Atypical Cadherin Dachsous Regulates The Cytoskeleton And Gastrulation Movements During Zebrafish Development

Nanbing Li
Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS
Division of Biology and Biomedical Sciences
Developmental, Regenerative, and Stem Cell Biology

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Atypical Cadherin Dachsous Regulates The Cytoskeleton
And Gastrulation Movements During Zebrafish
Development
by
Nanbing (Jade) Li

A dissertation presented to the
Graduate School of Arts & Sciences
of Washington University in
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of Doctor of Philosophy

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>μ</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
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<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>A</td>
<td>Animal</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>C&amp;E</td>
<td>Convergence and extension</td>
</tr>
<tr>
<td>CG</td>
<td>Cortical granules</td>
</tr>
<tr>
<td>CGE</td>
<td>Cortical granule exocytosis</td>
</tr>
<tr>
<td>D</td>
<td>Dorsal</td>
</tr>
<tr>
<td>Dchs</td>
<td>Vertebrate Dachsous</td>
</tr>
<tr>
<td>DD</td>
<td>Dorsal determinant</td>
</tr>
<tr>
<td>Dgo</td>
<td>Diego</td>
</tr>
<tr>
<td>Ds</td>
<td><em>Drosophila</em> Dachsous</td>
</tr>
<tr>
<td>Dsh</td>
<td><em>Drosophila</em> Dishevelled</td>
</tr>
<tr>
<td>Dvl</td>
<td>Vertebrate Dishevelled</td>
</tr>
<tr>
<td>ENU</td>
<td><em>N</em>-ethyl <em>N</em>-nitrosourea</td>
</tr>
<tr>
<td>EVL</td>
<td>Enveloping layer</td>
</tr>
<tr>
<td>Fj</td>
<td><em>Drosophila</em> Four-jointed</td>
</tr>
<tr>
<td>Fjx1</td>
<td>Vertebrate Four-jointed</td>
</tr>
<tr>
<td>Fmi</td>
<td>Flamingo</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>Fz</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>Hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>Kny</td>
<td>Knypek</td>
</tr>
<tr>
<td>MBT</td>
<td>Midblastula transition</td>
</tr>
<tr>
<td>Mpa</td>
<td>Minutes post activation</td>
</tr>
<tr>
<td>MO</td>
<td>Antisense morpholino oligonucleotide</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing center</td>
</tr>
<tr>
<td>MZ</td>
<td>Maternal zygotic</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>Pk</td>
<td>Prickle</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCP</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>SDCM</td>
<td>Spinning disc confocal microscope</td>
</tr>
<tr>
<td>sf-GFP</td>
<td>Super fold GFP</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TILLING</td>
<td>Targeting induced local lesions in genomes</td>
</tr>
<tr>
<td>Tri</td>
<td>Trilobite</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumor growth factor-β</td>
</tr>
<tr>
<td>V</td>
<td>Vegetal</td>
</tr>
<tr>
<td>Vang</td>
<td>Van Gogh/Strabismus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Vangl2</td>
<td>Vertebrate Vang-like 2/Trilobite</td>
</tr>
<tr>
<td>Wg</td>
<td>Wingless</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YSL</td>
<td>Yolk syncytial layer</td>
</tr>
<tr>
<td>YSN</td>
<td>Yolk syncytial nuclei</td>
</tr>
<tr>
<td>Z</td>
<td>Zygotic</td>
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</table>
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Dachsous (Dchs), an evolutionarily conserved atypical cadherin, regulates planar cell polarity, tissue size, and cell adhesion in Drosophila. However, its functions in vertebrates are just beginning to be elucidated. Inactivating one of two murine homologs, Dchs1, leads to multi-organ defects and postnatal lethality. Recent studies in humans suggest that mutations in DCHS1 cause pleiotropic Van Maldergem syndrome. My thesis work focuses on the functional characterization of zebrafish dchs1b and dchs2 genes. Mutations in dchs1b and dchs2 genes affect several aspects of embryogenesis, including gastrulation. Unexpectedly, dchs1b is also essential for the earliest vertebrate developmental stage, egg activation. We show that maternally contributed dchs1b coordinates cytoskeleton dependent processes including cortical granule exocytosis (GCE), cytoplasmic segregation, cell divisions, and maternal mRNA translocation in transcriptionally silent early embryos. Later, maternal zygotic (MZ) dchs1b mutants exhibit altered expression of several genes expressed in the dorsal organizer and mesendoderm, due to
impaired transport of a dorsal determinant and Nodal signaling. Mechanistically, aspects of the
MZdchs1b phenotype can be explained by defects in either actin and/or microtubule networks,
which both appear aberrantly bundled in mutants. Accordingly, disruption of actin cytoskeleton
in wild-type embryos phenocopied MZdchs1b mutant defects in cytoplasmic segregation and
CGE. Whereas, interfering with microtubules in wild-type embryos impaired dorsal organizer
and mesodermal gene expression without perceptible earlier phenotypes. During gastrulation,
both MZdchs1b and MZdchs2 mutants manifest defects in morphogenic movements: delayed
epiboly in mutants is caused in part by defects in actin and microtubule cytoskeleton and
adhesion defects, which is independent of planar cell polarity pathway; and cell polarity that
drives convergence and extension, which is under planar cell polarity pathway regulation. My
work establishes novel roles for vertebrate Dchs in actin and microtubule cytoskeletons
regulation in an unanticipated single cell context of the early zygote and conservation of function
for Dchs in regulation of planar cell polarity that could contribute to the pleiotropic defects
cauised by mutations in mammalian Dchs homologs.
Chapter 1: Introduction

1.1 Cadherins And Atypical Cadherins

Cadherins are a superfamily of proteins that function in many processes, including cell adhesion, cell sorting, cell movements, cell and tissue polarity, and growth (Halbleib and Nelson, 2006). Cadherins were first identified as calcium dependent cell-cell adhesion proteins (Gallin et al., 1983; Peyrrieras et al., 1983; Yoshida and Takeichi, 1982). Since the identification of the first cadherin, over a hundred family members have been described, all of which contain the characteristic cadherin repeats in the extracellular domain, a single transmembrane domain, and an intracellular domain (Nollet et al., 2000). Cadherins are divided into three subfamilies: classical cadherins, protocadherins, and atypical cadherins.

Classical cadherins were the first family identified and contain five extracellular cadherin repeats that are important for homophilic intercellular calcium dependent adhesion, although they can also interact in a heterophilic manner (Chen et al., 2005; Foty and Steinberg, 2005; Niessen and Gumbiner, 2002; Nose et al., 1988; Patel et al., 2006; Shimoyama et al., 2000). The intracellular domains of classical cadherins are highly conserved and can directly bind with cytoplasmic proteins such as β-catenin, α-catenin, and p120 (Aberle et al., 1994; Anastasiadis and Reynolds, 2001; Brembeck et al., 2006; Kobiela and Fuchs, 2004). These cytoplasmic interactors affect how the classical cadherins interact with the actin cytoskeleton to regulate processes such as cell sorting, cell movement, and orientation of cell division (Bertet et al., 2004; Foty and Steinberg, 2005; Kane et al., 2005; Lu et al., 2001; Nose et al., 1988; Shimizu et al., 2005; Yamashita et al., 2003; Zhang et al., 2010).
The protocadherin subfamily is the largest with over sixty members, but much less well studied than classical cadherins (Junghans et al., 2005; Sano et al., 1993; Wu and Maniatis, 1999). Protocadherins are primarily expressed in neural tissues. They feature six to seven extracellular cadherin repeats and have weak homophilic adhesive properties (Chen and Gumbiner, 2006). The cytoplasmic domains of protocadherins are diverse, unlike well-conserved classical cadherin cytoplasmic domains, and little is known about their binding partners (Hambsch et al., 2005; Wu and Maniatis, 1999). Some protocadherins have been found to be important during development: paraxial protocadherin (PAPC) is necessary for ectoderm and mesoderm separation and convergence and extension movements in *Xenopus* gastrulae; Protocadherin-10 facilitates somite formation; and neural fold protocadherin (NFPC) mediates ectodermal adhesion and neural tube closure in *Xenopus* (Bradley et al., 1998; Hirano et al., 1999; Murakami et al., 2006; Rashid et al., 2006; Unterseher et al., 2004; Yamamoto et al., 1998).

