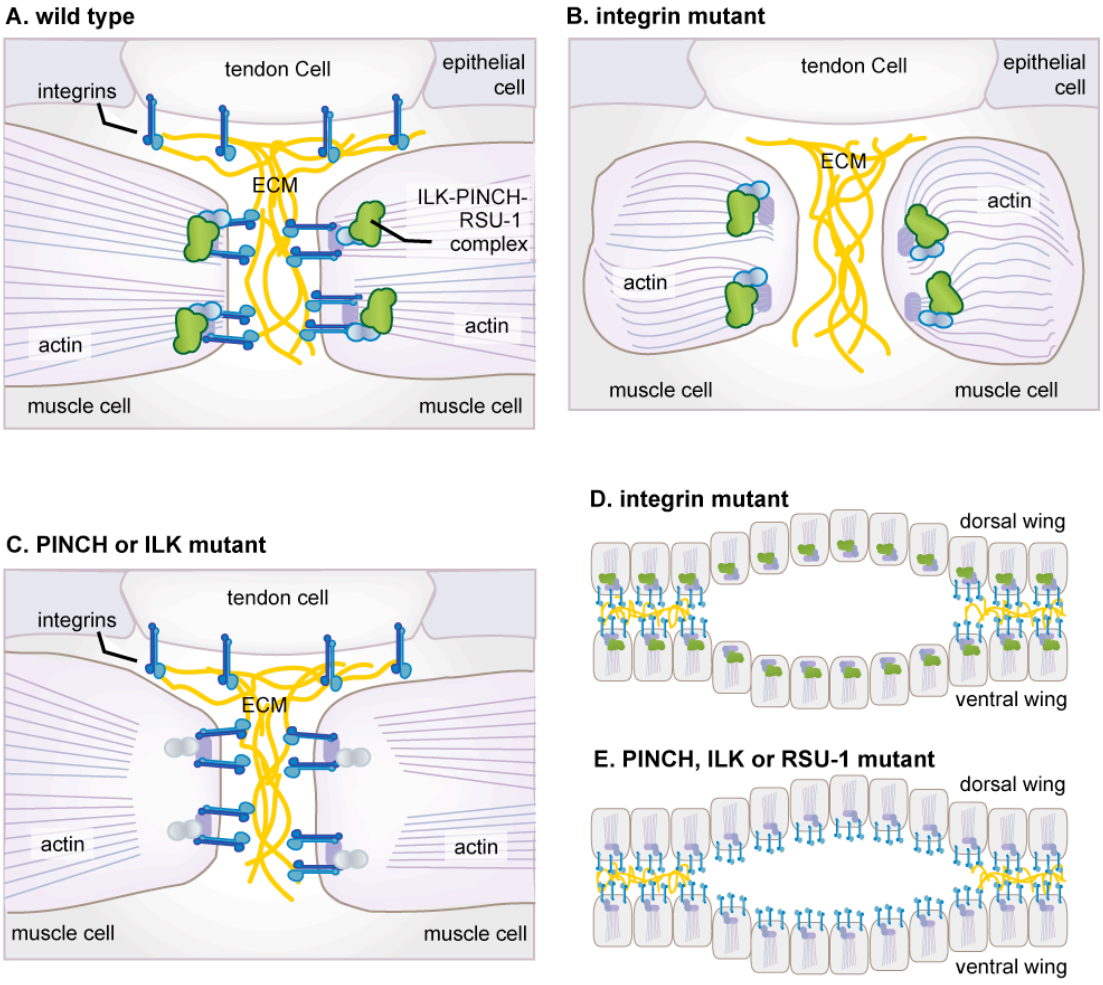


Figure 1.2: Integrin-mediated phenotypes in the *Drosophila* muscle and wing. (A) A diagram of a wild-type muscle attachment site demonstrates two muscle cells meeting a tendon cell to create the myotendinous junction. Cell adhesion is mediated by the attachment of the integrin receptors to the ECM and the assembly of proteins at the cell cortex. (B) In *myospheroid* (*mys*, βPS-integrin) mutants, loss of the receptor in *Drosophila* is embryonic lethal due to detachment of cells from the ECM and results in rounded up muscles. (C) In *steamerduck* (*stck*, PINCH) or *integrin linked kinase* (*ilk*, ILK) mutants, loss of the adaptor proteins in *Drosophila* is late embryonic/early larval lethal and results in loss of integrin-actin linkages due to detachment of the actin cytoskeleton from the muscle cell membrane. (D,E) In the *Drosophila* wing, loss of integrins, PINCH, ILK, or RSU-1 (*icarus*) results in wing blisters caused by the loss of adhesion between the two sheets of the wing epithelia.

ability of Talin to regulate integrin function on many levels (Muguruma et al., 1990). Recent work in *Drosophila* has identified Wech as another adaptor that localizes at muscle attachment sites (Loer et al., 2008). *wech* mutants, like *mys* (βPS-integrin) and *rhea* (Talin) mutants, demonstrate detachment of the muscle cell membrane from the ECM (Brown et al., 2002; Leptin et al., 1989; Newman and Wright, 1981; Wright, 1960).

PINCH and ILK are scaffolds that are required for integrin function

ILK (Integrin Linked Kinase) and PINCH (Particularly Interesting Cysteine-Histidine-rich Protein) are examples of another category of adaptor proteins that are downstream of integrins. ILK was isolated in a yeast-two-hybrid screen to identify proteins that bind the cytoplasmic tail of β-integrin (Hannigan et al., 1996). This specific interaction has not been shown in *Drosophila*, but biochemical evidence suggests that Wech may be the adaptor that links Talin and ILK (Loer et al., 2008). ILK is composed of an N-terminal ankyrin repeat (ANKR) domain, a pleckstrin homology (PH)-like domain and a C-terminal kinase-like domain. There has been a long-standing unresolved controversy about whether ILK is a kinase in mammalian systems, although there is no evidence suggesting kinase function in the fly (Fukuda et al., 2009; Hannigan et al., 2011; Wickstrom et al., 2010; Zervas et al., 2001). PINCH was first identified in a screen for antigens that bind to senescent cells, and soon after was categorized as an adaptor molecule involved in integrin function (Rearden, 1994; Tu et al., 1999; Wu, 1999). In mammals there are two *PINCH* genes (1 and 2), while in *Drosophila* there is one *PINCH* gene that has 3 transcripts that vary only in their very N-termini (a, b, and c). The PINCH protein is made up of 5 LIM domains and a C-terminal tail. Each LIM domain consists of

a conserved cysteine rich consensus sequence characterized by two zinc fingers that mediate protein-protein interactions (Kadrmas and Beckerle, 2004; Schmeichel and Beckerle, 1997). LIM1 of PINCH binds directly to the N-terminal ANKR domain of ILK (Tu et al., 1999). ILK also binds to another adaptor, Parvin, providing a link to the actin cytoskeleton (Nikolopoulos and Turner, 2002; Tu et al., 2001). The interactions between PINCH, ILK and Parvin are well conserved and have been studied in different systems (Fig 1.3) (Legate et al., 2006; Wickstrom et al., 2010).

Much of the work involving PINCH and ILK indicates that they have similar functions. In *Drosophila*, ILK is encoded by the integrin linked kinase (*ilk*) locus and PINCH by the *steamerduck* (*stck*) locus. *stck* and *ilk* null mutations are both embryonic lethal due to detachment of the actin cytoskeleton from the muscle cell membrane at muscle attachment sites (Fig 1.2 C, 1.4 B) (Clark et al., 2003; Zervas et al., 2001). This phenotype is different than defects observed in *mys*, *rhea,* or *wech* mutants, which display a loss of adhesion between the cell membrane and the ECM (Fig. 1.2 B) (Brown et al., 2002; Leptin et al., 1989; Loer et al., 2008). *stck* or *ilk* null mutant clones in the *Drosophila* wing result in blisters similar to integrin mutants, indicating a loss of adhesion between the two sheets of the wing epithelium (Fig 1.2 D, 1.4 C) (Clark et al., 2003; Zervas et al., 2001).

Mice with targeted gene disruptions in *PINCH* or *ILK* are both embryonic lethal at the peri-implantation stage, and embryoid bodies derived from *PINCH* and *ILK* null embryos both display abnormal epiblast polarity, impaired cavitation, and detachment of the endoderm and epiblast from the basement membrane (Li et al., 2005; Sakai et al., 2003). In order to study PINCH and ILK later in development, mice with conditional

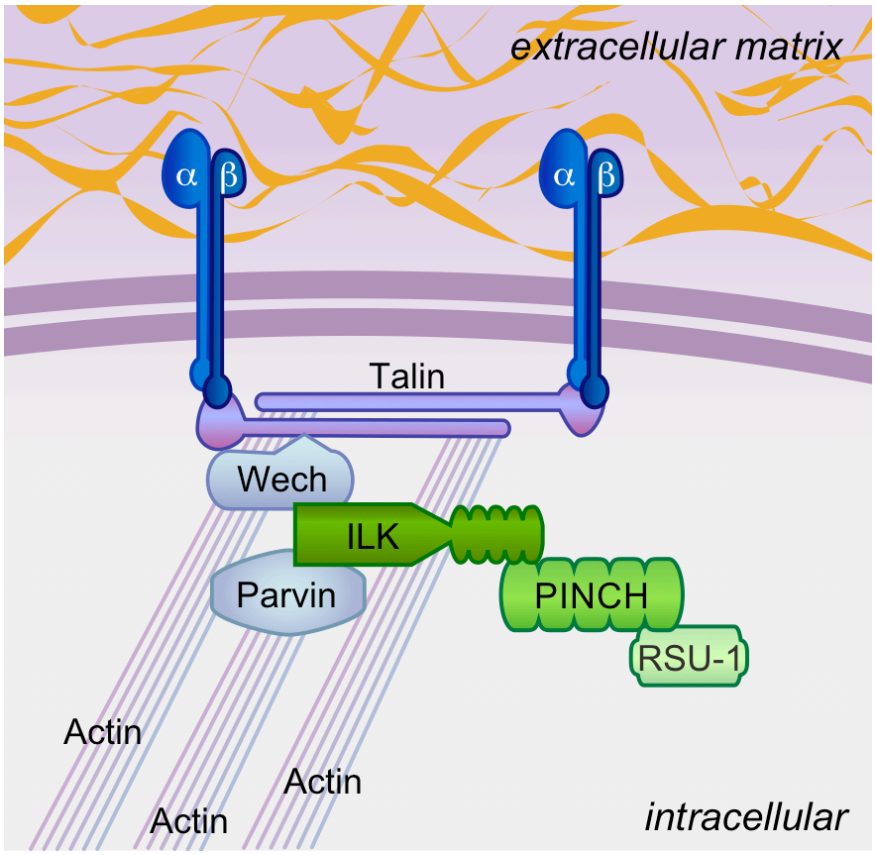


Figure 1.3: The PINCH-ILK-RSU-1 complex. The 5 LIM domain scaffold PINCH directly interacts with ILK via LIM1 and with RSU-1 via LIM5. Other essential components of integrin adhesion complexes, such as Talin, Wech, and Parvin, are shown to illustrate connections from the integrin receptors and to the actin cytoskeleton.

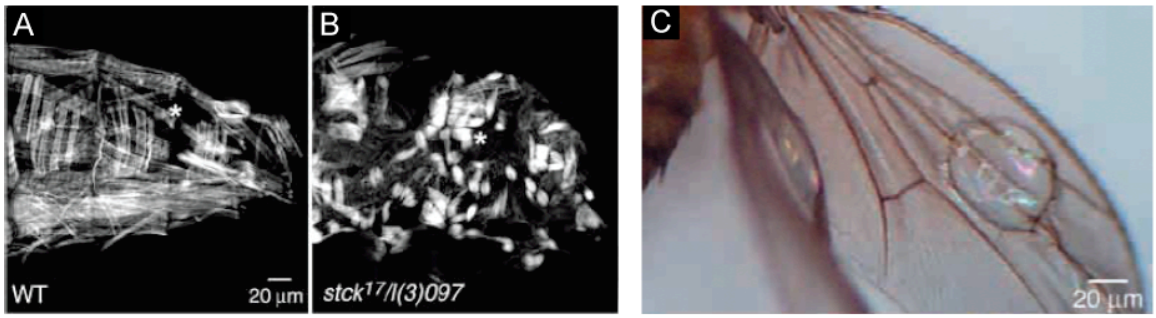


Figure 1.4: PINCH null phenotypes. (A,B) *stck* null mutants display late embryonic/early larval lethality due to detachment of the actin cytoskeleton from the muscle cell membrane. Wild-type and *stck* null embryos were stained with phalloidin to mark the actin cytoskeleton. (C) *stck* mutant clones in the wing result in wing blisters. The same phenotypes are observed in *ilk* mutant embryos and wing clones (Clark et al., 2003). Adapted with permission from Development | doi: 10.1242/dev.00492.

targeted gene disruptions have been generated. Loss of PINCH or ILK specifically in skeletal or cardiac muscle does not affect development, but animals do not survive due to defects in muscle function (Gheyara et al., 2007; Liang et al., 2009; Wang et al., 2008). PINCH and ILK have also been studied extensively in cell culture. Fibroblasts derived from mice harboring targeted gene disruptions in either *PINCH* or *ILK* display reduced cell spreading indicating a defect in integrin-mediated adhesion (Sakai et al., 2003; Stanchi et al., 2005). In HeLa cells, siRNA induced knockdown of PINCH or ILK causes defective cell spreading and reduced migration in Transwell motility chambers (Fukuda et al., 2003).

PINCH and ILK are highly conserved among species. Studies in *C. elegans* and zebrafish have complemented work in mouse, human and *Drosophila*, contributing to our understanding of these two proteins and their roles in integrin function. Two different *ILK* mutations have been described in zebrafish. *main squeeze* mutants display defects in cardiac muscle function and *lost contact* mutants display defects in skeletal muscle stability (Bendig et al., 2006; Postel et al., 2008). In *C. elegans unc-97* (PINCH) and *pat-4* (ILK) mutations have been described, each causing lethality due to defects in assembly of adhesion complexes in the muscle (Hobert et al., 1999; Mackinnon et al., 2002; Norman et al., 2007). Interestingly, both yeast-two hybrid screens and genetic screens to identify genes involved in muscle function, have identified novel PINCH binding partners, that have yet to be described in other organisms (unc-98, unc-95, LIM-8, and LIM-9) (Mercer et al., 2003; Qadota et al., 2007). Together, PINCH and the described associated protein complex in worms have been shown make a link to myosin thick filaments (Qadota and Benian, 2010).

PINCH and ILK expression have also been linked to more aggressive tumor phenotypes or poor prognosis in many different cancers (Cabodi et al., 2010). PINCH expression is upregulated in the stromal cells surrounding tumors and at the invasive margin in many common cancers (Wang-Rodriguez et al., 2002), and PINCH staining has been described as a prognostic indicator in both colorectal and pancreatic cancers (Gao et al., 2004; Scaife et al., 2010). Cell culture studies support the idea that PINCH and ILK functions could contribute to cancer phenotypes. RNAi studies in HeLa cells demonstrate that loss of PINCH or ILK causes reduced motility, reduced cell spreading, reduction of survival signaling, and increased apoptosis (Fukuda et al., 2003). These studies together suggest that altered PINCH and ILK expression may contribute to tumor progression. Understanding the regulation of PINCH and ILK with regard to integrin function will aid in understanding processes that are misregulated in cancer.

RSU-1 is a PINCH binding partner

Ras Suppressor-1 (RSU-1) is a recently identified binding partner for LIM5 in both *Drosophila* and mammalian PINCH (Dougherty et al., 2005; Kadrmas et al., 2004). RSU-1 was initially identified in a screen for proteins that could suppress the Ras transformed phenotype of cultured cell lines (Cutler et al., 1992). RSU-1 contains a 7 leucine rich repeat (LRR) sequence, which mediates the interaction with LIM5 of PINCH (Fig 1.3) (Dougherty et al., 2005). To date, no other binding partners for RSU-1 have been identified. In cell culture, RNAi-mediated knock down of RSU-1 reduces cell adhesion (Dougherty et al., 2005). Null mutants in the *Drosophila* homologue of RSU-1, *icarus* (*ics*), are viable but display wing blisters, indicating a loss of integrin-mediated adhesion (Fig. 1.2 D) (Kadrmas et al., 2004). RSU-1 colocalizes with PINCH at muscle

attachment sites in the *Drosophila* embryo and at focal adhesions in cell culture, but it is unknown how RSU-1 functions to maintain adhesion at these sites (Dougherty et al., 2005; Kadrmas et al., 2004).

RSU-1 negatively regulates the Jun N-terminal kinase (JNK) pathway in both cell culture and in *Drosophila* embryos (Dougherty et al., 2005; Kadrmas et al., 2004). PC12 cells display inherent Jun kinase activity that is inhibited by overexpression of RSU-1 (Masuelli and Cutler, 1996). Conversely, RNAi-mediated knock down of RSU-1 in 293T cells results in increased levels of phospho-JNK, the active form of the kinase (Dougherty et al., 2005). In *Drosophila*, *ics* mutations or introduction of one mutant copy of *stck* can partially suppress the dorsal closure and thoracic closure defects observed in JNK pathway mutants, demonstrating that both PINCH and RSU-1 are negative regulators of the JNK pathway (Kadrmas et al., 2004). Dorsal closure is also disturbed in integrin mutants indicating a possible cross talk between integrin and JNK signaling that could be mediated by PINCH and RSU-1. It remains to be determined if PINCH and RSU-1 are able to regulate the JNK cascade individually or if this regulation is dependent on their physical interaction. It is also unknown at what level of the signaling cascade this regulation is carried out, as experiments in *Drosophila* have only established a genetic interaction between PINCH, RSU-1 and members of the JNK cascade.

Domain analysis of PINCH

PINCH, ILK, and RSU-1 form a complex with reported roles in integrin-mediated adhesion. However, the precise mechanism by which they regulate the actin cytoskeleton has not been fully elucidated. PINCH is the central protein in this complex making contacts with ILK via LIM1 and with RSU-1 via LIM5 (Fig. 1.3). Due to the modular

nature of the LIM domain, it is feasible to alter individual LIM domains of PINCH while preserving the overall structure of the protein (Fig. 1.5). Domain analyses of PINCH and ILK have been performed in cell culture, *C. elegans*, and in *Drosophila* (Norman et al., 2007; Stanchi et al., 2005; Xu et al., 2005; Zervas et al., 2011; Zhang et al., 2002a; Zhang et al., 2002b). Much work has addressed the interaction between LIM1 of PINCH and the N-terminal ANKR domain of ILK, mostly using LIM1 deletion constructs. A point mutation in LIM1 of human PINCH that disrupts the interaction with ILK has also been characterized in cell culture (Velyvis et al., 2001; Xu et al., 2005). We have used this conserved residue to generate PINCH transgenic flies with disrupted ILK binding ($PINCH^{Q38A}$) to determine if the interaction between PINCH and ILK is required for any or all of their functions *in vivo* (Chapter 2). We also generated PINCH transgenic flies with a deletion in LIM1 ($PINCH^{\Delta LIM1}$) to determine if there are other functions for LIM1 other than binding to ILK (Chapter 3). Finally, little is known about RSU-1 and the functional significance of its interaction with LIM5 of PINCH. We identified and characterized a mutation in PINCH required to maintain the interaction with RSU-1 and generated PINCH transgenic flies carrying this mutation ($PINCH^{D303V}$). Due to the subtle phenotype of the *ics* (RSU-1) null flies, we also generated PINCH transgenic flies with a deletion of LIM5 ($PINCH^{\Delta LIM5}$) in order to determine if there are other roles for LIM5 other than binding to RSU-1 (Chapter 4).

Analysis of PINCH and its partners can be achieved with a high degree of precision in *Drosophila*. Using this model system, we have the ability to introduce tagged transgenes into a *stck* null background, which allows us to assess the properties of the mutant transgene in the absence of any endogenous wild-type PINCH. Furthermore, we

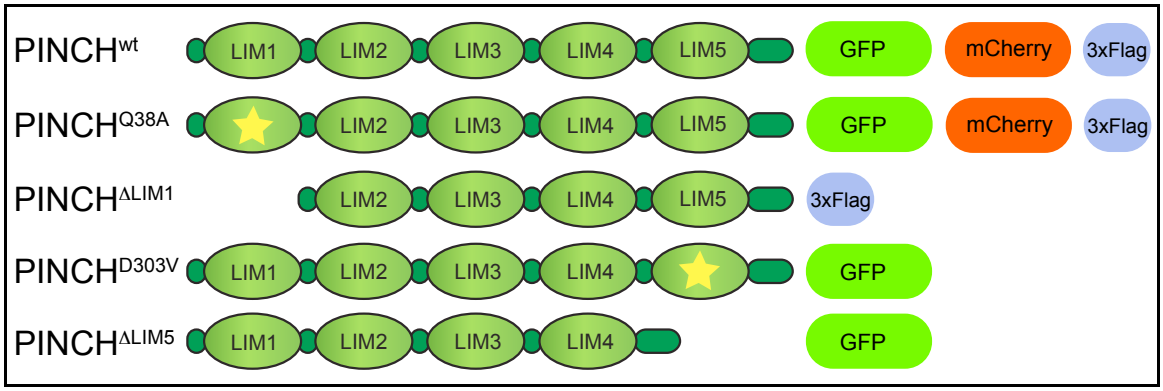


Figure 1.5: Domain analysis of PINCH. Diagram of PINCH transgenes introduced into *Drosophila* by p-element transposition include $PINCH^{wt}$, $PINCH^{Q38A}$, $PINCH^{\Delta LIM1}$, $PINCH^{D303V}$ and $PINCH^{\Delta LIM5}$. GFP, mCherry, and 3xFlag tags were used with some or all constructs. The genotype of all transgenes is *w*; P[w^+, PINCH*-tag], where * denotes the wild type or a specific mutation and the tag is either GFP, mCherry, or 3xFlag. All transgenic lines were generated using a genomic DNA fragment containing the *stck* gene under control of the endogenous *stck* promoter.

are able to determine the roles of these PINCH mutant transgenes *in vivo*, in whole tissues, and during different stages of development. This approach will allow us to assess function at stages where we know PINCH, ILK and RSU-1 function are important, and will also allow us to determine if there are other tissues where the functions of these proteins are required. Overall, the goal of this dissertation is to further understand how the PINCH-ILK-RSU-1 complex influences integrin function, and to determine how the individual interactions of PINCH with ILK and RSU-1 (and individual LIM domains of PINCH) contribute to integrin dependent adhesion.

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CHAPTER 2

DISRUPTION OF THE PINCH-ILK INTERACTION DOES NOT AFFECT INTEGRIN FUNCTION IN *DROSOPHILA*, BUT CAUSES LETHALITY IN COMBINATION WITH LOSS OF RSU-1

Abstract

Integrin-mediated adhesion is required for developmental processes and for the maintenance of differentiated tissues. Cellular adhesion is regulated by the many signaling and adaptor proteins that assemble at the cell cortex in response to integrin activation. Two such adaptor proteins that are required for maintaining actin-membrane linkages in *Drosophila* are PINCH and ILK. To investigate whether the interaction between PINCH and ILK is required for any or all of their functions *in vivo*, transgenic flies were generated carrying a variant of PINCH with a mutation in LIM1 that disrupts binding with ILK ($PINCH^{Q38A}$). We demonstrate that the $PINCH^{Q38A}$ transgene is able to rescue the lethality of the *stck* (PINCH) null mutant, that rescued flies are viable and fertile, and that normal distribution of β-integrin and stable actin linkages are observed in the embryonic body wall muscles. $PINCH^{Q38A}$ localizes appropriately at muscle attachment sites with the LIM5 binding partner, RSU-1, indicating that the PINCH-ILK interaction is not required for PINCH localization. ILK-GFP localizes appropriately in the absence of the PINCH-ILK interaction, in agreement with previous data demonstrating proper ILK localization in *stck* null animals. Western blot analysis of rescued embryos demonstrates that disruption of the PINCH-ILK interaction does not cause changes in the stability of PINCH or ILK, as observed in protein null embryos. Moreover, changes in PINCH and ILK protein stability were observed in viable *ics* (RSU-1) null mutants suggesting that RSU-1 may be playing a role in adhesion complex stability and function. Loss of RSU-1 in $PINCH^{Q38A}$ rescued flies causes a high degree of lethality during the larval stages and in pharate adults, indicating that in the absence of an interaction with ILK, RSU-1 plays an important role in maintaining viability. However,

this reduction in viable rescued adults is not due to a reduction of PINCH^{Q38A} protein in larvae. Our results demonstrate an importance for ILK-PINCH-RSU-1 complexes during *Drosophila* development and suggest that adhesion complexes possess redundant interactions to preserve critical functions downstream of integrins.

Introduction

Integrin-mediated adhesion is essential for proper development and for the maintenance of adult tissues (Bokel and Brown, 2002; Brower, 2003; Brown et al., 2002). Integrin receptors regulate cellular adhesion by mediating communication between the extracellular matrix (ECM) and the cell interior (Hynes, 2002). The association of the intracellular domains of integrins with cytoplasmic protein partners influences signal transduction cascades and the actin cytoskeleton to control cell adhesion (Brakebusch and Fassler, 2003; Delon and Brown, 2007). Understanding the functions and contributions of the protein complexes that link integrins to the actin cytoskeleton is fundamental to understanding how cellular adhesion is regulated.

In *Drosophila*, loss of integrin function results in perturbation of cell adhesion *in vitro* and *in vivo* (Jani and Schock, 2007; Pines et al., 2011). Moreover, many of the cytoplasmic factors that support integrin-mediated adhesion have been identified or characterized using various genetic methods in *Drosophila* (Loer et al., 2008; Prout et al., 1997; Walsh and Brown, 1998; Zervas et al., 2001). Two key genes identified in these studies are *steamerduck* (*stck*), which encodes PINCH (particularly interesting cysteine-histidine-rich protein) and *ilk*, which encodes integrin-linked kinase (ILK). Null mutations in both *stck* and *ilk* result in late embryonic or early larval lethality, and mutant clones of either gene in the wing result in wing blisters similar to what is observed when

integrin function is compromised (Brabant et al., 1996; Brower and Jaffe, 1989; Clark et al., 2003; Zervas et al., 2001). PINCH and ILK colocalize at muscle attachment sites in the embryo, suggesting that they function together in cell types where integrin-mediated adhesion is required (Clark et al., 2003). Consistent with this idea, both proteins are required to maintain the attachment of actin filaments to specialized regions of the plasma membrane where integrins are concentrated (Clark et al., 2003; Zervas et al., 2001). This type of linkage differs from what is observed in another class of integrin effectors, including *rhea* (Talin) and *wech,* which are required for anchorage of the cell membrane to the extracellular matrix (Brown et al., 2002; Loer et al., 2008).

Data in mammalian systems reinforce the idea that PINCH-ILK complexes operate as a functional unit. The N-terminal LIM domain of PINCH interacts directly with the N-terminal ankyrin repeat domain (ANKR) of ILK, and these proteins are recovered as a complex by immunoprecipitation along with Parvin, which binds both ILK and Actin (Li et al., 1999; Tu et al., 2001; Tu et al., 1999). ILK, PINCH and Parvin form a functional complex (IPP complex) that has been well studied in cell culture with regard to integrin function. It is thought that the binding of the IPP proteins precedes and is necessary to facilitate their localization and function at the cell membrane (Zhang et al., 2002). Depletion of either ILK or PINCH in cultured cells results in reduction in the levels of the other, indicating a mutual stabilization of these two proteins (Fukuda et al., 2003; Stanchi et al., 2009). Studies in cell culture have begun to address the contributions of the PINCH-ILK interaction using a version of PINCH with a point mutation in LIM1 that disrupts binding to ILK ($PINCH^{Q40A}$) (Velyvis et al., 2001; Xu et al., 2005; Zhang et al., 2002). The interaction between PINCH and ILK is required to maintain proper cell

spreading and survival, as well as normal protein levels of both PINCH and ILK (Xu et al., 2005; Zhang et al., 2002). Further evidence for a functional PINCH-ILK complex comes from targeted gene disruptions in mice. Targeted gene disruptions in *PINCH* or *ILK* result in embryonic lethal at the peri-implantation stage and embryoid bodies derived from *PINCH* and *ILK* null embryos display abnormal epiblast polarity, impaired cavitation, and detachment of the endoderm and epiblast from the basement membrane (Li et al., 2005; Sakai et al., 2003).

Although the data in *Drosophila* and mammalian systems largely support the idea that PINCH-ILK complexes function to maintain adhesion, protein localization, and protein levels, it has been difficult to tease apart their roles, particularly since elimination of one protein has an impact on the stability of the other partner. Nevertheless, there are also some findings that suggest divergent roles for PINCH and ILK. First, while the phenotypes from mice with targeted gene disruptions in *PINCH* or *ILK* are similar, they are not identical. The *PINCH* null mouse survives slightly longer (E6.5-E7.5) than the *ILK* null mouse (E5.5-E6.5). Furthermore, *PINCH* null embryoid bodies display additional defects in cell-cell adhesion of the endoderm and the epiblast and contain apoptotic cells within the endodermal layer that are not seen in embryoid bodies derived from *ILK* null embryos (Li et al., 2005; Sakai et al., 2003). These differences suggest that the knockout phenotypes do not arise from equivalent loss of PINCH-ILK complexes in either mouse. In *Drosophila*, *wech* mutants, which display defects in membrane-ECM adhesion similar to *mys* mutants, show reduced levels of ILK at muscle attachment sites whereas levels of PINCH remain similar to wild type, suggesting that the regulation of PINCH and ILK can be uncoupled at sites of adhesion (Loer et al., 2008). Results from a

recent study in which a domain analysis of ILK was performed in *Drosophila* demonstrate that ILK function is perturbed even when the ANKR domain that binds to PINCH is expressed in an *ilk* null background, indicating that ILK has functions that are not solely dependent on PINCH binding (Zervas et al., 2011). This study also demonstrated the ability of $PINCH^{\Delta LIM1}$ to partially localize to muscle attachment sites independently of ILK, providing further evidence of PINCH function that does not absolutely require ILK-binding. These data together indicate that while PINCH and ILK have largely overlapping functions, and they display mutually dependent protein stability, they also appear to make distinct contributions to integrin-mediated function.

