Exploring the interaction between Cindr and JNK signaling in *Drosophila*

By

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Chapter 1 – Introduction

Overview of chapter

The ability of cells to acquire and maintain stable positions is fundamentally important for the correct patterning of tissues in multicellular organisms. This feat of cell biology is brought about to a large extent by a vast number of inter- and intracellular protein-protein interactions. Failure of cells to appropriately regulate their adhesive properties can give rise to an array of diseases, including cancer. In this chapter, some universal properties of epithelia, the tissue type that is the focus of this thesis, will be considered. Previous work has identified the conserved *Drosophila* adaptor protein Cindr as an important regulator of epithelial tissue patterning (Johnson et al., 2011). This thesis seeks to build on that work; some of what is already known about this protein and its mammalian orthologues CD2AP and CIN85 will be summarized. We have recently identified JNK (c-Jun N-terminal Kinase) as a potential interactor with Cindr and as such, relevant background information about JNK signaling will be highlighted. This survey of the literature will focus on the (relatively) recently discovered role of JNK in dynamic, migratory cell behaviors including those associated with metastasis. Finally, based on this information, a hypothesis describing the functional significance of an interaction between Cindr and JNK will be formulated. A deeper understanding of such an interaction would be of great value in understanding the development of epithelial tissues and could also have important clinical implications.
The emergence of epithelia

Multicellular organisms depend on epithelia for diverse functions, including defense, compartmentalization of physiological systems, selective absorption, secretion and even chemosensation. Upon closer inspection, these diverse epithelia are all assembled according to a similar plan: they typically consist of tightly adherent cells with simple shapes and at least one layer of cells closely associated with a basement membrane. It is remarkable that cells with relatively simple individual structures can adhere and organize themselves into functional tissues that are well adapted for their respective functions. In a seminal review by Gumbiner (1996) on the role of cell adhesion in tissue architecture and morphogenesis, it was noted that “cell adhesion systems should be regarded as mechanisms that help translate basic genetic information into complex three-dimensional patterns of cells in tissues” and that such systems are critical for the correct structure and function of tissues and ultimately organs.

Two characteristic developmental motifs that can be seen replicated in many different emerging epithelial tissues across taxa are illustrated in figure 1.1. The first (A) shows formation of a highly condensed spheroid of tissue from a starting population of loose mesenchymal cells, or a transition from these loose mesenchymal cells to epithelial cells during epithelial compaction. Of note, although the individual epithelial cells have simple shapes, they have undergone changes in size, shape and position to form (in this case) a tube.
In the second (B), cells are shown to change shape and move, relative to each other, in order to form a tissue with a completely different structure, a common process of shaping tissues during embryogenesis. Gumbiner makes the important point that the maintenance of stable junctions is an active process and requires dynamic regulation of a multitude of adhesive elements at cell-cell and cell-ECM (extra cellular matrix) interfaces.

It is essential that cells are able to acquire and maintain correct positions relative to their neighbors and the extracellular environment. Failure to do so can affect not only the overall tissue architecture, but also contributes to pathogenesis of various diseases, including cancer. We have previously identified the adaptor protein Cindr, *Drosophila* orthologue of CD2AP and CIN85, as an important regulator of junctions and the cytoskeleton in emerging epithelia (Johnson et al., 2008, Johnson et al., 2011) and have recently observed that loss of Cindr can induce cell behaviors that reflect early events associated with metastasis.
Rearrangements and changes in cell shapes can give rise to different tissue structures. (B) Condensation or epithelial compaction with defined basement membrane (bm), zonula adhaerens (za), desmosomes (des) and tight junctions (tj). (B) Rearrangements and changes in cell shapes can give rise to different tissue structures. (Modified from Gumbiner, 1996).

Figure 1.1: Emergence of epithelia. Two fundamental mechanisms whereby adhesion-dependent underlie morphogenesis are illustrated: (A) Condensation or epithelial compaction with defined basement membrane (bm), zonula adhaerens (za), desmosomes (des) and tight junctions (tj). (B) Rearrangements and changes in cell shapes can give rise to different tissue structures. (Modified from Gumbiner, 1996).
A fundamental feature of all epithelia is the presence of apical adherens junctions (AJs), which are generated by calcium-dependent homophilic interactions between E-cadherin (epithelial cadherin) dimers of adjacent cells (reviewed by Cooper and Hausman, 2007). Additionally, the intracellular domains of E-cadherin interact with many other proteins at the plasma membrane, including p120, α- and β-catenin, providing a link to the actin cytoskeleton (figure 1.2). The extracellular interactions between opposing E-cadherin molecules provide a great deal of mechanical strength and are thus critical in the maintenance of epithelial integrity and polarity (Reviewed by Baum and Georgiou, 2011). Although dynamic regulation of the AJ has been shown to be important in remodeling epithelia, dysregulation of AJ proteins has also been implicated in metastasis (Pecorino, 2008). In a study of more than 100 patients diagnosed with lung cancer, a significant negative association was found between E-cadherin expression and lymph node metastasis (Sulzer et al., 1998). Additionally, loss of E-cadherin in tumor cells was associated with an increase in mortality rate (Sulzer et al., 1998).
Figure 1.2: E-cadherin in cell adhesion. Simplified representation shows E-cadherin mediated interaction between two adjacent cells (Baum and Georgiou, 2011).
The roles of Cindr and CIN85/CD2AP

The vertebrate orthologues of Cindr, CIN85 (Cbl-interacting protein of 85 kDa) and CD2AP (CD2-associated protein, also known as Mesenchyme-to-epithelium transition protein with SH3 domains), have been implicated in a wide variety of developmental, physiological and pathological processes (Dustin et al., 1998, Li et al., 2000, Schmidt et al., 2003). They function as adaptor-molecules and, as such, have been found to interact with a host of other proteins including the actin cytoskeleton (Welsch et al., 2001, Szymkiewicz et al., 2002). Their ability to orchestrate the assembly of large, multi-protein complexes has been shown to mediate diverse yet critical functions, including regulation of RTK (Receptor tyrosine kinase) signaling and renal slit diaphragm maintenance and formation of the immunological synapse between T-cells and antigen-presenting cells (reviewed by Dikic, 2002, Hattori et al. 2011, Dustin and Depoil, 2011).

The underlying structure of CIN85 and CD2AP reveals their high degree of similarity to each other (54% amino acid similarity) and to Cindr (figure 1.3). Of note, the longest isoform of all three proteins contain three SH3 (Src Homology 3) domains, which are known to mediate protein-protein interactions by binding to proline-rich domains (Pawson and Nash, 2003). Additionally, proline-rich motifs occur in all three proteins, and can conversely allow for interactions with SH3 domains of other proteins. Closer to the C-terminal region, the presence of coiled-coil (CC) domains suggest the ability
to form homotypic multimeric complexes (e.g. Cindr-Cindr or CIN85-CIN85) (Dikic, 2002). Thus, given the conservation of structure within the Cindr/CIN85/CD2AP adapter protein family, it is reasonable to speculate that proteins found to interact with Cindr may interact similarly with CD2AP and/or CIN85 in mammalian systems.
Figure 1.3: Conserved structure of Cindr, CIN85 and CD2AP. Diagrammatic representation highlights similarity in arrangement of SH3 domains which mediate protein-protein interactions. cc= coiled coil domain. (Modified from Johnson et al., 2008)
Even though the protein structure of CD2AP and CIN85 share a high degree of similarity, their functions are somewhat divergent and they were initially identified in quite different contexts. For example, CD2AP was the first to be identified in this family in a yeast two-hybrid interaction screen, and was named because of its interaction with the CD2 receptor of T cells (Dustin et al., 1998). It was also independently found expressed in the kidney and named Mesenchyme-to-epithelium transition protein with SH3 domains (METS-1), as a p130Cas binding partner (thus named CMS or Cas ligand with multiple SH3 domains) and even discovered in a mutant form implicated in chronic kidney disease (thus named FSGS3 or focal segmental glomerulosclerosis 3) (Szymkiewicz et al., 2002, Tang and Brieher, 2013). Similarly, CIN85 was identified as Ruk (Regulator of Ubiquitous Kinase), SETA (SH3 domain-containing gene expressed in tumorigenic astrocytes) and SH3KBP1 (SH3 domain kinase binding protein 1) (Mayer and Eck, 1995, Gout et al. 2000, Take et al. 2000, Soubeyran et al. 2002). Several of these name choices convey that the CD2AP/CIN85 family carries out important functions in diverse cellular contexts, recruiting proteins into complexes that fine-tune and coordinate a wide variety of different processes. Evidence for this is provided by the fact that CD2AP knockout mice display a range of degeneration-related phenotypes, including glomerular sclerosis of the kidney, cardiac defects and atrophy of the spleen and thymus (Shih et al., 1999). However, $CD2AP^{null}$ and $CIN85^{null}$ mice are viable throughout embryogenesis while double knockouts are not, indicating that some degree of functional redundancy exists (Shih et al., 1999, Shimokawa et al., 2009). Since Cindr is the sole orthologue of *Drosophila*, the problem of redundancy is eliminated.
Using *Drosophila* as a model organism can shed light on how CD2AP and CIN85 might function *in vivo* across the lifespan of the organism. This is especially powerful given the wealth of genetic and biochemical tools available to *Drosophila* researchers. One of these tools, the UAS-GAL4 system, has been especially valuable, because it allows for expression of transgenes that are dependent on the activation of a tissue-specific promoter (Brand and Perrimon, 1993). This can then, for example, allow one to target specific genes for knockdown in selected tissues by introducing an RNAi, without necessarily causing lethality.
Figure 1.4: CIN85 regulates RTK signaling. In this case, CIN85 dampens Epidermal Growth Factor Receptor (EGFR) signaling by facilitating endophilin-mediated endocytosis of the receptor. Note the three SH3 domains (A, B and C respectively), the proline rich (PR) and coiled coil (cc) domains of CIN85/Ruk (Regulator of Ubiquitous Kinase). This action is opposed by Dab2, AIP1/Alix and Sprouty 2 (modified from Havrylov et al., 2010).
An important property of adaptor proteins such as Cindr is the presence of motifs which facilitate protein-protein interactions (figure 1.4). Given that such interactions can involve many different partners, it is plausible that many different mechanisms exist whereby signal transduction could be regulated (Pawson and Nash, 2003). The role of CIN85 in regulating RTK (Receptor Tyrosine Kinase) signaling has been extensively studied, and a proposed mechanism is illustrated in figure 1.4. In association with the ubiquitin ligase Cbl and Endophilin, CIN85 facilitates endocytosis of activated receptors, while Cbl-mediated ubiquitination of the receptor targets the receptor for degradation (Szymkiewicz et al., 2002, Haglund et al., 2002). This serves to dampen RTK signaling at the level of the receptor in a sustained manner.