Dachsous (Ds in *Drosophila* and Dchs in vertebrates) and Fat are both giant atypical cadherins, and like classical cadherins, both are single-pass transmembrane proteins. Ds and Fat, however, have 27 and 34 extracellular cadherin domains respectively, compared to the 5 cadherin domains found in classical cadherins (Clark et al., 1995; Mahoney et al., 1991). The intracellular domains of Ds and Fat exhibit sequence homology with the binding site for β-catenin also found in classical cadherins. However, neither Ds nor Ft have been shown to associate physically with β-catenin (Clark et al., 1995; Mahoney et al., 1991). While I focus on Dachsous and Fat function in planar cell polarity (PCP) and cell-cell adhesion, they have also been implicated in regulating apical membrane organization, tissue growth, and morphogenesis (Ishiuchi et al., 2009; Mahoney et al., 1991).
Figure 1.1 Cadherin Superfamily Members
Representative schematics of members of classical cadherin, protocol cadherin, and atypical cadherin. Most cadherin superfamily members are single transmembrane proteins.
1.2 Cell Organization And Polarity

The two major cell types in all vertebrates, epithelia and mesenchymal cells, display very distinct characteristics. Epithelial cells are tightly packed with homophilic E-cadherin intercellular interactions at adherence junctions stabilizing cell-cell contacts and show both apical/basal and planar polarity (Takeichi, 2014; Yonemura, 2011). Although mostly stable in adult tissues, these epithelial cells are highly dynamic during development; and rearrangement of cells within epithelial sheets is a key mechanism of tissue and organ morphogenesis. Apical/basal polarity in epithelial cells is characterized by localization of specific proteins to the apical membrane, the side often orientated towards the lumen, such as aPKC, PAR, and Crumbs (Betschinger et al., 2003; Bilder and Perrimon, 2000; Colosimo et al., 2010; Kaplan et al., 2009). On the basaolateral sides of cuboidal epithelial cells, where cells interact with each other and the basal membrane, are adherence junctions composed of E-cadherin, β-catenin, and other adhesion proteins (Oda et al., 1993; Peifer et al., 1993; Tepass, 1996). Planar polarity coordinates cell polarity and cell behavior across a tissue perpendicular to apical/basal polarity. In epithelia, planar polarity drives tissue extension through polarized cell intercalations (Aigouy et al., 2010; Bertet et al., 2004; Blankenship et al., 2006). Mesenchymal cells are loosely packed, often migratory cells that also display planar polarity with a front and back or other features polarized in the tissue plane (Abercrombie et al., 1971; Hay, 2005; Ridley et al., 2003). An important regulator of planar polarity in both types of cells is the planar cell polarity (PCP) pathway.
Figure 1.2 Polarized Epithelia And Mesenchyme

A. Apical/basal and planar polarized epithelial cells.
B. Migrating mesenchymal cells with front and back; elongated intercalating mesenchyme with anterior and posterior polarity.
1.3 Planar Cell Polarity

1.3.1 Planar Cell Polarity In *Drosophila*

The PCP pathway was first discovered in insects (Lawrence, 1969; Gubb and Garcia-Bellido, 1982). Subsequent studies in the *Drosophila* eye ommatidia, wing, thorax, and abdomen setae showed that the PCP pathway controlled tissue polarity in these epithelial structures and that mutations in the core PCP pathway genes impair this polarity (Adler et al., 1997; Casal et al., 2006; Jenny et al., 2005; Usui et al., 1999; Yang et al., 2002). For example, in the wing, all epithelial cells secrete a cuticular protrusion called a hair or bristle. In wild-type (WT) flies, all hairs point distally, reflecting polarity of the cell. If the PCP pathway is disrupted, the orientation of the hairs becomes disorganized and yields a characteristic swirling pattern across the wing (Shulman et al., 1998; Strutt, 2001; Usui et al., 1999; Wong and Adler, 1993).

The “core” PCP pathway components, evolutionarily conserved genes that affect PCP in all tissues, polarize cells along a planar axis across a tissue via intracellular and intercellular interactions. Initiation of PCP signaling occurs via activation of the seven-pass transmembrane receptor Frizzled (Fz) by Wingless (Wg) and *Drosophila* Wnt homolog (dWnt4) in the wing (Wu et al., 2013). In other tissues, the relationship between Wg/dWnt4 has not been established and a global unidentified factor is thought to initiate PCP signaling. Activation of Fz triggers the recruitment of the cytoplasmic protein Dishevelled (Dsh) to the membrane (Adler et al., 1997; Theisen et al., 1994; Vinson et al., 1989). Another cytoplasmic protein, Diego (Dgo), also binds to Dsh and positively regulates PCP signaling by stabilizing the Fz/Dsh complex (Feiguin et al., 2001; Jenny et al., 2005; Wu and Mlodzik, 2008). Similar to the Fz/Dsh relationship, the four-pass transmembrane protein Van Gogh/Strabismus (Vang) recruits and forms complexes with the cytoplasmic protein Prickle (Pk) (Gubb et al., 1999; Taylor et al., 1998; Wolff and Rubin, 1998).
Pk has been shown to also interact with Dsh, but it decreases Dsh quantity, thus inhibiting its membrane accumulation (Tree et al., 2002). Another core PCP component is the seven pass transmembrane atypical cadherin Starry nigh/Flamingo (Fmi) (Chae et al., 1999; Usui et al., 1999). Fmi colocalizes with both the Fz/Dsh/Dgo and Vang/Pk complexes; Fmi, Fz, and Vang are required for one another’s proper localization on the apical membrane (Usui et al., 1999; Bastock et al., 2003; Das et al., 2004; Strutt and Strutt, 2008). Of these core PCP pathway components, \( f_{z} \) and \( v_{ang} \) mutant clones have non-autonomous effects, specifically, domineering non-autonomy such that the polarity of their WT neighbors in the wing and abdomen are perturbed (Casal et al., 2002; Vinson and Adler, 1987; Taylor et al., 1998; Adler et al., 2000).

The hallmark of Wnt/PCP signaling is formation of supramolecular membrane complexes of different composition at the opposite cell membranes, for example proximal and distal in the wing disc. The core PCP proteins are initially localized uniformly along apical cell membrane in the developing wing imaginal disc, but become asymmetrically distributed in cell membranes along the proximal-distal axis of the cell. In the polarized epithelial cells, the Fz/Dsh/Dgo complexes localize to the distal apical membrane and the Vang/Pk complexes localize to the proximal apical membrane, whereas Fmi is present at both cell edges (Tree et al., 2002; Axelrod, 2001; Strutt, 2001; Chen et al., 2008; Usui et al., 1999). The polarized distribution of the core PCP components in discrete subdomains on the membrane produces, and is likely a result of asymmetric intercellular contacts, which establishes cell polarity across a plane. Loss of any core PCP gene expression or function causes the other PCP proteins to become randomly distributed throughout the proximal-distal axis of the cell (Strutt et al., 2011; Axelrod, 2001; Chen et al., 2008; Usui et al., 1999). The asymmetrical localization of core PCP components potentiates planar cell polarity by regulating the cytoskeleton. For example, PCP effectors mediate actin
accumulations to form distally positioned wing hairs. (Vladar et al., 2009; Strutt et al., 1997; Winter et al., 2001). These PCP effectors, small GTPase RhoA, Inturned (In), and Fuzzy (FY), are tissue specific (Park et al., 1996; Collier and Gubb 1997; Strutt and Warrington 2008; Strutt et al., 1997). PCP signaling in some tissues requires activation of the receptor Fz by Wg and dWnt4 (Wu et al., 2013). However, in a model for a more global role for Fz in planar polarity, the extracellular domain of Fz acts as a ligand for Vang to allow neighboring cells to read Fz levels preceding cell-autonomous interactions (Wu and Mlodzik, 2008). Both models are under active discussion and experimental validation.