Despite the work by many labs demonstrating that PINCH and ILK are required for proper cell adhesion, the exact mechanism for how they reinforce actin-membrane linkage remains unclear. The current data suggest a model in which PINCH, ILK, and their direct binding partners regulate integrin-mediated function, and that the direct interaction between PINCH and ILK contributes to some or all of these functions. To test this model, we have utilized a described point mutation in PINCH that disrupts the interaction with ILK while otherwise preserving all aspects of PINCH structure and function ($PINCH^{Q38A}$) (Velyvis et al., 2001). We have generated flies carrying either $PINCH^{wt}$ or $PINCH^{Q38A}$ transgenes in order to determine whether the interaction between PINCH and ILK is required for viability, muscle structure, wing adhesion, protein localization, and protein stability. We extended our analysis to include the PINCH LIM5 binding partner RSU-1 (Ras Suppressor-1), which is encoded by the *icarus* (*ics*) locus in *Drosophila*. *ics* null flies are viable but display defects in wing adhesion, suggesting a role for PINCH-ILK-RSU-1 complexes in integrin function (Kadrmas et al., 2004).

This is the first study examining the PINCH-ILK interaction *in vivo* specifically using a point mutation rather than deletion of protein domains, allowing us to probe aspects of integrin-mediated function within the context of an intact organism and with minimal disruption of other complex members. Surprisingly, our results demonstrate that the interaction between ILK and PINCH is not required for proper muscle structure, wing adhesion or for maintaining proper localization and levels of PINCH and ILK. By comparing a point mutant to null alleles, we demonstrate that the ability of PINCH and ILK to stabilize protein levels of the other depends on the absolute presence of the proteins rather than simply the interaction between them. The null embryo analysis included additional adhesion complex components such as RSU-1. We find that not only are PINCH and ILK levels sensitive to loss of each other, but loss of RSU-1 can affect the levels of both PINCH and ILK. Furthermore, the levels of RSU-1 are reduced in both *stck* and *ilk* null embryos. These data indicate that other proteins within integrin associated complexes, such as RSU-1, may be contributing to integrin function together with PINCH and ILK. Finally, we find that the PINCH^{Q38A} rescued flies are sensitive to loss of RSU-1. This demonstrates a defect caused by disrupting the PINCH-ILK interaction, and suggests a functional importance for the PINCH-ILK-RSU complex. Our work demonstrates that networks of protein-protein interactions localize and stabilize components of integrin adhesion complexes with a redundancy reflecting the vital importance of integrin dependent processes.

Materials and methods

Fly stocks

w^{1118} were used as wild-type controls in all experiments. *ilk*^{1} and *ics*^{BG02577} stocks were obtained from the Bloomington Stock Center. *FRT82B stck*^{17}, *FRT82B stck*^{18}, and ILK-GFP flies were previously described (Clark et al., 2003; Zervas et al., 2001). *stck*^{17} germ line clone females were generated as previously described and were crossed to *stck*^{18}/TM3, Twist-GFP to generate *stck*$^{17/18}$ maternal/zygotic (m/z) null embryos (Clark et al., 2003).

Sequence alignment and accession numbers

Sequence alignment was performed with ClustalW using the following PINCH protein sequences (NCBI accession numbers): human PINCH1 (AAH05341), human PINCH2 (AAH65816), mouse PINCH1 (AAH05621), mouse PINCH2 (NP_659111), zebrafish PINCH (NP_001019560), *Drosophila* PINCH isoform A (NP_524278), *C.elegans* unc-97 (NP_508943).

Generation of PINCH Q38A reagents

A glutamine to alanine mutation at position 38 in the dPINCHa cDNA was introduced by PCR mutagenesis into a previously described pMT-PINCH^{wt}-His construct (Kadrmas et al., 2004). pMT-PINCH^{wt} and pMT-PINCH^{Q38A} were transfected into S2 cells using standard methods. Transgenic flies carrying the Q38A mutation were generated by p-element transformation using a previously described pCasper construct containing genomic PINCH^{wt}-GFP (Clark et al., 2003; Kadrmas et al., 2004). A C-

terminal 3xFlag tag replaced the GFP in both PINCH^{wt} and PINCH^{Q38A} constructs. DNA constructs were injected into embryos by Genetic Services Inc. (Cambridge, MA).

PINCH^{wt} and PINCH^{Q38A} rescue experiments

To test for rescue, crosses were set to introduce one or two copies of the transgene and two copies of different null *stck* alleles into the progeny. For example: PINCH^{wt}-3xFlag; *stck*^{17}/TM3 x PINCH^{wt}-3xFlag; *stck*^{18}/TM3. It is expected that full rescue would produce a ratio of 1/3 rescued adult progeny to 2/3 adult balanced progeny. Rescue was calculated as the observed rescued flies over 1/2 of the observed balanced progeny as predicted by the cross. To assess rescued progeny during development, late stage embryos were collected at two hour intervals from established rescued stocks or from crosses that were set using TM3, Twist-GFP balancers and were sorted for lack of GFP. Embryos were aged on grape juice agar plates at 25° C and followed through development, or were collected 48 hours after sorting for larval analysis. Graphpad Prism was used for all graphical analyses.

Pull downs, immunoprecipitations and Western blots

S2 cells lysates were prepared and His-tagged proteins were purified using a Ni-NTA column followed by Western blots. Adult fly lysates for IP were prepared in a dounce homogenizer with lysis buffer (Tris pH 7.9, 0.1% Triton-X 100) and protease inhibitors, and were incubated with Flag (M2) beads (Sigma), rinsed and boiled in 2x Laemmli sample buffer followed by Western blots. For other Western blots, equal numbers of adult flies or staged and sorted embryos or larvae were homogenized in 2x Laemmli sample buffer. Protein samples were run on SDS-PAGE gels and transferred to

nitrocellulose. Antibodies used were: anti-PINCH (1:5000) (Clark et al., 2003), anti-RSU-1 (1:5000) (Kadrmas et al., 2004), anti-ILK (1:500, BD #611802), anti-Lamin (1:5000, DSHB), anti-β integrin (DX.468.1, 1:1, DSHB)

Immunofluorescence and imaging

Staged embryos were fixed in 4% PFA or heat fixed according to previously published protocols (Clark et al., 2003). To preserve GFP or for staining with phalloidin, embryos were devitellinized in 80% ethanol. Antibodies used were anti-Flag (M2) preabsorbed against w^{1118} embryos (1:2000, Sigma), anti-RSU-1 preabsorbed against w^{1118} embryos (1:1000), anti-β-integrin (CF.6G11, 1:5, DSHB), anti-GFP (1:1000, Clontech), phalloidin 568 or 647 (1:100, Invitrogen). Secondary antibodies were Alexafluor anti-rabbit or anti-mouse 488 or 568 (1:250, Invitrogen). All images were obtained on an Olympus Fluoview 300 using a 20x or 60x objectives (University of Utah School of Medicine Cell Imaging Facility), and figures were assembled using Adobe CS4 programs.

Results

A conserved glutamine residue in LIM1 of PINCH is required for ILK binding

PINCH-ILK complexes are essential for integrin dependent cell adhesion of cultured cells, however this has not been demonstrated definitively within an intact organism. To test whether the PINCH-ILK interaction is required for integrin-mediated cell adhesion *in vivo*, we established a system for specific disruption of PINCH-ILK binding in *Drosophila*. The PINCH protein is composed entirely of LIM domains, with a

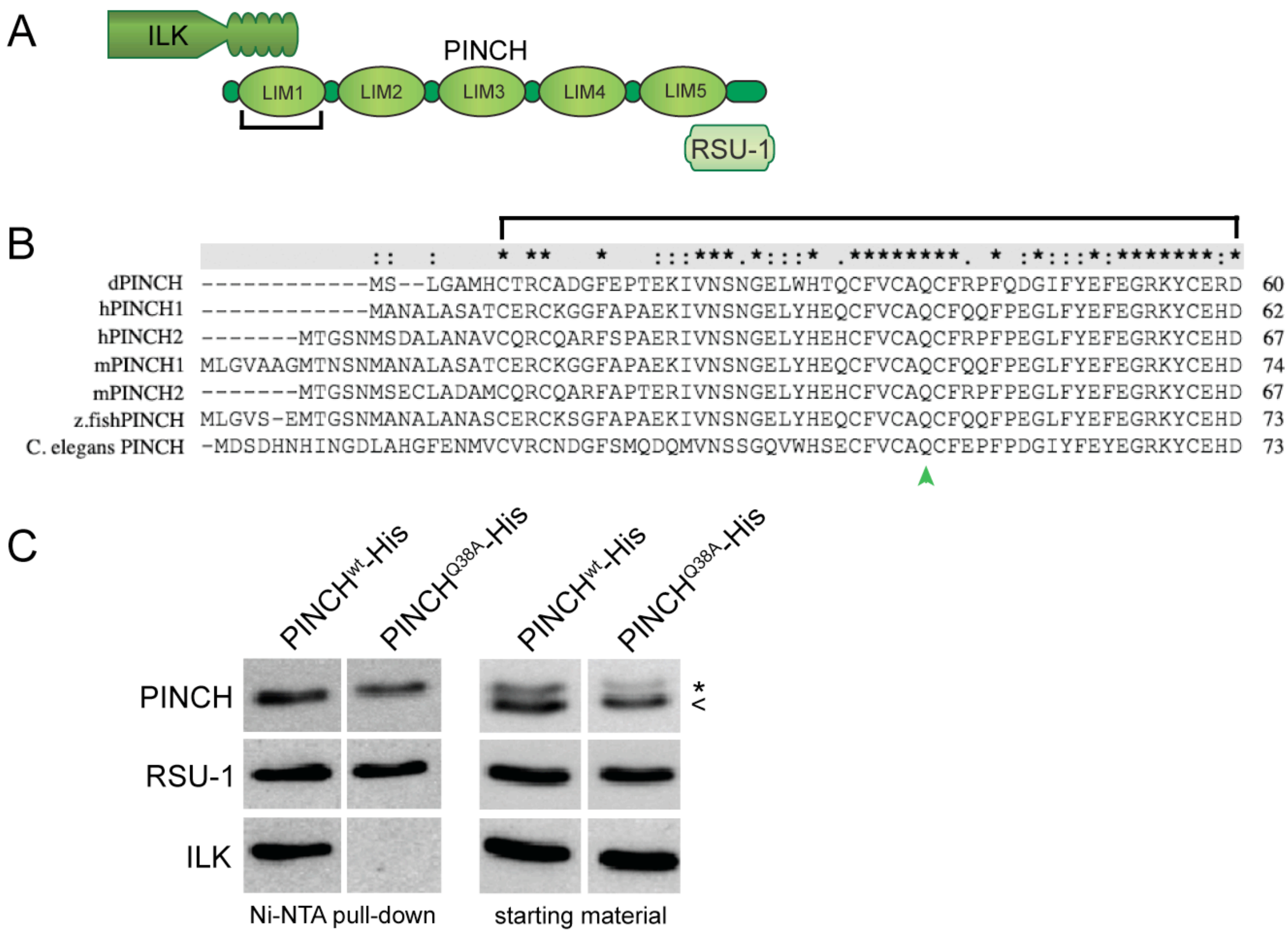
short N-terminus and a C-terminal tail (Fig. 2.1 A). The ILK N-terminal ANKR domain interacts with LIM1 of PINCH, and RSU-1 interacts with LIM5 of PINCH. Sequence alignment of PINCH proteins from *Drosophila*, human, mouse, zebrafish, and *C. elegans* reveals the high degree of sequence conservation of the N-terminal LIM domain of PINCH (LIM1) (Fig. 2.1 B). In mammalian cells, a variant of PINCH in which a universally conserved amino acid (glutamine 40) is mutated to alanine (Q40A) does not bind to ILK, fails to localize at focal adhesions, and causes defects in cell spreading and adhesion (Xu et al., 2005; Zhang et al., 2002). We engineered and expressed *Drosophila* PINCH^{wt}-His or PINCH^{Q38A}-His (the fly homologue of PINCH^{Q40A}) in *Drosophila* S2 cells, captured His-tagged proteins with their partners under native conditions using a Ni-NTA column, and blotted for known binding partners. PINCH^{wt}-His was associated with the LIM5 partner RSU-1 and the LIM1 partner ILK. In contrast, PINCH^{Q38A}-His was associated with RSU-1, but not ILK (Fig. 2.1 C). The data demonstrating that PINCH^{Q38A} retains the capacity to interact with RSU-1 provide evidence for conformational integrity of the protein and illustrates a critical role of glutamine 38 for specifying a docking site for ILK.

The interaction between PINCH and ILK is not

required for viability in *Drosophila*

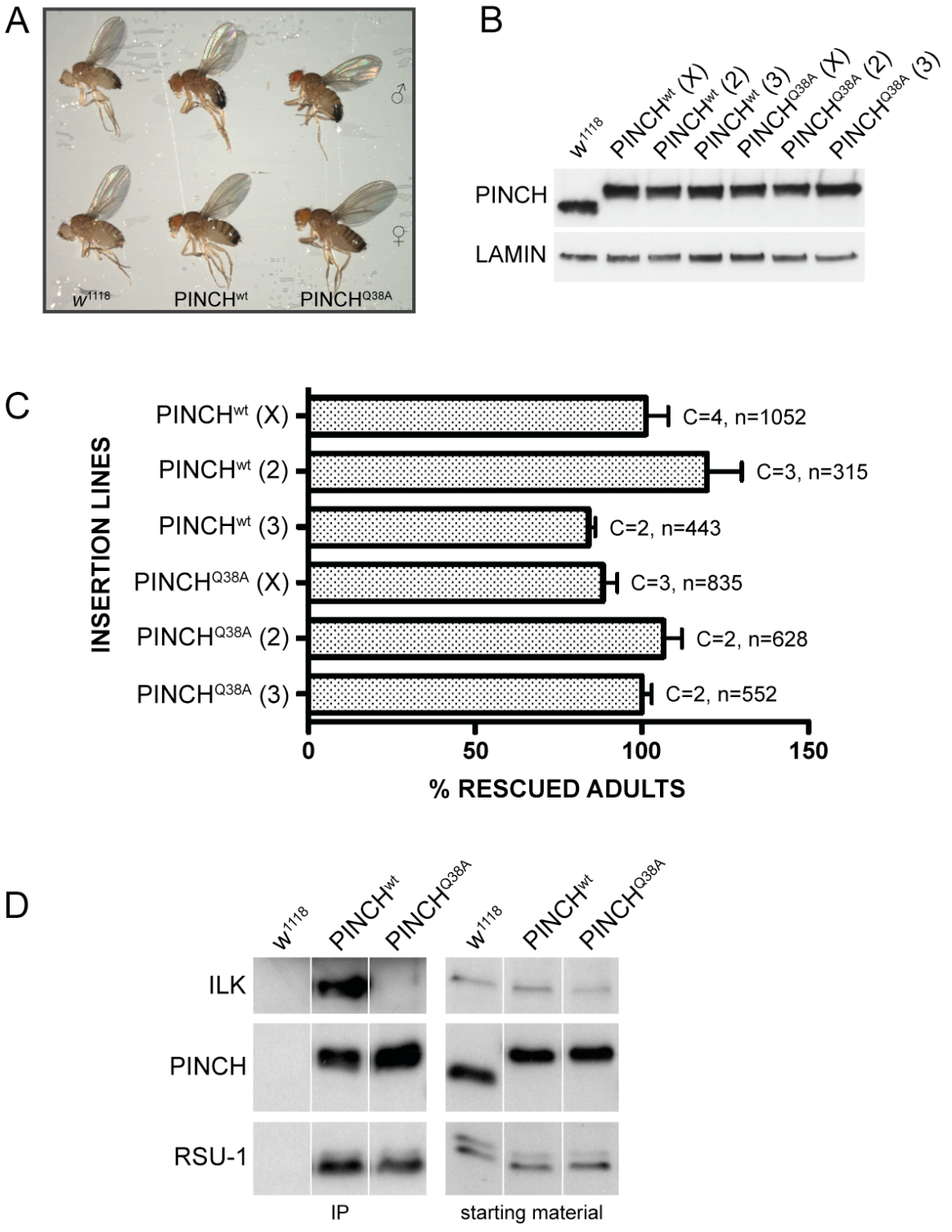
In order to evaluate the functional consequences of disrupting the PINCH-ILK interaction *in vivo*, we generated transgenic flies carrying either PINCH^{wt}-3xFlag or PINCH^{Q38A}-3xFlag (Clark et al., 2003). No dominant phenotypes were observed when either PINCH^{wt} or PINCH^{Q38A} was expressed in otherwise wild-type flies (data not shown). To determine whether PINCH that lacks ILK-binding capacity would rescue the

Figure 2.1. *Drosophila* PINCH^{Q38A} is a mutation that disrupts the interaction with ILK. (A) A PINCH schematic demonstrating the 5LIM domain structure with a short N-terminus and a C-terminal tail. LIM1 binds to the N-terminal ANKR domain of ILK, and LIM5 binds to RSU-1. (B) Sequence alignment of the N-terminus through LIM1 of various PINCH1 and PINCH2 sequences. The conserved glutamine is marked by a green arrowhead. "*" indicates positions which have a single, fully conserved residue, while ":" and "." indicate strong and weaker conservation defined by ClustalX. (C) Ni-NTA pull-down of PINCH^{wt}-His and PINCH^{Q38A}-His expressed in S2 cells. Western blots of purified proteins were probed with PINCH, RSU-1 and ILK antibodies. PINCH^{Q38A}-His does not show any detectible binding to ILK, but is still able to bind to RSU-1. Endogenous PINCH is noted with a <, PINCH-His species are noted with an *.


A
ILK
PINCH
LIM1
LIM2
LIM3
LIM4
LIM5
RSU-1
B
dPINCH ------------MS--LGAMHCTRCADGFEPTEKIVNSNGELWHTQCFVCAQCFRPFQDGIFYEFEGRKYCERD 60
hPINCH1 ------------MANALASATCERCKGGFAPAEKIVNSNGELYHEQCFVCAQCFQQFPEGLFYEFEGRKYCEHD 62
hPINCH2 -------MTGSNMSDALANAVCQRCQARFSPAERIVNSNGELYHEHCFVCAQCFRPFPEGLFYEFEGRKYCEHD 67
mPINCH1 MLGVAAGMTNSNMANALASATCERCKGGFAPAEKIVNSNGELYHEQCFVCAQCFQQFPEGLFYEFEGRKYCEHD 74
mPINCH2 -------MTGSNMSECLADAMCQRCQARFAPTERIVNSNGELYHEHCFVCAQCFRPFPEGLFYEFEGRKYCEHD 67
z.fishPINCH MLGVS-EMTGSNMANALANASCERCKSGFAPAEKIVNSNGELYHEQCFVCAQCFQQFPEGLFYEFEGRKYCEHD 73
C. elegans PINCH -MDSDHNHINGDLAHGFENMVCVRCNDGFSMQDQMVNSSGQVWHSECFVCAQCFEPFPDGIYFEYEGRKYCEHD 73
C
PINCH^{wt}-His
PINCH^{Q38A}-His
PINCH^{wt}-His
PINCH^{Q38A}-His
PINCH
RSU-1
ILK
*
<
Ni-NTA pull-down
starting material

late embryonic/early larval lethality of *stck* null mutants, we introduced either the PINCH^{wt} or PINCH^{Q38A} transgenes under control of the endogenous *stck* promoter into embryos that lack zygotic PINCH expression. The PINCH^{wt} transgene rescues the *stck* null mutant phenotypes, including both the embryonic lethality and adult wing blistering (Fig. 2.2 A). Surprisingly, expression of the PINCH^{Q38A} transgene also rescues the *stck* null mutant, indicating that the *in vivo* interaction between PINCH and ILK is not required for viability (Fig. 2.2 A). PINCH^{Q38A} rescued flies are fertile, and stocks were established and maintained for future experiments, indicating that it was not the maternal contribution of endogenous PINCH that allows for rescue. We confirmed rescue by Western blot in three independent insertion lines for both PINCH^{wt} and PINCH^{Q38A} and observed in each case the loss of endogenous PINCH expression and the presence of the Flag-tagged PINCH variant, which migrated at a higher molecular mass in adult rescued flies (Fig. 2.2 B). To evaluate whether there were subtle differences in developmental fitness between rescue with PINCH^{wt} and PINCH^{Q38A} transgenes, a quantitative analysis was performed to assess the numbers of viable progeny resulting from each rescue cross. The mean percent rescue was calculated for three different insertion lines of PINCH^{wt} and PINCH^{Q38A}, where each rescue cross was repeated two-four times (Fig. 2.2 C). We observe rescue between 85-120% for all lines tested, and consider this range to represent full rescue. The lack of interaction between PINCH^{Q38A} and ILK was confirmed by native immunoprecipitations from rescued transgenic fly lines (Fig. 2.2 D). Importantly, although PINCH^{Q38A} fails to recruit ILK *in vivo*, it does recruit the LIM5 binding partner, RSU-1, providing biochemical confirmation that PINCH protein structure is preserved.

Figure 2.2. PINCH^{wt}-3xFlag and PINCH^{Q38A}-3xFlag transgenes rescue the viability of the *stck* null mutant. (A) Adult rescued flies do not have wing blisters or any other overt phenotypes. PINCH^{wt} and PINCH^{Q38A} adult flies appear similar to each other, and to w^{1118} controls. (B) Western blots of adult rescued flies show a lack of endogenous PINCH and only show the higher migrating Flag-tagged species. Three different insertion lines are shown for both PINCH^{wt} and PINCH^{Q38A} on the X, 2nd, and 3rd chromosomes. (C) PINCH^{wt} transgenes rescue between 84-120% and PINCH^{Q38A} transgenes rescue between 88-107%. Bars represent the mean +/- S.E.M. of the % rescued flies counted over the number of crosses set. C denotes the number of times the rescue cross was set. n is the total number of flies counted across all crosses. (D) Flag IPs confirm disruption of the PINCH-ILK interaction in adult PINCH^{Q38A} rescued flies. PINCH^{wt} co-IPs with both PINCH and ILK, while PINCH^{Q38A} only co-IPs with RSU-1. *w*^{1118} lysate was used as a control to assess nonspecific binding. All proteins were present in the starting material.


A
♂
♀
w1118
PINCHwt
PINCHQ38A
B
w1118
PINCHwt (X)
PINCHwt (2)
PINCHwt (3)
PINCHQ38A (X)
PINCHQ38A (2)
PINCHQ38A (3)
PINCH
LAMIN
C
INSERTION LINES
PINCHwt (X)
PINCHwt (2)
PINCHwt (3)
PINCHQ38A (X)
PINCHQ38A (2)
PINCHQ38A (3)
C=4, n=1052
C=3, n=315
C=2, n=443
C=3, n=835
C=2, n=628
C=2, n=552
0
50
100
150
% RESCUED ADULTS
D
w1118
PINCHwt
PINCHQ38A
w1118
PINCHwt
PINCHQ38A
ILK
PINCH
RSU-1
IP
starting material

The PINCH-ILK interaction is not required for establishment

or maintenance of muscle cytoarchitecture

Because both PINCH and ILK have been shown to be required for muscle structure and function in *Drosophila* (Clark et al., 2003; Zervas et al., 2001), we performed high resolution fluorescence microscopy to evaluate the integrity of actin-membrane linkages in PINCH^{Q38A} rescued flies. The *Drosophila* embryonic somatic muscle consists of segments of muscle fibers, where two muscle segments meet specialized tendon cells that are part of the epidermis (Bokel and Brown, 2002; Brower, 2003; Schweitzer et al., 2010). The interaction of these two cell types is mediated by β-integrin dependent adhesion to ECM that is deposited between muscle and tendon cells. PINCH^{wt} and PINCH^{Q38A} rescued embryos were stained with β-integrin antibody to label adhesion sites at the muscle ends and with phalloidin to label the actin cytoskeleton (Fig. 2.3). We observe strong and coherent localization of β-integrin at the myotendinous junction (Fig. 2.3 A,C,D,F), with a normal distribution of actin fibers extending between these sites (Fig. 2.3 B,C,E,F) in both PINCH^{wt} and PINCH^{Q38A} rescued embryos, indicating that disruption of the PINCH-ILK interaction is not sufficient to alter muscle structure or stability of the actin cytoskeleton in the *Drosophila* embryo.

PINCH and ILK can localize independently

to muscle attachment sites

The normal actin-membrane interactions and adhesion observed in the muscle of PINCH^{Q38A} rescued embryos illustrates that the ability of PINCH to bind ILK is not essential for muscle integrity. Given the prior work in mammalian cells showing that disruption of the PINCH-ILK interaction compromises the ability of PINCH to

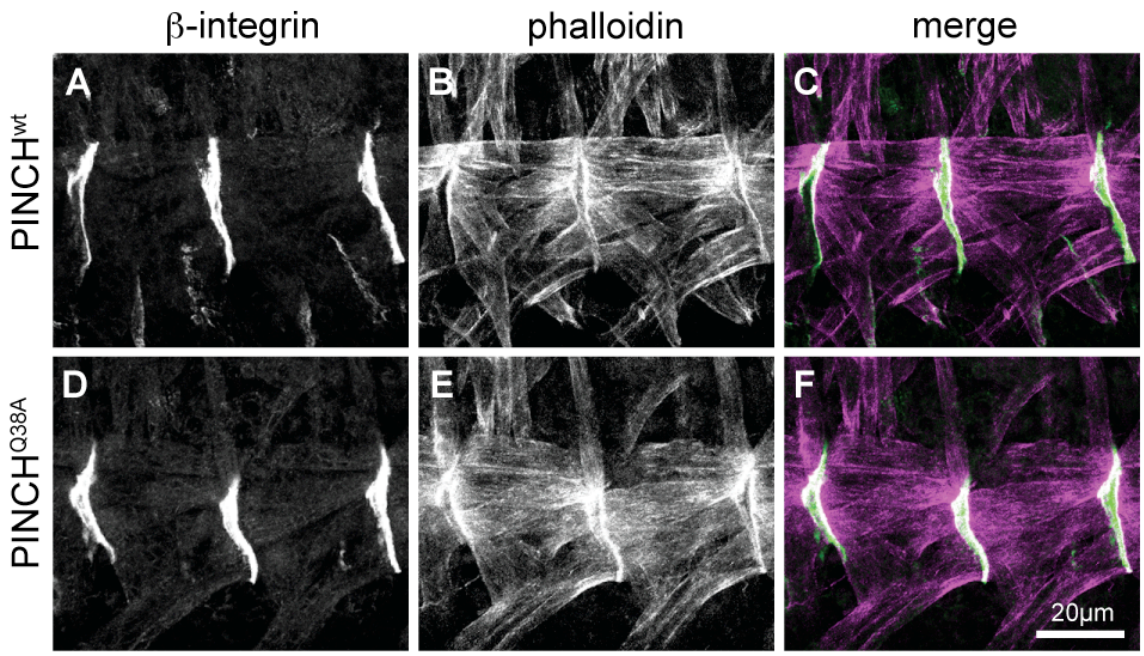


Figure 2.3. Muscle structure is preserved in PINCH^{Q38A} rescued embryos. Stage 16-17 embryos were stained with β-integrin antibody (A,C, D, F) and phalloidin (B,C,E,F) to mark the muscle attachment site (green) and the actin cytoskeleton (magenta). β-integrin localizes normally to the muscle cell membrane and phalloidin staining indicates that the actin cytoskeleton is stable and that the muscle fibers are properly distributed between muscle attachment sites in both PINCH^{wt} rescued embryos (A,B,C) and in PINCH^{Q38A} rescued embryos (D,E,F).

accumulate at integrin rich adhesion sites (Zhang et al., 2002), we tested whether PINCH^{Q38A} could rescue the *stck* null mutant even if it was not stably and appropriately localized at muscle attachment sites. Immunostaining of both PINCH^{wt} and PINCH^{Q38A} rescued embryos with the Flag (M2) antibody to label transgenic PINCH and myosin heavy chain (MHC) to label body wall muscle instead demonstrated that both PINCH^{wt} and PINCH^{Q38A} display robust localization at muscle termini in the *Drosophila* muscle (Fig. 2.4 A,B). At higher resolution, it is clear that both PINCH^{wt} and PINCH^{Q38A} colocalize with the LIM5 binding partner RSU-1 at muscle attachment sites (Fig. 2.4 C-D"). These findings illustrate that the PINCH-ILK interaction is not required for appropriate subcellular localization of PINCH or RSU-1 *in vivo*, and highlight the importance of identifying other factors that contribute to the targeting of PINCH to sites of adhesion.

Given that PINCH^{Q38A} is able to localize to muscle attachment sites independently of in interaction with ILK, we conducted a complementary experiment to evaluate whether proper ILK localization occurs in PINCH^{Q38A} rescued embryos. We introduced an ILK-GFP transgene into PINCH^{wt} and PINCH^{Q38A} rescued flies and observed that ILK-GFP does indeed accumulate at muscle attachment sites (Fig. 2.4 E,E",F,F"), colocalizing with both PINCH^{wt} and PINCH^{Q38A} (Fig. 2.4 E',E",F',F"). These results demonstrate that ILK localization is not dependent on direct binding to PINCH in *Drosophila* muscle. Furthermore, this result extends our earlier work and another recent report demonstrating appropriate ILK localization in *stck* maternal/zygotic (m/z) null embryos (Clark et al., 2003; Zervas et al., 2011).