Although the focus of this thesis is on MAPK (mitogen activated protein kinase) rather than RTK signaling, these findings demonstrate the capacity for an orthologue of Cindr (CIN85) to interface with cellular signal transduction machinery. In a similar vein, Cindr was shown to form a complex with ArfGAP3 and Asap, both of which act as Drosophila Arf GTPase-activating proteins. These in turn suppress the activity of Arf6, a regulator of actin-mediated cell motility that has also been implicated in metastasis and cell invasiveness (Sabe et al., 2009, Johnson et al., 2011).

In addition to the various roles of Cindr/CD2AP/CIN85 in signaling, a wealth of data shows that it acts to stabilize cell junctions. Much of the initial work characterizing CD2AP and CIN85 was carried out in kidney tissue, following the discovery of a link between loss of CD2AP and congenital nephrotic syndrome (Shih et al., 1999). The kidney is exquisitely well-adapted to carry
out the process of ultrafiltration, and an important component of the filtration system depends on the tight interdigitation of foot processes of podocytes in the glomerulus (Yaddanapudi et al. 2011). Reduced function of this highly efficient, selective barrier results in proteinuria and is one of the first steps in the development of chronic kidney disease (CKD) – which affects millions of people worldwide (Go et al., 2004, Hsu et al., 2006). CD2AP, along with its binding partners including Nephrin has been shown to be critical in maintaining the slit diaphragm, which is the specialized junction between the podocytes. Indeed, haploinsufficiency of CD2AP has been linked to CKD in humans (Kim et al., 2003) and a Cd2ap−/− mouse model has been successfully used to study renal failure (Shih et al., 1999, Huber et al., 2006). Importantly, previous work has shown that Cindr functions with Hibris (the Drosophila orthologue of Nephrin) and Roughest (the Drosophila orthologue of the Nephrin binding partner Neph1) to enable correct patterning of the eye (Johnson et al., 2011). This is of particular interest because it suggests considerable conservation in the function of Cindr and underscores the value of Drosophila as a model to study the precise roles and mechanisms of this important family of adaptor proteins in vivo.

Schmidt et al. (2003) were among the first to demonstrate a role for CIN85 in the cytoskeleton and, specifically, cell adhesion. Confocal microscopy of cultured astrocytes incubated with anti-CIN85/SETA, phalloidin or β-tubulin revealed significant colocalization of CIN85 with actin microfilaments and microtubules respectively. Notably, confluent HEK293 cells transfected with CIN85 were found to have significantly higher Electrical Cell-Substrate
Impedence (ECIS), which provides a quantitative measure of the extent of cell-cell adhesion in culture.

Similarly, CD2AP was found to localize to F-actin in podocytes (Welsch et al., 2001), and Lehtonen et al. (2002) showed a direct interaction between CD2AP and the actin cytoskeleton. A clearer picture of the functional significance of this interaction is emerging. Recently, Tang and Brieher (2013) showed that MDCK cells overexpressing CD2AP had more stable junctions and were thus more resistant to mechanical disruption. As one might expect from these data, cells with abundant CD2AP localized at the adherens junctions are more stable and less motile. Indeed, two independent wound-healing assays revealed significantly reduced migration in cells overexpressing CD2AP compared to untransfected cells (Johnson et al., 2011, Tang and Brieher, 2013). These results are consistent with previous in vivo data indicating the presence of Cindr puncta localized to the adherens junctions in the pupal eye (Johnson et al., 2008). Reduction in the levels of Cindr resulted in hypermotility and failure to acquire stable positions within the emerging fly eye. As described in this thesis, larval wing disc cells with reduced Cindr undergo delamination from their neighbors and can frequently be found some distance away from their original position. This phenotype is of great interest because of its resemblance to the early events of metastasis.

Understanding the mechanisms and signaling events that mediate the effects of loss of Cindr may provide clues about the basic cell biology of metastasis. As a promising point of departure, two independent yeast-2-hybrid screens
detected a direct protein-protein interaction between Cindr and Basket (the fly ortholog of c-Jun N-terminal kinase/JNK, a well-established mediator of cell migration and apoptosis) (Giot et al., 2003, Stanyon et al. 2004). Taken together, these data point towards the involvement of JNK signaling when the levels of Cindr are perturbed. However, this interaction has not been verified and there is a lack of data exploring and characterizing this potentially very important interaction in a model system. Thus, the broad aim of this study was to determine whether this interaction does in fact occur in *Drosophila* tissues and to provide an initial analysis of the functional significance of this relationship.

*The JNK signaling pathway*

*i. Overview of pathway*

Part of the evolutionarily ancient Mitogen Activated Protein Kinase (MAPK) superfamily, JNK has been extensively characterized as a critical signaling node that responds to a wide variety of stimuli. These include UV radiation, heat and osmotic shock, wounding and binding of Tumor Necrosis Factor-α (TNF-α) (Adler et al., 1995, Rosette and Karin, 1996, Rämet et al., 2002). Phosphorylation and activation of the transcription factors c-Jun and c-Fos downstream of activated JNK in this kinase cascade (figure 1.5) results in altered expression of an array of proteins that regulate diverse processes including cell migration, apoptosis and proliferation (You et al., 2013, reviewed by Stronach 2005). One of these targets is *puckered*, which encodes a phosphatase that in turn exerts negative feedback on JNK (Martin-Blanco,
The *Drosophila* homolog of JNK, encoded by *basket*, was first identified by Riesgo-Escovar et al. (1996), in a study that established JNK as an important regulator of morphogenesis, in addition to its previously discovered roles in the stress and immune response (reviewed by Karin and Gallagher, 2005). Basket itself is activated by phosphorylation by Hemipterous, which in turn is activated by Slipper. Thus, Basket functions as part of a conserved kinase cascade. Basket can also regulate a variety of cytoplasmic targets, although this thesis will focus on the more canonical transcriptional activity downstream of Basket (dJNK), mediated by c-Jun and c-Fos, and specifically the role of dJNK in regulating cell migration and apoptosis.
Figure 1.5: Summary of the JNK signal transduction cascade in Drosophila. A variety of extracellular stimuli bring about activation of a series of kinases, activating of Basket/dJNK, the fly orthologue of JNK. Subsequent activation of the transcription factors c-Jun and c-Fos modulate transcription of many targets, including Puckered. The *puckered* locus encodes a phosphatase that exerts negative feedback on dJNK. Also shown are cytoplasmic targets of dJNK. Many mammalian JNK kinases (JNKK) and kinase kinases (JNKKK) have been described, but the *Drosophila* orthologues central to this thesis are highlighted. In this thesis, Basket shall be referred to as dJNK (*Drosophila* JNK). (Image credit: Ruth Johnson).
ii. JNK in morphogenesis and wound healing