1.3.2 PCP In Vertebrates

The core components of the PCP pathway as well as Dachsous and Fat are conserved between Drosophila and vertebrates but it is unclear to what extent the signaling mechanisms of these pathways, the relationship between the pathways, and functions of the individual proteins within and outside of the pathways are also conserved. Conserved components of the core PCP pathway include Frizzled (Fz) and Dishevelled (Dvl), Trilobite (Tri/Vangl2), a homolog to Van Gogh/Strabismus, along with Vangl1), Prickle (Pk), and Flamingo/Clsr (Fmi/Clsr) (Djiane et al., 2000; Veeman et al., 2003, Kinoshita et al., 2003; Darken et al., 2002; Goto and Keller, 2002; Park and Moon, 2002; Jessen et al., 2002). Vertebrates typically contain multiple genes encoding each member of the PCP pathway, which presents experimental challenges. For example, two Vang-like genes exist in the genomes of both zebrafish and mice, and five Dishevelled (Dvl) genes exist in zebrafish and three in mice. Like in the Drosophila PCP pathway, where Wg and dWnt4 act as ligands, Wnt5 and Wnt11 are proposed to bind to Fz2 and Fz7 in zebrafish, Xenopus, and mice and are essential for PCP signaling (Andre et al., 2015; Wu et al., 2013; Heisenberg et al., 2000; Rauch et al., 1997; Kilian et al., 2003; Djiane et al., 2000). PCP
signaling in vertebrates also regulates the cytoskeleton through effectors such as Rok, small GTPases RhoA, Rac1, and Cdc42, to affect polarized structures in migrating cells such as protrusions (Habas et al., 2001; Tahinci and Symes 2003; Wallar and Alberts 2003; Kovar 2006; Vavylonis et al., 2006; Zhu et al., 2006; Tanegashima et al., 2008). Current differences between the PCP pathway in Drosophila and vertebrates include the proteins Knypek/Glypican 4 (Kny/Gpc4) and adhesion GPCR Gpr125/Adgra3, which were discovered in zebrafish and frog as components of the vertebrate PCP pathway (Topczewski et al., 2001; Ohkawara et al., 2003; Li et al., 2013). *Drosophila* also have glypican genes *dally* and *dally-like*, which are important during development and canonical Wnt signaling, however, they have not been implicated in PCP signaling (Baeg et al., 2001; Desbordes and Sanson, 2003; Kreuger et al., 2004). In zebrafish Kny acts as a positive regulator of PCP signaling, possibly through promoting binding of Wnt ligands to their Fz receptors (Topczewski et al., 2001; Ohkawara et al., 2003).

The vertebrate PCP pathway is key in regulating the essential process of gastrulation: convergence and extension (C&E). C&E is the process by which cells of all germ layers move, through directed migration, radial and mediolateral intercalation, to narrow mediolaterally and dorsoventrally, and to elongate anteroposteriorly the animal body axis (Keller et al., 2003; Solnica-Krezel, 2005). In zebrafish, cells from all three embryonic germ layers undergo C&E during gastrulation (Warga and Kimmel, 1992; Concha and Adams, 1998; Pezeron et al., 2008; Sepich et al., 2005). Mutations in the core PCP pathway components result in defective C&E movements during gastrulation leading to a shorter and wider embryonic body axis. For example, mutations in *tri/vangl2* and *kny/gpc4* both have similar phenotypes of short body axis and yolk extension as well as wide notochord and somites (Jessen et al., 2002; Topczewski et al.,
2001). Other gastrulation cell movements such as epiboly and internalization are not affected in PCP pathway mutants.

1.3.3 *Drosophila* Dachsous And Fat In Polarity

The Wnt/PCP pathway is a key regulator of C&E, but the factors that activate this signaling pathway to regulate polarity across a tissue have not been identified. Studies have linked the Fat (Ft) and Dachsous (Ds) signaling pathway, which yield mutant phenotypes similar to those of PCP components, to the regulation of planar polarity. However, whether Ft and Ds activate/regulate the PCP pathway or act in a parallel pathway remains unclear. Studies over the past fifteen years have led to two models with context dependent relationship between the two pathways. One model suggests that Ds signals to Ft, which in turn signals to the PCP pathway in a gradient dependent manner that is dependent on the cadherin kinase, Four-jointed (Fj) to coordinate polarity; another model suggests that Ds and Ft act in an independent pathway and confer polarity in parallel to the PCP pathway (Matis et al., 2014; Hale et al., 2015; Yang et al., 2002; Rawls et al., 2002; Casal et al., 2002; Casal et al., 2006; Donoughe and Dinardo, 2011; Simon et al., 2010).

Several lines of genetic evidence support that Ds acts upstream of the PCP pathway to control planar cell polarity. In particular, Adler et al. found that ds mutations resulted in tissue polarity defects similar to those of fz mutants, but with unique patterns in the wing hairs such as regions of reversed polarity (Adler et al., 1998). In studies of double mutants between ds and other core PCP pathways genes, the authors found that PCP signaling occurred in ds- mutants but in an abnormal way: the intracellular transduction of the Frizzled signal through downstream effectors In and Fy was not affected but the polarity of the cell was (Adler et al., 1998; Krasnow
and Adler, 1994). They also found that large *ds*- mutant clones often altered the polarity of surrounding WT cells, suggesting *ds* exhibits domineering non-autonomy. These data imply that *ds* changes the interactions between the core PCP pathway proteins and alters the direction of PCP signaling, which results in altered tissue polarity, placing Ds upstream of the core PCP pathway.

Additional data support the model that Ds and Ft function upstream of the core PCP pathway to confer tissue polarity. Studies in the *Drosophila* compound eye show that the orientation of the ommatidia is randomized in both Ds and Ft mutants; gradients of Ds and Ft directionally bias Fz signaling, and Ds and Ft act nonautonomously with *ds*- and *ft*- clones affecting wild-type tissue. Together these data suggest that Ds and Ft function in long-range signaling to affect cell polarity (Yang et al., 2002; Rawls et al., 2002). In addition, data from studies in the *Drosophila* abdomen and wings support a model where Fj phosphorylates both Ds and Fat; but while phosphorylation of Ds by Fj inhibits Ft binding, phosphorylation of Ft by Fj promotes Ds binding, which sets up opposing gradients to regulate polarity across tissues. However, how Ds and Ft gradients affect polarity is different in different tissues (Casal et al., 2002; Strutt and Strutt, 2002; Hale et al., 2015). For example, Casal et al. show that in the *Drosophila* abdomen the anterior and posterior compartments have opposing gradients of Ds and Fj, yet all abdominal bristles point posteriorly, leading to the conclusion that while cells respond to the gradients of Ds and Ft, they do so in a region-specific manner (Casal et al., 2002).

Although these are strong arguments supporting the model that Ds/Ft system signals to regulate polarity through the PCP pathway, no physical interactions between Ds or Ft and any core PCP proteins have been identified, and there is no direct data showing levels of Ds and Ft affect distribution of the core PCP proteins at a subcellular level. However, Ft/Ds/Fj gradients do
regulate polarization of apical acentrosomal microtubules prior to polarization of the core PCP pathway components. Because Dsh-containing vesicles are trafficked along these microtubules, microtubules provide a possible link between Ft/Ds/Fj and the core PCP pathway (Matis et al., 2014; Harumoto et al., 2010).

Casal et al., based on their studies of *Drosophila* abdomen, proposed the opposing model placing Ds and Ft in a pathway acting in parallel to the core PCP pathway to confer cell polarity (Casal et al., 2006). They showed that when core PCP components Fm and Fz are absent, the Ds/Ft system can still confer and propagate planar cell polarity, and that when Ds is absent, cells in the *Drosophila* abdomen seem to be more responsive to the core PCP signaling. A double mutant of *ds-* and *fmi-* is characterized by a more severe phenotype than either of the single mutants. This result also points to the two genes acting in separate pathways since there is an additive effect. However, such an additive effect in compound mutants could also be seen for interactions between two genes when the functions of neither are completely lost. Loss of either *ds* or *ft* result in altered polarization of the bristles, however a double mutant yields a phenotype similar to each single mutant, supporting previous data that Ds and Ft act in the same pathway. They also showed overexpression of Ds or Ft can repolarize bristles in a *fmi* and *fz* null background, indicating Ds and Ft can affect cell polarity independent of PCP signaling. They speculate that in other systems such as the eye the requirement for core PCP signaling may be so strong, it becomes impossible for the Ds system to reorganize planar polarity. Altogether, these observations support the notion that different tissues have different requirements for conferring planar cell polarity.