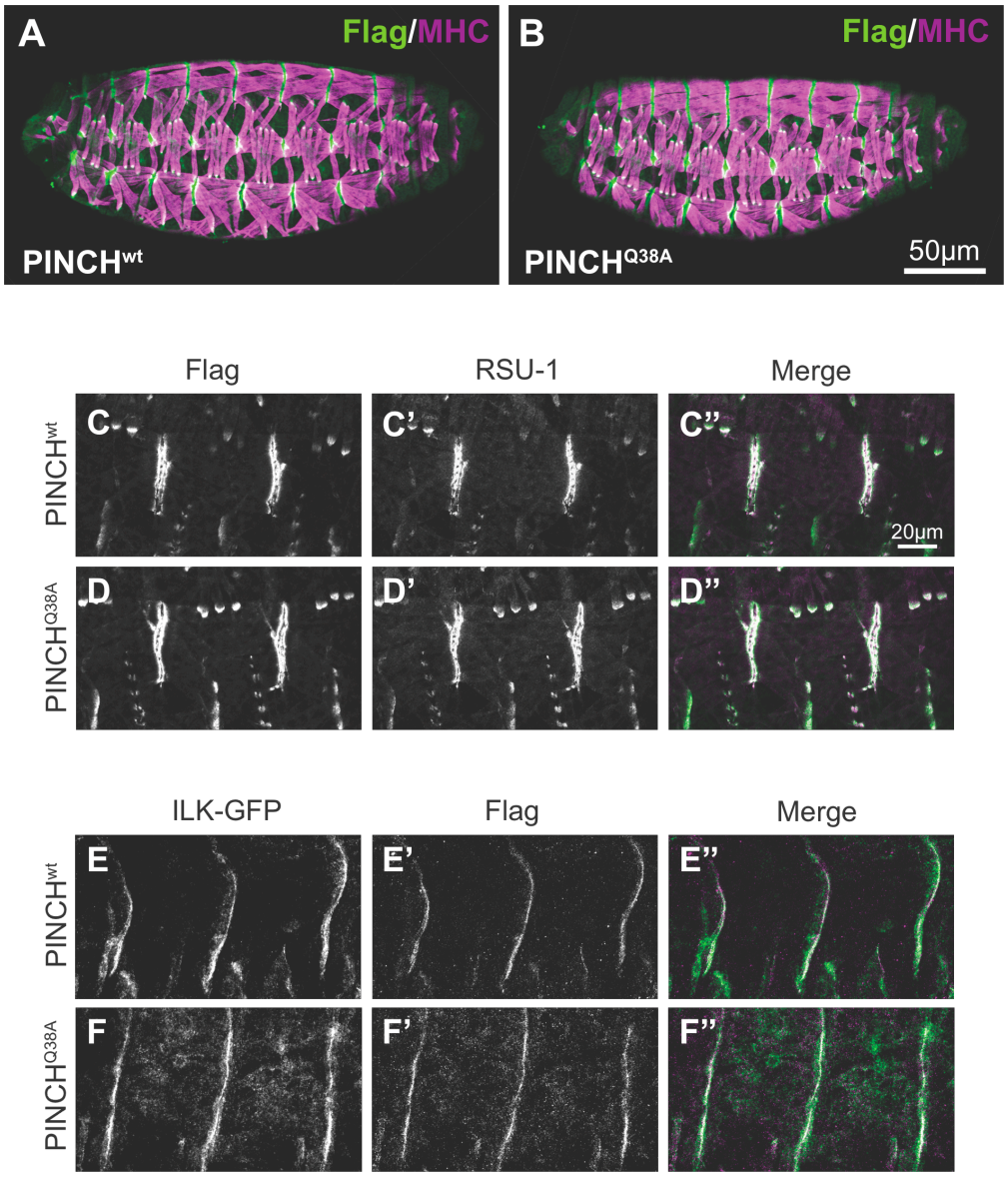


Figure 2.4. PINCH, RSU-1, and ILK are properly localized in PINCH^{Q38A} rescued embryos. (A,B) 20x images of stage 16-17 embryos stained with the Flag (M2) antibody to label PINCH transgenic protein (green) and myosin heavy chain to label the embryonic body wall muscle (magenta). PINCH^{wt} and PINCH^{Q38A} localization and muscle structure are normal throughout the embryo. (C-D") 60x images of stage 16-17 embryos stained with the Flag (M2) antibody (green) to label PINCH transgenic protein (C,C',D,D') and the RSU-1 antibody (magenta) (C',C",D',D") to show that they both localize in the absence of a PINCH-ILK interaction. Colocalization appears white in the merge (C",D"). (E-F") 60x images of stage 15-16 embryos stained with GFP antibody to label ILK-GFP (green) (E,E",F,F") and the Flag (M2) antibody to label PINCH transgenic protein (magenta) (E',E",F',F") show that PINCH and ILK colocalize in the absence of their direct interaction. Colocalization appears white in the merge. (E",F").

The PINCH-ILK interaction is not required to

stabilize PINCH or ILK protein levels

To determine if the PINCH-ILK interaction is required for maintaining protein stability, we performed Western blots with stage 16-17 $PINCH^{wt}$ and $PINCH^{Q38A}$ rescued embryos and observe that levels of PINCH and ILK are comparable (Fig. 2.5 A). This result indicates that loss of the interaction between PINCH and ILK is not sufficient to alter protein levels. The lack of effect on either $PINCH^{Q38A}$ or ILK protein stability is consistent with the viability observed in $PINCH^{Q38A}$ rescued flies. In cell culture, reductions in both ILK and PINCH protein levels are observed not only in $PINCH^{Q40A}$ expressing cells, but also in cells with reduced or absent ILK or PINCH (Fukuda et al., 2003; Stanchi et al., 2009; Xu et al., 2005). These changes in protein levels in knock down, null, and PINCH Q40A cells may contribute to the array of phenotypes observed in cell culture. Because we do not see changes in protein levels or overt phenotypic abnormalities in the $PINCH^{Q38A}$ embryos, we wanted to confirm the extent of this stabilization phenomenon in *stck* and *ilk* null embryos, which exhibit severe phenotypes resulting in lethality. Protein levels of adhesion complex components were assessed in stage 16-17 embryos. We observe that indeed PINCH protein levels are reduced in ilk^{1} mutant embryos and that ILK protein levels are reduced in $stck^{17/18}$ m/z mutant embryos (Fig. 2.5 B). This is in agreement with data from mammalian cell culture and demonstrates that the requirement for the presence of PINCH and ILK for their mutual stabilization is not species specific (Fukuda et al., 2003; Stanchi et al., 2009). We extended our analysis to look at protein levels of βPS-integrin as well as the PINCH binding partner RSU-1. The levels of βPS-integrin do not change compared to wild-type

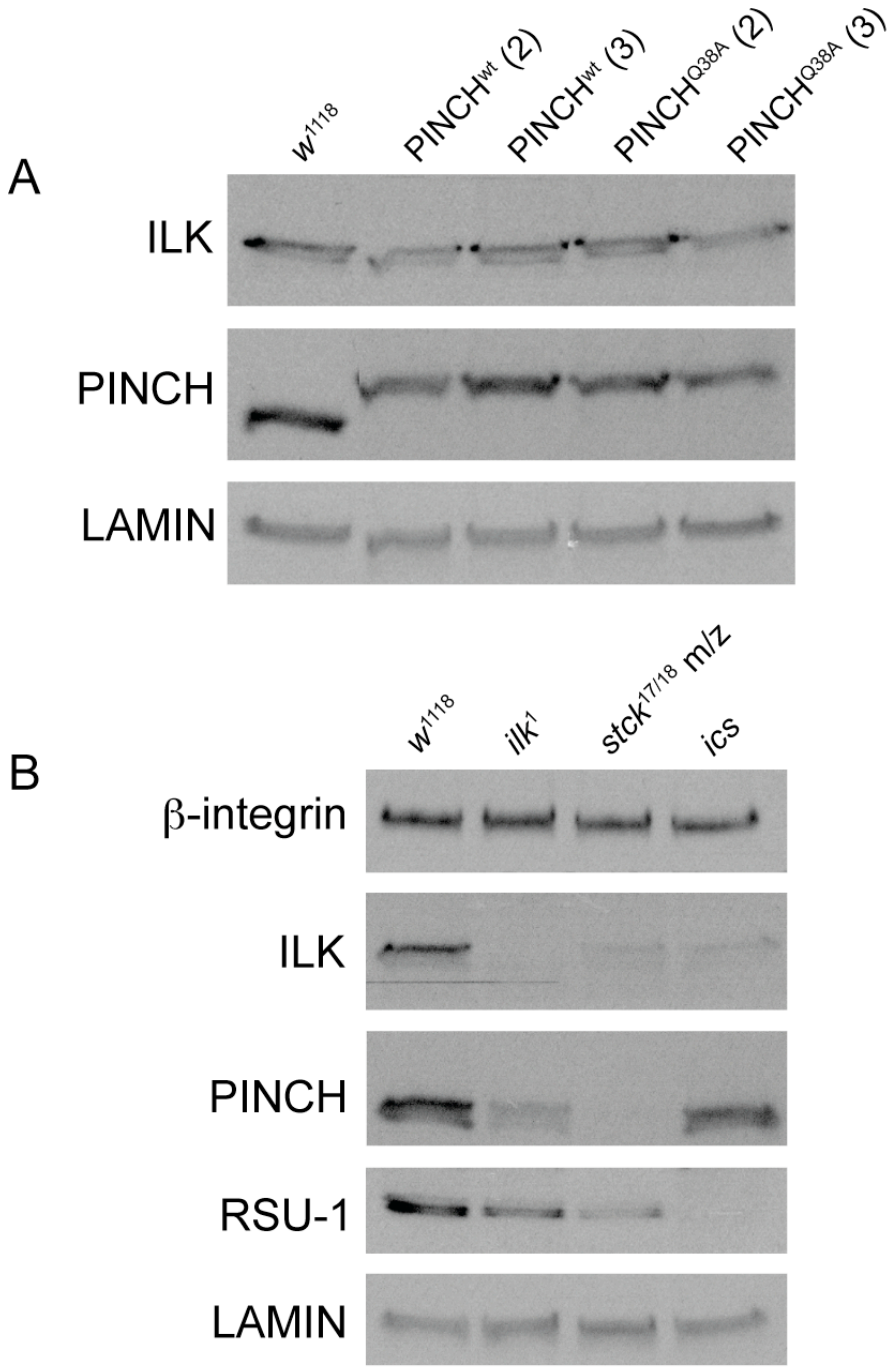


Figure 2.5. PINCH^{Q38A} rescued embryos do not exhibit the same changes in adhesion complex protein stability as null mutant embryos. (A) Stage 16-17 embryos were collected for two insertion lines each (2nd and 3rd chromosome) from PINCH^{wt} and PINCH^{Q38A} rescued stocks. Western blots were probed with PINCH, ILK, and Lamin antibodies. (B) Stage 16-17 null embryos of the genotypes indicated were collected and Western blots were probed with β-integrin, ILK, PINCH, RSU-1, and Lamin antibodies.

embryos in any of the mutants examined, indicating that the stability of this protein complex is independent of the levels of the receptor, and furthermore that not all adhesion complex proteins are affected. Interestingly, we observe a reduction of PINCH and ILK proteins in *ics* null mutants, as well as a reduction of RSU-1 protein in *stck*$^{17/18}$ m/z and *ilk*^{1} mutant embryos (Fig. 2.5 B). This demonstrates a fundamental difference between the loss of the interaction between PINCH and ILK in the PINCH^{Q38A} embryos and complete loss of either PINCH or ILK in null embryos, and suggests that other mechanisms may be in place to support PINCH and ILK stability. These data indicate that PINCH, ILK and RSU-1 function together as a stabilized complex in *Drosophila*, and that PINCH and ILK null phenotypes do not arise from loss of a single protein, but that other binding partners for PINCH and ILK could also contribute.

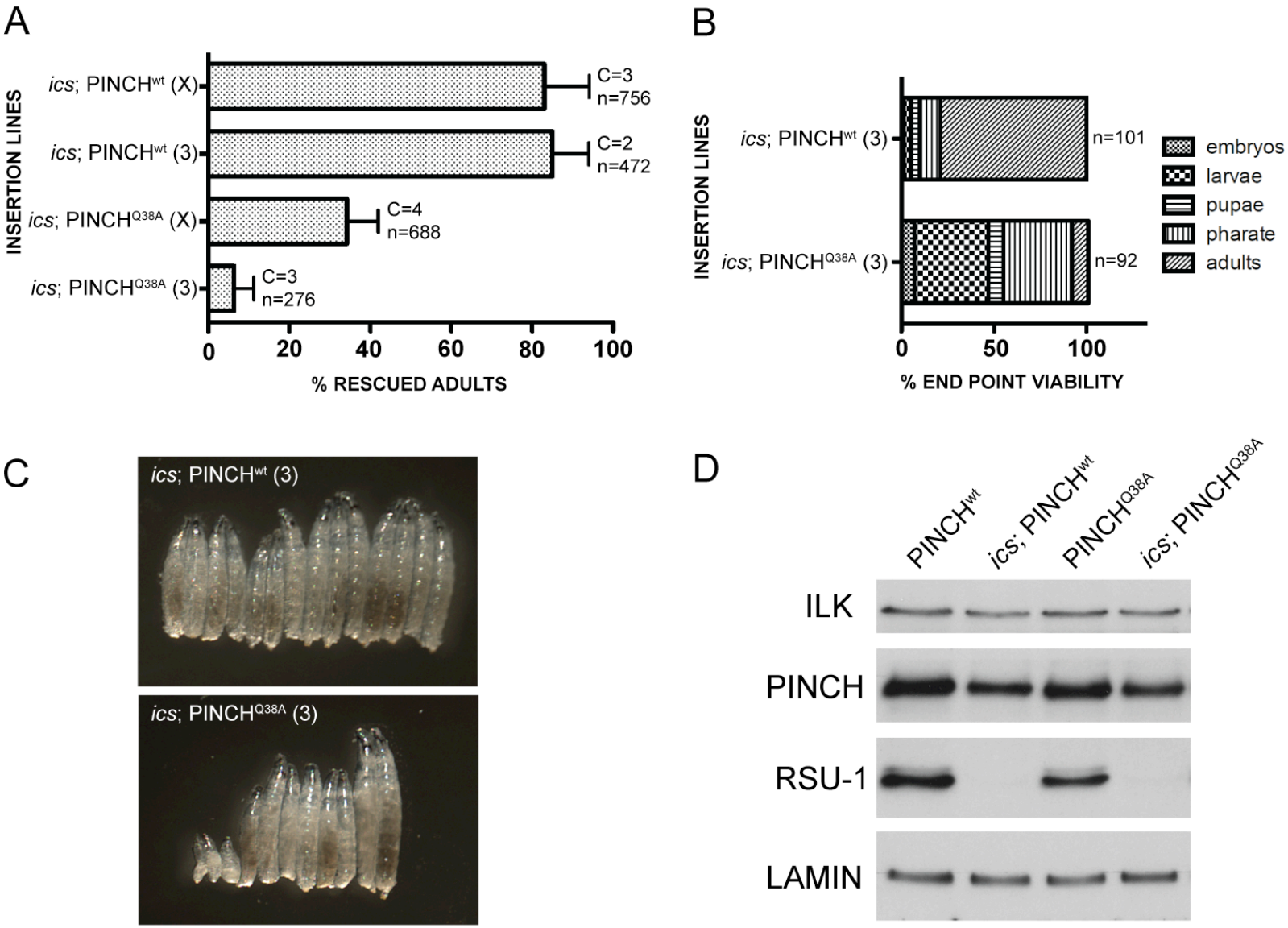
RSU-1 is critical for the viability of PINCH^{Q38A} rescued flies

The data thus far indicate that disrupting the interaction between PINCH and ILK bears no consequence on integrin-mediated functions, and moreover demonstrates that there must be some mechanism independent of ILK binding to enable PINCH function. RSU-1 is an attractive candidate to contribute to ILK independent PINCH function as it meets four important considerations: a) RSU-1 binds directly to PINCH. b) *ics* null mutants are viable but display wing blisters indicating a defect in integrin function. c) Loss of RSU-1 reduces protein levels of both PINCH and ILK. d) RSU-1 localizes to sites of adhesion in the *Drosophila* embryonic muscle (Fig. 2.4) (Kadrmas et al., 2004). We performed rescue crosses in which PINCH^{wt} or PINCH^{Q38A} transgenes were introduced into a *stck* and *ics* null background (*ics*; PINCH^{wt} and *ics*; PINCH^{Q38A}). Strikingly, we observe a great reduction of viable progeny in the *ics*; PINCH^{Q38A} rescued

animals (34% and 6%), whereas we observe full rescue of viability with *ics*; PINCH^{wt} (83% and 85%) (Fig. 2.6 A). Although there is a range in viable adult *ics*; PINCH^{Q38A} rescued progeny, these values are greatly reduced from both PINCH^{wt} and PINCH^{Q38A} rescue in the presence of RSU-1 (Fig 2.2 C, 2.6 A). These data suggest that in the absence of a PINCH-ILK interaction, RSU-1 plays an important role in maintaining PINCH function and highlight a functional deficit in PINCH^{Q38A} rescued flies that can be discerned in the context of an *ics* null background. Furthermore, the synthetic lethality observed when two mutations, which are normally viable alone, are introduced into the same animal (ics; PINCH^{Q38A}) supports the idea of a built in redundancy that would cope with a single mutation (*ics*; PINCH^{wt} or PINCH^{Q38A}).

To determine the lethal phase of *ics*; PINCH^{Q38A} animals, we collected late stage rescued embryos and monitored them over time (Fig. 2.6 B). No gross defect in hatching of embryos was observed, possibly due to the maternal contribution of PINCH (1% in *ics*; PINCH^{wt} vs. 7% in *ics*; PINCH^{Q38A} did not hatch). The majority of lethality in *ics*; PINCH^{Q38A} rescued animals occurs during the larval (4% in *ics*; PINCH^{wt} vs. 40% in *ics*; PINCH^{Q38A}) and pharate (11% in *ics*; PINCH^{wt} vs. 37% in *ics*; PINCH^{Q38A}) stages. Only 9% of *ics*; PINCH^{Q38A} rescued animals survive to adulthood compared to 79% observed in *ics*; PINCH^{wt} rescued animals (Fig. 2.6 B). Based on the increased lethality during larval stages, we monitored *ics*; PINCH^{Q38A} rescued larvae over time, and compared to *ics*; PINCH^{wt} rescued larvae, observe a growth defect as well as sluggish movement in a subset of animals. Approximately 66 hours after egg lay (AEL), *ics*; PINCH^{wt} rescued larvae appear relatively uniform in size, while *ics*; PINCH^{Q38A} rescued larvae display a range of sizes, including presumptive L1s that appear stumpy and sluggish and do not

Figure 2.6. Loss of RSU-1 reduces the viability of PINCH^{Q38A} rescued flies. (A) PINCH^{wt} transgenic flies lacking RSU-1 (*ics*; PINCH^{wt}) rescue at 83% and 85% while PINCH^{Q38A} transgenic flies lacking RSU-1 (*ics*; PINCH^{Q38A}) rescue at 34% and 6%. Bars represent the mean +/- S.E.M. of the % rescued flies counted over the number of crosses set. C denotes the number of times the rescue cross was set. n is the total number of flies counted across all crosses. (B) End stage analysis of *ics*; PINCH^{wt} and *ics*; PINCH^{Q38A} rescued animals demonstrates that the majority of lethality in *ics*/PINCH^{Q38A} animals occurs during the larval and pharate stages. (C) Heat fixed larvae at 66 hours AEL demonstrate a growth defect in *ics*; PINCH^{Q38A} larvae compared to *ics*; PINCH^{wt}. 14 L1s were counted for each genotype at 42 hours AEL. By 66 hours AEL, 14 *ics*; PINCH^{wt} L2s were observed while only 11 *ics*; PINCH^{Q38A} larvae had survived to the same time point. (D) Western blots of 66 hour larvae were probed with ILK, PINCH, RSU-1 and Lamin antibodies. There is no discernible difference in PINCH or ILK levels between *ics*; PINCH^{wt} and *ics*; PINCH^{Q38A} rescued larvae.


A
INSERTION LINES
ics; PINCHwt (X)
ics; PINCHwt (3)
ics; PINCHQ38A (X)
ics; PINCHQ38A (3)
C=3
n=756
C=2
n=472
C=4
n=688
C=3
n=276
0
20
40
60
80
100
% RESCUED ADULTS
B
INSERTION LINES
ics; PINCHwt (3)
ics; PINCHQ38A (3)
n=101
n=92
embryos
larvae
pupae
pharate
adults
0
50
100
% END POINT VIABILITY
C
ics; PINCHwt (3)
ics; PINCHQ38A (3)
D
PINCHwt
ics; PINCHwt
PINCHQ38A
ics; PINCHQ38A
ILK
PINCH
RSU-1
LAMIN

progress to L2s (Fig. 2.6 C). These results suggest a possible mechanism of action for RSU-1 in maintaining appropriate PINCH levels (Fig. 2.5 B), in which loss of RSU-1 in the *ics*; PINCH^{Q38A} flies reduces PINCH and/or ILK to a level that does not support viability. To test this, we performed Western blots of larval lysates at 66 hours AEL and do not observe a significant reduction in PINCH or ILK levels in *ics*; PINCH^{Q38A} rescued larvae compared to *ics*; PINCH^{wt} rescued larvae, indicating that the loss of viability observed in these flies is not due to altered levels of PINCH or ILK (Fig. 2.6 D). Alternatively, RSU-1 could be acting to maintain PINCH localization or function at adhesion sites, and under normal circumstance other proteins, including ILK, redundantly carry out this function. We conclude here that loss of RSU-1 in flies where the interaction between PINCH and ILK has been disrupted bears a functional consequence on growth, locomotion, and viability in *Drosophila*.

Discussion

Disrupting the PINCH-ILK interaction does not cause any overt defects in *Drosophila*

In this study, we have shown that disrupting the PINCH-ILK interaction bears no consequence on viability, wing adhesion, muscle structure, stability of the actin cytoskeleton, protein localization at muscle attachment sites, and protein stability of PINCH and ILK. This was surprising given the null phenotypes of both *stck* and *ilk* mutants and the requirement of PINCH and ILK for maintaining stable membrane-actin linkage. We conclude from these experiments that PINCH and ILK can carry out downstream integrin-mediated function independent of their direct interaction.

These results are in contrast to what has been reported in the mammalian literature. In C2C12 cells, PINCH^{Q40A} does not bind ILK and does not localize to focal adhesions (Zhang et al., 2002). In HeLa cells, PINCH^{Q40A} expression causes functional consequences in adhesion and survival signaling, and results in reduced ILK protein levels (Xu et al., 2005) similar to cells in which PINCH is knocked down by siRNA or in *PINCH* null cells (Fukuda et al., 2003; Stanchi et al., 2009). It is likely that mislocalization of PINCH and reduced levels of ILK contribute to the severe phenotypes of cells expressing PINCH^{Q40A}. We observe neither mislocalization nor reduction of PINCH or ILK in the PINCH^{Q38A} mutants suggesting that there is a mechanism in place to prevent these changes that would be catastrophic *in vivo*. These results support the idea that localization and function are tightly linked, and suggest that in *Drosophila* muscle there are multiple redundant interactions within adhesion complexes that can preserve protein localization, function, and stability more effectively than in cell culture.

Other domain analysis studies support the idea that the PINCH-ILK interaction is not solely responsible for PINCH and ILK localization. In mammalian cell culture, deletion of the C-terminal tail of PINCH (PINCH$^{\Delta CT}$) results in a similar phenotype with regard to localization and function seen with the PINCH^{Q40A} mutant. This indicates that it is not only LIM1 of PINCH that is capable of localizing PINCH to adhesion sites, but that the C-terminal tail is also necessary for this function even in the context of normal binding to ILK (Xu et al., 2005). Recent work in *Drosophila* has shown that a truncated version of ILK in which only the N-terminal ANKR domain is expressed is able to localize to muscle attachment sites, but is not sufficient to localize PINCH. This suggests that an interaction other than that with ILK ANKR participates in localizing PINCH

(Zervas et al., 2011). Furthermore, a version of PINCH in which LIM1 is deleted ($PINCH^{\Delta LIM1}$) demonstrates a reduced capacity to localize to muscle attachment sites, highlighting a key difference between $PINCH^{Q38A}$ and $PINCH^{\Delta LIM1}$ (Zervas et al., 2011) (MCE unpublished results). These results support the view that binding to ILK is likely not the only mechanism for PINCH localization, and that LIM1 and the C-terminal tail both play some role in normal recruitment of PINCH to sites of adhesion.

The authors of the recently published crystal structure of LIM1 of PINCH with the ANKR domain of ILK demonstrate that human $PINCH^{Q40}$ is a key residue in maintaining the PINCH-ILK interaction, but also discuss that upon binding with ILK, LIM1 undergoes a "twist" that could allow LIM1 to make additional interactions (Chiswell et al., 2008; Yang et al., 2009). In *C. elegans*, 3 LIM domain proteins (unc-95, LIM-8 and LIM-9) were identified in yeast two hybrid screens that bind to LIM1 of unc-97 (PINCH) (Qadota et al., 2007). unc-98, a C2H2 Zn finger protein, has been mapped to bind LIM1-2 of unc-97 (Mercer et al., 2003). More recently a Zn finger transcription factor, Wilms Tumor-1, was found to bind to LIM1 of PINCH in human podocytes stimulated with TGF β1 (Wang et al., 2011). These studies suggest that there are other roles for LIM1 in addition to mediating the PINCH-ILK interaction. Although clear functional homologues of these genes have not been shown in *Drosophila*, these new binding partners for LIM1 highlight the potential for identification of novel PINCH binding partners to further elucidate PINCH function.

RSU-1 plays a key role in maintaining viability in PINCH^{Q38A} flies

We have shown here that PINCH^{Q38A} flies are sensitive to loss of RSU-1, suggesting a novel function for RSU-1 in the absence of a PINCH-ILK interaction. We observe viability in the range of 6-34% in *ics*; PINCH^{Q38A} flies compared to 83-85% in *ics*; PINCH^{wt} flies (Fig. 2.6 A). Lethality of *ics*; PINCH^{Q38A} animals occurs mostly in larvae and pharate adults, and a growth defect and sluggish movement are observed in a subset of larvae. One explanation for this range of defects in larval growth and time of lethality could be slight differences in PINCH protein levels expressed from different transgene insertions. Although Western blots indicate similar transgenic PINCH protein levels in adults from several insertion lines (Fig. 2.2 A), it is possible that protein levels vary during development, and that slight differences in PINCH^{Q38A} expression exacerbated by loss of RSU-1 could account for the range seen in lethality.

The observation that a fraction of *ics*; PINCH^{Q38A} flies do survive to adulthood suggests that RSU-1 is not the sole protein maintaining PINCH function in the absence of an interaction with ILK. Other candidate genes that fit a subset of our criteria for testing RSU-1 may also contribute to PINCH function. For example, Tensin, Vinculin, and FAK are genes that are not required in *Drosophila* for viability, but localize to adhesion sites (Alatortsev et al., 1997; Grabbe et al., 2004; Lee et al., 2003; Torgler et al., 2004). Tensin and Vinculin also have reported roles in binding to Actin (Peng et al., 2011; Torgler et al., 2004; Ziegler et al., 2008). Like RSU-1, in *Drosophila*, these proteins are not absolutely required for adhesion on their own, but might support adhesion in combination with other adhesion complex components.

PINCH, ILK and RSU-1 are required for

protein stability in *Drosophila*

In agreement with data from mammalian cell culture, we show here that loss of PINCH affects ILK protein levels and vice versa, indicating that this is a conserved characteristic of proteins in this adhesion complex. This observation is important for interpreting results between different species, and demonstrates potential similarity in their regulation. We included RSU-1 in our analysis because of previous observations that loss of PINCH can affect RSU-1 levels and vice versa (Kadrmas et al., 2004). We confirmed this observation in late stage embryos, but also interestingly observed that loss of RSU-1 in *ics* embryos can affect the levels of ILK, and that ilk^{1} null embryos show a reduction in RSU-1 protein levels. These results extend the phenomenon of protein stability within this complex to include not only proteins with direct or primary interactions (PINCH-RSU-1 or PINCH-ILK), but to secondary protein interactions as well (ILK-RSU-1). Furthermore, these data support the idea that multiple proteins function together downstream of integrins. This secondary regulation is a phenomenon observed in other studies where loss of PINCH in cell culture reduces Parvin and Tensin protein levels (which both bind to ILK) (Fukuda et al., 2003; Stanchi et al., 2009).

These data also are indicative of the complexity of possible causes of the actin detachment phenotype of PINCH and ILK mutants. One view is that loss of ILK causes destabilization of Parvin, a direct actin binding protein, thereby causing actin detachment from the membrane. Similarly, loss of PINCH could cause actin detachment by reducing ILK levels, thereby causing destabilization of Parvin and actin linkages. Interestingly, our Western blots demonstrate that ILK protein levels appear similar in both *stck* and *ics*

mutant embryos (Fig. 2.5 B). This refutes the hypothesis that lethality of *stck* null mutants is caused solely by a reduction in ILK levels, since *ics* mutant embryos display a similar level of ILK but are viable. Alternatively, the cause of PINCH lethality could be independent of ILK, where loss of PINCH disrupts another yet unknown protein required to maintain contact with the actin cytoskeleton.

The viability observed in PINCH^{Q38A} flies may be attributed to other proteins localized with PINCH and ILK that support complex stability and help maintain adhesion with loss of only one interaction. This is in contrast to complete loss of either PINCH or ILK, and the disruption of all their protein contacts, which would be more catastrophic in the embryo. ILK makes many protein contacts at sites of adhesion and its loss could have functional consequences due to loss/reduction/mislocalization of other binding partners (Wickstrom et al., 2010). PINCH has two characterized binding partners in the fly, ILK and RSU-1. We have shown that disruption of the PINCH-ILK interaction bears no consequence on viability, and that loss of RSU-1 severely diminishes PINCH^{Q38A} viability but does not completely eliminate it. This suggests that PINCH has additional unidentified binding partners that maintain its function at adhesion sites. In *wech* mutants, there is a reduction of ILK localized at adhesion sites. However, PINCH levels are comparable to those of wild-type flies (Loer et al., 2008). This finding supports the idea that PINCH and ILK localization can be uncoupled at muscle attachment sites and suggests that PINCH levels and/or localization can be regulated independently of ILK.