JNK has also been implicated in key developmental events that require highly dynamic and coordinated changes in cell shape or position, including thoracic closure in *Drosophila*, and closure of the eyelid, neural tube and optic fissure in mice (Kuan et al., 1999, Zeitlinger and Bohmann, 1999, Colas and Schoenwolf, 2001, Weston et al., 2003). Functional Basket and its upstream kinase Hemipterous are required for dorsal closure: an event during embryogenesis where movements and changes in lateral ectodermal cell shape allow for ‘zippering up’ of the embryo (figure 1.6) (Riesgo-Escovar et al., 1996). Failure of dorsal closure is lethal, and such embryos remain open, hence the gene name *basket*. Embryonic dorsal closure has served as a valuable screening tool in working out many of the details of the JNK signaling pathway, since the embryos are readily accessible and the phenotype is striking.
Figure 1.6: Dorsal closure in Drosophila embryos. Scanning electron micrograph showing dorsal views of embryos undergoing: initiation (A), epithelial sweeping (B), zippering (C) and termination (D). The insets illustrate a lateral view of the respective stages. (Modified from Jacinto et al., 2000).
During dorsal closure, dynamic changes in the actin cytoskeleton are required for the opposing epithelial cells of the leading edge to move towards and fuse with each other (Young et al., 1993, Edwards et al. 1997, Jacinto et al., 2000). Indeed, fixed embryos were found to have a cable of actin along the circumference of the leading edge: much like the drawstring of a purse, this actin cable (and corresponding myosin) provides part of the force required to bring these opposing rows of cells together (Young et al., 1993, Kiehart et al., 2000). Additionally, a powerful live imaging approach of fly embryos revealed JNK-dependent assembly of prominent actin-based filopodia (Jacinto et al., 2000). These protrusions allow cells to ‘sense’ each other and facilitate spatial and temporal precision of leading edge fusion: loss of filopodia is associated with extreme misalignment of leading edge cells and failure of dorsal closure (Jacinto et al., 2000). These data underscore the importance of JNK activation in cell behaviors that require some dynamic change in shape or position. As such, JNK has also been involved in other analogous developmental events, including closure of the thorax (Zeitlinger and Bohmann, 1999). However, it has also been shown that JNK-dependent changes in cell behavior are not limited to morphogenesis, but are also crucial for certain processes in the adult. The most well established of these processes include wound healing and tissue repair, which are essential physiological functions that maintain homeostasis in response to injury. Loss of tissue continuity can expose organisms to life-threatening pathogens, and disrupted morphology can also perturb normal functioning of specialized structures. In vertebrates, this is
especially applicable to epithelial linings such as the epidermis, intestine and lung epithelium.

Intriguingly, it has been shown that the pattern of gene expression in healing wounds is recapitulated in malignant tumors (Chang et al., 2004). A better understanding of the molecular mechanisms involved in wound healing and tissue regeneration is of great clinical significance. Invertebrates such as Drosophila frequently demonstrate remarkable regenerative capacity, and have been used as models since the discovery that injured Drosophila imaginal discs can regenerate (Hadorn and Buck, 1962). Subsequently, it has been discovered that many of the fundamental mechanisms required for wound healing are conserved (Martin and Lewis, 1992, Brock et al., 1996, reviewed by Belacortu and Paricio, 2011). In addition to the role of JNK in morphogenesis discussed above, JNK signaling has also been shown to mediate wound healing in vertebrates and in Drosophila (Angel et al., 2001, Galko and Krasnow, 2004). Mattila et al. (2005) detected a marked increase in puckered (puc) expression in larval wings that had been experimentally injured in vivo. Since puc is an established transcriptional target of JNK, this result suggests that wounding of epithelial tissues induces JNK activation. Furthermore, Rämet et al. (2002) demonstrated a role for JNK in wound healing of the adult fly cuticle, suggesting that JNK involvement is not restricted to larval tissue regeneration.

Efficient wound healing requires highly dynamic, JNK-dependent changes to the actin cytoskeleton, but it is unclear how such processes are suppressed
under physiological (non-wounded) conditions. Excess activation of wound-healing machinery can result in the formation of scars (reviewed by Greenhalgh, 2003). Additionally, the similarities in gene expression seen in wound healing and malignancy have lead to concerns that aberrant wound healing may contribute to cancer (Freeman, 2008).

**iii. JNK signaling and stable cell junctions**

In epithelia, a fine balance exists between maintaining a barrier that keeps adjacent cells adherent, and dynamic turnover of junctions that facilitates remodeling and repair of tissues. Excessive plasticity of junctional dynamics can lead to the loss of integrity of cell adhesion at the tissue level, which can give rise to a number of disease states.

Recently, a number of important discoveries concerning the role of JNK signaling in the regulation of junction stability have been made. These have helped shed light on the mechanisms of important developmental processes as well as positioning JNK within the realm of cancer cell biology (You et al., 2013). In a study using human model intestinal epithelial cells, Naydenov et al. (2009) observed disassembly of adherens junction proteins E-cadherin and β-catenin in confluent calcium-depleted cells in culture. Calcium depletion is a classic technique to drive remodeling of cell junctions. Intriguingly, the disassembly was completely abolished by pharmacological inhibition of JNK function using the drug SP 600125. Additionally, in the cells where lack of calcium triggered dissolution of the adherens junctions, a highly
significant increase in the levels of phosphorylated JNK was detected using western blot analysis.

One mechanism that may explain the above findings was proposed by the Andreadis lab (one of the first to explore JNK as a regulator of cell-cell adhesion), which demonstrated that JNK binds to and phosphorylates the E-cadherin binding partner β-catenin (Lee et al. 2009). Such phosphorylation has been previously implicated in the disassembly of adherens junctions (Behrens et al., 1993). These results imply recruitment of active JNK to the plasma membrane, at least under certain conditions. The mechanisms whereby JNK might be targeted to the adherens junction in this manner are unclear. An intriguing possibility is that JNK may be sequestered at the plasma membrane and, under certain circumstances, is permitted to interact with adherens junction proteins.

Taken together, these data point to JNK as an important regulator of junctional dynamics, and specifically as an antagonist of stable junctions. In some contexts, it is essential that cells temporarily relax the rigid paracellular junctions, such as during wound repair. However, excessively permissive junctions may disrupt the structure and function of epithelia, and compromise homeostasis. A particularly catastrophic consequence of this plays a role in metastasis.

While cell-culture based studies have shed light on many of the details and functions of JNK signaling, it is important to consider in vivo experiments in
order to understand the physiological relevance of such signaling in a three dimensional context. This is especially true when considering complex and dynamic cell behaviors such as migration, where cells interact with each other in a spatially heterogeneous environment. To this end, the fly larval wing imaginal disc has served as a valuable model tissue. The utility of the *Drosophila* larval wing disc for observing changes in cell position, morphology and protein expression has been well-established and is illustrated in figure 1.7. Specifically, the UAS-GAL4 system allows for selective expression of transgenes, in this case under control of the *patched* promoter (Brand and Perrimon, 1993). The *patched* expression is within a stripe of tissue that abuts the anterior-posterior boundary of the wing imaginal disc (figure 1.7). Intriguingly, the invasion depicted in cells with activated proto-oncogenic Src has been shown to be directed by JNK-mediated actin dynamics (Rudrapatna et al., 2013). Additionally, Ma et al. (2013) demonstrated that ectopic activation of JNK was sufficient to trigger migration of epithelial cells from the *patched* stripe. This migration coincided with expression of matrix metalloproteases that degrade the basement membrane, allowing cells that have escaped their initial position to invade surrounding tissue. Using a similar experimental strategy, we have recently observed similar migration of wing disc cells expressing cindrRNAi, suggesting the hypothesis that Cindr may act to oppose JNK in stable epithelial tissues.
fracadherin-dependent adhesion is reduced in structure. Src modulates the integrity of these adhesion sites: E-adehens junctions, which are important for cell adhesion and tissue tumors in the eye epithelia (discussed above) (Brumby et al., 2011).

Conversely, migration has been used to model tumor metastasis. Invasion extracellular matrix, and eventually apoptose (Fig. 3). This invasive phenotype induced by enhancment of the invasion phenotype. Conversely, the migration of the A-P boundary of the wing disc (Singh et al., 2010). Co-phenotype induced by enhancement of the invasive phenotype. Conversely, the migration of the A-P boundary of the wing disc (Singh et al., 2010; Vidal et al., 2010). In a separate study, the capacity of Src to contribute to Ras functions downstream of Csk and Src to mediate cell invasion. In

Figure 1.7: Cartoon of larval wing disc experimental approach. Expression of transgenes in a defined, labeled stripe of tissue (the patched expression domain) is shown in green. These cells are surrounded by wild-type tissue, which provides a reliable internal control. Cells in the patched domain only expressing RFP remain in stable positions, while cells with reduced Csk (C-terminal SRC kinase, an inhibitor of Src) migrated away from their original positions. This can be visualized because only cells of the ptc domain lineage express the RFP marker. Activation of JNK signaling was found to enhance this migration (Modified from Miles et al., 2011, Vidal et al., 2006)
Figure 1.8: Transcriptional readouts of JNK activity. Wild type wing discs displayed basal levels of hid-lacZ (A), puc-lacZ (C) and Mmp1 (E) expression, while ectopic Rho1 induced strong expression of hid-lacZ (B), puc-lacZ (D) and Mmp1 (F) (Modified from Neisch et al., 2010).
As illustrated in figure 1.8 above, hid, puc and mmp1 expression have been shown to be transcriptionally upregulated in response to JNK activation in Drosophila (Neisch et al., 2010). The expression of these (and several other) proteins serves as a reliable readout (also used in this thesis) of JNK activation and sheds light on the resulting cell behaviors. For example, Hid (Head Involution Defective) plays an important role in promoting apoptosis, Puc acts as a phosphatase that inhibits JNK in a negative feedback loop, and Mmp1 (Matrix Metalloprotease 1) degrades the basement membrane and can facilitate invasion of cells to other positions (Martin-Blanco et al., 1998, Takatsu et al., 2000, Yoo et al., 2002, Uhlirova and Bohmann, 2006). Thus it appears that ectopic expression of the small GTPase Rho1 in wing discs resulted in robust activation of JNK compared to wild type wings, and this was consistent across three independent transcriptional readouts (figure 1.8). Importantly, activation of JNK coincided with migration of cells from their primary position (in this case, in the defined stripe of cells expressing Dpp, along the midline of the wing disc), a result that has also been reported by other groups (Vidal et al., 2006, Ma et al., 2013). These invasive cell behaviors are especially interesting because they recapitulate a number of elements associated with metastasis (this thesis).

