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The adaptor protein Cindr regulates JNK activity to maintain epithelial sheet integrity

Hannah W.R. Yasin [1], Samuel H. van Rensburg [1], Christina E. Feiler [1,2], Ruth I. Johnson *

Biology Department, Wesleyan University, 52 Lawn Avenue, Middletown, CT, USA

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ABSTRACT

Epithelia are essential barrier tissues that must be appropriately maintained for their correct function. To achieve this a plethora of protein interactions regulate epithelial cell number, structure and adhesion, and differentiation. Here we show that Cindr (the *Drosophila* Cin85 and Cd2ap ortholog) is required to maintain epithelial integrity. Reducing Cindr triggered cell delamination and movement. Most delaminating cells died. These behaviors were consistent with JNK activation previously associated with loss of epithelial integrity in response to ectopic oncogene activity. We confirmed a novel interaction between Cindr and *Drosophila* JNK (dJNK), which when perturbed caused inappropriate JNK signaling. Genetically reducing JNK signaling activity suppressed the effects of reducing Cindr. Furthermore, ectopic JNK signaling phenocopied loss of Cindr and was partially rescued by concomitant *cindr* over-expression. Thus, correct Cindr-dJNK stoichiometry is essential to maintain epithelial integrity and disturbing this balance may contribute to the pathogenesis of disease states, including cancer.

1. Introduction

Multicellular organisms depend on epithelia for diverse functions including protection, compartmentalization of physiological systems, selective absorption, secretion and sensory reception. Given the importance of these functions, it is essential to better understand the mechanisms and molecules that, together, maintain the strength and integrity of epithelia. Diverse epithelia are assembled according to a similar plan: they typically consist of closely adherent polarized cells with simple shapes and at least one layer of cells closely associated with a basement membrane (Rodriguez-Boulan and Macara, 2014). Disrupting the internal organization of epithelial cells or their organization within the tissue can compromise the function of the epithelium. In addition the majority of fatal cancers are of epithelial origin (Weinberg, 2013). This is in part due to their exposure to carcinogens that can cause genetic mutations and because most epithelia retain mitotic potential to facilitate their rapid repair. If oncogenic mutations occur that deregulate proliferation, disrupt cell death and compromise cell polarity or adhesion, tumors and even metastases can occur (Halaoui and McCaffrey, 2015; Hanahan and Weinberg, 2011; Chaffer and Weinberg, 2011; Wogan et al., 2004; Martin-Belmonte and Perez-Moreno, 2012). A better understanding of how epithelia are maintained and regulated is therefore a priority.

Here we describe a role for the cytoplasmic *Drosophila* adaptor protein Cindr in maintaining the integrity of a pseudostratified epithelium, the fly wing. Cindr and its vertebrate orthologs Cd2ap and Cin85 contain multiple SH3 domains and several other protein interaction motifs that confer the ability to assemble multi-protein complexes that mediate diverse yet critical functions. Cindr and Cd2ap have previously been implicated in regulating the cytoskeleton and promoting stable cell adhesion via interactions with actin, the actin capping proteins (CPs), and GTPase activating proteins (GAPs) that target Arf6 and Cdc42 (Johnson et al., 2008, 2011, 2012; Faul et al., 2007; Bruck et al., 2006; Tang and Brieher, 2013; Zhao et al., 2013; Elbediwy et al., 2012; Yaddanapudi et al., 2011; Welsch et al., 2005; Mustonen et al., 2005; Lehtonen et al., 2002). Additionally an interaction between Cindr and Anillin at the cleavage furrow of mitotic cells is critical for cell proliferation (Haglund et al., 2010). In contrast the complexes assembled by Cin85 have mainly been implicated in regulating ubquitination and endocytosis of receptor tyrosine kinases (RTK) (Dikic, 2002,

List of abbreviations: CP, actin-capping protein; AJ, adherens junction; APF, after puparium formation; A/P, anterior/posterior; Bsk, basket; β-Gal, β-Galactosidase; JNK, c-Jun N-terminal kinase; Chic, chickadee; Dcr-2, dicer-2; D/V, dorsal/ventral; dJNK, Drosophila JNK; Jra or dJun, Drosophila Jun; Ecad, ecadherin; GMA, GFP: moesin-Actin-binding-domain; GFP, green fluorescent protein; GAPs, GTPase activating proteins; Hid, hid involution defective; MMP, matrixmetalloprotease; Ptc, patched; Puc, puckered; RTK, receptor tyrosine kinase; Slpr, slipper; TdT, terminal deoxynucleotidyl transferase; TUNEL, mediated dUTP nick end labeling; ZA, zonula adherens

* Corresponding author.
E-mail address: rijohnson@wesleyan.edu (R.I. Johnson).
[1] These authors contributed equally to this work.
[2] Present address: Center of Plant Molecular Biology, Eberhard-Karls-University of Tuebingen, Auf der Morgenstelle 32, Tuebingen, Germany.