The ability of PINCH to stabilize the actin cytoskeleton could be independent of its interaction with ILK

The data demonstrating that the PINCH-ILK interaction is not required for integrin function suggest that PINCH may stabilize integrin-actin linkages independent of binding to ILK, and supports the idea that there are multiple redundant pathways or interactions that support adhesion in *Drosophila*. One known connection to the actin cytoskeleton is via Parvin, a direct ILK binding partner. It is possible that PINCH regulates Parvin, even in the absence of an interaction with ILK. However, yeast-3-hybrid data from C. *elegans* shows that PINCH and Parvin are only able to physically interact when ILK is present, supporting the idea that Parvin functions through its interaction with ILK and not via PINCH (Norman et al., 2007).

The work presented here highlights the need for identification of novel PINCH binding partners. PINCH is categorized as a molecular scaffold with no enzymatic activity of its own, and is thought to act as a docking platform for other proteins to carry out downstream functions. Our work suggests that PINCH binds to other proteins that can anchor the actin cytoskeleton independently of its interaction with ILK. Other partners for PINCH have been described in mammalian cell culture including Hic-5, NCK-2, Beta thymosin-4, and PP1α (Bock-Marquette et al., 2004; Eke et al., 2010; Mori et al., 2006; Tu et al., 1998). However, functional homologues of these proteins or direct interactions with PINCH have not been described in *Drosophila*. Interestingly, all of these binding partners bind to LIM4 or LIM5 of PINCH, allowing the possibility that they interact with or are regulated by RSU-1 as well. Our future work will focus on understanding the role

of the ILK-PINCH-RSU complex with the goal of identifying other PINCH interactions that contribute to integrin function in *Drosophila*.

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CHAPTER 3

ANALYSIS OF PINCH LIM1

Abstract

The LIM domain structure of PINCH suggests that it acts as a protein binding platform to integrate signals downstream of integrins. However, few interactions have been described and well characterized, especially in *Drosophila*. In order to determine if other functions exist for LIM1 of PINCH other than binding to ILK, PINCH$^{\Delta LIM1}$ transgenic flies were generated in the same manner as PINCH^{wt} and PINCH^{Q38A} transgenic flies. We demonstrate that PINCH$^{\Delta LIM1}$ is expressed, but at lower levels than endogenous PINCH, and that PINCH$^{\Delta LIM1}$ retains the structural capacity to interact with the LIM5 binding partner RSU-1. The PINCH$^{\Delta LIM1}$ transgene does not fully rescue the lethality of the *stck* (PINCH) null mutant, but rescued animals do survive longer than the *stck* null mutant to larval and pupal stages, indicating some level of rescue and function attributed to expression of the transgene. The PINCH$^{\Delta LIM1}$ transgene localizes to muscle attachment sites in the *Drosophila* embryo, but not exactly in the same manner as PINCH^{wt} transgenes. Taken together these results indicate that LIM1 is not absolutely required for PINCH localization to sites of adhesion, but that LIM1 may possess functions other than binding to ILK. Understanding the limits of PINCH$^{\Delta LIM1}$ function during *Drosophila* development may uncover novel post embryonic roles for PINCH and aid in the identification of novel LIM1 binding partners.

Introduction

The interaction between LIM1 of PINCH and the N-terminal ankyrin repeat (ANKR) domain of ILK is well conserved and has been well studied in many organisms. The PINCH-ILK interaction and the domains required for binding were identified using a yeast two hybrid strategy and validated by immunoprecipitation using cell culture lysates

(Tu et al., 1999). Higher resolution NMR and crystal structure studies have identified individual residues, such as Q40 in human PINCH1, required to maintain the PINCH-ILK interaction (Chiswell et al., 2008; Velyvis et al., 2001; Yang et al., 2009). Cultured mammalian cells in which PINCH was knocked down by siRNA or where $PINCH^{Q40A}$ was expressed in PINCH knock down cells display reduced ILK levels and demonstrate defects in cell shape and survival signaling (Fukuda et al., 2003; Xu et al., 2005). These studies indicate a crucial role for the interaction between PINCH and ILK. However, in *Drosophila*, disruption of the PINCH-ILK interaction using $PINCH^{Q38A}$ does not affect viability or integrin function unless another PINCH binding partner, RSU-1, is also absent (Chapter 2). This is in contrast to what is observed in *stck* and *ilk* null flies, which are embryonic lethal due to disruption of actin-membrane linkages (Clark et al., 2003; Zervas et al., 2001). These data suggest different regulation of PINCH-ILK complex function in different cell types and/or species.

In cultured mammalian cells, both $PINCH^{Q40A}$ and $PINCH^{\Delta LIM1}$ fail to localize to focal adhesions (Zhang et al., 2002b; Zhang et al., 2002c) leading to the hypothesis that the PINCH-ILK interaction is required for PINCH localization. However, in *Drosophila*, $PINCH^{Q38A}$ is still able to localize to muscle attachment sites illustrating that the direct association with ILK is not absolutely necessary for the appropriate subcellular localization of PINCH (Chapter 2). Recent work in *Drosophila* demonstrates that $PINCH^{\Delta LIM1}$ is able to localize to sites of adhesion although this localization does not resemble wild-type PINCH localization (Zervas et al., 2011). This difference in localization in *Drosophila* raises the possibility that LIM1 may have other functions than just binding to ILK. Indeed, a recent publication demonstrates a novel binding partner for

LIM1 of PINCH, Wilms Tumor-1 (WT-1), a zinc finger transcription factor, expressed in human podocytes (Wang et al., 2011). The idea of other functions for LIM1 is also supported by the crystal structure of PINCH LIM1 bound to the ANKR domain of ILK, where upon binding LIM1 undergoes a conformational "twist" which could potentially allow for other interactions in a region different than that responsible for binding to ILK (Chiswell et al., 2008). While this has not been confirmed, the idea is intriguing and warrants further study.

In order to determine if additional roles exist for LIM1 of PINCH besides binding to ILK in *Drosophila*, we generated PINCH$^{\Delta LIM1}$ transgenic flies. Preliminary results indicate that the transgene is not able to rescue the *stck* null mutant, even when multiple copies are introduced. Analysis of protein levels indicate that PINCH$^{\Delta LIM1}$ is expressed, but at reduced levels compared to PINCH^{wt} rescued animals. Introduction of multiple copies of the transgene has the potential to increase protein levels and provide more confidence in results observed. An initial lifespan analysis revealed that lethality of PINCH$^{\Delta LIM1}$ rescued animals occurs during the larval and pupal stages, well past the lethal phase of *stck* null mutants. Images of rescued larvae demonstrate a growth defect in PINCH$^{\Delta LIM1}$ rescued animals. PINCH$^{\Delta LIM1}$ protein localizes to muscle attachment sites in the *Drosophila* embryo, but appears more punctate than in PINCH^{wt} or PINCH^{Q38A} recued embryos. These results demonstrate that the deletion of LIM1 is more severe than simply disrupting the PINCH-ILK interaction, and suggests that LIM1 has functions other than binding to ILK. Furthermore, in agreement with recently published data, the ability of PINCH$^{\Delta LIM}$ to localize to muscle attachment sites supports the idea that binding to ILK is not the only mode for PINCH localization. These initial results are promising

and justify further investigation aimed at understanding the functional differences between PINCH^{Q38A} and PINCH$^{\Delta LIM1}$ with the goal of uncovering novel roles for LIM1.

Materials and methods

Fly stocks

w^{1118} or PINCH^{wt}-3xFlag rescued flies were used as controls. PINCH^{wt} and PINCH^{Q38A} transgenic flies were previously described (Chapter 2). *stck*^{17} and *stck*^{18} were previously described and used to generate the *stck* null background for the rescue crosses (Clark et al., 2003).

Generation of PINCH$^{\Delta LIM1}$ flies and rescue crosses

A fragment containing the PINCH LIM1 deletion was generated by PCR and ligated into the pCasper-PINCH^{wt}-3xFlag vector used to generate PINCH^{wt}-3xFlag transgenic flies under control of the endogenous PINCH promoter. Contructs were injected into wild-type embryos (Genetic Services, Inc., Cambridge, MA), and individual lines were isolated and mapped. Rescue crosses were set as previously described introducing two-four copies of the transgene in to a *stck* null background (Chapter 2). Graphical representation of lifespan analysis was done with Graphpad Prizm software.

Immunoprecipitations and Western blots

Adult fly lysates for IP were prepared in a dounce homogenizer with lysis buffer (Tris pH 7.9, 0.1% Triton-X 100) and protease inhibitors, and were incubated with Flag (M2) beads (Sigma), rinsed and boiled in 2x Laemmli sample buffer followed by Western blots. For other Western blots, equal numbers of adult flies or staged and sorted embryos or larvae were homogenized in 2x Laemmli Sample buffer. Protein samples were run on

SDS-PAGE gels and transferred to nitrocellulose. Antibodies used were: anti-PINCH (1:5000) (Clark et al., 2003), anti-Flag (M2, Sigma, 1:2000), anti-ILK (1:500, BD #611802), anti-RSU-1 (1:5000) (Kadrmas et al., 2004), and anti-Lamin (1:5000, DSHB).

Immunofluorescence and imaging

A rescue cross introducing two copies of the transgene was set and monitored over time. Stills from a movie of 48 hour progeny illustrate rescued PINCH$^{\Delta LIM1}$ larvae. A rescue cross introducing four copies of the transgene was set, and late stage embryos were heat fixed and stained as previously described (Chapter 2). The Flag (M2) antibody (1:2000, preabsorbed against w^{1118} embryos, Sigma) was used to identify only transgenic PINCH$^{\Delta LIM1}$ as there is likely residual maternal PINCH at this stage. An Alexafluor anti-mouse 568 secondary was used (Invitrogen, 1:250). Movies were obtained on an Olympus SZX12 dissecting scope under UV light. Confocal images were taken on an Olympus Fluoview 300, with a 20x air objective. Images were processed using Adobe CS4 programs.

Results

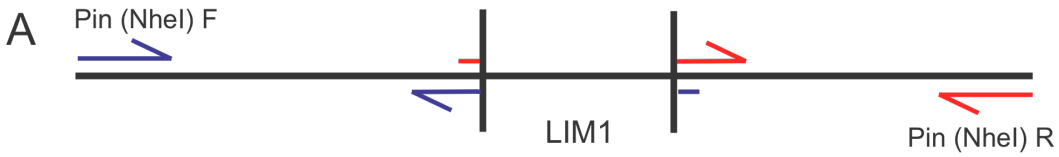
The PINCH$^{\Delta LIM1}$ transgene does not rescue the lethality of the PINCH null mutant

In order to determine whether the only function for LIM1 of PINCH is to bind ILK, PINCH$^{\Delta LIM1}$-3xFlag transgenic flies were generated. Results from this set of transgenic flies can be directly compared to PINCH^{wt} and PINCH^{Q38A} rescued flies which were generated and characterized in the same manner (Chapter 2). Mutagenic primers were designed and an overlap extension PCR strategy was used to generate a fragment of

PINCH in which LIM1 and the linker between LIM1 and LIM2 were deleted (Fig. 3.1 A,B). The resulting fragment was ligated into the PINCH^{wt}-3xFlag construct used to generate PINCH^{wt} transgenic flies and injected into wild-type embryos. Individual lines were isolated and transgenic insertions were mapped to specific chromosomes (Table 3.1). In order to test for rescue, crosses were set to introduce two copies of the PINCH$^{\Delta LIM1}$ transgene into a *stck* null background. Ten of 14 individual lines were tested and none of the crosses produced any viable progeny, indicating that PINCH$^{\Delta LIM1}$ is unable to fully rescue the *stck* null mutant (Table 3.2). This is in contrast to the PINCH^{Q38A} transgene, which is able to fully rescue the *stck* null mutant (Chapter 2). This initial result indicates that the PINCH^{Q38A} transgene, which contains a point mutation disrupting the interaction with ILK, is functionally distinct from the PINCH$^{\Delta LIM1}$ transgene, which certainly disrupts the interaction with ILK, but may have other functions.

The PINCH$^{\Delta LIM1}$ transgene is expressed and retains the ability to bind RSU-1

One possible explanation for the lack of rescue using the PINCH$^{\Delta LIM1}$ transgene is that the transgenic protein is unstable. This was observed in PINCH$^{\Delta LIM5}$-GFP transgenic flies, in which it was thought that the combination of the GFP tag along with a large deletion of the protein rendered it unstable (Chapter 4). In consideration of this, and based on previous results demonstrating that PINCH^{wt}-3xFlag and PINCH^{Q38A}-3xFlag transgenics express protein at levels comparable to endogenous PINCH (Chapter 2), we generated PINCH$^{\Delta LIM1}$ transgenics with a 3xFlag tag to help ensure appropriate levels of



B PIN (NheI) F: ATCATTTGGCAGCATAATAATCATC
PIN (NheI) R: GCTGAAAAGCAGAGTAATAGCAAC

ΔLIM1 Forward: GCCATGCAT//TGCAACAAGTGCGGCGAG
ΔLIM1 Reverse: CTTGTTGCA//ATGCATGGCACCCAGCGA

Figure 3.1: PCR scheme to generate LIM1 deletion fragment. (A) Two sets of primers were designed to generate 5' (blue) and 3' (red) fragments of LIM1 with engineered NheI sites and an overlapping region surrounding LIM1. Both fragments were used as template in a second PCR reaction using the Pin NheI F/R primers to generate a single overlap product in which LIM1 was deleted. The resulting fragment was digested with NheI and ligated into the pCasper vector containing the PINCH^{wt}-3xFlag sequence under control of the endogenous PINCH promoter. (B) Sequences of the PCR primers used to generate the fragments described above. The // denotes the LIM1 border with a tail sequence generated on the opposite side of the LIM1 for both 5' and 3' fragments.

Table 3.1

PINCH$^{\Delta LIM1}$ transgenic lines

Insertion ID	Chromosomal Location	Homozygous Viable
ΔLIM1 A2.3	X	YES
ΔLIM1 A10.1	X	YES
ΔLIM1 A45.1	X	YES
ΔLIM1 A2.2	2	YES
ΔLIM1 A4.3	2	YES
ΔLIM1 B3.1	2	YES
ΔLIM1 B37.5	2	YES
ΔLIM1 A4.2	3	YES
ΔLIM1 A21.1	3	YES
ΔLIM1 A26.2	3	YES
ΔLIM1 A50.1	3	NO
ΔLIM1 B37.3	3	YES
ΔLIM1 B43.2	3	NO
ΔLIM1 B47.3	3	YES

Each individual PINCH$^{\Delta LIM1}$ insertion line is listed with its original identification. Insertion lines were mapped to single chromosomes. In order to confirm that some insertion sites were not in critical regions of the genome, we determined whether each transgene was homozygous viable in a *stck* wild-type background. Homozyogous viable lines were used to test for rescue of the *stck* null mutant.

Table 3.2

PINCH$^{\Delta LIM1}$ rescue data

Insertion ID	**% Rescue *stck*^{18}/*stck*^{17}**
ΔLIM1 A2.3	0%
ΔLIM1 A10.1	0% (n=266)
ΔLIM1 A45.1	0% (n=218)
ΔLIM1 A2.2	0%
ΔLIM1 A4.3	0%
ΔLIM1 B3.1	0%
ΔLIM1 B37.5	0%
ΔLIM1 A4.2	0%
ΔLIM1 A21.1	0% (n=228)
ΔLIM1 B43.2	0%
ΔLIM1 A2.2/A21.1	0%
ΔLIM1 A2.3/A21.1	0%

Ten individual insertion lines were used to test for rescue of the PINCH null mutant using the *stck*^{17} and *stck*^{18} alleles. Crosses where two copies of the transgene were introduced into the progeny did not produce any viable rescued adults (n=# of progeny counted from the rescue cross). Two additional crosses were set to introduce four copies of the transgene into the progeny, and also did not produce any viable rescued adults. Examples of crosses set for two and four copy rescue: ΔLIM1$^{A21.1}$, *stck*^{17}/TM3,Sb x ΔLIM1$^{A21.1}$, *stck*^{18}/TM3, Sb and ΔLIM1$^{A2.2}$; ΔLIM1$^{A21.1}$, *stck*^{17}/TM3,Sb x ΔLIM1$^{A2.2}$; ΔLIM1$^{A21.1}$, *stck*^{18}/TM3,Sb. Rescued progeny would have the following genotypes: ΔLIM1$^{A21.1}$, *stck*^{17}/ΔLIM1$^{A21.1}$, *stck*^{18} or ΔLIM1$^{A2.2}$; ΔLIM1$^{A21.1}$, *stck*^{17}/ΔLIM1$^{A21.1}$, *stck*^{18}.

transgenic PINCH. PINCH$^{\Delta LIM1}$-3xFlag is predicted to be approximately the same size as endogenous PINCH (Fig. 3.2 A), so to distinguish endogenous PINCH from transgenic PINCH, we probed Western blots with both PINCH and Flag (M2) antibodies. Lysates from PINCH$^{\Delta LIM1}$ transgenic flies in a *stck* heterozyogous background (PINCH$^{\Delta LIM1}$ H) were prepared along with PINCH^{wt} and PINCH^{Q38A} lines either in a *stck* heterozyogous background or rescued lines in a *stck* null background (PINCH$^{wt/Q38A}$ H or R). Of note, PINCH transgene expression is reduced in the presence of endogenous PINCH, and is upregulated once it is the only PINCH species present (Fig. 3.2 B,C lanes 2-5). We observe robust PINCH expression in the blot probed with the PINCH antibody. While the PINCH^{wt} and PINCH^{Q38A} Flag tagged species are visible as higher migrating bands in the control lanes, stronger bands are observed in the PINCH$^{\Delta LIM1}$ lanes due to levels of both endogenous and transgenic PINCH migrating together (Fig. 3.2 B). To determine the contribution of transgenic PINCH, we probed blots with the Flag (M2) antibody (Fig. 3.2 C). PINCH$^{\Delta LIM1}$ expression is observed in all lines tested but levels are reduced compared to the PINCH^{wt} H sample. PINCH$^{\Delta LIM1}$ levels are comparable or greater than levels in the PINCH^{Q38A} H sample. PINCH^{wt} and PINCH^{Q38A} rescued lines do however express greater levels of PINCH that that seen in any of the PINCH$^{\Delta LIM1}$ lines. This experiment demonstrates that PINCH$^{\Delta LIM1}$ is expressed, but it is unclear from this data whether there is enough transgenic protein to support viability.

It is possible that the deletion of LIM1 could alter the overall structure of PINCH causing defects that were not intended. To test this, we performed Flag co-immunoprecipitations of PINCH-3xFlag species and blotted for PINCH and its known

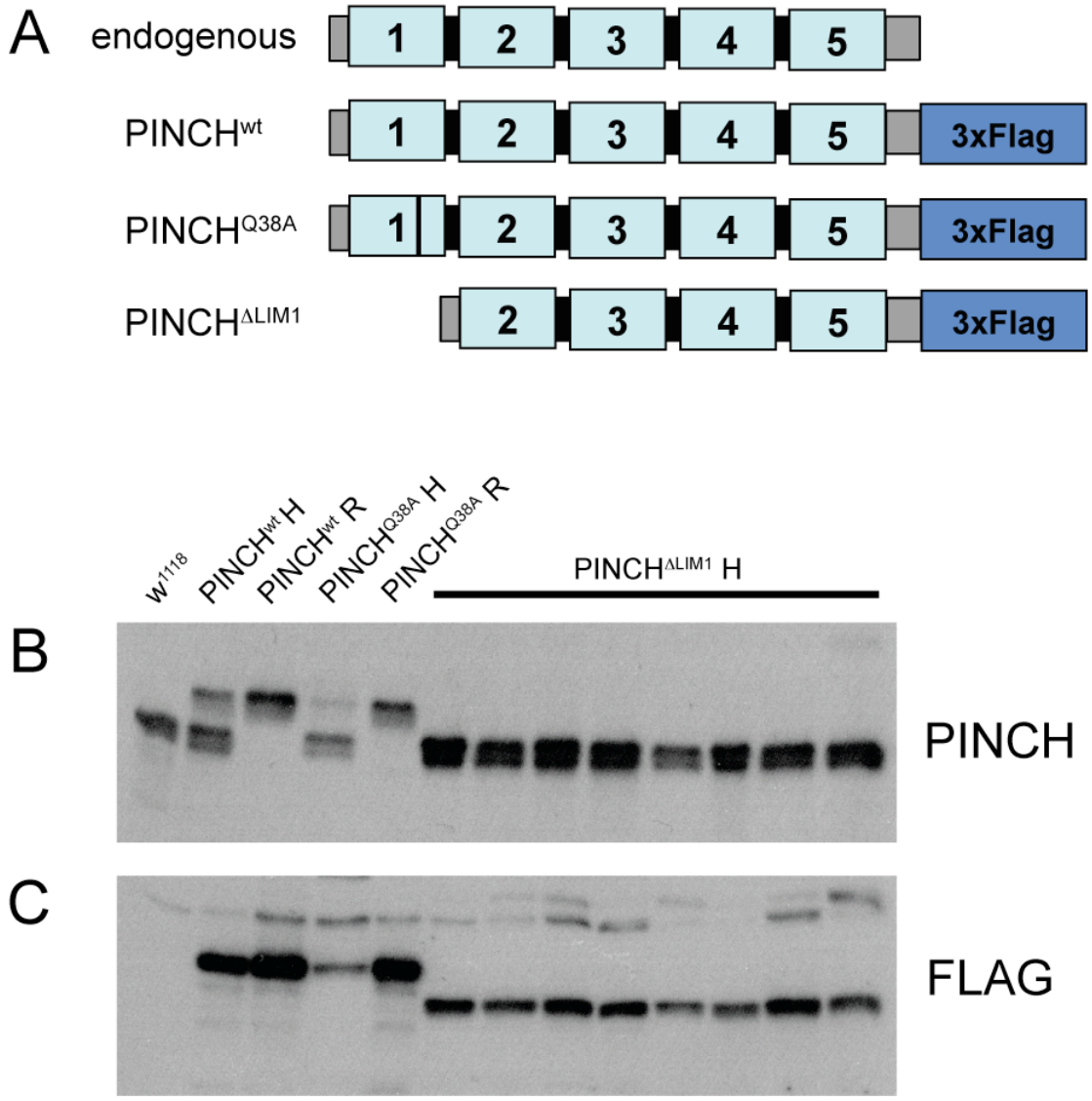


Figure 3.2: The PINCH$^{\Delta LIM1}$ transgene is expressed in a *stck* heterozygous background. (A) Schematic of endogenous PINCH protein and transgenic lines used for Western blots. PINCH^{wt} and PINCH^{Q38A} are a higher molecular weight than endogenous PINCH due to the presence of the 3x-Flag tag. PINCH$^{\Delta LIM1}$ transgenes are a similar molecular weight to endogenous PINCH due to lack of LIM1 and the addition of the 3x-Flag tag. (B) Western blots of a set of PINCH$^{\Delta LIM1}$ transgenic lines in a *stck* heterozygous background demonstrate robust PINCH expression when probed with the PINCH antibody. w^{1118} flies, and PINCH^{wt} and PINCH^{Q38A} transgenic lines were used as controls to compare expression. H denotes transgenic lines in a *stck* heterozygous background and R denotes transgenic rescued lines in a *stck* null background. (C) The same samples were probed with the Flag (M2) antibody to determine the contribution of transgenic PINCH only. PINCH$^{\Delta LIM1}$ transgenic lines in a *stck* heterozygous background express less protein than PINCH^{wt} H lines and comparable levels to PINCH^{Q38A} H lines. PINCH$^{\Delta LIM1}$ transgenic expression is less than both PINCH^{wt} R and PINCH^{Q38A} R lines.

binding partners, ILK and RSU-1. We have previously shown that PINCH^{Q38A} does not co-IP ILK, but does enrich for RSU-1 (Chapter 2). PINCH$^{\Delta LIM1}$ readily immunoprecipitates from adult fly lysates comfirming that it is expressed in a *stck* heterozygous background. PINCH$^{\Delta LIM1}$ does not co-IP ILK, but retains the interaction with RSU-1 in a similar manner as seen in PINCH^{Q38A} rescued flies, while PINCH^{wt} is able to co-IP both ILK and RSU-1 (Fig. 3.3). These data indicate that the overall structure of PINCH has been preserved, and further support the idea that PINCH$^{\Delta LIM1}$ does not rescue the *stck* null mutant due to a requirement for LIM1.

PINCH$^{\Delta LIM1}$ rescued animals can survive past the lethal phase observed in *stck* null mutants

Without definite confirmation of sufficient transgenic protein levels to support viability, we asked if PINCH$^{\Delta LIM1}$ can carry out some function by determining if rescued embryos survive past the lethal phase observed for *stck* null mutants (late embryos/early larvae). To test this, two hour collections of embryos from a PINCH$^{\Delta LIM1}$,*stck*^{17}/Twist-GFP stock were sorted based on the presence or absence of GFP to denote PINCH$^{\Delta LIM1}$ transgenes in a *stck* heterozygous background (H) or PINCH$^{\Delta LIM1}$ rescued (R) animals respectively. At 48 hours after egg lay, PINCH$^{\Delta LIM1}$ rescued larvae were observed crawling among their heterozygous counterparts (Fig. 3.4 A,B). This is well past the lethality observed by 24 hours after egg lay in *stck* null mutants. Rescued larvae are smaller and do not move as vigorously as their heterozygous counterparts. Furthermore, between 48-72 hours we observe some dead larvae among the live ones (data not shown). Thus, PINCH$^{\Delta LIM1}$ rescued animals display enhanced function and viability relative to *stck* null mutants.

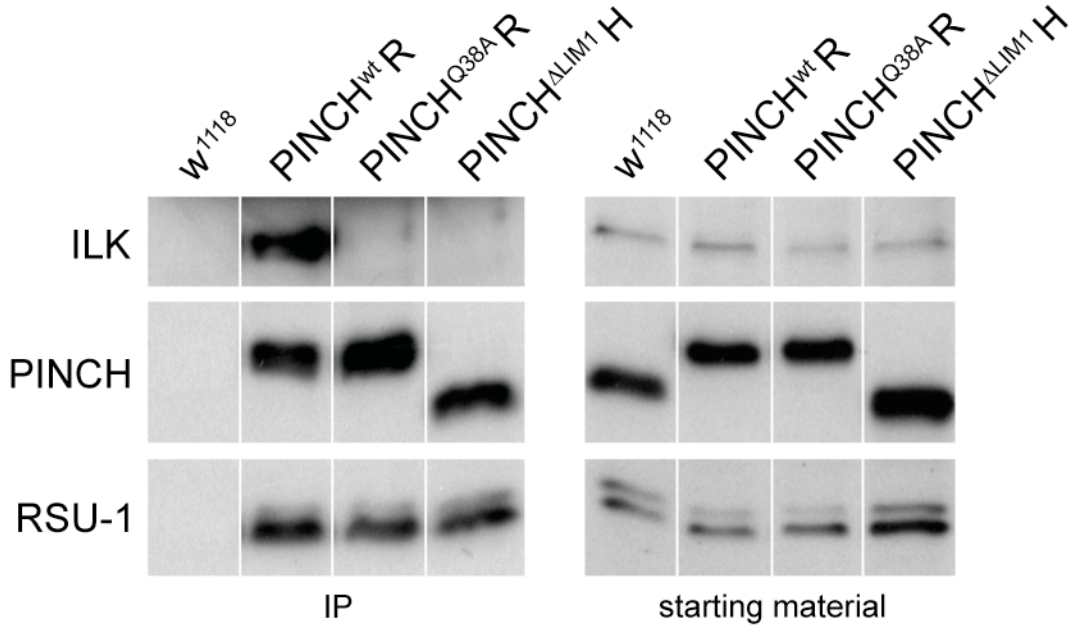


Figure 3.3: PINCH$^{\Delta LIM1}$ transgenes retain stable protein structure. Flag Co-immunoprecipitations were performed from adult fly lysates to enrich for Flag tagged transgenic proteins from PINCH^{wt}, PINCH^{Q38A}, and PINCH$^{\Delta LIM1}$ lines. PINCH-Flag was enriched from all three lines. PINCH^{wt} was able to co-IP both the LIM1 binding partner ILK and the LIM5 binding partner RSU-1. PINCH^{Q38A} and PINCH$^{\Delta LIM1}$ both were not able to co-IP ILK, but did co-IP RSU-1. As shown with the point mutation in PINCH^{Q38A} (Chapter 2), PINCH with a deletion of LIM1 retains appropriate structure to maintain an interaction with RSU-1. w^{1118} flies were used as a control to show the absence of non-specific binding in the IP. All proteins were present in roughly equal amounts in the starting material.