Cancer and the role of JNK

Metastasis can be defined as “malignant growth forming at one site in the body, the cells of which derive from a malignancy elsewhere in the body” (Weinberg, 2007). It is one of the hallmarks of cancer proposed by Hanahan
and Weinberg (2000, 2011). Metastasis is of particular salience because of its major contribution to deaths from cancer: once aberrant cells have migrated from a primary tumor to multiple secondary tumors throughout the body, treatment is substantially more challenging. Indeed, metastases from primary tumors are responsible for 90% of deaths in patients with cancer and of these cancers, at least 80% are carcinomas (i.e. epithelial) (Weinberg, 2007). As already mentioned, we have recently observed cell behaviors of cells with reduced Cindr that are consistent with some (but not all) of the early features of metastasis, including displacement from original position, expression of basement membrane-degrading proteases and altered cell morphology (H. Yahin, R. Johnson and this thesis). Determining the mechanisms responsible for such early metastasis-like behavior could prove to be of great clinical significance, because currently the majority of tumors have already undergone metastasis when patients are diagnosed with cancer (Pecorino, 2008). Understanding the underlying cell biology of metastasis may therefore provide important clues that could ultimately facilitate earlier diagnosis.

Accumulating evidence has implicated JNK in cancer, although the precise mechanisms remain controversial. Zhang et al. (2007) explored the role of JNK signaling in human squamous cell carcinoma (SCC), an invasive cancer with a high prevalence (Chen et al., 2001). Their goal was to assess blockade of this pathway as a potential therapeutic target for epithelial cancers. They discovered moderate or strong induction of phosphorylated JNK (pJNK) in 81% of human SCC samples. Blockade of JNK signaling in SCC skin grafts at the level of the receptor (TNF-α) prevented invasive
neoplasia and maintained tissue integrity. Significantly, a follow up study demonstrated that experimental coactivation of JNK and Ras (a known oncogene) was sufficient to transform human epidermal cells into malignancy (Ke et al., 2010). JNK activation has also been detected in many other human cancers, such as renal cell carcinoma, prostate carcinoma, osteosarcoma and glioma (Tsuiki et al., 2003, Yang et al., 2003, Papachristou et al., 2003, Liu et al., 2009, Ke et al., 2010). Of note, JNK and downstream Jun and Fos activity were found to correlate with development of chemotherapy-resistance in breast tumors (Schiff et al., 2000). Finally, JNK has been identified as a target of PTEN (Phosphatase and tensin homolog), one of the most common tumor suppressors found mutated in human cancers, which suggests that dysregulation of JNK signaling is an important theme in many cancers (Vivanco, 2007). Taken together, these data implicate JNK activity in several human cancers.

The JNK family in vertebrates is encoded by \textit{Jnk1}, \textit{Jnk2} and \textit{Jnk3} respectively, and these three genes undergo alternative splicing to give rise to many different isoforms of JNK (Davis, 2000). This high degree of redundancy has made it challenging to determine the precise role of JNK signaling in tumorigenesis (Zhang et al., 2007). Since Basket is the sole JNK orthologue in flies, \textit{Drosophila} has already served as a valuable model organism in addressing this issue (Neisch et al., 2010). Similarly, fly genetic screens can analyze numerous loci for modifiers of \textit{in vivo} metastasis-like phenotypes, an approach which is less feasible in mammalian models (Pagliarini and Xu, 2003). Encouragingly, JNK signaling in \textit{Drosophila} was identified as a “central
driver of tumorigenesis": JNK upregulation was necessary and sufficient for invasive growth of epithelial cells expressing the well-known oncogene $\text{Ras}^\text{ACT}$ (Active/Oncogenic Ras) (Brumby et al., 2011). In support of this conclusion, a conserved potential tumor suppressor Sds22 was found to inhibit JNK-mediated cell invasion in Drosophila (Jiang et al., 2011). Human Sds22 is also mutated in many epithelial cancers (Narayan et al., 2003, Cengiz et al., 2007). Similarly, the human E3 ligase complex factor SPOP was found to be upregulated in 99% of renal cell carcinomas: Drosophila-SPOP is known to increase JNK by promoting degradation of the JNK phosphatase Puckered (Liu et al., 2009). The above results support the view that inappropriate disinhibition of JNK and concomitant loss of tissue integrity is a conserved mechanism frequently underlying tumorigenesis.

Aims

Although JNK activity is crucial under certain developmental or physiological conditions, it is perhaps equally if not more important that such activity be tightly regulated, both temporally and spatially. While this pathway has received considerable attention, the precise mechanisms whereby regulatory precision is achieved remain elusive. As shown above, evidence has accumulated that in the Drosophila larval wing model of cell migration, ectopic JNK signaling triggers migration of cells in a manner that parallels our observation in cindrRNAi tissue (Ma et al., 2013, Rudrapatna et al., 2013, Ma et al., 2014). The aim of the present study is to investigate the role of the adaptor protein Cindr in the model organism Drosophila melanogaster, and
specifically, to explore its putative interaction with the well-known signaling protein, JNK. We hypothesized that Cindr acts to inhibit JNK activity in order to maintain stable junctions and cell positions. This hypothesis generated several predictions, some of which are the subject of this thesis. Specifically:

i. Cindr binds to Basket \textit{in vivo}, and this interaction is direct

ii. Loss of Cindr triggers activation of JNK signaling

iii. Overexpressed Cindr attenuates the effects of ectopic JNK activity

iv. The effects of a reduction of Cindr are mediated by JNK
Chapter 2 - Methods and materials

Fly husbandry and genotypes used

Fly stocks were maintained on standard media at 18°C and experimental crosses on nutrient rich media at 25°C for larval dissection or 21°C for adult wing dissection to maximize adult survival. Embryos for co-immunoprecipitation experiments were gathered on apple agar plates spread with a layer of baker’s yeast paste: crosses were housed in standard embryo cages (Flystuff - Genesee scientific, San Diego, CA). The genotypes of flies used for this thesis are summarized below (table 1).

Table 1: List of fly stocks used with abbreviated genotype and source

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Description</th>
<th>Source/Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ptc-GAL4</td>
<td>Driver line</td>
<td>BDSC</td>
</tr>
<tr>
<td>ptc-GAL4, UAS-GFP ; puc^{ESG} - lacZ / SM5-TM6b</td>
<td>Reporter</td>
<td>Dr. Ruth I Johnson (RIJ), Wesleyan University</td>
</tr>
<tr>
<td>ptc-GAL4, UAS-GFP ; hid-lacZ / SM5-TM6b</td>
<td>Reporter</td>
<td>S Van Rensburg, Wesleyan University</td>
</tr>
<tr>
<td>yw; hid-lacZ/TM3</td>
<td>Reporter</td>
<td>Andreas Bergmann, University of Massachusetts Medical School</td>
</tr>
<tr>
<td>TRE-RFP-16</td>
<td>Reporter</td>
<td>Dirk Bohmann, University of Rochester</td>
</tr>
<tr>
<td>UAS-GMA, UAS-Dcr2 / FM7 ; ptc-Gal4, UAS-GFP / CyO</td>
<td>Reporter</td>
<td>RIJ</td>
</tr>
<tr>
<td>UAS-slpr^{IA-SS}</td>
<td>UAS expression construct (UAS)</td>
<td>Beth Stronach, University of Pittsburgh</td>
</tr>
<tr>
<td>UAS-cindr-PC^{TAP,2532} / TM6b</td>
<td>UAS</td>
<td>RIJ (Johnson et al., 2008)</td>
</tr>
<tr>
<td>UAS-slpr^{IASS}, UAS-cindrPC^{TAP}/SM5-TM6b</td>
<td>UAS</td>
<td>RIJ</td>
</tr>
<tr>
<td>UAS-puc^{Tt56}, cindrRNAi2.21+23/SM5-TM6b</td>
<td>UAS/ Transgenic RNAi</td>
<td>RIJ</td>
</tr>
<tr>
<td>UAS-cindrRNAi^{2.21A}, UAS-cindrRNAi^{2.23A} / TM6b</td>
<td>Transgenic RNAi</td>
<td>RIJ (Johnson et al., 2008)</td>
</tr>
<tr>
<td>UAS-lacZ</td>
<td>UAS</td>
<td>BDSC</td>
</tr>
<tr>
<td>UAS-GFP / TM6b</td>
<td>UAS</td>
<td>BDSC</td>
</tr>
<tr>
<td>Canton S</td>
<td>Wild type</td>
<td>BDSC</td>
</tr>
</tbody>
</table>
Co-immunoprecipitation (Co-IP) and Western Blot