More recently, evidence from studies of denticle patterning in the *Drosophila* larval ventral epidermis further supports the model that the core PCP pathway and the Ds/Ft pathway
act in parallel to each other (Donoughe and DiNardo, 2011). They reported that the requirement
for Ds signaling increases as the larvae grows such that the phenotype of a third instar larva
lacking maternal and zygotic Ds function is much more severely affected than a first instar larva.
They also found that the core PCP signaling still functions in loss of Ds system, suggesting that
the core PCP pathway does not absolutely depend on signaling from the Ds/Ft pathway.
Conversely, overexpression of the Ds extracellular domain in a fz null background can reorient
adjacent cells, revealing that Ds can signal in the absence of PCP signaling, which confirms the
findings of Casal et al., 2002. Interestingly, the authors also found that in ds maternal zygotic
(MZ) mutants as well as fz maternal zygotic mutants F-actin protrusion placement defects were
present in the wing epithelium cells suggesting similar downstream effectors that regulate the
cytoskeleton for both systems. Taken together, these lines of evidence from multiple tissues
place Ds and Ft in a separate pathway from core PCP components at least in some tissues.
However, the mechanism of how cells interpret signals from Ds and Ft to elaborate planar
polarity independent of PCP signaling remains to be determined in different systems.

1.3.4 Vertebrate Dachsous And Fat

While clear evidence exists that the atypical cadherins Ds and Fat influence tissue polarity in
Drosophila, relatively little is known about the functions of Dchs and Fat homologs in
vertebrates. The first insights into the vertebrate Dchs1, Fat1, and Fat4 proteins came from
studies in the mouse (Ciani et al., 2003; Saburi et al., 2008; Mao et al., 2011), where analysis of
Dchs and Fat mutant mice revealed a plethora of developmental defects. Phenotypes
characteristic of PCP signaling defects, however, were not seen. Mouse mutants for core PCP
genes have phenotypes such as craniorachischisis, defects in orientation of coat hair, stereocilia
polarity disruption in the inner ear, and abnormal cell polarization during gastrulation (Kibar et
Fat1 mutant mice are perinatally lethal with partially penetrant holoprosencephaly and anophthalmia phenotypes. Whereas the latter defects are similar to Hedgehog (Hh) mutant phenotypes, Hh signaling defects were not detected (Ciani et al., 2003). Fat4 mutant mice have cystic kidney disease, likely due to disrupted oriented cell division and tubule elongation (Saburi et al. 2008). The authors proposed a link between mammalian Fat4 to the PCP pathway, however other than cystic kidneys, mutant mice do not show other classic PCP pathway defects. In 2011, Mao et al. found that unlike in Drosophila, where Ds and Ft are expressed in the epithelia, in mice Dchs1 and Fat4 are also expressed in mesenchymal tissues. Some possibly PCP related phenotypes exhibited by these mutant mice include shorter cochleae in Dchs1, Fat4, and Dchs1Fat4, but without differences in stereocilia orientation and smaller cystic kidneys with less branching. These modest phenotypes may be due to the existence of multiple copies of Dchs and Fat with possible overlapping functions. The authors also show that Dchs1 mutants have stronger staining in the kidney for Fat4 and vice versa, consistent with the ligand/receptor relationship between Dchs and Fat shown in previous Drosophila and mammalian studies. These data establish function for Dchs and Fat during vertebrate development; however, characterizations of these mutant mice have been concentrated on postnatal animals, giving little information on the effects of these mutations during embryogenesis. Studies in early development may provide to us the elusive link between Dchs, Fat, polarity, and the PCP pathway in vertebrates.

1.4 Hippo Signaling

1.4.1 Drosophila Hippo
In *Drosophila* Ds and Fat have also been implicated in regulation of tissue size and organ morphogenesis acting through the Hippo pathway. The Hippo pathway is a kinase cascade that inhibits transcription to regulate growth and morphogenesis through faster proliferation and resistance to pro-apoptotic signals, with Salvador, Hippo, and Warts kinases at the core of the pathway (Harvey et al., 2003; Jia et al., 2003; Kango-Singh et al., 2002; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003). When the Hippo pathway is activated, Yorkie, the transcriptional co-activator is sequestered in the cytoplasm and its transcriptional activity is suppressed (Huang et al., 2005; Dong et al., 2007; Oh and Irvine, 2008; Ren et al., 2009). Intercellular interactions of Ds and Fat have been shown to regulate Yorkie activity (Bennett and Harvey, 2006; Willecke et al., 2006; Silva et al., 2006; Cho et al., 2006; Willecke et al, 2008; Rogulja et al., 2008). Ds intracellular domain, irrespective of Ft, binds to Riquiqui, which promotes Yorkie activity by inhibiting Warts kinase while Fat intracellular domain regulates the abundance of Warts kinase (Bennett and Harvey, 2006; Willecke et al., 2006; Silva et al., 2006; Cho et al., 2006; Willecke et al, 2008; Rogulja et al., 2008; Degoutin et al., 2013).

1.4.2 Vertebrate Hippo

In vertebrates, the core Hippo signaling pathway is conserved, as well as its role in regulating tissue growth and organ size through transcriptional regulators YAP, functional homolog of Yorkie, and TAZ (Sudol, 1994; Sudol et al., 1995; Kanai et al., 2000; Dong et al., 2007). TAZ/YAP respond to phosphorylation by LATS1 and LATS2, the mammalian homologs of the *Drosophila* kinase Warts (Tao et al., 1999; Hao et al., 2008; Zhang et al., 2008; Huntoon et al., 2010). Other conserved components of the pathway are MST1 and MST2, homologs of Hippo kinase; and SAV1, a homolog of Salvador (Creasy and Chernoff 1995; Avruch et al., 2006; Praskova et al., 2008; Harvey and Tapon, 2007; Tapon et al., 2002). Although these core
components of Hippo signaling are conserved, the relationship between Dchs and Fat with the Hippo pathway has not been verified in vertebrates.

Other than regulating tissue growth and morphology, TAZ/YAP can respond to additional signals, and the Hippo pathway also affects other processes during development and disease. In early murine development, localization of TAZ/YAP in a cell determines the very first cell fate decision in the preimplantation embryos: to become the inner cell mass with active Hippo signaling and TAZ/YAP distributed in the cytoplasm or the apical/basal polarized trophectoderm cells where Hippo signaling is inactive and TAZ/YAP accumulates in the nuclei (Nishioka et al., 2009; Anani et al., 2014; Hirate et al., 2013; Kono et al., 2014). Whereas at the 8-cell stage, each embryonic cell displays apicobasal polarity, after one cell division, at the 16-cell stage, the outer cells maintain apicobasal polarity, but the inner cells are apolar (Shirayoshi et al., 1983; Fleming, 1987; Morris et al., 2010). Inactive Hippo signaling in the trophectoderm allows TAZ/YAP to regulate TEAD transcription factors in the nucleus and promote trophectoderm fate. Consistently, deletion of Tead4 resulted in an embryo that failed to establish trophectoderm cells (Nishioka et al., 2008; Yagi et al., 2007). Deletion of both TAZ and YAP results in embryo death prior to inner cell mass and trophectoderm specification. However, deletion of either TAZ or YAP alone does not cause any preimplantation defects, revealing their functional redundancy in this process (Nishioka et al., 2009; Hossain et al., 2007; Morin-Kensicki et al., 2006). In humans, misregulation of TAZ/YAP intracellular distribution as well as levels can lead to a variety of cancers with the severity of misregulation correlated with cancer progression (Cordenonsi et al., 2011; Harvey et al., 2013). GPCR signaling can also regulate TAZ/YAP through Rho-GTPases and ROCK activation, which inhibits LATS1/2 kinases allowing nuclear translocation of TAZ/YAP (Yu et al., 2012; Yu et al., 2013; Kim et al., 2013;
Dupont et al., 2011). Mechanical cues, such as those generated by the cytoskeleton also mediate TAZ/YAP activity through regulation of Rho-GTPases and ROCK (Aragona et al., 2013; Dupont et al., 2011). Conversely, TAZ/YAP can also regulate the actin cytoskeleton during collective cell migration, cell polarization, and formation of the 3D body shape (Bertrand et al., 2014; Low et al., 2014; Lucas et al., 2013; Porazinski et al., 2015). With such a plethora of functions for the Hippo pathway and TAZ/YAP, their upstream regulators must play important roles in vertebrate development and disease. Hence, whether and in what way Dchs/Fat affect TAZ/YAP activity in vertebrates remains to be further explored.