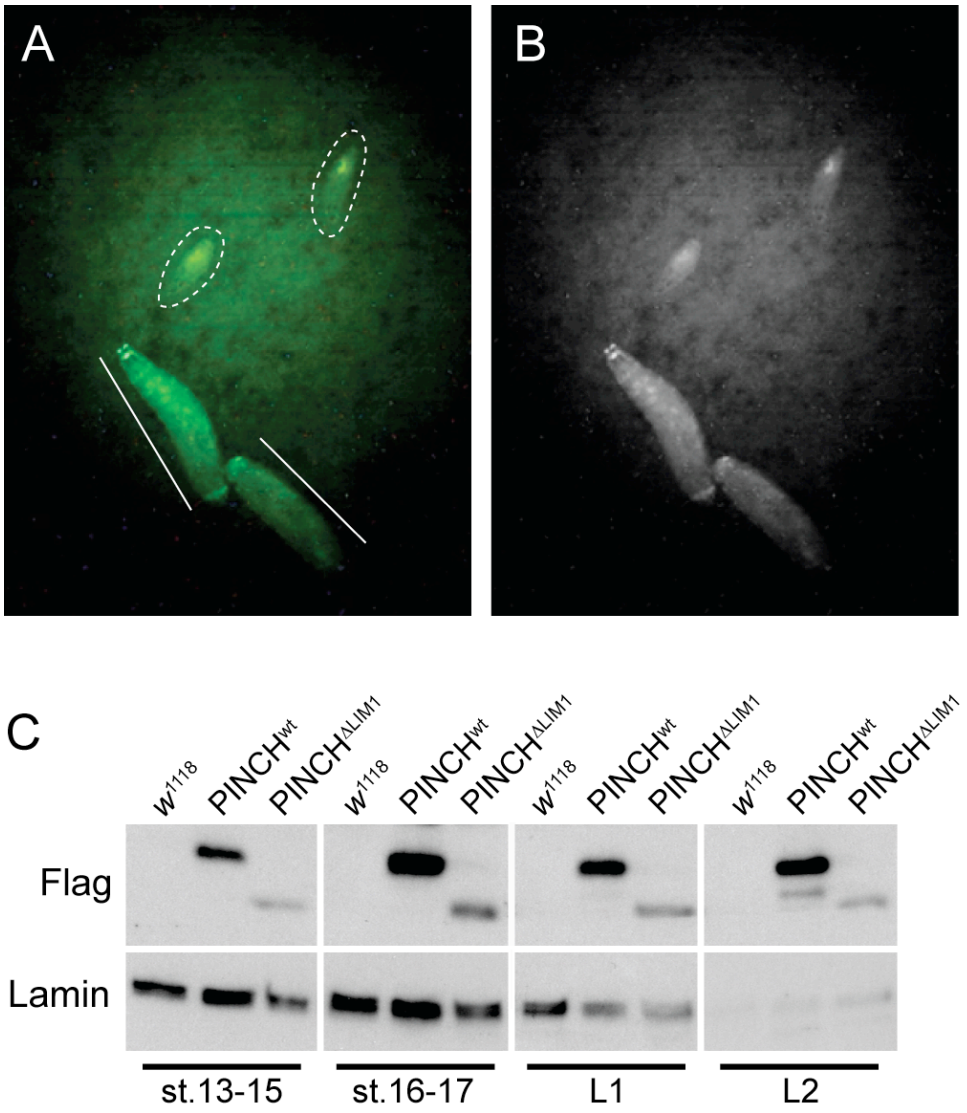


Figure 3.4: PINCH$^{\Delta LIM1}$ rescued animals display a growth defect and reduced levels of transgenic PINCH. (A) Stills from a movie taken under UV light demonstrate PINCH$^{\Delta LIM1}$ R (dotted outline) and PINCH$^{\Delta LIM1}$ H (solid line along length) larvae at approximately 48 hours after egg lay. Rescued larvae appear smaller and are identified by the lack of GFP present in the balancer chromosome of PINCH$^{\Delta LIM1}$ H flies. Rescued animals display bright autofluorescence in the gut and have an over all yellow appearance. (B) A black and white version of the same image shows rescued larvae with more contrast. (C) Western blots of w^{1118}, PINCH^{wt} rescued and PINCH$^{\Delta LIM1}$ rescued animals over 4 stages of *Drosophila* development probed with the Flag (M2) antibody. In all stages examined there is a great reduction of PINCH$^{\Delta LIM1}$ protein compared to PINCH^{wt}. Lamin levels of PINCH$^{\Delta LIM1}$ appear lower in the embryonic stages, but are equivalent in L1 transgenic samples and possibly elevated in PINCH$^{\Delta LIM1}$ L2 samples compared to PINCH^{wt}. Larval samples appear to have undergone some degradation. Stages shown are: 12-13 (dorsal closure), 16-17 (late embryo), L1 (1st instar larvae), L2 (2nd instar larvae).

In order to determine levels of PINCH$^{\Delta LIM1}$ in rescued animals, embryos and larvae lacking GFP were collected from the same stock of PINCH$^{\Delta LIM1}$,*stck*^{17}/Twist-GFP flies. *w*^{1118} and PINCH^{wt} rescued embryos and larvae were also collected at the same time points. Using the Flag (M2) antibody to probe Western blots, we observe a reduced level of PINCH$^{\Delta LIM1}$ protein at all stages examined (Fig. 3.4 C). Although, lamin control levels are not equivalent in all samples, this experiment suggests that in rescued animals, PINCH$^{\Delta LIM1}$ protein levels may be reduced compared to PINCH^{wt} and could be one explanation for the lethality observed in the PINCH$^{\Delta LIM1}$ animals.

Increased dosage of PINCH$^{\Delta LIM1}$ transgenes results in higher protein levels, but does not fully rescue the *stck* null mutant

To determine whether increased levels of PINCH$^{\Delta LIM1}$ could affect rescue, stocks were generated that contained two different homozygous viable PINCH$^{\Delta LIM1}$ insertions on the 2nd and 3rd chromosomes in a *stck* heterozygous background. Western blots of adult fly lysates demonstrate a dose dependent increase in PINCH$^{\Delta LIM1}$ protein levels from stocks containing one, two, or three copies of the transgene (Fig. 3.5 A). We repeated rescue crosses introducing four copies of the transgene into progeny and again did not observe rescue to adulthood of the *stck* null mutant (Table 3.2). It is possible that levels of PINCH$^{\Delta LIM1}$ in rescued animals are still not at a level that supports viability, but further experiments will be required to determine the levels of PINCH$^{\Delta LIM1}$ by introducing multiple copies of the transgene into a *stck* null background during development compared to PINCH^{wt}, as was done in Figure 3.4 C.

We performed an initial endpoint analysis in the same manner as was done for *ics*; PINCH^{Q38A} animals (Chapter 2) using transgenic lines that introduced four copies of

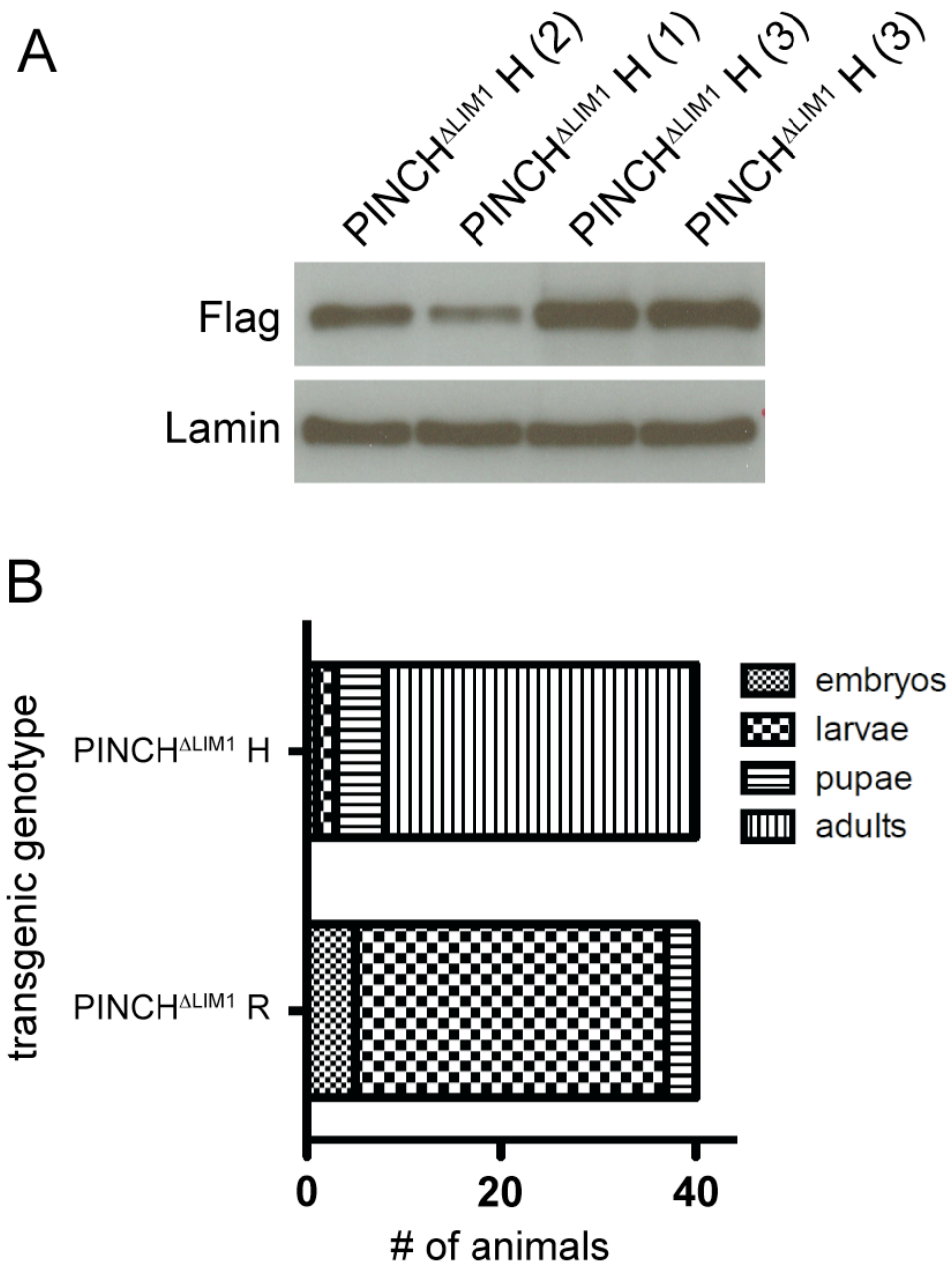


Figure 3.5: Analysis of increased PINCH$^{\Delta LIM1}$ transgene copy number. (A) Western blots of adult fly lysates from PINCH$^{\Delta LIM1}$ transgenic flies in a *stck* heterozygous background (PINCH$^{\Delta LIM1}$ H). Transgene copy number is indicated in parentheses after each sample on the blot. The Flag (M2) antibody was used to detect only transgenic PINCH. A dose dependent increase is observed in transgenic lines with either one, two, or three copies of PINCH$^{\Delta LIM1}$. (B) Endpoint viability assay of either PINCH$^{\Delta LIM1}$ H or PINCH$^{\Delta LIM1}$ R (rescued) animals generated by crossing lines shown in lane 3 and 4 of (A). Of 40 embryos sorted for each genotype, most PINCH$^{\Delta LIM1}$ H animals survive to adulthood (32/40) while no PINCH$^{\Delta LIM1}$ R viable adults are observed. The majority of PINCH$^{\Delta LIM1}$ R animals survive to the larval stages (32/40) and few to the pupal stage (3/40).

PINCH$^{\Delta LIM1}$ into rescued progeny. 40 PINCH$^{\Delta LIM1}$ R and 40 PINCH$^{\Delta LIM1}$ H embryos were sorted based on the absence or presence of the Twist-GFP balancer and placed on separate grape juice agar plates and monitored over time. The majority of PINCH$^{\Delta LIM1}$ animals in a *stck* heterozygous background survive to adulthood (32/40) due to the presence of endogenous PINCH. In contrast, the majority of PINCH$^{\Delta LIM1}$ rescued animals do not survive past the larval stage, and this lethality is spread over nine days after hatching (Fig. 3.5 B and data not shown). This observation is in agreement with the initial analysis demonstrating live PINCH$^{\Delta LIM1}$ rescued larvae containing two copies of the transgene (Fig. 3.4 A,B). While the majority of lethality was observed during the larval phases, 3/40 PINCH$^{\Delta LIM1}$ rescued animals in this experiment also survived to the pupal stage, indicating that PINCH$^{\Delta LIM1}$ is sufficient to support viability in a subset of mutants through embryogenesis and the larval phases.

PINCH$^{\Delta LIM1}$ is able to localize to muscle attachment sites

PINCH$^{\Delta LIM1}$ retains some PINCH function even though it is unable to rescue the lethality of the PINCH null mutant to adulthood. In order to determine whether this preserved function was dependent on PINCH localization at muscle attachment sites, we immunostained stage 16-17 embryos with the Flag (M2) antibody. Using an antibody against the Flag epitope eliminates any signal that may result from residual maternal PINCH. PINCH$^{\Delta LIM1}$ localizes to muscle attachment sites in the embryo, although it appears punctate and not as tightly localized compared to PINCH^{wt} rescued embryos (Fig. 3.6 A,B). More importantly, this result supports the claim that PINCH is able to localize to sites of adhesion in the absence of an interaction with ILK. The observation that the staining pattern is different than in PINCH^{Q38A} rescued embryos suggests that

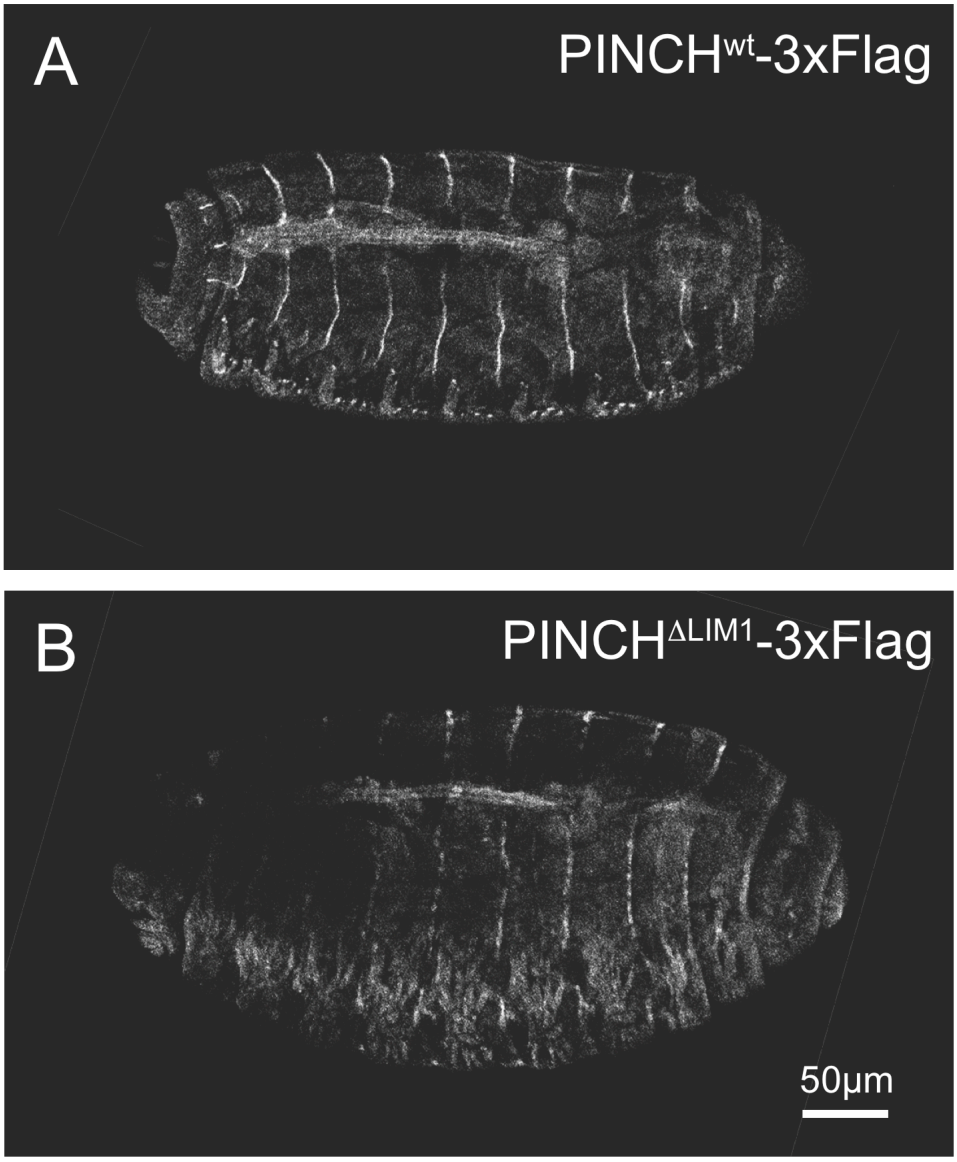


Figure 3.6: PINCH$^{\Delta LIM1}$ localizes to muscle attachment sites in the *Drosophila* embryo. Embryos containing four copies of the PINCH$^{\Delta LIM1}$ transgene, were stained with the Flag (M2) antibody. PINCH^{wt} localizes to muscle attachment sites in stage 15-16 embryos (A). PINCH$^{\Delta LIM1}$ also localizes to muscle attachment sites but the signal appears weak and punctate compared to PINCH^{wt} (B).

additional features of LIM1, independent of ILK binding, may contribute to PINCH localization or function.

Discussion

Appropriate PINCH levels must be present to support viability

In this chapter, we have demonstrated that the PINCH$^{\Delta LIM1}$ transgene is able to rescue the late embryonic/early larval lethality of the *stck* null mutant, but does not rescue to adulthood. This is in contrast to results shown with the PINCH^{Q38A} transgene, which is able to fully rescue the *stck* null mutant (Chapter 2). These results indicate that the deletion of LIM1 disrupts the interaction with ILK, but may disrupt other unknown PINCH functions as well. While we were unable to explain definitively the reason for incomplete rescue, the appropriate expression of PINCH$^{\Delta LIM1}$ must be resolved before other functional interpretations can be considered. We have begun to address this, but further experiments must be performed. Results from the PINCH^{D303V} and PINCH$^{\Delta LIM5}$ analyses suggest that the choice of tag in combination with deletion of entire domains of PINCH can contribute to instability of the protein (Chapter 4). In order to address these concerns, a 3x-Flag tag was used instead of a GFP tag. Protein levels of both PINCH^{wt}-3xFlag and PINCH^{Q38A}-3xFlag in rescued flies is similar to the levels observed for endogenous PINCH, indicating that the smaller tag made a difference in expression levels of the protein (Chapter 2). Even with attempts to improve expression with a different tag, preliminary experiments demonstrated PINCH$^{\Delta LIM1}$ transgenic protein levels were reduced compared to PINCH^{wt} rescued protein levels (Fig. 3.2 B, 3.4 C). The introduction of four copies of the PINCH$^{\Delta LIM1}$ transgene to increase protein levels is promising (Fig. 3.5 A), and a similar analysis of assessing protein levels of PINCH$^{\Delta LIM1}$ rescued animals

during development will be performed. If appropriate levels of PINCH$^{\Delta LIM1}$ can be confirmed as compared to PINCH^{wt} control animals, then further analysis of the functional consequences of PINCH$^{\Delta LIM1}$ are justified.

PINCH$^{\Delta LIM1}$ is capable of carrying out some PINCH function

Even without absolute confirmation of appropriate PINCH$^{\Delta LIM1}$ protein levels, our results demonstrating that the PINCH$^{\Delta LIM1}$ transgene is able to rescue the late embryonic/early larval lethality of *stck* null mutants is intriguing. We observe viable larvae well past the lethal phase of *stck* null mutants. Our initial lifespan analysis, where four copies of the transgene were introduced, demonstrates that most PINCH$^{\Delta LIM1}$ rescued animals survive to the larval stages and few survive to the pupal stage (Fig. 3.5 B). We do observe a growth defect in PINCH$^{\Delta LIM1}$ rescued animals that may be attributed to either the loss of LIM1 or to reduced levels of transgenic protein overall (Fig. 3.4 A,B,C). Future experiments will be designed to determine if additional copies of the transgene increase protein levels and extend viability over time.

Preliminary experiments to assess localization of PINCH$^{\Delta LIM1}$ demonstrate that it has the capacity to localize to muscle attachment sites in the *Drosophila* embryo, but does not resemble the staining pattern of PINCH^{wt} transgenes (Fig. 3.6). This result is in agreement with recently published work demonstrating that PINCH$^{\Delta LIM1}$ localizes to muscle attachment sites in the *Drosophila* embryo and that PINCH$^{\Delta LIM1}$ expression increases over time, appearing more like wild-type PINCH in the larval muscle (Zervas et al., 2011). This study did not specify how the PINCH$^{\Delta LIM1}$-GFP transgene was driven (either by an endogenous promoter or using the Gal4-UAS system). We have not assessed localization in the larval muscle, but future experiments will address the

localization of PINCH$^{\Delta LIM1}$ in order to determine whether localization could be contributing to the larval lethality observed. Furthermore, future experiments will attempt to determine the cause of the larval/pupal lethality in order to understand the differences between loss of the interaction with ILK (PINCH^{Q38A}) and deletion of LIM1 entirely (PINCH$^{\Delta LIM1}$). The PINCH$^{\Delta LIM1}$ data from our lab and from the Brown lab (Zervas et al., 2011), in combination with results from the PINCH^{Q38A} transgene demonstrate that PINCH is able to localize in the absence of an interaction with ILK, and suggest another interaction that supports PINCH localization at adhesion sites.

LIM1 of PINCH may have other functions besides binding to ILK

The results here demonstrate that PINCH$^{\Delta LIM1}$ does not fully rescue the *stck* null phenotype, but retains enough function to allow viability through the larval and pupal stages. This suggests that even with reduced levels of the transgene that LIM1 may have other functions than just binding to ILK. This is supported but not definitively confirmed by the differences in overall viability and protein localization observed between rescue with PINCH^{Q38A} transgenes and PINCH$^{\Delta LIM1}$ transgenes. Once concerns of overall expression levels have been addressed, it will be interesting to understand what these potential differences could be and how they affect integrin function overall.

Evidence in the literature demonstrates that other binding partners for LIM1 of PINCH exist in other species. In *C. elegans*, yeast-two hybrid and genetic screens have identified unc-98, unc-95, LIM-8, and LIM-9 as novel PINCH LIM1 binding partners (Mercer et al., 2003; Qadota et al., 2007). Together, these proteins and PINCH comprise a protein complex in worms that makes a link to myosin thick filaments and plays a role in muscle function (Qadota and Benian, 2010). The recent identification of WT-1 as a

novel binding partner for LIM1 of PINCH in human podocytes is extremely promising and supports the claim that LIM1 has other functions than binding to ILK (Wang et al., 2011). Furthermore, this report brings to light potential functions for PINCH that may not be related to integrin function, as the interaction between PINCH and WT-1 occurs in the nucleus upon TGF-Beta induced injury. This is not the first report of PINCH function in the nucleus and these studies together could aid in uncovering novel post-embryonic functions for PINCH (Campana et al., 2003; Hobert et al., 1999; Zhang et al., 2002a). Unfortunately, there is no WT-1 homologue in *Drosophila*, but this study supports the necessity of identifying novel binding partners for PINCH, and extends the possible functions of PINCH in the cell. Taken together, these studies highlight the potential of using screens and other methods to learn more about the interactions of molecular scaffolds such as PINCH, and how they carry out functions via their binding partners.

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CHAPTER 4

ANALYSIS OF PINCH LIM5 AND THE INTERACTION WITH RSU-1

Abstract

The PINCH-RSU-1 interaction has been described both in mammalian cells and in *Drosophila* and is mediated by LIM5 of PINCH and the LRR repeat domain of RSU-1. RSU-1 has reported roles in maintaining cell adhesion and in the regulation of the JNK cascade in both cultured cells and in *Drosophila*. Despite these described roles, the specific mechanism for RSU-1 function is unknown. In *Drosophila*, *ics* (RSU-1) null flies are viable and fertile, but do exhibit a mild wing blister phenotype. Using *stck* (PINCH) and *ics* null embryos, we demonstrate that RSU-1 localization requires the presence of PINCH, but that PINCH localization does not require the presence of RSU-1. In order to determine which functions of RSU-1 are dependent on the interaction with PINCH, a mutation in PINCH that disrupts the interaction with RSU-1 was identified ($PINCH^{D303V}$), and transgenic flies were generated carrying this mutation. To determine if there are other functions for LIM5 of PINCH other than binding to RSU-1, transgenic flies were generated with a deletion of LIM5 ($PINCH^{\Delta LIM5}$). The $PINCH^{D303V}$ transgene is able to rescue the lethality of the *stck* null mutant only when two or more copies are present. Rescued flies are not healthy, display wing blisters, and rescued stocks are difficult to maintain, indicating some defect caused by disrupting the PINCH-RSU-1 interaction. In contrast, the $PINCH^{\Delta LIM5}$ transgene is unable to rescue the *stck* null mutant. Preliminary evidence suggests that $PINCH^{\Delta LIM5}$ is not expressed at sufficient levels to support viability, but a role for LIM5 other than binding to RSU-1 cannot be excluded. Further analysis of the $PINCH^{D303V}$ rescued flies demonstrates that they express comparable levels of transgenic PINCH compared to a $PINCH^{wt}$ rescued line. However expression of both $PINCH^{wt}$ and $PINCH^{D303V}$ transgenes are reduced compared to

endogenous PINCH. PINCH^{D303V} rescued flies display reduced levels of RSU-1 compared to PINCH^{wt} rescued flies indicating that disruption of the PINCH-RSU-1 interaction affects RSU-1 protein stability. RSU-1 does not localize to muscle attachment sites in PINCH^{D303V} rescued flies indicating that the interaction between PINCH and RSU-1 is required for RSU-1 localization. Finally, introducing the *ics* null mutation into PINCH^{D303V} rescued flies further reduced viability, indicating that the presence of RSU-1 is important for viability in PINCH^{D303V} rescued animals. Given the mild phenotype of *ics* null flies, it was surprising that PINCH^{D303V} rescued flies exhibited worse phenotypes. One possible explanation is that the PINCH^{D303V} mutation could be disrupting an unknown PINCH binding partner that is required to maintain adult viability.

Introduction

The 5LIM domain structure of PINCH suggests that it is a molecular scaffold, mediating protein-protein interactions that carry out downstream functions in the cell (Kadrmas and Beckerle, 2004). Although, the structure of PINCH conceptually could allow multiple interactions, very few have been described. LIM1 of PINCH has been shown to bind with high affinity to ILK in many organisms, and this interaction has been studied in various cell types in culture as well as in *Drosophila* (Clark et al., 2003; Tu et al., 2001; Tu et al., 1999). LIM4 of PINCH1 interacts weakly with NCK2, providing a link to growth factor signaling in mammalian cells, but this interaction has not been confirmed in other species (Tu et al., 1998). LIM5 of PINCH has been shown to bind with high affinity to the LRR protein Ras Suppressor-1 (RSU-1), in both mammalian cell culture and in *Drosophila* (Dougherty et al., 2005; Kadrmas et al., 2004). In *Drosophila*, RSU-1 is encoded by the *icarus* (*ics*) locus. Other proteins such as Thymosin β4, Hic5,

and PP1α have been described as LIM5 binding partners in mammalian systems, although these interactions have not been extensively characterized, nor have they been shown to be conserved in other species (Bock-Marquette et al., 2004; Eke et al., 2010; Mori et al., 2006).

Although RSU-1 was identified in 1992 (Cutler et al., 1992), the PINCH-RSU-1 interaction was identified in 2004 simultaneously by two labs using a Gal4-based yeast two-hybrid screen in mammalian cells and using a PINCH TAP-tag purification followed by mass spectrometry in *Drosophila* embryos and S2 cells (Dougherty et al., 2005; Kadrmas et al., 2004). The finding that PINCH and RSU-1 physically interact broadened the roles of RSU-1 as a Ras suppressor and JNK regulator to include that of an integrin effector, and introduced new questions about the role of RSU-1 as it relates to PINCH function.

Indeed, previous work in cell culture has demonstrated a role for RSU-1 in the regulation of Jun Kinase (JNK) signaling (Dougherty et al., 2005; Masuelli and Cutler, 1996). This finding was supported by work in *Drosophila* showing that both PINCH and RSU-1 are able to negatively regulate the JNK cascade (Kadrmas et al., 2004). Active JNK signaling is required for the developmental process of dorsal closure to occur properly, and mutations in many of the pathway genes result in embryonic lethality as evidenced by a dorsal open phenotype (Harden, 2002; Xia and Karin, 2004). Introduction of one mutant *stck* allele or the *ics* null mutation suppressed the dorsal open phenotype seen in *misshapen* hypomorphs, indicating that both PINCH and RSU-1 are capable of negative regulation of the JNK cascade. In stage 13 embryos undergoing dorsal closure, RSU-1 protein is reduced in *stck* mutants and PINCH protein is reduced in *ics* mutants,

indicating a mutual dependence for protein stability (Kadrmas et al., 2004). This observation is further supported by experiments in 293T cells where expression of an RSU-1 specific shRNA not only reduced RSU-1 protein levels but also reduced PINCH levels (Dougherty et al., 2005). PINCH and RSU-1 have been shown to colocalize in cell culture, and RSU-1 localizes to muscle attachment sites in *Drosophila* in the same pattern as PINCH (Dougherty et al., 2005; Kadrmas et al., 2004). Mammalian cells with reduced levels of RSU-1 demonstrate a marked loss of cell-matrix adhesion indicating that RSU-1 contributes to integrin function (Dougherty et al., 2005). In *Drosophila*, *ics* null flies are viable and fertile but display wing blisters, indicating a requirement for RSU-1 in maintaining adhesion of the wing epithelium (Kadrmas et al., 2004). This phenotype overlaps with *stck* null phenotypes in that null *stck* clones, specifically in the wing, result in blisters. However, the *stck* null phenotype overall is much more severe, resulting in lethality due to loss of actin-membrane linkages in the late embryo or early larvae (Clark et al., 2003). Work from Chapter 2 has shown that in *Drosophila*, RSU-1 may have more of an accessory role in healthy animals, but has a more critical function in maintaining viability in flies where the PINCH-ILK interaction has been disrupted.