*Canton S* adults were incubated at 25°C in an embryo cage with an agar plate and baker's yeast paste to encourage laying. Embryos were gathered and dechorionated in 50% bleach for 2 minutes, rinsed thoroughly in ddH$_2$O to remove excess bleach and washed twice with wash buffer (20 mM HEPES, pH 7.5, 5% glycerol, 0.2% BSA). Washed embryos were resuspended and smashed in 400µl cold lysis buffer with 20 mM HEPES, pH 7.5, 125 mM NaCl, 1.5 mM MgCl$_2$, 5% glycerol, 1 mM EDTA, 1 mM DTT, 1mM Na$_3$VO$_4$, 1mM beta-glycerolphosphate, 25 mM NaF, 1.5% Triton-X, 1 Complete Protease Cocktail (Roche, Indianapolis, IN) tablet per 10ml, followed by a 30 minute incubation at 4°C. The lysate was spun down for 1 minute at 5000 rpm and the supernatant transferred to a new tube on ice with 20µl Protein A/G Ultralink Resin (Thermo Scientific, Rockford, IL) to pre-clear the lysate (1 hour with rocking at 4°C). Pre-cleared lysate was spun down at 15000 RPM for 2 minutes, and the supernatant transferred to a fresh tube on ice. Each tube of lysate was then incubated (with rocking at 4°C overnight) with antibody (experimental) or the IgG of the species corresponding to the antibody. Antibodies used included rabbit anti-Cindr (30µl for IP reaction or 1:500 for western blot, Johnson et al., 2008), rabbit anti-JNK (30µl for IP reaction or 1:1000 for western blot, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-active JNK (Promega Corporation, Madison, WI) or mouse anti-myc (1:1000, Cell Signaling Technology, Beverly, MA). Following incubation, 30µl anti-Cindr or anti-JNK was added to each tube: the IP reaction then proceeded for 2
hours with rocking at 4°C, followed by centrifugation at 15000 RPM for 15 seconds. The supernatant was discarded, each pellet of beads was washed three times in cold lysis buffer, and finally resuspended in 20µl 2x Laemmli Sample Buffer (Bio-rad, Hercules, CA) prior to freezing at -20°C prior to SDS-PAGE gel electrophoresis.

Samples were boiled for 10 minutes and chilled on ice prior to spinning down at 15000 RPM for 15 seconds. Each entire sample was loaded into one well of a 10% Mini-PROTEAN TGX gel (Bio-rad). Gels were run at 200V for ~ 35 minutes, prior to transfer on to a PVDF Immobilon-P membrane (Millipore, Billerica, MA) for 1 hour at 100V. A dual-color Precision Plus Protein Prestained Standard (Bio-rad) was used to verify transfer and to determine the molecular weight of loaded proteins. The membrane was rinsed in TBS-Tween (24.2g TRIS 87.66g NaCl, 0.2g NaN₃, 5ml TWEEN20 per 1L 10 x stock at pH7.5) (3 x 10 minutes) and blocked in 5% milk in TBS-Tween for at least 4 hours with slow rocking at 4°C. Similarly, the membrane was then incubated with antibody solution overnight followed by rinsing with TBS-Tween (3 x 5 minutes) and blocking in 5% milk at room temperature for 45 minutes. The membrane was then incubated in secondary antibody (1:1000 HRP conjugated anti-rabbit or anti-mouse, Cell Signaling Technology) for 1 hour at room temperature with slow rocking followed by rinsing in TBS-Tween (3 x 10 minutes). Chemiluminescent detection was then carried out to visualize the bands (Clarity Western ECL substrate: equal parts peroxide reagent and luminol enhancer reagent, Bio-rad). Images were captured using

*Larval wing dissection and analysis*

Wandering third instar (L3) larvae were rinsed (x3) in ddH2O in a nine well glass plate and transferred to 0.01M PBS (Sigma) on ice. Dissection using standard Biology forceps (0.05 x 0.02 mm tip) (Fine Science Tools, Foster City, CA) was carried out in a drop of refrigerated PBS on a Sylgard dish. Following removal of the fat body, each inverted carcass with the wing discs was fixed on ice (250µl 16% formaldehyde in PBS per 1ml aliquot) for 35 minutes for immunohistochemistry; or 2% glutaraldehyde in PBS for 15 minutes for the X-gal reaction. Each carcass was then transferred to fresh PBS (3 x10 minutes for immunohistochemistry or 3 x 5 minutes for the X gal reaction) and then PBT (PBS with 375µl 20% TritonX, and 0.25g BSA per 50ml working solution) (1 x 10 minutes). For immunohistochemistry: carcasses separated in wells by genotype were incubated with primary antibody overnight (4°C), rinsed in PBT (3 x 10 minutes) and incubated with secondary antibody (1-2 hours, 4°C), followed by rinsing (3 x 10 minutes PBT, 1 x 10 minutes PBS) and immersion in mounting media (0.5% n-propylgalate in 80% glycerol) for at least one hour to overnight at 4°C. Following mounting, wings were imaged using confocal microscopy (Zeiss LSM 510 metaconfocal with Zen software). For X gal reaction: fixed and rinsed carcasses were incubated in 0.2% X-gal solution (150 mM NaCl, 10 mM Na_2HPO_4, 3mM K_4[Fe(II)(CN)_6], 3mM K_3[Fe(II)(CN)_6], 1mM MgCl_2, 0.1% TritonX) for a minimum
of one hour to overnight at 37°C. In each experiment, experimental and control genotypes were subjected to identical conditions and incubated in different wells of the same glass dish for the same duration. Carcasses were then rinsed in PBS (4 x 5 minutes), before immersion in mounting media as described above.

**Adult wing dissection and analysis**

Adults were preserved in 70% EtOH until the time of dissection, then rinsed in 70% ethanol (x 3) and placed in molecular biology grade absolute ethanol. Dissection was carried out in a 9 well glass plate, in 100% EtOH: the wings were removed by forceps and transferred in EtOH to a glass slide, before being mounted in Euparal (BioQuip Products, Rancho Dominguez, CA). Adult wings were imaged using a 5 X objective on a Zeiss Axioplan light microscope, a Tucsen H series camera and ISCapture V3.0 software Images were analysed using ImageJ (National Institutes of Health) to determine relative wing areas by tracing a polygon around areas for analysis and using the Measure function.

**Statistical analysis**

Adult wing areas and larval wing pixel intensities were recorded in Microsoft Excel for Mac 2011 and exported to GraphPad Prism Version 6.0a, which was used to generate graphs, compute SEM and carry out Student’s t test and
ANOVA (Analysis of Variance). Results were considered statistically significant if $p<0.05$. 
Chapter 3: Results and discussion

Reducing Cindr disrupts wing patterning

Previous work has shown that Cindr is required for correct patterning of the *Drosophila* eye, and a variety of binding partners have been identified (Johnson et al., 2008, Johnson et al., 2012, Shulman et al., 2014). However, the precise mechanisms whereby this important adaptor protein functions in development, and how it may go awry in disease, are unclear. The work described in this thesis harnessed the wing epithelium as a model: reduction in the levels of Cindr (by expressing *cindrRNAi*) in the *patched* (*ptc*) expression domain using the UAS-GAL4 system (Brand and Perrimon, 2003) results in strikingly altered adult wing morphology (Figure 3.1). Figure 3.2 provides quantitative confirmation (using the Measure function in ImageJ) that much of the tissue within this region has been lost, as evidenced by the decrease in area between the L3 and L4 wing veins.

The above result indicates that Cindr is not only important for correct development of the eye, but may also have a more general function in the development and maintenance of stable epithelia. This result also paves the way for the adult wing to be used as a quantitative system to study a range of genetic interactions with Cindr. One limitation of this approach is that many genetic manipulations, including reducing Cindr, frequently result in death earlier in the life cycle. However, from those that do survive, a stable phenotype is observed. In the case of loss of Cindr, there is a reduction in
tissue in the zone expressing the RNAi. This suggests that the cells with reduced Cindr have undergone apoptosis at a rate that exceeds cell division (assuming relatively normal division rate); that a large proportion of cells with reduced Cindr have left their initial position; or both. The last conclusion is supported by work in our lab demonstrating massive migration and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling, an apoptosis assay that detects DNA fragmentation) activity in the larval wing disc with reduced Cindr in the ptc stripe (Hannah Yasin, Honors Thesis, 2015). It is thus reassuring that the phenotype observed during larval development is not rescued by adulthood, suggesting that Cindr is indispensable for correct development of epithelial tissues.
Figure 3.1: Loss of Cindr disrupts normal wing patterning. Adult wings obtained from flies expressing either lacZ under control of the patched-GAL4 expression domain (i.e. ptc>lacZ) (A) or cindrRNAi (B) throughout development. The region between wing veins L3 and L4 corresponding to most of the patched (ptc) expression domain has been highlighted in the control (A). This region is markedly reduced when Cindr is reduced (this thesis).
Figure 3.2: Loss of Cindr causes loss of wing tissue. The adult wing images were imported into ImageJ and polygons traced on to the L3-L4 region and the entire wing, allowing area to be determined using the Measure function. Quantification of relative (proportion of L3-L4 to entire wing area) areas indicated significant reduction (p<0.0001) in the ptc domain expressing cindrRNAi compared to the control animals which expressed lacZ. The area between wing veins L3 and L4 was taken as a proxy for the ptc domain. For each wing, this area was normalized by division by the entire wing area, to account for individual differences in wing and fly size. Thus, the L3-L4 region constitutes approximately 20% of the measured wing area in control animals but only 8% in animals expressing cindrRNAi.
A key aim of the project described in this thesis was to explore the interaction between Cindr and the JNK signaling pathway. As already outlined, JNK is an important regulator of cell migration and is also involved in apoptosis. Additionally, two independent Yeast 2-hybrid screens identified Cindr and JNK as direct interactors (Giot et al., 2003, Stanyon et al. 2004). Since such screens are known to generate false positive hits, it was important that such an interaction be verified. Additionally, while Yeast 2-hybrid screens provide information about whether two proteins interact, they do not necessarily speak to physiologically relevant protein-protein interactions in Drosophila. The approach taken in this project was to carry out a battery of co-immunoprecipitation experiments using Drosophila embryo tissue.