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2003) as well as adhesion proteins of the slit diaphragm, a specialized junction of the vertebrate kidney (Tossidou et al., 2010). When, where and how Cindr, Cd2ap and Cin85 assemble appropriate protein complexes to accomplish these diverse functions remains a challenge. Genetically tractable *Drosophila* tissues serve as models to examine the nature and function of these complexes and address these challenges.

In exploring how Cindr maintains the integrity of the fly wing epithelium, we uncovered novel interactions with JNK (c-Jun N-terminal kinase) signaling. We found that reducing Cindr in the developing wing epithelium caused dramatic cell delamination: some cells migrated short distances and many cells died. These cell behaviors were mediated in part by ectopic JNK signaling that was triggered in cells with lower concentrations of Cindr. JNK signaling is a critical kinase cascade that responds to a variety of stimuli including DNA damage, environmental stress, wounding and Tumor Necrosis Factor-α (Adler et al., 1995; Rosette and Karin, 1996; Ramet et al., 2002; Igaki et al., 2002; Moreno et al., 2002). Appropriate responses to these stimuli include apoptosis, cell migration and even cell proliferation, effects that are mediated by expression of an array of genes targeted by transcription factors including c-Jun and c-Fos, which in turn are activated by JNK (Picco et al., 2013; Stronach, 2005; Rios-Barrera and Riesgo-Escovar, 2012).

The relationship between JNK activity and the maintenance of epithelial integrity is complex. JNK drives cytoskeletal changes and migratory cell behaviors necessary to repair epithelial wounds (Rios-Barrera and Riesgo-Escovar, 2012; Repiso et al., 2011). Here we show that this JNK function contributes to the movement of cells that lack Cindr. In addition, JNK is an important antagonist of stable epithelial junctions (You et al., 2013). A fundamental feature of epithelia is the presence of apical AJs that are generated by homophilic interactions between epithelial cadherin (Ecad) dimers of adjacent cells (Harris and Tepass, 2010). The intracellular domains of Ecad interact with proteins at the plasma membrane including Catenins that provide links to the actin cytoskeleton. Extracellular interactions between apposing Ecad molecules and internal interactions with the cytoskeleton provide mechanical strength critical for epithelial integrity and support epithelial cell structure (Baum and Georgiou, 2011). However, activated JNK leaves AJs susceptible to disassembly *in vitro* because β-catenin is phosphorylated by JNK, which compromises its interaction with cadherins and consequently their tethering to the cytoskeleton (Lee et al., 2009, 2011; Naydenov et al., 2009). Hence ectopic JNK activity in cells lacking Cindr could account for changes in adhesion that release cells from the epithelium, as described below. However, JNK activity also commonly triggers apoptosis (Davis, 2000; Lin, 2003; Liu and Lin, 2005) and we found that JNK mediated the death of *cindr*-depleted cells. Hence Cindr-JNK interactions are necessary to regulate adhesion and the cytoskeleton to maintain cells within the epithelium and spare them from death. Indeed we found that *Drosophila* JNK (named Basket, Bsk) resides in complexes with Cindr that we argue are essential to curtail JNK activity.