To further understand the function of RSU-1 as a PINCH binding partner, we determined protein localization and dependence in *ics* and *stck* null animals. Furthermore, we performed a screen to identify point mutations in PINCH that disrupt the interaction with RSU-1 (D303V) and confirmed this in *Drosophila* S2 cells. We generated $PINCH^{D303V}$-GFP transgenic flies in order to determine if disruption of the PINCH-RSU1 interaction bears any consequence on viability, protein levels, localization and on the ability of both PINCH and RSU-1 to regulate JNK signaling. Given that *ics* null flies only

display a wing phenotype, we predicted that the PINCH^{D303V} flies would not display any severe defects, but would have wing blisters if the PINCH-RSU-1 interaction was required for integrin function in the wing. In order to determine whether the only role for LIM5 is to bind RSU-1 or if there are potential other roles for LIM5, we also generated transgenic flies where LIM5 of PINCH was deleted (PINCH$^{\Delta LIM5}$-GFP).

Our results demonstrate that RSU-1 is absent from muscle attachment sites in *stck*$^{17/18}$ maternal/zygotic null embryos, indicating that RSU-1 requires PINCH for its localization. However, PINCH localizes independently of RSU-1 at muscle attachment sites in *ics* null embryos. The PINCH^{D303V} transgene fully rescues the viability of the *stck* null mutant when two or more copies are introduced into the rescue cross. Although we observe full rescue, viable flies are unhealthy, display wing blisters, and are difficult to maintain as stocks. In contrast, the PINCH$^{\Delta LIM5}$ transgene is unable to rescue the *stck* null mutant, suggesting a functional difference between the two transgenes. PINCH^{D303V} is expressed at lower levels than endogenous PINCH, and RSU-1 levels are reduced in PINCH^{D303V} rescued flies to a greater extent than in PINCH^{wt} rescued flies. PINCH^{D303V} is able to localize to muscle attachment sites, and while RSU-1 is still detected in these animals, it does not localize indicating that the interaction between PINCH and RSU-1 is required to localize RSU-1 to muscle attachment sites. PINCH$^{\Delta LIM5}$ embryos do not survive past first instar larvae, and analysis of protein levels indicates that the transgenic protein may be unstable over time. We cannot, however, rule out a defect caused by the LIM5 deletion. Taken together these results provide initial evidence that disrupting the interaction between PINCH and RSU-1 has some functional consequences and further

investigation and optimization of reagents will be required to fully study the PINCH-RSU-1 interaction.

Materials and methods

Fly stocks

w^{1118} or PINCH^{wt}-GFP 4A flies are used as a controls in all experiments (Kadrmas et al., 2004). *ics* flies were described previously (Kadrmas et al., 2004). *stck*^{17} germ line clones were generated as previously described and were crossed to *stck*^{18}/TM3, Sb, Ser, Tw-GFP males to generate maternal and zygotic (m/z) null embryos (Clark et al., 2003).

Western blots

Adult flies were homogenized in 2x Laemmli Sample buffer and run on SDS-PAGE gels as previously described (Chapter 2). Antibodies used were RSU-1 (1:5000) (Kadrmas et al., 2004), PINCH (1:5000-1:10,000) (Clark et al., 2003), and Lamin (1:5000, DSHB). Secondary antibodies used were either HRP conjugated anti-mouse or anti-rabbit (1:5000) followed by detection with ECL (GE).

Immunofluorescence

Embryos were heat fixed using previously published protocols (Clark et al., 2003). Primary antibodies used were RSU-1 (preabsorbed against w^{1118} embryos, 1:1000 or affinity purified, 1:100) (Kadrmas et al., 2004), Actin C4 (1:500, MP Biomedicals), and GFP (1:500, Invitrogen). Secondary antibodies used were Alexafluor anti-rabbit 488 or 568 and anti-mouse 488 or 568 (Invitrogen).

Identification of PINCH^{D303V}

A low fidelity PCR strategy was used to introduce mutations into the PINCH LIM5 sequence. A mutant pool of DNA was cloned into a yeast-two hybrid bait vector and was introduced into yeast along with the wild-type RSU-1 prey (Kadrmas et al., 2004). Using a pink-white selection strategy, where white clones indicate a positive interaction and pink clones indicate a lack of interaction, individual pink clones were picked and sequenced. PINCH^{D303V} met the criteria for a good candidate to disrupt RSU-1 binding as it was a single point mutation in a residue not required to maintain LIM domain structure but was conserved among species.

Generation and validation of PINCH^{D303V} reagents

The mutation replacing aspartic acid at position 303 with valine (D303V) in *Drosophila* PINCH isoform A was introduced using a PCR mutagenesis strategy as previously described (Chapter 2). pMT-PINCH^{wt}-His and pMT-PINCH^{D303V}-His cDNA constructs were generated and expressed in *Drosophila* S2 cells according to standard protocols. Ni-NTA pull-downs and Western blots were performed as previously described (Chapter 2).

Generation of transgenic flies and rescue experiments

Fragments containing the D303V mutation or LIM5 deletion were generated by PCR and ligated into the original pCasper-PINCH^{wt}-GFP vector used to generate PINCH^{wt}-GFP transgenic flies (Kadrmas et al., 2004). Contructs were injected into wild-type embryos (Model System Genomics, Duke University). Rescue crosses were set as

previously described introducing one-four copies of the transgene in to a *stck* null animal (Chapter 2).

Results

RSU-1 localization at muscle attachment sites is dependent on PINCH

The PINCH-RSU-1 interaction has been described as strong and stoichiometric (Kadrmas et al., 2004). It is unknown whether all PINCH in the cell is bound to RSU-1 or vice versa, but the lack of other known binding partners for RSU-1 suggests that it functions by binding to PINCH. Previous work has shown PINCH and RSU-1 colocalization at muscle attachment sites in *Drosophila* embryos and at focal adhesions in cell culture, providing further evidence that they function together in the cell (Dougherty et al., 2005; Kadrmas et al., 2004). In order to determine if RSU-1 localization was dependent on PINCH, we stained $stck^{17/18}$ m/z null embryos for RSU-1 and actin (Fig. 4.1). We find that in $stck^{17/18}$ m/z embryos that RSU-1 is unable to localize to muscle attachment sites where it properly localizes in w^{1118} embryos, indicating that RSU-1 localization requires PINCH (Fig. 4.1 A,A",B,B"). Conversely, we labeled *ics* embryos for PINCH and actin and demonstrate that PINCH still localizes at muscle attachment sites in a similar pattern as in w^{1118} embryos indicating that other interactions, such as ILK, are capable of anchoring PINCH (Fig. 4.1 C,C",D,D"). This observation is supported by the fact that *ics* null flies are viable and fertile and that loss of RSU-1 does not have any catastrophic effects on PINCH function.

Figure 4.1: RSU-1 localization requires PINCH, but PINCH localization does not require RSU-1. w^{1118} (A, A', A") and $stck^{17}/stck^{18}$ m/z null (B, B', and B") stage 16-17 embryos were stained with RSU-1 (A, A",B, B") and actin (A', A". B',B") antibodies to label muscle attachment sites and body wall muscles. RSU-1 does not localize to muscle attachment sites in $stck^{17}/stck^{18}$ m/z null embryos (B), where it normally localizes in w^{1118} embryos (A). w^{1118} (C, C',C") and *ics* (D, D', D") stage 16-17 embryos were stained with PINCH (C,D, C",D") and actin (C',C",D',D") antibodies. PINCH localizes to muscle attachment sites in the absence of RSU-1 (D) in a similar manner as localization in w^{1118} embryos (C).

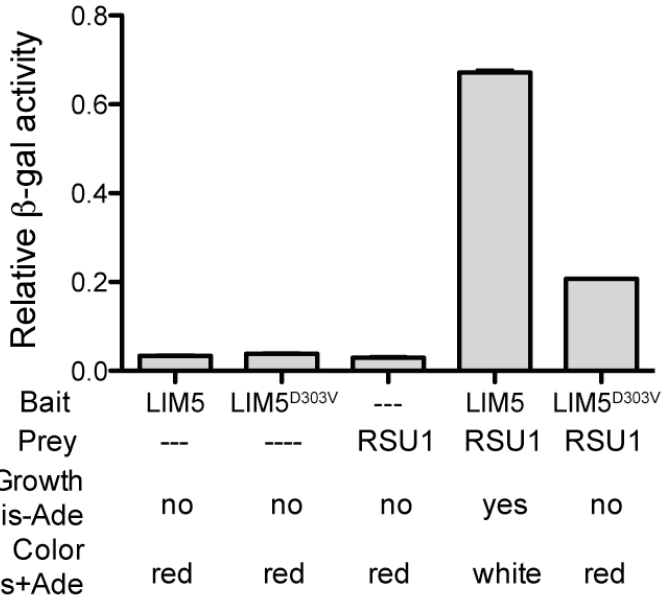
RSU-1
actin
merge
A
A'
A"
B
B'
B"
50μm
PINCH
C
C'
C"
D
D'
D"
50μm
w1118
stck17/stck18 m/z
w1118
ics

PINCH^{D303V} does not interact with RSU-1

Previous work in our lab has demonstrated a role for both PINCH and RSU-1 in the negative regulation of the JNK cascade, and that loss of either PINCH or RSU-1 affects the protein levels of the other (Kadrmas et al., 2004). We were interested in understanding whether the regulation of JNK pathway members and of protein levels was dependent on the interaction between PINCH and RSU-1. In order to further address the role of the PINCH-RSU-1 interaction *in vivo*, we performed a PCR based mutagenesis screen followed by yeast two hybrid analysis. We isolated a point mutation in LIM5 of PINCH changing an aspartic acid at position 303 of *Drosophila* PINCH isoform A to a Valine (D303V). Under the conditions tested by yeast two-hybrid, LIM5 of PINCH containing the D303V mutation showed a greatly reduced interaction with RSU-1, compared to the robust interaction observed between wild-type LIM5 and RSU-1 (Fig. 4.2 A). Sequence alignment of LIM5 from various species demonstrates that D303 is highly conserved and is unique to LIM5 of PINCH (Fig. 4.2 B,C). To test whether this mutation was relevant in the context of full length PINCH, PINCH^{wt} and PINCH^{D303V} His-tagged cDNAs were generated and expressed in S2 cells. Ni-NTA pull-downs were performed, followed by Western analysis to look for proteins of interest. Both PINCH^{wt} and PINCH^{D303V} mutants were expressed in S2 cells, and were enriched in the pull-down. We note that PINCH^{wt}-His is able to pull down the LIM1 binding partner ILK and the LIM5 binding partner RSU-1, but that PINCH^{D303V}-His is only able to pull down ILK, but not RSU-1 (Fig. 4.2 D). This *in vitro* experiment demonstrates that PINCH^{D303V} is expressed and retains an interaction with another known PINCH binding partner, giving us confidence that this mutant could be used for further *in vivo* analysis.

Figure 4.2: Identification and validation of the PINCH^{D303V} mutation. (A) A graph representing yeast-two hybrid data demonstrates a robust interaction between wild-type LIM5 and RSU-1 and a reduced interaction between LIM5^{D303V} and RSU-1. Growth on -Ade/-His plates, and white colony color indicates a positive interaction, while lack of growth on -Ade/-His plates and pink colony color indicates a lack of interaction. (B) Sequence alignment of *Drosophila*, mouse, and human PINCH demonstrates that D303V is conserved among species. (C) The PINCH LIM domain consensus sequence demonstrates that the Aspartic Acid at position 303 in LIM5 is unique among equivalent positions in the other LIM domains of PINCH. (D) Ni-NTA pull downs of PINCH^{wt}-His and PINCH^{D303V}-His demonstrate that PINCH^{D303V}-His does not interact with RSU-1 but retains the capacity to bind to ILK. All proteins were present in the starting material. PINCH-His species are marked with *, while endogenous PINCH is marked with a <.

A



B

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dPINCH  (287) SVCDTKMTQKSKFYEYDEKPVCKKCYDRFPNELRRRLRTAHEMTMKKNT-
mPINCH-1(291) STCNTKLTLKNKFVEFDMKPVCKKCYEKFPLELKKRLKKLSETLGRK---
mPINCH-2(284) STCNMKLTLKNKFVEFDMKPVCKRCYERFPLELKKRLKKLSDLSSRKAQP
hPINCH-1(279) STCNTKLTLKNKFVEFDMKPVCKKCYEKFPLELKKRLKKLAETLGRK---
hPINCH2 (284) STCNSKLTLKDKFVEFDMKPVCKRCYEKFPLELKKRLKKLSELTSRKAQ
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C

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dPINCH/LIM1 CTRCADGFEPTEKIVNSNGELWHTQCFVCAQCFRPFQD-GIFYEFE--------GRKYCERDF
dPINCH/LIM2 CNKCGEF--VIGRVIKAMSASWHPQCFRCQLCAKELAD-CGFIKNQ--------NRALCHECN
dPINCH/LIM3 CQKCHG--LIDEEPLRFRGEVYHGYHFSCTACGTELDSTAREVKSRPGLAANDMNELYCLRCH
dPINCH/LIM4 CGACRR--PIEERVVTALGKHWHVEHFVCAKCEKPFLG-HRHYEKR--------GLAYCETHY
dPINCH/LIM5 CFVCNQ--VIGGDVFTALNKAWCVHHFACSVCDTKMTQKSKFYEYD--------EKPVCKKCY
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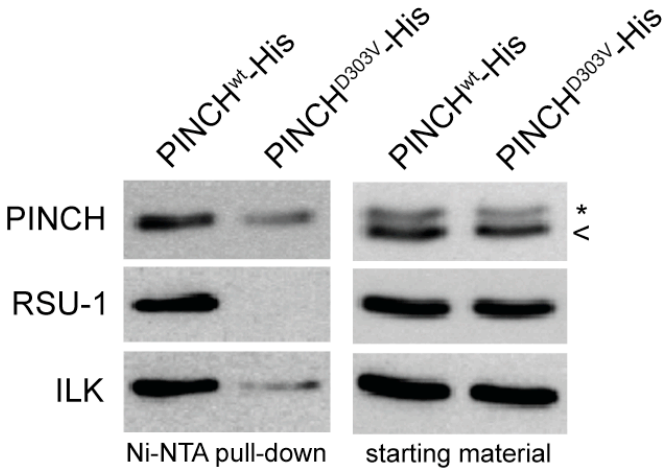
D



PINCH^{D303V} and PINCH$^{\Delta LIM5}$ demonstrate different abilities to rescue the PINCH null

To test the *in vivo* function of PINCH-RSU-1 complexes, we generated transgenic flies carrying the PINCH^{D303V} mutation. Knowing that *ics* null flies have a very mild phenotype, we also generated transgenic flies where the entire LIM5 domain was deleted (PINCH$^{\Delta LIM5}$). Differences between PINCH^{D303V} and PINCH$^{\Delta LIM5}$ would potentially allow us to determine if there are other functions for LIM5 besides binding to RSU-1. Mutant fragments of *Drosophila* PINCH were generated using an overlap extension PCR strategy to introduce either the point mutation or deletion (Fig. 4.3 A,B). These fragments were then cloned into a previously described genomic PINCH^{wt}-GFP construct under control of the endogenous PINCH promoter, and injected into *Drosophila* embryos. Resulting transgenic lines were isolated, and mapped, and crosses were performed to generate stocks to test for rescue of the *stck* null mutant (Table 4.1). Initial rescue crosses were performed introducing one copy of the PINCH^{D303V}-GFP or PINCH$^{\Delta LIM5}$ transgenes into a PINCH null background, using two different combinations of *stck* null alleles (*stck*^{18}/*l(3)097* and *stck*^{17}/*stck*^{18}). Surprisingly, the PINCH^{D303V} transgene gave variable and mediocre rescue (2%-59%) and the PINCH$^{\Delta LIM5}$ did not rescue at all (Table 4.2). PINCH^{D303V} rescued flies displayed severe wing blisters at high frequency (data not shown).

Western blots of adult fly lysates from one PINCH^{D303V} rescued line (D4) and a PINCH$^{\Delta LIM5}$ transgenic line in which endogenous PINCH was present (A4) demonstrate low levels of transgenic PINCH compared to endogenous PINCH levels (Fig. 4.4 A). Of note, the previously described PINCH^{wt}-GFP 4A rescued flies also express lower levels

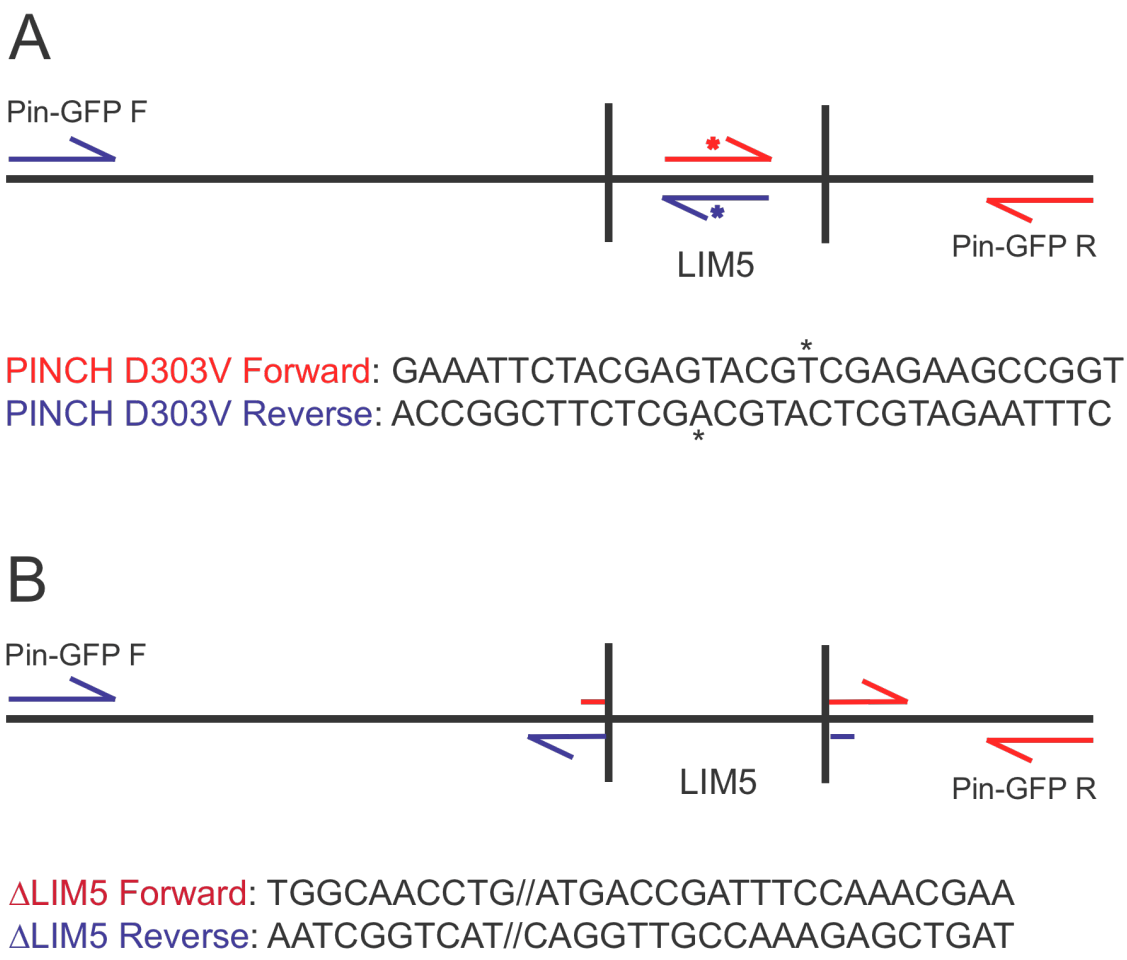


Figure 4.3: PCR scheme to generate the PINCH^{D303V} mutation and the PINCH$^{\Delta LIM5}$ deletion fragment. (A) A schematic demonstrating two sets of primers to generate 5' (blue) and 3' (red) fragments of LIM5 containing the D303V mutation. The nucleotide change to introduce the mutation is marked with *. Both fragments were used as template in a second PCR reaction using the PIN-GFP F/R primers to generate a single overlap product. The resulting fragment was digested with appropriate restriction enzymes and ligated into the pCasper vector containing the PINCH^{wt}-GFP sequence under control of the endogenous PINCH promoter. (B) A schematic demonstrating two sets of primers to introduce the LIM5 deletion. The // denotes the LIM5 border with a tail sequence generated on the opposite side of the LIM5 for both 5' and 3' fragments. The remaining steps were completed in the same manner as the PINCH^{D303V} construct.

Table 4.1

PINCH^{D303V} and PINCH$^{\Delta LIM5}$ transgenic lines.

Insertion ID	Chromosomal Location	Homozygous Viable?
D303V B5	X	YES
D303V D4	X	YES
D303V B10	2	NO
D303V B5	3	YES
D303V B31	3	NO
ΔLIM5 A4	3	NO
ΔLIM5 A17	3	YES
ΔLIM5 A19	3	YES
ΔLIM5 A25	2	YES
ΔLIM5 B1	3	YES
ΔLIM5 B32	2	YES

Each individual insertion line is listed with its original identification. Insertion lines were mapped to single chromosomes and it was determined whether each transgene was homozygous viable in a *stck* wild- type background.

Table 4.2

PINCH^{D303V} and PINCH$^{\Delta LIM5}$ rescue data.

Insertion ID	% Rescue (one copy) *stck*^{18}/*l(3)097*	% Rescue (one copy) *stck*^{18}/*stck*^{17}	% Rescue (two copy) *stck*^{18}/*stck*^{17}
D303V D4	57% (n=187)	48% (n=221)	78% (n=279)
D303V B10	59% (n=186)	17% (n=409)	
D303V B5[1]	22% (n=159)	30% (n=243)	85% (n=657)
D303V B31	3% (n=187)	2% (n=411)	
ΔLIM5 A17	0% (n=250)	0% (n=244)	
ΔLIM5 A19	0% (n=92)	0% (n=234)	
ΔLIM5 A25	0% (n=234)	0% (n=224)	
ΔLIM5 B32	0% (n=299)	0% (n=250)	

[1]B5 was originally mapped to chromosome 2, but out crossing and remapping revealed two insertions, one on the X and one on 3 (noted in Table 1). Two copy rescue was performed with the 3rd chromosome insertion

Four individual insertion lines for each transgene were used to test for rescue of the *stck* null mutant using two combinations of PINCH null alleles (*stck*^{17}/*l(3)097* and *stck*^{17}/*stck*^{18}). Variable rescue was observed when one copy of PINCH^{D303V} transgene was introduced into the rescue cross (2%-59%) and was ameliorated by increasing the dosage to two copies of the transgene in the rescue cross (48% to 78% and 30% to 85%). The PINCH$^{\Delta LIM5}$ transgene did not rescue the lethality of the *stck* null mutant in all crosses set. Examples of crosses set for one and two copy rescue: D303V^{B5},*stck*^{18}/TM3,Sb x *stck*^{17}/TM3, Sb and D303V^{B5},*stck*^{18}/TM3,Sb x D303V^{B5},*stck*^{17}/TM3, Sb. Rescued progeny would have the following genotypes: D303V^{B5},*stck*^{18}/ *stck*^{17} or D303V^{B5},*stck*^{18}/ D303V^{B5},*stck*^{17}.

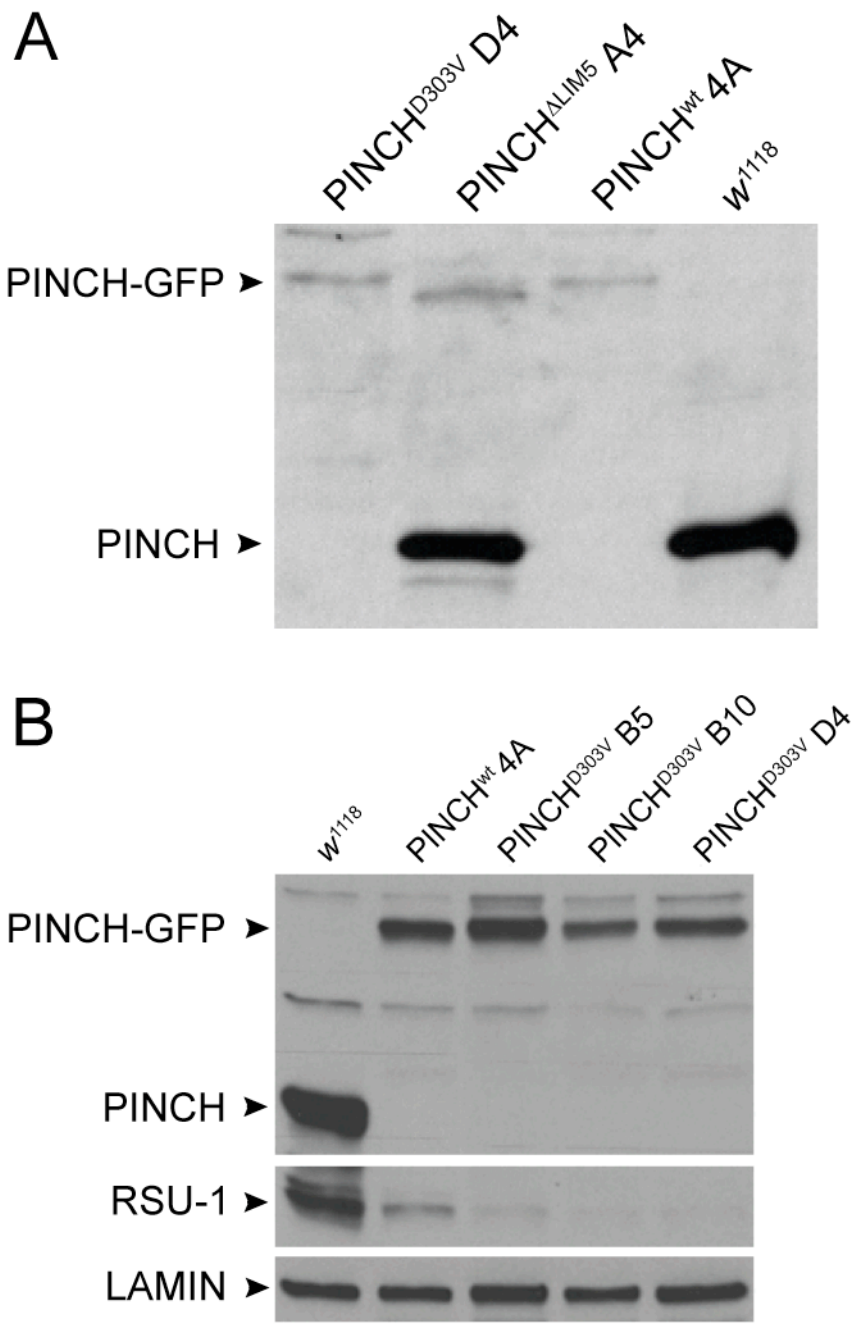


Figure 4.4: Protein levels of PINCH^{D303V} and PINCH$^{\Delta LIM5}$ are reduced compared to endogenous PINCH. (A) Western blots of lysates from PINCH^{wt} and PINCH^{D303V} rescued flies compared PINCH$^{\Delta LIM5}$ in a background containing endogenous PINCH. w^{1118} lysate is a control for endogenous levels of PINCH. (B) Western blots of lysates from PINCH^{D303V} rescued flies representing three insertion lines. Levels of transgenic PINCH are comparable to PINCH^{wt} control lysates, but all GFP tagged transgenic lines express at reduced levels compared to endogenous PINCH. Levels of RSU-1 are reduced in PINCH^{D303V} rescued flies compared to a PINCH^{wt} rescued control.

of transgenic PINCH than endogenous levels seen in w^{1118} flies. This low level of PINCH^{wt}-GFP is sufficient to rescue the *stck* null mutant and to maintain a rescued stock. Furthermore, PINCH^{wt}-GFP flies display a weak wing blister phenotype presumably from low level expression of the transgene. We observe a band corresponding to PINCH$^{\Delta LIM5}$ that is slightly smaller than the full length PINCH-GFP species indicating that the transgene is expressed and demonstrating the presence of the LIM5 deletion (Fig. 4.4 A). To determine PINCH and RSU-1 levels in all PINCH^{D303V} rescued flies, we performed Western blots using lysates from adult rescued flies, and probed for PINCH, RSU-1, and Lamin (Fig. 4.4 B). We observe reduced levels of transgenic PINCH compared to endogenous PINCH, although PINCH^{D303V} levels are comparable to PINCH^{wt} levels. Interestingly, we observe lower levels of RSU-1 in all PINCH^{D303V} lines compared to PINCH^{wt} rescued flies, indicating that disruption of the PINCH-RSU-1 interaction could affect RSU-1 protein stability. In order to determine whether we had indeed disrupted the interaction with RSU-1 *in vivo*, we stained late stage embryos with GFP and RSU-1 (Fig. 4.5). PINCH^{wt} rescued embryos demonstrate that PINCH and RSU-1 colocalize at muscle attachment sites (Fig. 4.5 A, A',A"). However in the PINCH^{D303V} rescued embryos, PINCH localizes at muscle attachment sites (Fig. 4.5 B,B"), but there is a lack of detectable localization of RSU-1 (Fig. 4.5 B',B"). This result confirms what has been previously been observed in *stck* null embryos that RSU-1 requires PINCH for its localization at muscle attachment sites, and that the D303V mutation is sufficient to disrupt binding and localization of RSU-1.