1.5 Dchs And Fat In Cell-Cell Adhesion

Unlike most cadherins, neither Ds nor Ft mediates homophilic cell-cell adhesion; rather Ds and Ft together appear to promote cell-cell adhesion via heterophilic interactions (Takeichi, 1995; Matakatsu and Blair, 2004; Ishiuchi et al., 2009). For example, Matakatsu and Blair showed that Drosophila S2 cells expressing either ds or ft alone do not form homophilic cell aggregates. However, when ds and ft were co-transfected, or when cells expressing ds or ft were mixed, cell aggregates formed efficiently. These aggregates were inhibited by EGTA, suggesting the ability of Ds and Ft to associate depends on calcium like in the case of classical cadherins. Ds and Ft also stabilize each other on the cell membrane both in vitro and in vivo (Takeichi, 1995; Matakatsu and Blair, 2004). In cells transfected with either Ds or Ft, antibody staining showed that most of the Ds and Ft protein localized to “internal vesicle-like structures” with very low protein levels detected at cell membranes. In contrast, in mixed cultures of cells transfected with ds or ft, Ds and Ft localization at the cell-cell interfaces was much stronger. In third instar wing discs, overexpression of Ds heightened anti-Ft antibody staining and conversely, overexpression
of Ft heightened anti-Ds antibody staining. These results indicate that heterophilic intercellular interactions between Ds and Ft stabilize the proteins at the cell membrane, supporting the proposed ligand-receptor relationship between Ds and Ft by Ma et al., 2003.

Work in vertebrate systems supports the model that Dachsous and Fat mediate heterophilic interactions in an evolutionarily conserved manner. For example, Fat4 and Dachsous1 co-localize to the apical portion of neural progenitor cells in the cerebral cortices (Ishiuchi et al., 2009). Antibody staining of cerebral cortices from an E14.5 mouse embryo showed both Fat4 and Dachsous1 were distributed throughout the cortex but were more concentrated together at the apical cell surfaces. L cells, which are cadherin null fibroblasts, transfected with either Fat4 or Dachsous1 alone did not form cell aggregates, and each protein localized diffusely throughout the cells. However, once Fat4-expressing and Dachsous1-expressing cell populations were mixed, cell aggregates formed and both proteins became concentrated at the “heterotypic cell boundaries” (Ishiuchi et al., 2009). siRNA mediated knockdown of Fat4 in the embryonic cortices causes a reduction in Dachsous1 expression, but also a modest upregulation of Fat4 expression (Ishiuchi et al., 2009). These data suggest that in mammals, Fat 4 stabilizes Dchs1 at the cell membrane; Dchs1 negatively regulates Fat4 expression; and that Dchs1 and Fat4 form heterophilic dimers to mediate cell-cell adhesion, as proposed for *Drosophila*.

### 1.6 Zebrafish Development

In order to assess Dachsous function *in vivo* during early vertebrate development we use zebrafish as a model system. Vertebrate embryonic development following gametogenesis is initiated by fertilization and egg activation, followed by cell cleavages resulting in an
unstructured blastula, which undergoes gastrulation to form the three germ layers and
subsequently the basic animal body plan. In zebrafish, other than gametogenesis, these events
occur externally, providing, along with other attributes of this model system, an excellent setting
for studying early development in a vertebrate system (Streisinger, 1984).

Zebrafish oogenesis consists of five stages with identifying characteristics at each stage.
In zebrafish as in other vertebrates, germline cysts yield multiple oocytes with somatic follicle
cells surrounding each primary oocyte throughout oogenesis (Kloc et al., 2004; Saito et al., 2007;
Marlow and Mullins, 2008). Polarity manifests within the oocyte by the evolutionarily conserved
transient structure, the Balbiani body, being asymmetrically positioned vegetally during stage I
oogenesis (Heasman et al., 1984 Pepling et al., 2007; Billett and Adam, 1976; Kloc et al., 2004;
de Smedt et al., 2000). In zebrafish as in other animals, the Balbiani body consists of
endoplasmic reticulum, Golgi, mitochondria, and maternally contributed proteins and RNAs
(Pepling et al., 2007; Billett and Adam, 1976; Kloc et al., 2004; Bukovsky et al., 2004; Wilk et
al., 2005; de Smedt et al., 2000). The formation of Balbiani body is dependent on the function of
bucky ball encoding an RNA binding protein (Marlow and Mullins, 2008). Whereas
asymmetrical translocation of the Balbiani body is dependent on the microtubule cytoskeleton as
in oocytes with disruption of microtubule actin croslinking factor 1 Balbiani body is assembled
but not vegetally localized (Dosch et al., 2004; Marlow and Mullins, 2008; Bontems et al., 2009;
Gupta et al., 2010; Roper and Brown, 2004). Cortical granules form during stage II oogenesis
and are localized to the cortex of stage III oocytes (Becker and Hart, 1999; Marlow, 2010;
Kanagaraj et al., 2014). During stage IV oogenesis, a specialized follicle cell on the animal pole
of the oocyte forms the micropyle, the site for sperm entry (Amanze and Iyengar, 1990; Marlow
and Mullins, 2008). The oocyte undergoes both nuclear maturation, which entails the first
meiosis and extrusion of the polar body; and cytoplasmic maturation that renders an immature opaque oocyte translucent, which is attributed to processing of major yolk proteins (Ferrell, 1999; Ferrell et al., 2009; Masui, 2001; Eppig, 1996; Wallace and Selman, 1990; Selman et al., 1993). Once maturated and ovulated from the follicle cell layer, the oocyte is considered an egg (Marlow, 2010).

In zebrafish, egg activation upon fertilization triggers cortical granule exocytosis, expansion of the chorion and formation of the cytoplasmic blastodisc at the animal pole. Cortical granules release their contents at the surface of the egg; this process prevents polyspermy, promotes actin reorganization at the cortex, and contributes to the expansion of the chorion (Tsaadon et al., 2006; Wong and Wessel, 2006; Hart, 1990). Cortical granule exocytosis is dependent on the dynamics of the actin cytoskeleton, as both stabilizing or destabilizing of F-actin with drugs lead to abnormalities in the exocytosis process (Wolenski and Hart, 1988; Ivanenkov et al., 1987). Simultaneously, cytoplasmic streaming, which moves the cytoplasm that is intermixed with yolk towards the animal pole to form the blastodisc, is also dependent on the actin cytoskeleton in addition to myosin as the blastodisc does not form when F-actin is destabilized by drug treatment (Hart and Becker, 1994; Becker and Hart, 1999; Fernandez et al., 2006; Leung et al., 2000). Microtubules, another component of the cytoskeleton, form transient networks at vegetal region of the yolk cell during egg activation and function to initiate asymmetric translocation of the maternal dorsal determinants. Underscoring the significance of the vegetally-localized dorsal determinants, removal of this region results in axis formation deficiencies (Jesuthasan and Strahle, 1997; Mizuno et al., 1998; Detrich et al., 1998).

After fertilization, a series of synchronous, meroblastic cleavages occur every 15 minutes to partition the nascent blastodisc (Kimmel et al., 1995). Cleavage furrows are dependent on the
microtubule cytoskeleton and occur perpendicular to the previous cleavage for the five cleavages (Lee et al., 2004). Starting with egg activation and during the cleavage period, the microtubules in the superficial yolk cytoplasmic layer, which surrounds the yolk cell, undergo reorganization to form transient parallel arrays and function to transport the dorsal determinants from the vegetal pole towards the animal pole asymmetrically on the future dorsal side of the embryo (Lu, 2011; Jesuthasan and Strahle, 1997; Detrich et al., 1998; Gore and Sampath, 2002). Disruption of the parallel microtubule array through drug treatment also disrupts the transport of dorsal determinants and consequently axis formation (Lu, 2011; Gore and Sampath, 2002; Jesuthasan and Strahle, 1997).