The western blot in figure 3.3 shows that Cindr but not control rabbit IgG immunoprecipitated with JNK. A band of protein recognized by the anti-JNK antibody was detected at approximately 50 kDa (based on a colorimetric size marker with a 50kDa band), closely corresponding to the 49 kDa molecular weight of Drosophila JNK (dJNK) (Riesgo-Escovar et al., 1996). Similarly, in an independent experiment, Cindr co-immunoprecipitated with phosphorylated (i.e. active) dJNK (figure 3.4). While this does not clarify whether Cindr exclusively or preferentially interacts with the activated form of the JNK protein, it opens the possibility that the phosphorylation status of JNK
may play a role in mediating the Cindr-JNK interaction. Future work is needed to resolve this issue.

An inverse experiment was carried out in order to further confirm the Cindr-dJNK interaction (figure 3.5): dJNK was immunoprecipitated and Cindr was detected at ~100 kDa, consistent with the published molecular weight of Cindr (Johnson et al., 2008). Since anti-Cindr is a polyclonal antibody, doing the co-immunoprecipitation experiment both ways as described above helps eliminate the possibility of detecting spurious protein-protein interactions.

Finally, serial immunoprecipitation of Cindr and dJNK revealed that ubiquitinated dJNK may form a complex with Cindr (figure 3.6). Specifically, Cindr was immunoprecipitated as described above and subsequently, the resulting eluent containing putative Cindr-JNK complexes was further purified by immunoprecipitating Ubiquitin. The resulting lysate was then subjected to western blot analysis to probe for the presence of dJNK, which was indeed detected when Cindr and then Ubiquitin were immunoprecipitated but not when no antibody was introduced. This control is necessary because it rules out non-specific binding of Cindr, dJNK or Ubiquitin to the resin beads used during the immunoprecipitation protocol. The positive detection of dJNK in the final eluent (figure 3.6) allows us to speculate that the Cindr-JNK complexes also contain ubiquitin and that Cindr may facilitate ubiquitination and subsequent degradation of dJNK. This hypothesis was informed by the known role of CIN85 (the mammalian orthologue of Cindr) in promoting ubiquitination and degradation of Receptor Tyrosine Kinase elements. However, this result
serves as a promising starting point for future experiments which are necessary to generate a more detailed model. Some of these are proposed in chapter 4. In brief, it is important that we confirm whether dJNK is indeed ubiquitinated and whether such ubiquitination is functionally relevant (i.e. whether the levels of JNK are modulated in response to modified levels of Cindr).

These co-immunoprecipitation data support the hypothesis that Cindr and dJNK interact in vivo, but they do not indicate whether the interaction is direct or not. Notably, Cindr could interact with JNK via some intermediary protein(s). Future work will use the GST pull-down approach to address this question. However, taken together with the independent Yeast 2-hybrid data, a direct interaction is highly likely, although intermediate yeast proteins could potentially have mediated the detected protein-protein interactions. More importantly, this interaction appears to be stable across experimental paradigms.
Figure 3.3: Cindr co-immunoprecipitates with dJNK. Western blot analysis detected the presence of dJNK when Cindr was immunoprecipitated, but not when control rabbit (Rab) IgG was used. This indicates that dJNK specifically forms a complex with Cindr in CantonS (wild type) embryos, and does not bind with high affinity to the generic Rab IgG or the beads. Representative image shown from experiment done in duplicate.
Figure 3.4: Cindr co-immunoprecipitates with phosphorylated dJNK. Western blot analysis detected the presence of active JNK (pJNK) when Cindr was immunoprecipitated, but not when control rabbit (Rab) IgG was used. This result suggests that when Cindr interacts with dJNK, dJNK is phosphorylated. Representative image shown from experiment done in duplicate.
Figure 3.5: dJNK co-immunoprecipitates with Cindr. Western blot analysis detected the presence of Cindr close to the 100kD size marker when dJNK was immunoprecipitated, but not when control rabbit (Rab) IgG was used. This experiment was the inverse of that shown in Fig. 3.3, and provides additional support that Cindr forms a complex with dJNK in CantonS embryos. Representative image shown from experiment done in duplicate.
Figure 3.6: Cindr, dJNK and Ubiquitin co-immunoprecipitate. CantonS embryo lysate was purified for the presence of Cindr as well as Ubiquitin in a relay co-IP experiment. dJNK was detected in the resulting lysate (+), but not when Cindr and Ubiquitin were not immunoprecipitated (-). This opens the possibility that dJNK may be targeted for ubiquitination in a protein complex that includes Cindr.
Reducing Cindr triggers JNK activation

A central hypothesis proposed in this thesis is that Cindr acts to inhibit dJNK activity in order to maintain stable epithelia. As discussed in chapter 1, activation of dJNK is associated with dynamic cell behaviors such as migration, while Cindr is required to acquire and maintain stable cell-cell contacts. Thus, this thesis sought to determine whether one way in which Cindr acts to maintain stable cell junctions is by suppressing dJNK activity. In order to test this hypothesis, Cindr was targeted for knockdown by expressing cindrRNAi in the patched stripe of larval wing discs, and dJNK activity was assayed via transcriptional reporters puc-lacZ and hid-lacZ described in chapter 1. The product of the lacZ gene is β-galactosidase (β-gal), and the expression levels of this enzyme are under control of the puckered and hid promoters respectively. I used the X-gal reaction to assay the levels of β-gal expression: in the presence of β-gal, the chromogenic lactose analog X-gal undergoes hydrolysis and oxidation to produce galactose and the blue pigment 5,5'-dibromo-4,4'-dichloro-indigo (Liu et al., 1988, Burn 2012).

Both puc and hid were transcriptionally upregulated when cindrRNAi was expressed (fig 3.8 B and D) but not when only GFP was expressed in the ptc stripe (fig 3.8 A and C). Importantly, the strategy taken for these experiments was to only perturb the levels of Cindr mildly by expressing cindrRNAi rather than to strongly reduce them (such as by expressing cindrRNAi with Dicer-2). This is because Cindr is required to maintain stable cell-cell junctions, and complete loss of this function would cause cells to detach, activating stress
signaling including the JNK pathway indirectly. Thus, we are able to isolate the direct effects of perturbing the Cindr-dJNK interaction. Another advantage of this particular transgenic approach is that a direct comparison can be made with control tissue both anterior and posterior to the *ptc* stripe within the same wing (figure 3.7). Indeed, the relative pixel intensity for both transcriptional reporters was significantly higher in *ptc* tissue expressing *cindrRNAi* compared to *GFP* (fig 3.9 A and B). This quantification was obtained by subtracting the background in a sample posterior to the *ptc* from a sample in the center of the transgenic *ptc* region.

These data suggest that reduction of Cindr in epithelial tissue induces activation of JNK. Of note, Hid (Head Involution Defective) is a conserved pro-apoptotic protein that inhibits dIAP (*Drosophila* Inhibitor of Apoptosis), thus promoting caspase activation (Haining et al., 1999, Goyal et al., 2000). Additionally, we have recently observed an increase in activated caspase and TUNEL signal in larval wing tissue expressing *cindrRNAi* (Hannah Yasin, Honors Thesis, 2015, see figure 3.10). This confirms that reduction of Cindr induces apoptosis. Taken together, these data support the hypothesis that loss of Cindr triggers activation of dJNK and promotes apoptosis. This underscores the importance of Cindr in maintaining stable epithelia: while dJNK activity is important in various situations discussed previously, inappropriate activation can induce non-physiological apoptosis and ultimately impair the patterning of tissues (as seen in fig 3.1).
Figure 3.7: *Patched (ptc)* expression domain. Cartoon shows expression of *GFP* indicated in green in the patched domain along the anterior-posterior (A-P) boundary of a larval wing. Expression of *ptc* is confined to the region shown, and expression of the transgene (in this case *GFP*) is under the control of the *ptc* promoter. Magnified region shows pixel intensity sampling of small, rectangles at the dorsal-ventral (D-V) boundary: the rectangle with the dotted line represents a control sample of the same size in the posterior compartment of the wing, adjacent to the *ptc* domain (Modified from Miles et al., 2006).
Figure 3.8: Loss of Cindr triggers JNK activation. X Gal assay of transcriptional reporters of JNK activity, *puc-lacZ* (A and B) and *hid-lacZ* (C and D) indicated increased activity when Cindr was reduced in the *ptc* domain (B and D) compared to control animals expressing GFP (A and C).
Figure 3.9: Loss of Cindr increases JNK activity. Quantification of X gal reaction intensity of larval wings indicates that expression of cindrRNAi but not GFP triggered an increase in puc-lacZ (A) and hid-lacZ (B) transcription. For each wing disc, the pixel intensity at the Dorsal-Ventral boundary was sampled and subtracted by an adjacent, posterior sample of the same size to correct for background detection. In (A), the result for GFP is slightly negative, indicating that in at least some wings, the control/posterior sample had a slightly higher background pixel intensity than the sample in the patched domain expressing GFP (*: p<0.0001).
Strongly reducing Cindr causes cell migration

The dJNK transcriptional activity detected in the larval wings (figure 3.8) was associated with minimal cell migration but rather induction of apoptosis. Specifically, the pigmented tissue corresponding to the presence of β-Gal is visible as a distinct stripe with minimal cells migrating outside the patched domain (figure 3.8). However, transcriptional activation of pro-apoptotic hid (figure 3.9 B), supports the view that apoptosis has been initiated. This result is consistent with previous reports that the extent of JNK activation can determine whether cells undergo apoptosis (low JNK activation) or migration (high JNK activation) (Ma et al. 2013). As mentioned in the previous section, the transgenic approach to reduce Cindr was intentionally mild, but strong enough to trigger robust JNK activation (figure 3.9). The fact that the larval wing epithelium seemed relatively intact suggests that the JNK activity observed was directly related to reduction of Cindr and not due to cell stress associated with delamination or displacement.