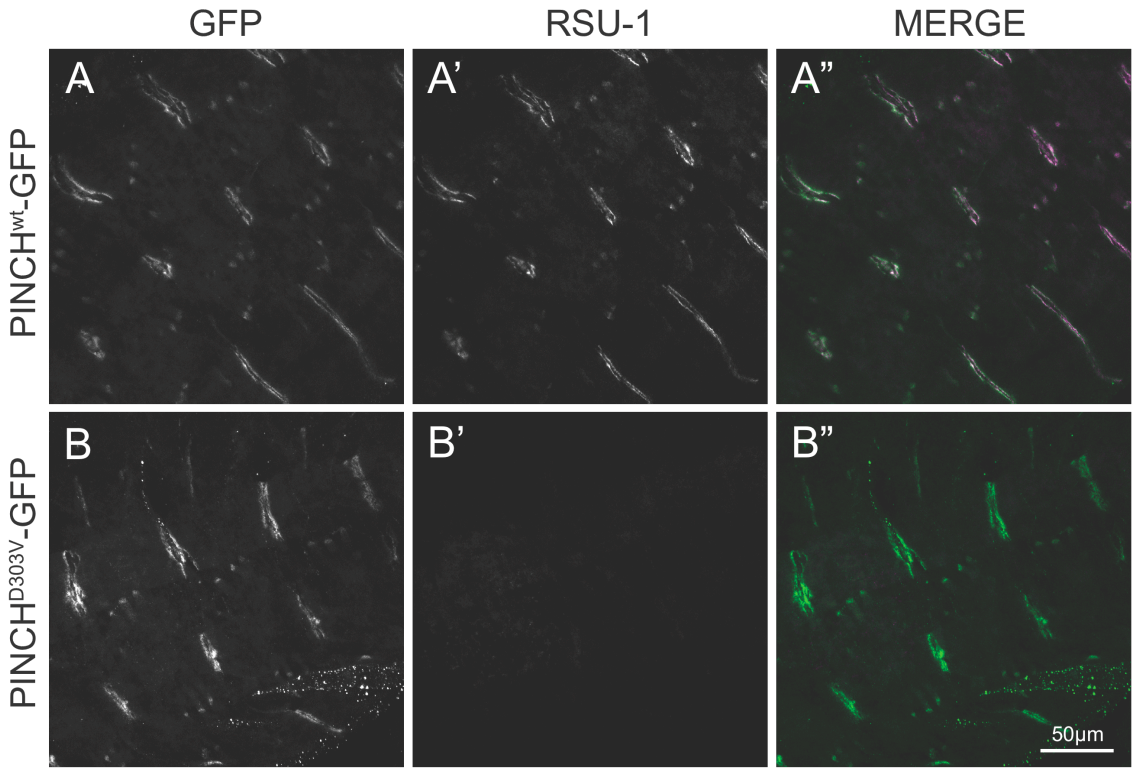


Figure 4.5: RSU-1 localization to embryonic muscle attachment sites is dependent on the interaction with PINCH. PINCH^{wt} (A, A', A") and PINCH^{D303V} (B,B',B") rescued embryos were stained with antibodies against GFP (to mark transgenic PINCH) (A,A",B,B") and RSU-1 (A',A",B',B"). RSU-1 does not localize to muscle attachment sites in PINCH^{D303V} rescued embryos (B'B"), where as RSU-1 localization is normal in PINCH^{wt} rescued embryos (A',A").

PINCH$^{\Delta LIM5}$ transgenic animals demonstrate reduced protein levels

In order to characterize the lethality of the PINCH$^{\Delta LIM5}$ transgenic animals, we collected rescued embryos and monitored them over time. We observe that PINCH$^{\Delta LIM5}$ transgenic animals are not surviving past the first larval instar (L1), and that surviving L1s are sluggish and stumpy in appearance (data not shown). One explanation for this lethality is that once the maternal contribution of PINCH is depleted, that PINCH$^{\Delta LIM5}$ does not support viability due to a critical role for LIM5. Alternatively, once the maternal contribution of PINCH is depleted, if PINCH$^{\Delta LIM5}$ is unstable or not expressed at sufficient levels we would expect to see lethality around the time of a *stck* null mutant (as late embryos or early L1). In order to distinguish between these two possibilities, we collected PINCH^{wt} and PINCH$^{\Delta LIM5}$ rescued embryos and surviving L1s and performed Western blots to determine PINCH levels (Fig. 4.6). In samples from PINCH^{wt} stage 12-13 embryos, stage 16-17 embryos, and in L1, we observe a lack of endogenous PINCH and only the higher migrating PINCH^{wt}-GFP species. In comparison, PINCH$^{\Delta LIM5}$ samples, collected from a rescue cross, demonstrate residual maternal PINCH that decreases over time. Levels of PINCH$^{\Delta LIM5}$-GFP are reduced compared to PINCH^{wt}-GFP and the levels of PINCH$^{\Delta LIM5}$-GFP do not increase over time as the maternal contribution is depleted, suggesting that the protein is unstable and therefore unable to support viability in the absence of endogenous PINCH (Fig. 4.6). We repeated rescue crosses introducing three copies of the PINCH$^{\Delta LIM5}$ transgene and again did not observe any rescued progeny (data not shown). We conclude from these experiments that the lethality observed in PINCH$^{\Delta LIM5}$ rescue is more likely due to the loss of endogenous PINCH protein and insufficient expression of the transgene. We cannot however rule out that

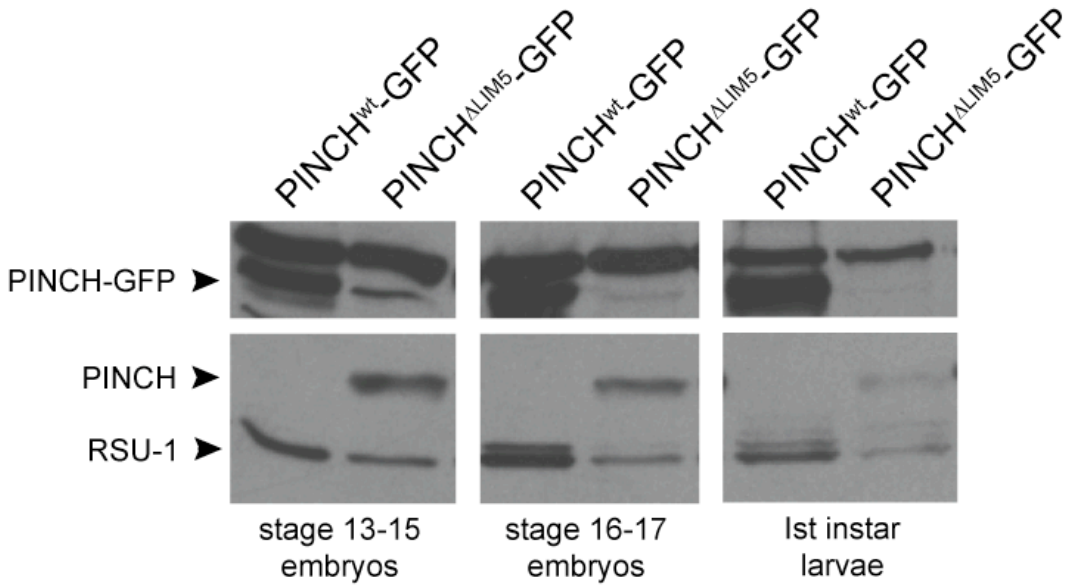


Figure 4.6: PINCH$^{\Delta LIM5}$ protein levels decrease during during development. A PINCH^{wt} rescued line and a PINCH$^{\Delta LIM5}$ rescue cross were set in laying pots, and rescued embryos and first instar larvae were collected. Western blots of stage 12-13 embryos demonstrate maternal PINCH present in PINCH$^{\Delta LIM5}$ and reduced levels of transgenic PINCH compared to PINCH^{wt} rescued animals. Over time, endogenous maternal PINCH is depleted, although not completely absent by the first larval instar. A decrease is also observed over time in PINCH$^{\Delta LIM5}$ transgenic protein levels indicating a lack of upregulation of transgene expression in rescued animals.

lethality is due to a defect caused by loss of LIM5. Any future work involving deletion of LIM domains will require modifications to the strategy used to generate transgenic flies to ensure more appropriate protein levels.

PINCH^{D303V} rescue is dose dependent and is not improved by loss of RSU-1

In order to determine whether the partial rescue observed with the PINCH^{D303V} flies was due to reduced transgenic PINCH levels, we repeated the rescue crosses introducing two copies of the transgene into progeny (Table 4.2). We observe an increase in viable progeny (48% to 78% and 30% to 85%) in the two insertion lines tested indicating that increased dosage of PINCH^{D303V} is favorable for viability. While we see improved rescue overall, stocks were still very difficult to maintain. One possible explanation for these results is that while an increased dose of PINCH improves viability, transgenic protein levels are still reduced compared to endogenous levels of PINCH. To test this idea, we introduced four copies of the transgene into rescued progeny. We observe similar rescue introducing four copies of the transgene into rescued flies (75%) as we do with two copies on the X or 3rd chromosome (78% and 85%), indicating that we have achieved the rescue capable with this specific transgene (Table 4.3). Stocks with four copies of the PINCH^{D303V} transgene do not seem to be any healthier than those generated with two copies, suggesting that alternatively there may be some consequence to disrupting the PINCH-RSU-1 interaction *in vivo*.

We considered that the normal function of PINCH is to inhibit RSU-1, and that loss of the PINCH-RSU-1 interaction renders a normally bound and inactive RSU-1 free to do harm in the cell. This situation is potentially worse than the complete loss of RSU-

Table 4.3

PINCH^{D303V} rescue with increased copy number of the transgene
and in the absence of RSU-1

Insertion ID	**% Rescue (two copy)** ***stck*^{18}/*stck*^{17}**	**% Rescue (four copy)** ***stck*^{18}/*stck*^{17}**
D303V D4	78% (n=279)	
ics; D303V D4	1% (n=182)	
D303V B5	85% (n=657)	
ics; D303V B5	43% (n=237)	
D303V D4/B5		75% (n=437)
ics; D303V D4/B5		29% (n=311)

PINCH^{D303V} transgenes on the X and 3rd chromosomes were combined into individual stocks used to test for rescue. Progeny from these crosses contain 4 copies of the transgene. Increasing the PINCH^{D303V} dosage from two to four copies did not affect the percent rescue observed (78% and 85% with two copies to 75% with four copies). Removal of RSU-1(*ics*) in the rescue crosses introducing either two or four copies of the transgene resulted in reduced numbers of viable rescued progeny (78% to 1%, 85% to 43%, and 75% to 29%).

1, where we observe healthy adult flies that only display a defect in wing adhesion. To test whether the weak viability of PINCH^{D303V} rescued flies can be ameliorated by loss of RSU-1, we introduced the *ics* mutation into stocks carrying two or four copies of the transgene and tested for rescue (Table 4.3). In all cases, we observe a reduction in viable progeny when the *ics* mutation is present. Furthermore, stocks of PINCH^{D303V} flies lacking RSU-1 (*ics*; PINCH^{D303V}) could not be maintained. This result suggests that the defect observed in PINCH^{D303V} rescued flies is not due to unbound RSU-1 in the cell.

Based on the data demonstrating that PINCH protein levels are reduced in *ics* mutant flies, we hypothesized that loss of RSU-1 in PINCH^{D303V} rescued flies (which already have reduced PINCH) further reduces PINCH to levels that cannot support viability. Western blots of adult rescued flies demonstrate that *ics*; PINCH^{D303V} flies do indeed display reduced PINCH protein levels compared to PINCH^{D303V} flies where RSU-1 is present (Fig. 4.7). This is more evident in the *ics*; PINCH^{D303V} D4 samples and is supported by the rescue percents calculated with and without the *ics* mutation (78% and 1%) (Table 4.3). Further investigation of this regulation is required and will include appropriate loading controls as well as including an *ics*; PINCH^{wt} sample for comparison. We cannot exclude that in the case where reduced levels of transgenic PINCH-GFP are still able to support viability, that slight variations in expression may cause greater effects than predicted.

The observation that *ics*; PINCH^{D303V} rescued flies display reduced viability compared to PINCH^{D303V} rescue alone is inconsistent with the mild phenotype observed in *ics* null flies. The results presented so far suggest another possible hypothesis to explain the weak viability observed in PINCH^{D303V} rescued flies. We cannot exclude the

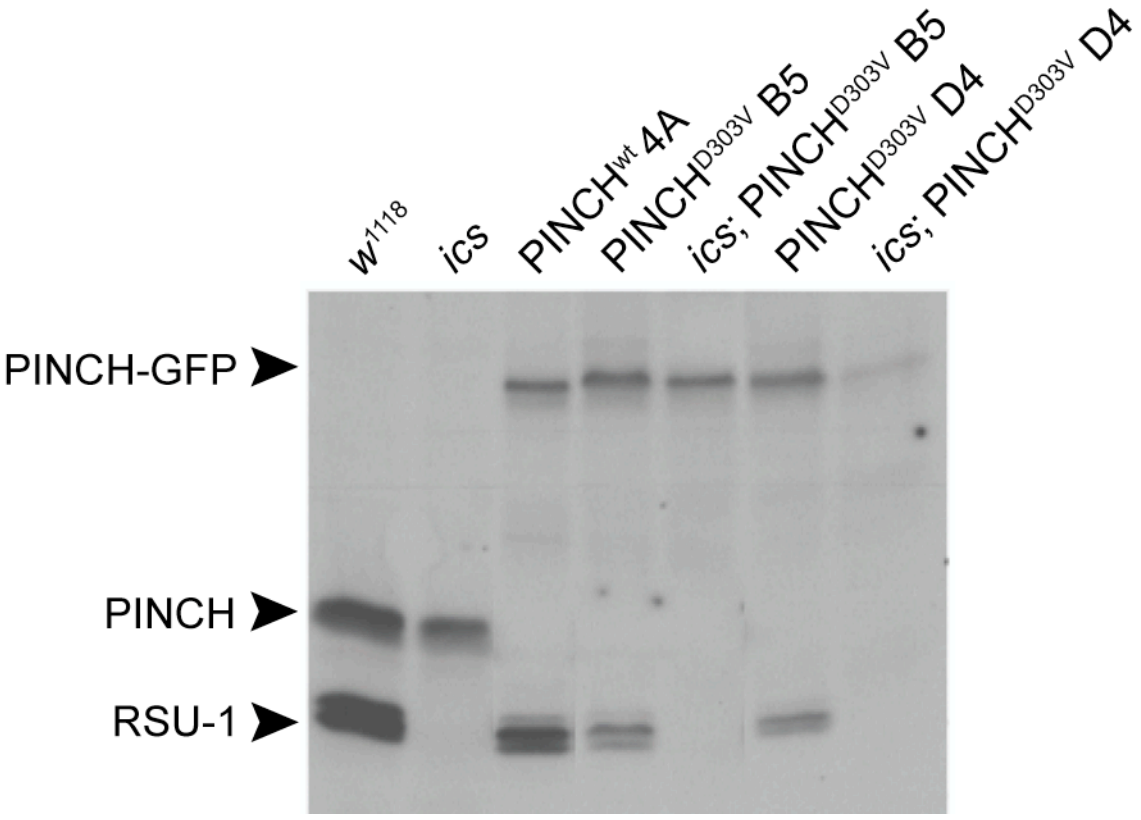


Figure 4.7: PINCH^{D303V} levels are reduced in rescued progeny that lack RSU-1. Western blots of adult fly lysates demonstrate that PINCH^{D303V} rescued flies that lack RSU-1 (*ics*; PINCH^{D303V}) have reduced PINCH protein compared to PINCH^{D303V} rescued flies that express RSU-1. The effect is much greater in the PINCH^{D303V} D4 line compared to the B5 line. This experiment also confirms results from Figure 4.4, where PINCH^{D303V} rescued lines express less RSU-1 overall compared to a PINCH^{wt} rescued control.

possibility that the D303V mutation disrupts another unknown PINCH binding partner that may or may not also interact with RSU-1. In an *ics* null animal, this interaction with PINCH is preserved. However, in a PINCH^{D303V} rescued animal, the interaction with RSU-1 and the unknown protein is sufficient to cause a reduction in viability. If this unknown protein also interacts and is somehow regulated by RSU-1, then the loss of RSU-1 may be sufficient to cause the decrease in viability observed in *ics*; PINCH^{D303V} rescued flies.

Together, these results demonstrate that disruption of the PINCH-RSU-1 interaction appears to affect the general health of adult rescued flies, and that loss of RSU-1 further reduces the viability observed in PINCH^{D303V} rescued flies. In conclusion, although PINCH^{D303V} transgenic flies display reduced protein levels compared to endogenous PINCH, levels are comparable to PINCH^{wt} transgenic flies indicating sufficient protein to support viability. Alternatively, we propose a novel role for LIM5 of PINCH involving a binding partner in addition to RSU-1. Further investigation of this idea will require the implementation of alternate strategies to ensure appropriate protein expression and to confirm or refute the existence of other LIM5 binding partners.

Discussion

The ability of PINCH^{D303V} and PINCH$^{\Delta LIM5}$ to rescue the *stck* null could be dependent on transgene expression

The PINCH^{D303V} transgene is able to fully rescue the lethality of the *stck* null mutant when at least two copies of the transgene are introduced in the rescue cross. However, adult flies display some defect, as it is difficult to maintain viable stocks of rescued flies. In contrast, the PINCH$^{\Delta LIM5}$ transgene is not able to rescue the lethality of

the *stck* null even in the presence of multiple copies of the transgene. While these results suggest that LIM5 could have functions other than binding to RSU-1, the inability of these transgenes to express at levels comparable to wild-type PINCH may be contributing to their lack of viability. One argument against this is that levels of $PINCH^{wt}$-GFP are also much lower than levels of endogenous PINCH, but that we are able to maintain stocks of these flies. While PINCH levels are low in all GFP-tagged transgenic flies, the $PINCH^{wt}$ flies may be at a slight advantage over $PINCH^{D303V}$ flies such that even a slight perturbance in $PINCH^{D303V}$ levels could result in a reduced viability.

Reduced levels of transgenic PINCH-GFP has made interpretation of results using different mutations and deletions very difficult. To address this, we have generated transgenic flies using a 3xFlag tag instead of GFP, and have demonstrated that these transgenes express at levels comparable to endogenous PINCH (Chapter 2). Appropriate transgenic PINCH levels assist in the interpretation of results based on the specific mutations introduced, and eliminate some concern that reduced levels are contributing to altered function or viability. Any future work involving transgenes that disrupt the interaction between LIM5 and RSU-1 will be made using tags (such as 3xFlag) that allow for appropriate PINCH expression.

Based on our initial analysis, the $PINCH^{\Delta LIM5}$ transgene appears to have en even greater reduction in protein levels than that seen with either $PINCH^{wt}$ or $PINCH^{D303V}$ transgenes. It is possible that deletion of entire domains of PINCH could cause protein instability. We observe that $PINCH^{\Delta LIM5}$ is expressed, but that levels seem to decrease over time even as the maternal contribution of PINCH is depleted (Fig. 4.6). This issue can be addressed by generating transgenic flies using the same genomic PINCH but with

a 3xFlag tag to improve expression. Alternatively, the generation of UAS-PINCH$^{\Delta LIM5}$ transgenic flies could also overcome the issue of decreased protein stability, as use of the Gal4-UAS system allows the over-expression of transgenes in a spacial and/or temporal manner.

PINCH LIM5 may have other functions than binding to RSU-1

We did not expect that PINCH^{D303V} rescued flies would exhibit any severe defects since *ics* null flies are viable and fertile and only display wing blisters. However, the weak viability of PINCH^{D303V} resuced flies suggests an alternative explanation where PINCH^{D303V} disrupts another LIM5 binding partner. Indeed, other protein interactions for LIM5 have been described in mammalian cells such as Thymosin β4, Hic5, and PP1α, but they have not been verified in *Drosophila* (Bock-Marquette et al., 2004; Eke et al., 2010; Mori et al., 2006). Furthermore, published work in mammalian cell culture has shown that LIM5 and the C-terminal tail are important for PINCH function (Xu et al., 2005). In order to address the possible other functions for LIM5, we can test other LIM5 mutations identified in the screen that disrupt the interaction with RSU-1 and determine if they behave in a similar manner as the D303V mutation. Another mutation identified was K305E, which is only two amino acids away from D303V, and could confirm or refute what we have observed so far. Furthermore, we have employed a similar mutagenesis strategy to identify mutations in RSU-1 that abolish binding with PINCH. RSU-1^{N102D}-3xFlag transgenic lines have been generated and initial characterization of these flies demonstrates that transgenes are expressed at levels comparable to or even greater than endogenous RSU-1 and can rescue the *ics* null wing blister phenotype. These reagents

also provide additional tools to determine whether it is the PINCH-RSU-1 interaction that is important for function or whether other PINCH binding partners are being affected.

Future investigation of the roles for PINCH and RSU-1

in regulation of the JNK cascade

It was proposed that by generating PINCH^{D303V} transgenic flies that we could test whether the PINCH-RSU-1 interaction was required for the regulation of JNK signaling in the fly. The technical issues raised by these flies did not allow us to perform these experiments. Preliminary work studying the roles of PINCH and RSU-1 were in embryos undergoing dorsal closure. It will be interesting to investigate how PINCH and RSU-1 affect JNK signaling in the developing wing, a two layered epithelium that requires integrin-mediated adhesion. PINCH and RSU-1 are expressed in the wing disc and *ics* flies display wing blisters indicating that there may be differences in how RSU-1 functions in the muscle and wing (Kadrmas et al., 2004). Future experiments can be carried with PINCH^{D303V} transgenic flies with increased protein expression or with the RSU-1^{N102D}-3xFlag transgenic flies. Comparison of results using different strategies may help create a clearer picture of the functions of the PINCH-RSU-1 interaction *in vivo*.

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CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

Thesis summary

Integrin-mediated adhesion and migration are required for developmental processes and for the maintenance of adult tissues. The details of how adhesion is regulated at the cellular level are critical for our understanding of these biological processes. While the integrin receptors do not have any catalytic activity of their own, it is the binding of protein complexes to the cytoplasmic tail of integrins that is responsible for downstream signaling events and regulation of the actin cytoskeleton.

PINCH, ILK, and RSU-1 are three such downstream proteins that form a physical complex downstream of integrins, and have well described and overlapping roles in integrin-mediated adhesion. The N-terminal LIM1 domain of PINCH binds directly to the N-terminal ANKR domain of ILK and these two proteins demonstrate many similar functions with regard to integrin function in various experimental systems including cell culture, mice, worms, and flies (Legate et al., 2006; Wickstrom et al., 2010). The C-terminal LIM5 domain of PINCH binds to the LRR motif of RSU-1 (Dougherty et al., 2005). In *Drosophila*, *ics* (RSU-1) mutants are viable and fertile, but display wing blisters, which is also a phenotype caused by loss of PINCH specifically in the wing (Clark et al., 2003; Kadrmas et al., 2004). Despite what has been currently published for RSU-1, it is unclear how RSU-1 functions and is regulated in the cell and whether either of these involves the interaction with PINCH. Since PINCH is the central protein in this complex, making a contact with both ILK and RSU-1, a domain analysis of PINCH was designed to study the role of PINCH with ILK and RSU-1 to understand how these three proteins contribute to integrin function in *Drosophila*.

Summary and interpretation of results

PINCH^{Q38A}

We hypothesized that the interaction between PINCH and ILK would be required for some or all of their functions. To test this, we generated transgenic flies carrying either PINCH^{wt}-3xFlag or PINCH^{Q38A}-3xFlag constructs under control of the native PINCH promoter. To our surprise, specific disruption of the PINCH-ILK interaction did not cause any overt phenotypes in rescued flies and PINCH localization and function were normal (Fig. 2.2, 2.4). Only when the *ics* mutation was introduced into PINCH^{Q38A} rescued animals were consequences observed in development and overall viability (Fig. 2.6). These findings shed new light on PINCH, ILK, and RSU-1 function. First, these results demonstrate that in *Drosophila,* PINCH can carry out all the functions tested here independently of an interaction with ILK, and indicate that PINCH has alternate modes for localization to adhesion sites and for stabilization of the actin cytoskeleton. Second, these results demonstrate some functional role for RSU-1 that is only observed under circumstances where PINCH-ILK binding is compromised. We demonstrate that the reduction in viability observed in *ics*; PINCH^{Q38A} flies is not due to a reduction in PINCH protein. We have not ruled out the possibility that loss of RSU-1 causes mislocalization of PINCH^{Q38A} or that we have uncovered a novel role for RSU-1. We demonstrate that disruption of the PINCH-ILK interaction is not sufficient to alter protein stability of either PINCH or ILK, which is in contrast to changes in protein stability observed in *stck*, *ilk*, and *ics* null mutants (Fig. 2.5). This result supports the idea that complete loss of one protein likely disrupts many protein contacts, and is indicative of the complexity of interactions that are possible. These results suggest that, *in vivo*, adhesion sites have a

built in redundancy that is capable of coping with the loss of one interaction, but that defects are observed when more than one interaction is disrupted. This idea is supported by the large number of molecules that assemble at adhesion sites, included some that are not required on their own, such as RSU-1, but that could have a larger role in supporting adhesion in combination with other proteins.

PINCH$^{\Delta LIM1}$

To test whether the only function of LIM1 of PINCH was to bind ILK, we generated PINCH$^{\Delta LIM1}$-3xFlag transgenic flies and tested for rescue in the same manner as for PINCH^{Q38A}. We found that the PINCH$^{\Delta LIM1}$ transgene does not fully rescue the *stck* null mutant, suggesting that there are other vital roles for LIM1 of PINCH (Table 3.2). While we were not able to determine the exact other functions for LIM1, we did observe that PINCH$^{\Delta LIM1}$ rescued animals survive longer than the *stck* null mutant, indicating that the transgene retains some function and allows animals to survive to the larval or pupal stages (Fig. 3.5). The ability of PINCH$^{\Delta LIM1}$ to partially localize to adhesion sites in *Drosophila* also suggests that there are interactions capable of localizing PINCH other than with ILK (Fig. 3.6). Recent work from other labs also provides preliminary evidence for other functions for LIM1 that is in agreement with out work (Zervas et al., 2011). Binding partners for LIM1 have been identified in *C. elegans*, and in human cultured podocytes (Qadota et al., 2007; Wang et al., 2011). While these interactions have not been shown in *Drosophila*, these results are intriguing and demonstrate the possibility of novel binding partners for PINCH.

PINCH^{D303V} and PINCH$^{\Delta LIM5}$

We hypothesized that disruption of the PINCH-RSU-1 interaction would not cause any functional defects as the *ics* null mutant is viable and fertile. In order to test this, we performed a screen to indentify point mutations in PINCH that would disrupt binding with RSU-1 (Fig. 4.2). We generated PINCH^{D303V}-GFP transgenic flies and found that they could rescue the *stck* null phenotype, but only when at least two copies of the transgene were introduced (Table 4.2). Although we observed full rescue, the resulting adult flies were not healthy, and it was difficult to maintain rescued stocks. This weak viability was exacerbated by loss of RSU-1 indicating that it was not unbound RSU-1 that was causing any defect in viability, and the loss of RSU-1 was actually harmful (Table 4.3). The PINCH^{D303V} transgenic protein was expressed at much lower levels than endogenous PINCH, which could be one explanation for their reduced fitness (Fig. 4.4). However, PINCH^{wt}-GFP flies also expressed similarly low levels of transgenic PINCH, leaving open the possibility that the PINCH^{D303V} mutation could be disrupting another unknown PINCH interaction, and could explain the weak viability that is exacerbated by loss of RSU-1. Despite the unknown reasons for reduced viability in PINCH^{D303V} flies, we were able to conclude from this work that RSU-1 localization at muscle attachment sites is not only dependent on the presence of PINCH, but requires the direct interaction with PINCH (Fig. 4.1 and 4.5).

We also generated PINCH$^{\Delta LIM5}$-GFP transgenic flies in order to determine if there were other functions for LIM5, and found that they were unable to rescue the *stck* null mutant (Table 4.2). Our preliminary analysis suggests that the combination of the deletion and the GFP tag rendered the protein unstable, and therefore made it difficult to

test its function. While we were unable to draw definite conclusions from these studies, the results are intriguing and warrant further study.

Technical challenges

Initially all transgenic flies generated for this study (with the exception of PINCH$^{\Delta LIM1}$) contained a C-terminal GFP tag. We had previously generated PINCH^{wt}-GFP transgenic flies and demonstrated their ability to rescue the *stck* null mutant (Kadrmas et al., 2004). While it was known that these lines expressed at levels lower than endogenous PINCH, the rescue data provided sufficient rationale to generate the transgenic PINCH mutants in the same manner. The PINCH^{D303V} and PINCH$^{\Delta LIM1}$ flies in particular raised concerns about whether using a GFP tag was the best approach, as it was difficult to interpret whether the results observed were due to poor expression of the transgene or due to a functional consequence of the specific PINCH mutation. This issue was also confused by the knowledge that protein levels of the adhesion complex members tested in this study were sensitive to loss of other complex members, making interpretation of the cause of lethality in null mutants very difficult (Fig. 2.5). To address this, we initially generated more PINCH^{wt} and PINCH^{Q38A} lines with mCherry and 3xFlag tags. Both of these tags could be tracked using antibodies and in the case of mCherry, the endogenous fluorescent protein could be detected under appropriate conditions. We observed reduced levels of transgenic PINCH in mCherry tagged lines that were similar to levels observed in GFP tagged lines. Both PINCH^{wt} and PINCH^{Q38A} transgenes with GFP or mCherry tags fully rescued the *stck* null, which still makes them useful reagents. The PINCH^{wt} and PINCH^{Q38A} lines with the 3xFlag tags, however, expressed at levels equivalent to endogenous PINCH. The expression of transgenes at a level that would be

observed in a wild-type animal eliminated some concerns that results could be due to changes in levels of PINCH rather than the specific mutations that were introduced. The improved expression of 3x-Flag tagged PINCH transgenes provided the rationale for which tag to use to generate the PINCH$^{\Delta LIM1}$ lines. Indeed, while we still do not observe rescue to adulthood with the PINCH$^{\Delta LIM1}$ transgene, we have demonstrated that it is expressed and possesses some function as it can rescue the embryonic lethality of the *stck* null mutant.