Around the 10th cell division, a series of events occur, which together are termed midblastula transition (MBT): the yolk syncytial layer (YSL) forms, cell divisions across the embryo become asynchronous, zygotic transcription is activated, and cells become motile (Kane and Kimmel, 1993). The YSL is an extraembryonic tissue positioned between the blastoderm and the yolk cell and plays crucial roles in patterning and morphogenesis of the embryo (Mizuno et al., 1999; Chen and Kimelman, 2000; Carvalho and Heisenberg, 2010; Sakaguchi et al., 2002). The YSL produces homeodomain protein, bozozok/dharma as well as ligands to induce formation of the dorsal organizer mesoendoderm formation (Nodal ligands: Squint and Cyclops), and functions in gastrulation movements (Shimizu et al., 2000; Sirotkin et al., 2000; Mizuno et al., 1996; Rodaway et al., 1999; Solnica-Krezel and Driever, 1994; Lachnit et al., 2008; Carvalho et al., 2009). The YSL undergoes similar movements as the blastoderm during gastrulation, including epiboly, convergence and extension (Solnica-Krezel and Driever, 1994; Carvalho et al., 2009; D’Amico and Cooper, 2001).
After MBT, cells continue to divide asynchronously and are categorized as enveloping layer (EVL), the outer most layer of cells with large and flat morphology that forms around two hours post fertilization (hpf), and deep cells, which comprises all cells between the EVL and YSL (Betchaku and Trinkaus, 1978; Kimmel and Law, 1985; Kimmel et al., 1990). Gastrulation results in the formation of the three embryonic germ layers from deep cells, a morphologically uniform population of cells, the specification of many cell fates, and the establishment of the basic animal body plan. By the onset of gastrulation, future cell fates in different regions can be mapped to the organ and tissue level. Fate mapping experiments revealed that endodermal progenitors are located at the blastoderm margin, ectodermal progenitors at the animal pole, with mesodermal progenitors in-between (Kimmel et al., 1995). Gastrulation is marked by massive morphogenetic cell movements that place the embryonic mesoderm, and endoderm germ layers beneath ectoderm, and subsequently shape them into the embryonic body. During this process, dorsal/ventral, anterior/posterior, and left/right body axes become defined and specific cell lineages within each of the germ layers are born (Roszko et al., 2009). While some of the cell movements are organism specific, four morphogenetic movements occur during gastrulation in all vertebrates: epiboly, internalization, convergence and extension (C&E) (Keller et al., 2003).

C&E is the process by which cells of all germ layers move and rearrange, by directed migration, radial and planar mediolateral intercalation, to narrow and to elongate anteroposteriorly the animal body axis (Keller et al., 2003; Solnica-Krezel, 2005). The molecular mechanisms that drive C&E are complex and involve many signaling pathways. For example, the the noncanonical Wnt/PCP, the Bone Morphogenetic Protein (BMP), and G-protein-coupled receptor signaling pathways all have been proposed to regulate the complex migratory cell behaviors during C&E. In general, these pathways function in different domains of the embryo.
to control these movements, and crosstalk between these pathways ensures proper spatiotemporal coordination between all the cell movements. After gastrulation follows the segmentation period when somites and rudimentary organs form, the embryo continues to elongate, and becomes motile by 24 hpf.

**1.7 Dachsous In Development And Disease**

As discussed earlier, Dachsous has been shown to regulate multiple aspects of development in *Drosophila*, mice, and humans. Recent studies uncover its roles in disease. In *Drosophila* loss of Ds function leads to both Hippo pathway associated imaginal disc over growth phenotypes as well as planar polarity associated misorientation of larval ventral denticles, which are used for locomotion (Grusche et al., 2011; Matakatsu and Blair, 2012; Baena-Lopez et al., 2008; Clark et al., 1995). Morpholino knock down of *dchs2* in zebrafish larvae lead to defects in polarized intercalation of cartilage precursor cells resulting in craniofacial abnormalities (Le Pabic et al., 2014). As mentioned above, loss of function of Dchs1 in mice, the homolog most similar to *Drosophila* Ds, leads to pleotropic phenotypes in multiple organs (Mao et al., 2011; Rock et al., 2005). Homozygous mutant pups are born with similar size as their siblings but with slightly bent spines and curly tails. Dchs1 mutant mice can survive for a few weeks and form hair with normal orientation, but do not grow in size. Mutant mice exhibit shorter cochleae in the ear with normal polarization of stereocilia as well as a modest increase in neural tube width-to-height ratio. Additionally, Dchs1 mutant mice present with slightly shorter sternum and intestine; smaller lungs and smaller cystic kidneys with less branching; two ossification sites in the sternum and vertebral column instead of one; and defects in heart morphology (Mao et al., 2011). Dchs1b mutant mice do not exhibit classical PCP mutant phenotypes: open neural tube,
misoriented hair, etc. Albeit, the shorter cochleae and increased neural tube width-to-height ratio could be PCP related phenotypes. Similarly, mutant mice do not exhibit characteristic Hippo signaling deficiency phenotypes, conversely, multiple organs are reduced in size.

In humans, mutations Dchs1 have been linked to Van Maldergem, an autosomal recessive syndrome that presents with pleiotropic phenotypes (Cappello et al., 2012). Patients with Van Maldergem syndrome exhibit malformations in craniofacial, auditory, renal, skeletal, and limb development as well as neuronal differentiation leading to periventricular neuronal heterotopia (Cappello et al., 2012). The periventricular neuronal heterotopia phenotype could be recapitulated by RNAi knock down of Dchs1 in mice and is due to defects in migration of neuronal cells rather than in proliferation or cell fate specification (Cappello et al., 2012). The pleiotropic phenotypes in mice and humans point to an important role for Dchs in early development, likely prior to organ formation to affect multiple organ systems, and different roles for Dchs in multiple organs.

1.8 Significance

The studies reviewed here poise a fascinating web of possible genetic and functional interactions for Dchs in developmental processes. Answering these questions of how Dchs functions during vertebrate development will help to determine which roles are conserved from Drosophila to vertebrates and possibly shed light on how its inactivation leads to pleiotropic defects in patients with Van Maldergem syndrome.

In this thesis, I describe experiments that uncovered many roles of Dachsous1b and Dachsous2 in zebrafish embryogenesis, starting from novel roles during the earliest stages of
vertebrate development to the conserved function of cell polarity regulation. In Chapter 2 I describe the novel role of Dchs1b in egg activation, cell division, and cell fate specification through regulation of actin and microtubule cytoskeleton. Then in Chapter 3, I demonstrate conservation of Dachsous function in cell adhesion and planar cell polarity during zebrafish gastrulation. Lastly, I will discuss how this body of work establishes Dachsous function during vertebrate development through regulating the cytoskeleton and planar cell polarity as well as future directions for this project.
Chapter 2: Dachsous1b Cadherin Regulates Actin And Microtubule Cytoskeleton During Early Zebrafish Embryogenesis

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2.1 Abstract

Dachsous (Dchs), an atypical cadherin, is an evolutionarily conserved regulator of planar cell polarity, tissue size, and cell adhesion. In humans, DCHS1 mutations cause pleiotropic Van Maldergem syndrome. Here, we report that mutations in zebrafish dchs1b and dchs2 disrupt several aspects of embryogenesis, including gastrulation. Unexpectedly, maternal zygotic (MZ) dchs1b mutants show defects in the earliest developmental stage, egg activation, including abnormal cortical granule exocytosis (CGE), cytoplasmic segregation, cleavages, and maternal mRNA translocation, in transcriptionally quiescent embryos. Later, MZdchs1b mutants exhibit altered dorsal organizer and mesendodermal gene expression, due to impaired dorsal determinant transport and Nodal signaling. Mechanistically, MZdchs1b phenotypes can be explained in part by defective actin or microtubule networks, which appear bundled in mutants. Accordingly, disruption of actin cytoskeleton in wild-type embryos phenocopied MZdchs1b mutant defects in cytoplasmic segregation and CGE. Whereas, interfering with microtubules in wild-type embryos impaired dorsal organizer and mesodermal gene expression without perceptible earlier phenotypes. Moreover, the bundled microtubule phenotype was partially rescued by expressing either full-length Dchs1b or its intracellular domain, suggesting Dchs1b affects microtubules and some developmental processes independent of its known ligand Fat. Our results establish novel roles for vertebrate Dchs in actin and microtubule cytoskeleton regulation in the unanticipated context of the single-celled embryo.
2.2 Introduction