With the above data in hand, we were then able to further decrease the levels of Cindr by expressing Dicer-2 (Dcr-2) along with cindrRNAi, under control of the patched promoter. The ribonuclease Dcr-2 acts to cleave double stranded (ds) RNA, facilitating formation of small interfering (si) RNAi that are subsequently incorporated into the RNAi-induced silencing (RISC) complex (reviewed by Tijsterman and Plasterk, 2004). Thus, increasing the availability of Dcr-2 acts to amplify the effect of the RNAi transgene expressed. Figure 3.10 shows that this more stringent reduction in the levels of Cindr triggers
migration of cells from their original position. Interestingly, an increase in *Drosophila* Profilin (encoded by *chickadee*) expression was detected in many of the cells with reduced Cindr. Profilin is a conserved actin-binding protein that is known to facilitate assembly of actin filaments and play role in cell migration (Verheyen and Cooley, 1994). Furthermore, *chickadee* has been identified as a JNK-responsive locus that plays an important role in JNK-mediated cytoskeletal dynamics (Jasper et al., 2001). Therefore, these data suggest that loss of Cindr triggers cell migration in a manner that is likely at least partially JNK-dependent.

Together with the transcriptional reporter data (figures 3.8, 3.9), these results support the hypothesis that Cindr acts to suppress JNK activity. Furthermore, these data underscore the potential clinical significance of the interaction between Cindr and JNK: the *cindrRNAi* phenotype recapitulates several of the cell behaviors associated with cancer. Notably, the cell migration observed (figure 3.10) and upregulation of matrix-degrading metalloproteases (Hannah Yasin, Honor’s thesis, 2015), both of which are JNK-dependent, are consistent with one of the hallmarks of cancer: invasion and metastasis (Hanahan and Weinberg, 2011). A caveat to this conclusion is that the development of cancer is known to be a multi-step process, and many redundant defense mechanisms must be thwarted during tumorigenesis. A crucial example is that another hallmark of cancer is the evasion of apoptosis (Hanahan and Weinberg, 2011), and we have shown that reducing Cindr triggers apoptosis (figure 3.9 B, figure 3.10 D). Thus, stringent reduction of Cindr is sufficient to compromise epithelial integrity and affect development of
tissues (figure 3.1) but not to cause tumorigenesis *per se*. However, the data may help understand some of the early events that allow cell-cell junctions to destabilize and cells to escape their initial position, events which precede formation of diagnosable and often untreatable tumors.
Figure 3.10: Loss of Cindr triggers cell migration, apoptosis and profilin expression. Adherens junctions remain intact in cells expressing GFP (A) or lacZ (C) while disruption of cell position and detection of Profilin was observed in cells expressing cindrRNAi and Dicer-2 (B). Red channel (anti-Profilin) shown in (A’) and (B’) for (A) and (B) respectively. Extensive cell migration to the posterior compartment and strong TUNEL apoptotic signal in cells expressing cindrRNAi and Dicer-2 (D) Arrows: cells with elevated Profilin (B’) and migrating cells (D). (C and D courtesy of Hannah Yasin and Ruth Johnson).
Reducing JNK activity rescues cindrRNAi phenotype

From the data so far, we can conclude that Cindr forms a complex with JNK, that loss of Cindr activates JNK transcriptional activity as well as gives rise to mispatterning of the adult wing. However, these data do not support whether the increase in transcriptional activity and mispatterning are a result of Cindr-JNK interaction or are more correlational. In order to address this concern, JNK activity was modified in a background with reduced Cindr. Specifically, the JNK phosphatase encoded by puckered was overexpressed along with cindrRNAi, under control of patched. As shown in figure 3.1, reduction of Cindr resulted in striking loss of tissue corresponding to the ptc domain. However co-expression of puc was sufficient to rescue the cindrRNAi phenotype, as evidenced by the significant increase in L3-L4 relative intervein area (figure 3.11 C). Since Puckered interacts directly with JNK, dephosphorylating and thus deactivating it (McEwan and Peifer, 2005), it can be concluded that the cindrRNAi phenotype in the fly wing requires activation of JNK. As summarized in chapter 1, JNK is an important mediator of both apoptosis and cell migration. Thus, it is encouraging that experimental suppression of JNK signaling also suppresses the cell behaviors that underlie loss of Cindr. Indeed, confocal microscopy revealed that experimental inhibition of JNK signaling at various levels in the kinase cascade attenuated cindrRNAi-induced cell migration and cell death (figure 3.10, Hannah Yasin, Honors Thesis, 2015).
Figure 3.11: Inhibition of JNK rescues effect of cindrRNAi in the adult wing. Adult wings were obtained from flies expressing either cindrRNAi (A) or cindrRNAi with puckered (puc) under control of patched-GAL4 (B) in the ptc domain throughout development. Quantification of the relative L3-L4 intervein areas indicates a significant increase in area when puc is expressed in addition to cindrRNAi (p<0.0001).
Conclusion

In conclusion, taken together, the data presented in this thesis support the hypothesis that Cindr interacts with dJNK and inhibits its activity in order to maintain stable epithelia. The co-immunoprecipitation data confirm previous Yeast 2-hybrid screen results, increasing our confidence in direct protein-protein interactions between Cindr and dJNK. Transcriptional reporters revealed that loss of Cindr upregulated dJNK transcriptional activity, including activity associated with apoptosis and cell migration. Finally, it was demonstrated that tissue mispatterning induced in wing tissue with reduced Cindr could be ameliorated by simultaneously suppressing dJNK activity.
Chapter 4 - Discussion and Future directions

Testing three models to explain Cindr-mediated dJNK inhibition

While this thesis supports the hypothesis that Cindr inhibits dJNK signaling, the mechanism whereby this occurs is unclear. A variety of possibilities exist, and these might not be mutually exclusive. Cindr is an adaptor protein with multiple interaction domains. Cindr is also known to localize to specific subcellular regions such as the cell membrane (Johnson et al., 2008). We have also demonstrated that Cindr forms a complex with dJNK, suggesting that they interact in vivo. Taken together, it is plausible that Cindr sequesters dJNK, thereby reducing the overall pool of free cytoplasmic dJNK. One way to test this would be to determine whether subcellular localization of dJNK is modified when the levels of Cindr are perturbed.

A second possibility is also partially addressed in this thesis, and that is that Cindr may promote ubiquitination and subsequent proteolysis of dJNK protein. I carried out a relay co-IP experiment that suggested in the Cindr-dJNK complex, dJNK was ubiquitinated (figure 3.6). While this is by no means conclusive, it does provide a useful starting point. The ubiquitination model predicts that loss of Cindr would decrease the efficiency of dJNK degradation, and thus the levels of dJNK would increase, in turn enabling higher levels of dJNK activation. I have attempted to test this prediction by western blot analysis of wing discs with reduced Cindr, but was unable to resolve a significant difference in dJNK levels (data not shown). This was likely due to
insufficient total protein concentration obtained by the technically challenging larval wing dissection, since many larval wing discs need to be isolated as rapidly and precisely as possible. Thus, we are exploring an alternative strategy whereby lysate is obtained from readily-obtainable embryos ubiquitously expressing cindrRNAi. We have also subsequently obtained more sensitive anti-JNK and anti-pJNK antibodies that may enable us to more accurately resolve and quantify differences in protein levels. Additionally, our lab is currently carrying out a genetic screen to identify potential E-3 ligases that may be recruited by Cindr to target JNK for degradation.

A third model is that Cindr recruits a phosphatase that in turn dephosphorylates and inactivates dJNK. This thesis describes several pieces of data that indirectly address this model. Firstly, loss of Cindr triggers an increase in JNK activity, suggesting disinhibition of dJNK. Secondly, co-IP experiments demonstrated an interaction between Cindr and phosphorylated JNK, indicating that Cindr might target activated JNK. Finally, overexpression of the phosphatase Puckered rescued the cindrRNAi phenotype in the adult wing. Our lab is also engaged in a genetic screen to identify other phosphatases that might be recruited by or function with Cindr to regulate dJNK activity.