2. Materials and methods

2.1. Drosophila genetics

All fly crosses were raised at 25 °C unless otherwise noted. We generated the fly lines *UAS-GMA*, *UAS-Dcr-2*; *ptc-GAL4*, *UAS-GFP* (X, II) and *ptc-GAL4*, *UAS-GFP*; *UAS-GMA* (*II*, *III*) with transgenic stocks obtained from the Bloomington Stock Center (BL-6874, BL-2017, BL-24646, BL-31774, BL-31776). We nicknamed these stocks GDPG and PGG respectively. The transgenic lines used to reduce Cindr are described in Johnson et al. (2008). *UAS-cindr*$^{RNAi2.21+23}$ combines two identical *RNAi* transgenes that target nucleotides 1016 to 1518 (nucleotide positions relate to the longest, predominantly expressed *cindr* transcript). A second line, *UAS-cindr*$^{RNA3.73+81}$ combines two identical *RNAi* transgenes that target nucleotides 1664 to 2246. Both *UAS-cindr*$^{RNAi2.21+23}$ and *UAS-cindr*$^{RNA3.73+81}$ reduce expression of long and intermediate Cindr isoforms (described in Johnson et al., 2008), but *UAS-cindr*$^{RNAi2.21+23}$ is three times more effective than *UAS-cindr*$^{RNA3.73+81}$ in reducing *cindr* transcripts. We therefore used *cindr*$^{RNAi2.21+23}$, abbreviated throughout the manuscript to *UAS-cindr*^{RNAi2}, for most studies. To assay the effects of reducing *cindr*, we crossed GDPG females to *UAS-cindr*^{RNAi2} males and dissected male third larval instar progeny, which had higher levels of *Dcr-2* expression than females. Stable stocks carrying *UAS-cindr*^{RNAi2} and *UAS-p35* (BL-5072) were generated and crossed to GDPG to block cell death in the *ptc*-domain of the progeny. Stable stocks carrying *UAS-cindr*^{RNAi2} and *UAS-bsk*^{DN} (BL-9311) or *bsk*^{1} (BL-3088) or *Jra*^{3} (Fanto et al., 2000, gift from Ursula Weber) were established and crossed to GDPG to assess whether reducing JNK signaling activity modified *cindr*^{RNAi2}-phenotypes. Again, only male larval progeny were dissected. *UAS-slpr*$^{WT\text{-}HA\text{-}55}$ (Garlena et al., 2010, gift from Beth Stronach) was combined with *UAS-cindrPC*^{TAP} (Johnson et al., 2008) to generate stable fly lines and crossed to PGG to test whether ectopic Cindr could quash ectopic JNK activity. When PGG was used as the maternal parent, both male and female larval progeny were dissected.

To assay JNK activity we generated stable fly lines that carried *ptc-GAL4, UAS-GFP* and *puc*^{E69} (*puc-lacZ*, BL-6762), *hid-lacZ* (Fan et al., 2010, gift from Andreas Bergman) or *TRE-RFP-16* (Chatterjee and Bohmann, 2012, gift of Dirk Bohmann) and crossed these to *UAS-cindr*^{RNAi2} males. For better detection of *TRE-RFP-16* expression, crosses were raised 29 °C. The *ptc-GAL4, UAS-GFP*; *puc*^{E69} line was also used to test whether *UAS-cindrPC*^{TAP} suppressed *UAS-slpr*$^{WT\text{-}HA\text{-}55}$-induced JNK activity.

High expression of *cindr*^{RNAi2} was mainly pupal-lethal. To generate pupal *cindr*^{RNAi2} tissue for live cell imaging, we maintained cultures of *UAS-cindr*^{RNAi2} crossed to GDPG at 18 °C until the progeny pupated. Male pupae were gathered at 0 h after puparium formation (APF), maintained at 25 °C, and imaged 14 h later. Live imaging is described below.

2.2. Dissection, immunofluorescence and microscopy

Wandering third instar larvae were dissected in PBS and fixed in 4% formaldehyde using standard procedures. Progeny of crosses utilizing GDPG or PGG were imaged within 24 h of dissection. For immunofluorescence, primary antibodies were rabbit anti-Cindr (1:300, Johnson et al., 2008), rat anti-*Drosophila* Ecadherin (1:50, DSHB DCAD2), rabbit anti-cleaved caspase-3 (Cell Signaling Technology, #9661S), mouse anti-MMP1 (1:10, DSHB 3B8D12-S), mouse-anti-Profilin (1:1, DSHB chi 1J), and rat anti-Twist (1:500, Roth et al., 1989, gift from E. Wieschaus). To visualize actin, Rhodamine Phalloidin was included in both fixative and primary antibody incubations (1:100–1:200, Molecular Probes R415). Secondary antibodies were conjugated to Alexafluor 488, Alexafluor 647 or Cy3 (Jackson ImmunoResearch). Cell death was determined using an In Situ Cell Death Detection Kit (TMR Red, Roche). Tissue was imaged with a Zeiss LSM 501 metaconfocal and Zen software.

To detect *puc-lacZ* or *hid-lacZ* expression, dissected wing discs were fixed in 2% gluteraldehyde and β-Gal activity detected using standard methods. Wing discs were imaged using a Zeiss Axioplan light microscope, Tucsen H series camera and ISCapture V3.0 software.

Adult wings were preserved in 100% Ethanol and then mounted in Euparal. These were imaged with a Zeiss Axioplan light