Dachsous is an evolutionarily conserved large cadherin whose role in vertebrate embryogenesis is only beginning to be understood. Dachsous features 27 extracellular cadherin repeats, a single-pass transmembrane and an intracellular domain (Clark et al., 1995). In Drosophila, where dachsous was first identified, it functions in tissue growth control upstream of Hippo signaling (Clark et al., 1995) and planar cell polarity (PCP), the process of polarizing cells within the tissue plane, acting in part through an unconventional myosin, Dachs (Cho and Irvine, 2004; Mao et al., 2006). Studies in Drosophila and cell culture demonstrated that Dachsous mediates PCP and cell adhesion via heterophilic intercellular interactions with another cadherin, Fat (Ishiuchi et al., 2009; Matakatsu and Blair, 2004; Takeichi, 1995). In Drosophila, phosphorylation of cadherin repeats by the Golgi-localized kinase Four-jointed modulates these interactions (Ishikawa et al., 2008; Simon et al., 2010). Non-mutually exclusive models for Dachsous function in planar polarity posit that it acts upstream and/or parallel to the core PCP components (Adler et al., 1998; Casal et al., 2006; Casal et al., 2002; Donoughe and DiNardo, 2011; Ma et al., 2003; Matis et al., 2014; Rawls et al., 2002; Yang et al., 2002).

Less is known about the two vertebrate homologs, Dachsous1 (Dchs1) and Dachsous2 (Dchs2). Mice homozygous for a N-terminal deletion of Dchs1 die postnatally, exhibiting abnormalities in multiple organs (Mao et al., 2011); and defects in migration of hindbrain branchiomotor neurons (Zakaria et al., 2014). Mutations in human DCHS1 were recently linked to recessive Van Maldergem syndrome, with pleiotropic phenotypes including neuronal periventricular heterotopia (Cappello et al., 2013). These data establish a requirement for Dchs1 during vertebrate organogenesis, but the underlying cellular mechanisms are unknown.
Here we examine Dchs roles in vertebrate development using zebrafish, whose genome contains three dchs genes, dchs1a, dchs1b, and dchs2, with dchs1a and dchs1b likely resulting from genome duplication (Taylor et al., 2003). Through mutational analyses, we uncovered essential overlapping and unique roles for dchs1b and dchs2 during embryogenesis. Unexpectedly, maternal dchs1b activity is uniquely required for egg activation, focusing our investigation on early dchs1b developmental functions.

Vertebrate embryogenesis is initiated by egg activation and fertilization, followed by cell cleavages generating the blastula, which then gastrulates to form the germ layers and basic body plan (Solnica-Krezel, 2005; Stern, 1992). Zebrafish eggs, composed of intermixed cytoplasm and yolk, exhibit animal-vegetal polarity (Houston, 2013; Wallace and Selman, 1990). Egg activation triggers cortical granule exocytosis (CGE) and cytoplasmic streaming to form the blastodisc at the animal pole. Cortical granules (CG) release their contents at the egg cortex, contributing to chorion expansion and surface remodeling (Fuentes and Fernandez, 2010; Hart, 1990; Tsaadon et al., 2006; Wong and Wessel, 2006). Stabilizing or destabilizing F-actin established the dependence of both CGE and cytoplasmic streaming on a dynamic actin cytoskeleton (Becker and Hart, 1999; Fernandez et al., 2006; Hart and Fluck, 1995; Ivanenkov et al., 1987; Leung et al., 2000; Wolenski and Hart, 1988). Maternally deposited dorsal determinants (DDs), including wnt8a mRNA, reside at the vegetal pole (Kosaka et al., 2007; Lu et al., 2011). Embryonic patterning requires these vegetally located molecules, as their removal surgically (Jesuthasan and Stahle, 1997; Mizuno et al., 1999) or by maternal-effect mutations, impairs dorsal axis specification (Ge et al., 2014; Nojima et al., 2010). During early cleavages, a dynamic vegetal microtubule network mediates asymmetric transport of DDs (Lu et al., 2011; Nojima et al., 2004; Tran et al., 2012), which accumulate in a few marginal blastomeres to establish the Nieuwkoop
center, a key regulator of axis determination (Gore and Sampath, 2002; Jesuthasan and Stahle, 1997; Lu et al., 2011). Disruption of these microtubule arrays impairs DD transport and axis formation (Ge et al., 2014; Gore and Sampath, 2002; Jesuthasan and Stahle, 1997; Lu et al., 2011; Nojima et al., 2004; Tran et al., 2012).

Midblastula transition (MBT) occurs around the 10th cell division, when marginal blastomeres collapse into the yolk forming the yolk syncytial layer (YSL), zygotic transcription starts, and cell divisions become asynchronous (Kane and Kimmel, 1993; Kimmel et al., 1995). The YSL is crucial for embryonic patterning and morphogenesis (Carvalho and Heisenberg, 2010; Fekany et al., 1999; Mizuno et al., 1999). Dorsal YSL and marginal blastomeres constitute the Nieuwkoop center where DDs promote nuclear accumulation of maternal β-catenin, which activates zygotic transcriptional regulators, including Bozozok/Dharma and secreted Nodal morphogens, to induce the gastrula organizer and specify mesendoderm (Carvalho and Heisenberg, 2010; Lachnit et al., 2008; Mizuno et al., 1999; Rodaway et al., 1999; Shimizu et al., 2000; Sirotkin et al., 2000; Solnica-Krezel and Driever, 2001).

We generated zebrafish MZdchs1b and MZdchs2 mutants and found they exhibit epiboly and convergence and extension (C&E) defects during gastrulation, while only MZdchs1b mutants display egg activation and cell fate specification defects. Signifying that MZdchs1b phenotypes are due to cytoskeletal abnormalities, actin and microtubule networks in MZdchs1b mutants appeared excessively bundled, defects that were partially rescued by expressing either full-length or Dchs1b intracellular domain. Accordingly, pharmacologic interference with actin or microtubule dynamics in WT embryos phenocopied mutant defects in egg activation or dorsal mesoderm specification, respectively. Together, these results uncover novel roles for Dchs1b in
embryonic patterning and morphogenesis through regulation of actin and microtubules, likely independent of its intercellular ligand Fat.
2.3 Results

2.3.1 Generation Of Nonsense Mutations In Zebrafish dchs1b And dchs2 Genes

Quantitative RT-PCR (qRT-PCR) revealed that zebrafish dchs1a, dchs1b, and dchs2 genes were expressed maternally and zygotically (Fig. 2.1A). Notably, dchs1b transcripts were more abundant maternally, whereas expression of both dchs1a and dchs2 peaked during zygotic stages (Fig. 2.1A). Whole mount in situ hybridization (WISH) of dchs transcripts revealed similar ubiquitous distribution during embryogenesis and enrichment in neural tissues at 24 hpf (Fig. 2.1B and 2.2A). To investigate the unique and overlapping functions of the three dchs genes we generated two nonsense mutations in dchs1b and one nonsense mutation in dchs2 through TILLING (Targeting Induced Local Lesions IN Genomes) (Draper et al., 2004; Wienholds and Plasterk, 2004). dchs1b\textsuperscript{fh274} (C11527T; Q924) and dchs1b\textsuperscript{fh275} (C11683T; Q976) mutations both generated amber stop codons, whereas dchs2\textsuperscript{stl1} (T6528A; Y201) mutation yielded the ochre stop codon, with all three mutations predicted to truncate the proteins early in the extracellular domains (Fig. 2.1C and D). qRT-PCR analysis revealed significant reduction of dchs1b and dchs2 mRNA levels in both alleles of MZdchs1b and MZdchs2 mutants respectively compared to WT (Fig. 2.1E and 2.2B). Notably, dchs1a and dchs2 transcript abundance in MZdchs1b mutants and dchs1a and dchs1b transcript in MZdchs2 mutants were unchanged (Fig. 2.2C-E). These results are consistent with nonsense-mediated degradation of mRNA encoded by all mutant alleles (Chang et al., 2007), indicating null or severe hypomorphic mutations.