Endogenous dJNK activation and Cindr localization

One of the central aims of this thesis was to examine how Cindr acts to inhibit dJNK signaling to maintain stable cell positions. However, the opposite
scenario is also intriguing: in cells which are undergoing normal, JNK-dependent dynamic changes, is Cindr delocalized or similarly modified? We used transgenic tools to artificially reduce the levels of Cindr in larval wing discs, which have relatively low endogenous dJNK activity. When the level of Cindr was reduced, robust dJNK-mediated transcriptional activity was detected, suggesting that dJNK was disinhibited in the absence of Cindr. It stands to reason that in cells that are undergoing dynamic changes in response to endogenous dJNK activity, the localization and/or levels of Cindr or activity of Cindr complexes may be modified. A promising model system to test this is dorsal closure, a dJNK-dependent process described previously. Using the TRE-RFP (Tetradecanoylphorbol acetate Response Element) reporter as a marker of the cells in the leading edge (with known activated dJNK) and the Cindr antibody, it will be possible to determine changes in the localization or levels of Cindr in a physiologically relevant context (Chatterjee and Bohmann, 2012). Taking a genetic approach, we could also determine whether overexpression of Cindr in the leading edge cells reduces the efficiency of dorsal closure. For example, complete failure of dorsal closure would result in lethality, while inefficient dorsal closure could result in delayed progression to the larval stages. This would suggest that in cells that require dJNK signaling, excess Cindr dampens this signaling. As such, it is also possible that a reduction in the TRE-RFP (a function of dJNK activity) signal intensity will be observed.
Further characterization of adult wings

Although analysis (with a light microscope) of the adult wing does not provide the cellular-level precision of confocal imaging of dissected imaginal discs, it does provide important information about the outcome of genetic manipulations. For example, the experimental reduction in some protein may perturb structure and function during development, but other redundant mechanisms may result in a seemingly normal, viable adult. Gratifyingly, loss of Cindr in the *patched (ptc)* domain resulted in extensive cell migration and cell death in the larval wing disc and the resulting adult wing discs were markedly aberrant. As already discussed, this phenotype could be completely rescued by also overexpressing *puc*. It would be interesting to determine whether similar results could be achieved by reducing dJNK signaling by other means in the background of reduced Cindr. The change in the area of the Ptc domain could then be quantified for a battery of different JNK-related genotypes, providing increased confidence in the validity of the results. One challenge associated with these experiments is that many of the progeny with reduced Cindr and/or dJNK activity do not survive to adulthood. Potential solutions to this include lowering the temperature of incubation (since expression of the RNAi, under control of the UAS-GAL4 system, is temperature sensitive) or using alternative driver lines which may result in a milder phenotype than with the *patched-GAL4* driver. It could also be valuable to take an approach in which a qualitative assessment of the adult wing structure is made (Neisch et al., 2010). This could be useful for wings in which
the structure is too disrupted for accurate measurements of wing area to be made, or in which differences in area are subtle but other structural abnormalities are present. Such data could still be made semi-quantitative by using a rating scale based on the severity of the resulting phenotypes, in order to make statistical inferences across many different genotypes.

*Test whether dJNK activity regulates expression of Cindr*

This project sought to examine how Cindr acts to directly regulate the activity of dJNK. As discussed previously, *puckered* is one of several targets expressed in response to dJNK activation, via the intermediate transcription factors Jun and Fos (Martin-Blanco et al., 1998). These transcription factors dimerize to form the AP-1 transcriptional complex (Pulverer et al., 1991). Interestingly, Puckered acts as a MAP Kinase Phosphatase that deactivates dJNK.

Importantly, an analysis of the human CD2AP gene promoter in renal epithelial cells by Lu et al. (2009) demonstrated that the AP-1 transcriptional complex (a direct product of dJNK activation) activates the CD2AP gene promoter. Importantly, the observed upregulation of CD2AP correlated with a significant decrease in apoptosis (Lu et al., 2009). While the mechanism responsible for this protective effect remains unclear, it is possible that CD2AP may interact with and inhibit JNK, which in turn plays a key role in promoting apoptosis. However, the hypothesis that the Cindr/CD2AP/CIN85 family is transcriptionally regulated by JNK needs to be tested *in vivo*. The cell
culture work of Lu et al. (2009) provides a valuable starting point, but their results need to be verified in a more physiologically relevant context. I hypothesize that in epithelial tissues with high JNK activity, the levels of Cindr are increased as part of a negative feedback mechanism. If this hypothesis is supported, the data in this thesis suggests that the increased levels of Cindr will act to restrain dJNK activity as part of a negative feedback loop.

The larval wing epithelium experimental system could serve as a powerful way to achieve this. I propose that dJNK activity be induced in the ptc domain by ectopic upstream kinases Slipper (Slpr) or a constitutively activated version Hemipterous (Hep). I have already verified that ectopic Slpr results in expression of puc, a transcriptional target of dJNK. The α-Cindr antibody and confocal microscopy could then be used to determine whether the levels of Cindr are modified in response to dJNK. Additionally, a biochemical approach could be used, whereby dJNK is ubiquitously activated in embryos prior to western blot analysis to quantify potential changes in the levels of Cindr. This approach could provide important quantitative information in terms of the expression of cindr and suggest the possibility of a powerful negative feedback mechanism, whereby dJNK activation increases Cindr expression, with Cindr in turn inhibiting dJNK.
Test whether Cindr-JNK interaction is general

The work described in this thesis specifically dealt with the role of the interaction between Cindr and JNK in regulating epithelial cell junctions. It would be interesting to explore this interaction in other experimental paradigms. One potential approach could be to focus on the well-established role of JNK signaling in the oxidative stress-response and organism longevity (Ma et al., 2014). Wang et al. (2003) provided compelling evidence that in *Drosophila*, ectopic neuronal dJNK is protective under conditions of oxidative stress, and even promotes longevity. It is unclear precisely why JNK activity can affect the lifespan of an organism, but it appears that medial neurosecretory cells (mNSCs), which help maintain metabolic homeostasis, may play an important role (reviewed by Biteau et al., 2011). Since the JNK pathway is well conserved in the animal kingdom, these results may have important implications for research on the biology of aging.

A simple initial experiment could be carried out in which *cindr* is overexpressed in neurons using the pan-neuronal driver line elav-GAL4 (Wang et al. 2003). We could then compare the average lifespan of these flies to controls expressing *lacZ*. It is plausible that a modest decrease in survival will be seen in flies with overexpressed *cindr*, if indeed Cindr acts to inhibit dJNK in neurons in addition to epithelial cells. An alternative or additional approach would be to induce dJNK activity in neurons by overexpressing the upstream kinase encoded by *hep*, since this effect is known to significantly increase lifespan (Wang et al. 2003). We could then overexpress *cindr* in this
genetic background and determine whether this dampens the protective effect of dJNK. While the converse experiment, where the levels of Cindr are reduced by expressing cindrRNAi, would be compelling, it is likely that such flies would be extremely unhealthy, because of disruption of functions independent of dJNK signaling.

In a parallel experiment to the above, dJNK could be pharmacologically activated using paraquat, an agent known to generate oxidative stress and activate dJNK (Arking et al., 1991, Chatterjee and Bohmann, 2012). Flies with genetically-reduced dJNK activity die significantly more quickly than controls when exposed to paraquat (Ma et al., 2014). We could use a similar protocol to test whether changing the levels of Cindr leads to differential survival, which would also speak to an interaction between Cindr and dJNK. A follow-up study by Wang et al. (2005) showed that the significant increase in lifespan in flies with (modest) dJNK upregulation was mediated by insulin signaling. This is consistent with seminal work that was carried out in *C. elegans* (Kenyon, 2001). Intriguingly, Nephrin, an important CD2AP binding partner, is essential for the insulin response in human glomerular podocytes (Coward et al., 2007). Furthermore, podocytes cultured under diabetic conditions were found to have reduced and delocalized CD2AP (Ha et al., 2015). This is of particular interest because diabetic nephropathy is the primary cause of chronic kidney disease worldwide (Parving et al., 2011). Taken together, these findings suggest a possible link between the Cindr-JNK interaction, insulin signaling and ultimately survival: exploring such an interaction could
potentially shed light on the mechanisms of aging as well as the pathogenesis of diabetes and diabetic nephropathy.

Conclusion

The data presented in this thesis supports the hypothesis that Cindr interacts with dJNK, suppressing its activity in order to maintain stable cell-cell junctions. A series of co-immunoprecipitation experiments confirmed previous independent yeast 2-hybrid data suggesting that Cindr interacts with dJNK. These experiments also expanded on what is known, because these experiments were conducted using *Drosophila* tissue. However, additional experiments are needed in order to determine whether the Cindr-dJNK interaction is direct. Notwithstanding, this thesis has demonstrated the functional relevance of the Cindr-dJNK interaction, since reducing Cindr triggered activation of JNK as well as apoptosis. These findings further reinforce the previously known role for Cindr in maintaining epithelial stability, but extend what is known in that these experiments were conducted using a completely different model tissue (i.e. the larval and adult wing as opposed to the eye). As mentioned, it would be interesting to determine whether Cindr functions in other epithelial and non-epithelial tissues. Furthermore, it is important to determine whether the interaction described in this thesis is conserved in vertebrates. This is particularly important given the fact that loss of Cindr results in abnormal development and may contribute to early metastatic cell behaviors.


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