Future studies

PINCH^{Q38A} and PINCH$^{\Delta LIM1}$

We have shown that loss of RSU-1 affects the viability of PINCH^{Q38A} rescued flies and that this lethality is not due to changes in PINCH^{Q38A} protein levels compared to PINCH^{wt}. What remains to be determined is whether the loss of RSU-1 causes mislocalization of PINCH^{Q38A} in larvae, which is the first stage where a high percentage of lethality is observed. If PINCH^{Q38A} is mislocalized, this would indicate that RSU-1 is acting as an anchor in the absence of an interaction with ILK, and would also imply that RSU-1 makes other contacts with proteins at the cell cortex. If PINCH does localize normally, this implies a novel function for RSU-1 at muscle attachment sites in *Drosophila*. It will be interesting to determine if the lethality caused by loss of RSU-1 is unique or if other adhesion proteins that are not required for viability (such as Vinculin, FAK, or Tensin) could produce the same result. The finding that loss of RSU-1 in PINCH^{Q38A} flies causes lethality demonstrates some defect in the PINCH^{Q38A} rescued flies. It will be interesting to see if PINCH^{Q38A} rescued flies are more sensitive than PINCH^{wt} rescued flies in various muscle function tests or when raised at different

temperatures. Finally, this study focused on PINCH, ILK, and RSU-1 localization and function at muscle attachment sites in *Drosophila*. It will be interesting to see if our findings hold true or if there is alternate regulation and/or localization in other tissues where these proteins function, such as in the developing and adult wing.

The results demonstrating that $PINCH^{\Delta LIM1}$ rescue is different than $PINCH^{Q38A}$ is intriguing and warrants further study. Once we have determined whether or not appropriate levels of $PINCH^{\Delta LIM1}$ are expressed then it will allow us to design future experiments with more confidence. Future experiments will focus on a more careful and expanded analysis of the lethal phase of $PINCH^{\Delta LIM1}$ rescued animals, and a more detailed examination of the localization of $PINCH^{\Delta LIM1}$ at muscle attachment sites. We observed partial localization that does fully resemble $PINCH^{wt}$ or $PINCH^{Q38A}$ localization, and it will be interesting to determine in exactly which cell types $PINCH^{\Delta LIM1}$ is expressed or if the subcellular localization of $PINCH^{\Delta LIM1}$ is different than $PINCH^{wt}$ and $PINCH^{Q38A}$ transgenes. Finally, other binding partners for LIM1 have been identified in other organisms, including proteins involved in muscle function in *C. elegans* (Qadota et al., 2007) and a nuclear binding partner in human podocytes (Wang et al., 2011). While there are no clear cut homologues for these genes in *Drosophila*, a careful examination of conserved domains could provide candidate genes to test for interactions with *Drosophila* PINCH. Finally, the identification of novel binding partners for PINCH and RSU-1 is an active area of investigation in the lab. Although, our search has not been fruitful to this point, alternative purification methods or larger scale proteomic approaches may provide new candidates for not only direct PINCH binding

partners, but for complex proteins that are part of a larger network of interactions required for integrin function.

PINCH^{D303V} and PINCH$^{\Delta LIM5}$

The PINCH^{D303V} and PINCH$^{\Delta LIM5}$ transgenic lines were the most technically challenging to work with of all lines generated for this study. However, the preliminary results obtained with these lines, and the demonstration of a function for RSU-1 in the PINCH^{Q38A} rescued flies warrant further study of the PINCH-RSU-1 interaction. Any future work to study this interaction will involve one or more different approaches. First, it will be necessary to generate new PINCH^{D303V} and PINCH$^{\Delta LIM5}$ transgenic flies with the 3xFlag tag. This should improve protein expression and give greater confidence in the results observed. Alternatively, UAS-PINCH^{D303V} or UAS-PINCH$^{\Delta LIM5}$ transgenic lines could be made, where forced expression of transgenes in a tissue specific manner could provide a better way to study PINCH mutants that may not be stable when expressed from the endogenous promoter. Second, to eliminate concerns that the D303V mutation could disrupt another PINCH interaction, transgenic flies have been generated that carry a point mutation in RSU-1 that disrupts binding with PINCH (RSU-1^{N103D}-3xFlag). This complementary approach will aid in the interpretation of defects caused by disrupting the PINCH-ILK interaction.

We were never able to test the requirement of the PINCH-RSU-1 interaction in the regulation of JNK signaling. This is still an important question, and will be addressed once the appropriate reagents have been established. Specifically, the consequences of disrupting the PINCH-RSU-1 interaction will be tested during dorsal closure, and in the wing epithelium.

Perspectives

Integrin function is required for development and the maintenance of adult tissues. Understanding the roles of the large protein complexes that assemble downstream of integrins is critical to understanding integrin function. Due to the vast number of molecules that assemble at adhesion sites, making multiple contacts, it is difficult to study the specific contributions of individual proteins. We chose to study the contributions of PINCH, ILK, and RSU-1, three proteins that are downstream of integrins and whose mutant phenotypes display overlapping but not identical defects in adhesion. This is the first study aimed at understanding the interactions between PINCH, ILK, and RSU-1 *in vivo* using specific point mutations (and comparing them to deletions of specific domains). We have demonstrated that the PINCH-ILK interaction is not required for viability, that RSU-1 may have a novel role in maintaining PINCH-ILK-RSU-1 complex function, and that LIM1 may have additional roles than just binding to ILK. Furthermore, our preliminary results warrant further study of the PINCH LIM5 interaction with RSU-1, with the goal of identifying additional roles for RSU-1.

We have demonstrated that this type of domain analysis is feasible and informative in *Drosophila*. This approach can be used to study other LIM domains of PINCH for which there has yet to be any described function in *Drosophila* (LIM2-4). Conversely, the reagents that have been established can be used to test the requirements for any novel PINCH functions that may be identified in the future, or to test the requirements for novel binding partners. Extensive domain analysis studies of β-integrin, Talin and ILK in *Drosophila* have been recently published, and our current and future

work aimed at dissecting the roles of PINCH will complement these studies (Franco-Cea et al., 2010; Pines et al., 2011; Zervas et al., 2011).

The work presented in this thesis has implications for further understanding the complexity of integrin function in normal and disease states. PINCH function has been described in many tissues including skeletal muscle, cardiac tissue, the kidney, and the brain (Kovalevich et al., 2011). Furthermore, alterations in expression have been observed in many different cancers (Cabodi et al., 2010). Understanding how PINCH functions with its binding partners, ILK and RSU-1, will contribute to future studies aimed at understanding the role of these proteins in diverse tissues.

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APPENDIX A

GENOTYPES AND DESIGNATIONS

Table A.1

Drosophila gene names, protein names and null alleles

Gene	CG designation	Protein	Null Alleles
myospheroid (*mys*)	CG1560	βPS integrin	NA
rhea	CG6831	Talin	NA
wech	CG42396	Wech	NA
parvin	CG32528	Parvin	NA
integrin linked kinase (*ilk*)	CG10504	ILK	ilk^{1}
steamerduck (*stck*)	CG7954	PINCH	$stck^{17}$ $stck^{18}$ *l(3)09716*
icarus *(ics*)	CG9031	RSU-1	$ics^{BG02577}$

A list of genes and proteins mentioned in this dissertation. NA indicates that null alleles of these genes were not used in any experiments. $ics^{BG02577}$ is referred throughout the text as *ics* since it is the only described null allele. CG designations were obtained from flybase.org.

Table A.2

PINCH transgene designations and genotypes

Transgene	**Genotype**
PINCH^{wt}	*w*, P[w^+, PINCH^{wt}-3xFlag] (X) *w*; P[w^+, PINCH^{wt}-3xFlag] (2 or 3) *w*; P[w^+, PINCH^{wt}-GFP] (3)
PINCH^{Q38A}	*w*, P[w^+, PINCH^{Q38A}-3xFlag] (X) *w*; P[w^+, PINCH^{Q38A}-3xFlag] (2 or 3)
PINCH$^{\Delta LIM1}$	*w*, P[w^+, PINCH$^{\Delta LIM1}$-3xFlag] (X) *w*; P[w^+, PINCH$^{\Delta LIM1}$-3xFlag] (2 or 3)
PINCH^{D303V}	*w*, P[w^+, PINCH^{D303V}-GFP] (X) *w*; P[w^+, PINCH^{D303V}-GFP] (2 or 3)
PINCH$^{\Delta LIM5}$	*w*, P[w^+, PINCH$^{\Delta LIM5}$-GFP] (X) *w*; P[w^+, PINCH$^{\Delta LIM5}$-GFP] (2 or 3)
PINCH^{wt} rescued	*w*, P[w^+, PINCH^{wt}-3xFlag]; *stck*^{18}/*stck*^{17} (X) *w*; P[w^+, PINCH^{wt}-3xFlag]; *stck*^{18}/*stck*^{17} (2) *w*; P[w^+, PINCH^{wt}-3xFlag], *stck*^{18}/*stck*^{17} (3)
PINCH^{Q38A} rescued	*w*, P[w^+, PINCH^{Q38A}-3xFlag]; *stck*^{18}/*stck*^{17} (X) *w*; P[w^+, PINCH^{Q38A}-3xFlag]; *stck*^{18}/*stck*^{17} (2) *w*; P[w^+, PINCH^{Q38A}-3xFlag], *stck*^{18}/*stck*^{17} (3)
ics; PINCH^{wt} rescued	*w*, P[w^+, PINCH^{wt}-3xFlag]; *ics*; *stck*^{18}/*stck*^{17} (X) *w*; *ics*; P[w^+, PINCH^{wt}-3xFlag], *stck*^{18}/*stck*^{17} (3)
ics; PINCH^{Q38A} rescued	*w*, P[w^+, PINCH^{Q38A}-3xFlag]; *ics*; *stck*^{18}/*stck*^{17} (X) *w*; *ics*; P[w^+, PINCH^{Q38A}-3xFlag], *stck*^{18}/*stck*^{17} (3)
PINCH$^{\Delta LIM1}$ in a *stck* heterozygous background (H)	*w*; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag]; *stck*^{18}/TM3 (2) *w*; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag], *stck*^{18}/TM3 (3) *w*; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag]; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag], *stck*^{18}/TM3 (2 and 3)
PINCH$^{\Delta LIM1}$ rescued (R)	*w*; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag]; *stck*^{18}/*stck*^{17} (2) *w*; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag], *stck*^{18}/*stck*^{17} (3) *w*; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag]; P[w^+, PINCH$^{\Delta LIM1}$ -3xFlag], *stck*^{18}/PINCH$^{\Delta LIM1}$ -3xFlag], *stck*^{17} (2; 3)

Table A.2 Continued

PINCH^{D303V} rescued	*w*, P[w^+, PINCH^{D303V}-GFP]/+; *stck*^{18}/*stck*^{17} (X) *w*, P[w^+, PINCH^{D303V}-GFP]/+; *stck*^{18}/*l(3)097* (X) *w*; P[w^+, PINCH^{D303V}-GFP], *stck*^{18}/ *stck*^{17} (3) *w*, P[w^+, PINCH^{D303V}-GFP]; *stck*^{18}/ *stck*^{17} (X) *w*; P[w^+, PINCH^{D303V}-GFP], *stck*^{18}/P[w^+, PINCH^{D303V}-GFP], *stck*^{17} (3) *w,* P[w^+, PINCH^{D303V}-GFP]; P[w^+, PINCH^{D303V}-GFP], *stck*^{18}/P[w^+, PINCH^{D303V}-GFP], *stck*^{17} (X; 3)
ics; PINCH^{D303V} rescued	*w*, P[w^+, PINCH^{D303V}-GFP]; *ics*; *stck*^{18}/ *stck*^{17} (X) *w*; *ics*; P[w^+, PINCH^{D303V}-GFP], *stck*^{18}/P[w^+, PINCH^{D303V}-GFP], *stck*^{17} (3) *w,* P[w^+, PINCH^{D303V}-GFP]; *ics*; P[w^+, PINCH^{D303V}-GFP], *stck*^{18}/P[w^+, PINCH^{D303V}-GFP], *stck*^{17} (X; 3)

Appropriate genotypes of transgenic flies used in this dissertation. All transgenes are in a *white* background and were introduced into the *Drosophila* genome by P-element transposition. Different fly genotypes were generated using a variety of transgenes with *stck* and *ics* alleles. The chromosomal insertion(s) of the transgene(s) in each genotype is(are) denoted in parentheses.

APPENDIX B

GFP, mCHERRY, AND 3xFLAG TAGGED TRANSGENIC LINES

Table B.1

PINCH^{wt}-GFP and PINCH^{Q38A}-GFP transgenic lines.

Insertion Line	ID	Chromosome	Homozygous Viable	Rescue of PINCH null
PINCH^{wt}–GFP	2B[a]	X	YES	YES
PINCH^{wt}–GFP	3B[a]	2	NO	YES
PINCH^{wt}–GFP	4A[a]	3	YES	YES
PINCH^{Q38A}–GFP	4[b]	X	YES	ND
PINCH^{Q38A}–GFP	3	2	YES	YES
PINCH^{Q38A}–GFP	5	2	YES	YES
PINCH^{Q38A}–GFP	6	2	YES	YES
PINCH^{Q38A}–GFP	2	3	YES	YES
PINCH^{Q38A}–GFP	7	3	NO	YES
PINCH^{Q38A}–GFP	8	3	YES	YES
PINCH^{Q38A}–GFP	9	3	YES	ND[c]
PINCH^{Q38A}–GFP	10	3	YES	ND[c]

[a] Generated previously by JLK (Kadrmas et al., 2004).
[b] Stock has notched wings when transgene is homozygous.
[c] Chromosome 3 insertions that were not tested for rescue could not be recombined with *stck*^{17} or *stck*^{18} alleles. ND=no data

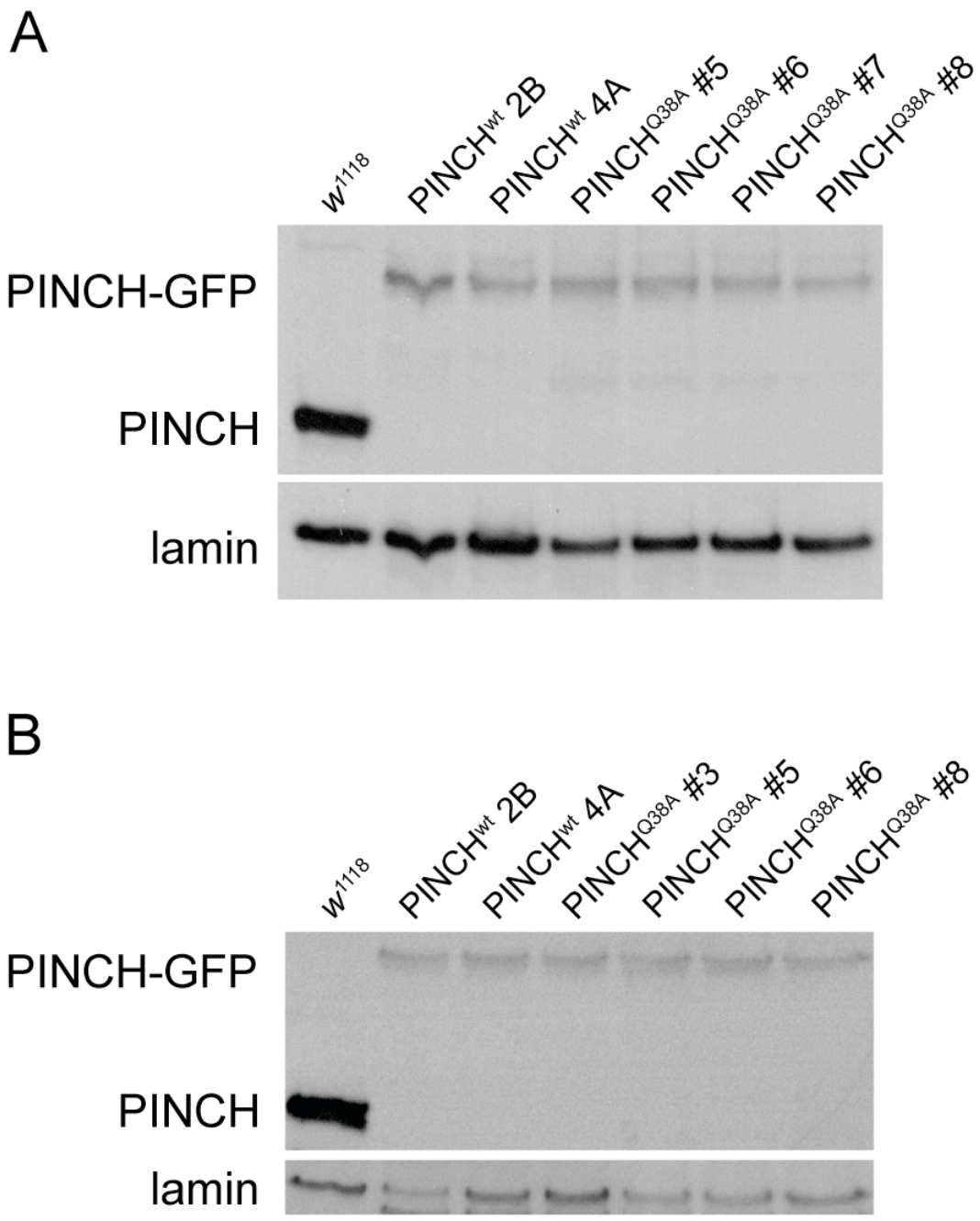

Figure B.1: PINCH^{wt}-GFP and PINCH^{Q38A}-GFP rescued lines. (A, B) Western blots of adult rescued flies demonstrate lack of endogenous PINCH seen in w^{1118} control samples and the higher migrating GFP tagged PINCH. Although under control of the endogenous PINCH promoter, GFP-tagged transgenes express at much lower levels than endogenous PINCH. PINCH^{wt}-GFP and PINCH^{Q38A}-GFP transgenes are expressed at similar levels to each other in adult rescued flies. Blots were probed with the PINCH (C) antibody and with lamin antibody as a loading control.

Table B.2

PINCH^{wt}-mCherry and PINCH^{Q38A}-mCherry transgenic lines.

Insertion Line	ID	Chromosome	Homozygous Viable	Rescue of PINCH null
PINCH^{wt} –mCh	A36.1	X	YES	YES
PINCH^{wt} –mCh	A33.2	2	YES	YES
PINCH^{wt} –mCh	A40.3	2	YES	YES
PINCH^{wt} – mCh	A43.1	2	YES	YES
PINCH^{wt} – mCh	C31.1	2	YES	YES
PINCH^{wt} – mCh	C33.1	2	YES	YES
PINCH^{wt} – mCh	A24.1	3	YES	YES
PINCH^{wt} – mCh	C33.2	3	YES	YES
PINCH^{Q38A} – mCh	1	X	YES	YES[a]
PINCH^{Q38A} – mCh	6	X	YES	YES[a]
PINCH^{Q38A} – mCh	4	2	YES	YES
PINCH^{Q38A} – mCh	5	2	YES	YES
PINCH^{Q38A} – mCh	2	3	NO	YES[a]
PINCH^{Q38A} – mCh	7	3	YES	ND[b]

[a] not full rescue

[b] Chromosome 3 insertions that were not tested for rescue could not be recombined with *stck*^{17} or *stck*^{18} alleles. ND=no data

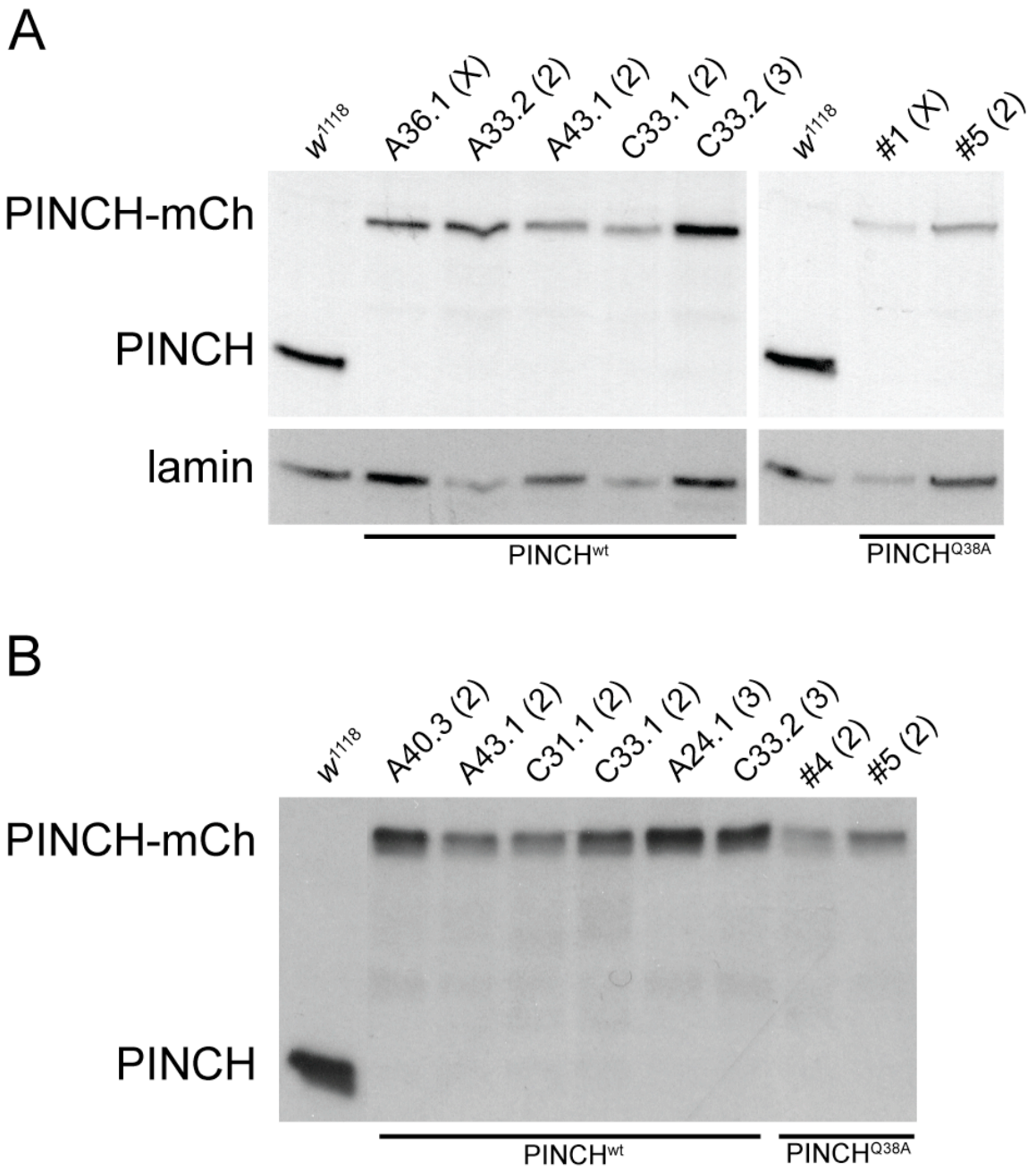

Figure B.2: PINCH^{wt}-mCherry and PINCH^{Q38A}-mCherry rescued lines. (A, B) Western blots of adult rescued flies demonstrate lack of endogenous PINCH seen in w^{1118} control samples and the higher migrating mCherry tagged PINCH. Although under control of the endogenous PINCH promoter, mCherry-tagged transgenes express at lower levels than endogenous PINCH. There is some variability in levels of PINCH^{wt}-mCherry and PINCH^{Q38A}-mCherry transgenes, which may correlate with overall fitness or ability to rescue the PINCH null mutant. Blots were probed with the PINCH (C) antibody and with lamin as a loading control.

Table B.3

PINCH^{wt}-3xFlag and PINCH^{Q38A}-3xFlag transgenic lines.

Insertion Line	ID	Chromosome	Homozygous Viable	Rescue of PINCH null
PINCH^{wt}–3xFL	6[b]	X	YES	YES
PINCH^{wt}–3xFL	10	X	YES	YES
PINCH^{wt}–3xFL	2	2	YES	YES
PINCH^{wt} – 3xFL	9[b]	2	YES	YES
PINCH^{wt} – 3xFL	3	3	YES	YES
PINCH^{wt} – 3xFL	4[b]	3	NO	YES
PINCH^{wt} – 3xFL	7	3	YES	ND[a]
PINCH^{wt} – 3xFL	8	3	YES	YES
PINCH^{Q38A} – 3xFL	4[b]	X	YES	YES
PINCH^{Q38A} – 3xFL	9	X	YES	YES
PINCH^{Q38A} – 3xFL	3	2	YES	YES
PINCH^{Q38A} – 3xFL	6[b]	2	YES	YES
PINCH^{Q38A} – 3xFL	1[b]	3	NO	YES

[a] Chromosome 3 insertions that were not tested for rescue could not be recombined with *stck*^{17} or *stck*^{18} alleles. ND=no data

[b] Lines that were used for experiments in Chapter 2

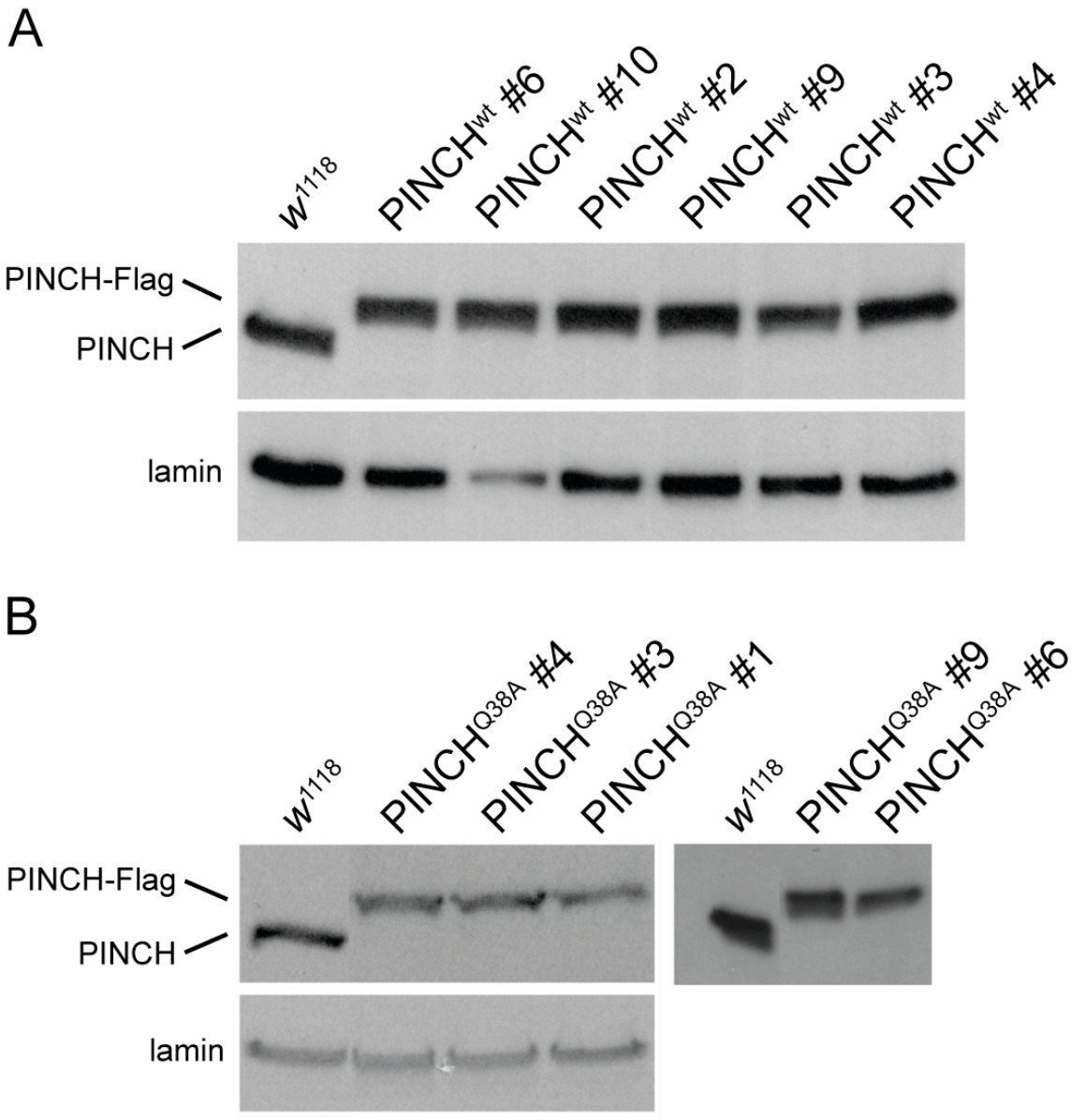

Figure B.3: PINCH^{wt}-3xFlag and PINCH^{Q38A}-3xFlag rescued lines. (A, B) Western blots of adult rescued flies demonstrate lack of endogenous PINCH seen in w^{1118} control samples and the higher migrating 3xFlag tagged PINCH. Flag tagged transgenes express at similar levels to endogenous PINCH. Additional blots and more in depth analysis of a subset of these transgenic lines are in Chapter 2. Blots were probed with the PINCH (C) antibody and with lamin as a loading control.

APPENDIX C

FIXATION AND IMMUNOFLUORESCENCE CONDITIONS

Table C.1

Fixation and immunofluorescence conditions for late stage *Drosophila* embryos

Antigen/Tag/Marker	Species	Fix	Concentration	Notes
endogenous GFP/mCherry	NA	PFA	NA	-no MeOH
mCherry (dsRed)	rabbit	PFA	1:500	-Clontech #632496
GFP	rabbit	PFA	1:1000	-Invitrogen #A6455
phalloidin (actin)	NA	PFA	1:100	-no MeOH
β-integrin	mouse	PFA	1:5	-DSHB CF.6G11
actin	mouse	PFA/heat	1:500	-clone C4
myosin heavy chain	rabbit	PFA/heat	1:500	-Dan Kiehart Lab
phospho-tyrosine	mouse	PFA/heat	1:1000	-clone 4G10
Flag (M2)	mouse	heat/PFA	1:2000	-Sigma F3165 -preabsorb against w^{1118} embryos -better with heat fix
PINCH (C)	rabbit	heat	1:250	-affinity purified B82 (dPIN318) -Works better with endogenous PINCH
RSU-1 (C)	rabbit	heat	1:1000	-high background -use remaining affinity purified RSU-C (B87) OR -preabsorb against w^{1118} embryos