2.3.2 MZdchs1b Mutants Display Pleiotropic Defects During Embryogenesis

Zygotic dchs1b and dchs2 mutants showed no overt developmental anomalies and developed into fertile adults. Morphological analysis of in vitro fertilized time-matched progeny of WT and dchs1b or dchs2 mutant parents revealed an overall developmental delay of MZ mutants (Fig.
Figure 2.1 Spatiotemporal Expression And Mutations In Zebrafish dchs Genes Leading To Pleiotropic Defects During Embryogenesis

A. qRT-PCR analysis of all three zebrafish dchs genes’ expression at maternal and zygotic stages normalized to gapdh transcripts.

B. Whole mount in situ hybridization (WISH) of dchs1b in WT embryos at 4-cell, shield, 90% epiboly, and 24 hpf stages. Inset in 24 hpf panel shows a cross section of the hindbrain.

C. Sanger sequencing trace for dchs2 A to T mutations.

D. Schematic of Dchs protein with mutations denoted by asterisks.

E. qRT-PCR analysis of dchs1b expression in MZdchs1b relative to WT embryos at maternal and zygotic stages.

F. Bright field images of WT, MZdchs1b, MZdchs2\textsuperscript{att/att}, and MZdchs1b\textsuperscript{Rh275/Rh275}, MZdchs2\textsuperscript{att/att} time-matched embryos at 1, 6, and 10. Red boxes: distortions in the yolk cell. Large arrowhead: yolk masses in the blastoderm. Small red arrowhead: anterior (A). Small blue arrowhead: posterior (P).

G. ntl WISH for stage-matched WT, MZdchs1b, and MZdchs2\textsuperscript{att/att} embryos at 70% epiboly. Inset depicts time-matched MZdchs1b embryo presenting with a gap in the ntl expression domain.

H. Quantification of axial mesoderm length in WT (n=111), MZdchs1b (n=414), and MZdchs2\textsuperscript{att/att} (n=486) embryos.

I. hgg1 and dlx3 WISH analysis of WT, MZdchs1b, and MZdchs2\textsuperscript{att/att} stage-matched embryos at 2-somite stage (12 hpf).

J. Quantification of the mediolateral width of dlx3 domain for WT (n=6), MZdchs1b (n=27), and MZdchs2\textsuperscript{att/att} (n=18), shown by black line.
2.1F). MZ mutants required 5.5 compared to 4 hours for WT to progress from the shield to yolk plug closure stage, indicating slower epiboly. Examination of the relative positions of cell type specific markers to diagnose C&E movements in stage-matched mutant and WT gastrulae (Jessen et al., 2002) revealed a mediolaterally wider and anteroposteriorly shorter notochord marked by no tail/brachyury (ntl) expression in mutant gastrulae at 70% epiboly (Schulte-Merker et al., 1992), suggesting defective C&E movements (Fig. 2.1G and H). At early segmentation, the hgg1 expressing prechordal plate was positioned anterior to the arc-shaped dlx3 domain demarcating neuroectoderm. Whereas in mutants, the hgg1 domain overlapped with or was positioned posterior to the dlx3 domain, which was also mediolaterally wider, typical of impaired prechordal mesoderm migration or C&E movements (Heisenberg et al., 2000; Marlow et al., 1998; Topczewski et al., 2001) (Fig. 2.1I and J).

Next we investigated whether delayed gastrulation was due to earlier defects. Whereas MZdchs2stl1/stl1 mutants progressed through cleavage and blastula stages normally, compared to time-matched WT embryos, MZdchs1bfh275/jh275 mutants displayed defects beginning from fertilization including smaller blastodiscs with non-uniform cleavages, producing variably sized blastomeres (Fig. 2.1F and 2.6C). We detected globular yolk-like inclusions in the blastodiscs of MZdchs1bfh275/jh275, hereafter called MZdchs1b mutants, but not in WT blastodiscs (Fig. 2.1F). These defects varied in penetrance and expressivity with the most severe resulting in lethality by 24 hpf. Typically, fewer than 30% of mutants survived beyond 24 hpf compared to 80% of WT embryos (Fig. 2.4A). Images in Figure 1F represent moderate mutant phenotypes.

MZdchs1bfh274/jh274 mutants showed a similar array of abnormalities, indicating these defects are specific to loss of maternal and zygotic dchs1b function (Fig. 2.4D). MZdchs1bfh275/jh275;MZdchs2stl1/stl1 compound mutant phenotypes resembled those of single MZdchs1b embryos, albeit
Figure 2.2 dchs Expression In Zebrafish

A. Whole mount *in situ* hybridization of *dchs2* in WT embryos at 4-cell (1 hpf), shield (6 hpf), 90% epiboly (9 hpf), and 24 hpf stages.

B. Quantitative RT-PCR analysis of expression levels of *dchs2* in MZ*dchs2*<sup>stl1/stl1</sup> mutants relative to WT embryos at maternal and zygotic stages.

A. Relative expression by qRT-PCR of *dchs1a* in WT, MZ*dchs1b*<sup>fh275/fh275</sup>, and MZ*dchs1b*<sup>stl1/stl1</sup> embryos.

B. Relative expression by qRT-PCR of *dchs2* in WT and MZ*dchs1b*<sup>fh275/fh275</sup> embryos.
occurring with higher penetrance and more uniform expressivity (Fig. 2.1F, 2.8A, E, G and data not shown). Detailed analyses of \textit{dchs} functions during gastrulation will be described elsewhere. Hereafter, we further investigate the early developmental roles of MZ\textit{dchs}1b.

2.3.3 Largely Normal Progression Of Oogenesis In \textit{Mdchs}1b Mutants

As defects were already apparent in MZ\textit{dchs} eggs upon fertilization, we investigated potential \textit{dchs} roles in oogenesis. Zebrafish oogenesis consists of five stages with characteristic features that appeared largely normal in \textit{dchs}1b mutant oocytes. Microtubule organizing centers (MTOCs) were present at stage Ia and lost by stage Ib in WT and mutant oocytes (Fig. 2.3A and B). Apical basal polarity of follicle cells shown by F-actin and β-catenin enrichment on the follicle cell surface juxtaposed to the oocyte was comparable between WT and mutant stage Ib oocytes (Fig. 2.3C and 2.4E). Moreover, the presence of a single Balbiani body in stage Ib and II oocytes of WT and mutants indicated \textit{dchs} mutant oocytes are polarized (Fig. 2.3D and 2.4F). The number of acetylated α-tubulin labeled microtubules in stages Ia and Ib was significantly reduced in mutant oocytes compared to WT but by stage II was comparable (Fig. 2.3E, F and 2.4G). In unactivated and unfertilized WT and \textit{Mdchs}1b mutant eggs, vitelline envelope morphology, CG size, number, and distribution at the cortex were comparable (Fig. 2.3G). Lastly, as in WT, polar bodies were extruded from mutant eggs (Fig. 2.3H). Taken together, these data indicate Dchs1b is dispensable for zebrafish oogenesis.

2.3.4 Delayed CGE In \textit{Mdchs}1b Mutants

Upon activation, delayed CGE in \textit{Mdchs}1b mutant eggs was evident from stereomicroscopic analysis (data not shown). We next labeled CGs in fixed eggs using fluorescent dye-conjugated MPA (\textit{Maclura pomifera} agglutinin) (Becker and Hart, 1999; Dosch et al., 2004; Mei et al., 2009; Talevi et al., 1997). At 1 minute post activation (mpa), CGE in WT and \textit{Mdchs}1b eggs
Figure 2.3 *Mdchs1b* Oogenesis Is Largely Unaffected

A. γ-tubulin immunostaining reveals a perinuclear MTOC (pink arrow) in the stage Ia oocyte that is lost during stage Ib of oogenesis in WT and *dchs1b* mutants.

B. Quantification of MTOC in oocytes from 2 WT and 3 mutant ovaries.

C. Rhodamine phalloidin labels actin filaments in the cortical ooplasm and in the follicle cell layer. β-catenin localizes to the oocyte cortex or membrane in stage Ib oocytes.

D. H&E stained ovary sections of WT and *Mdchs1b* ovaries reveal normal polarization of stage IB oocytes as indicated by the presence of the Balbiani body (black arrowhead).

E. WT and *Mdchs1b* mutant Stage II oocytes stained with antibody against acetylated α-tubulin.

F. Quantification of acetylated microtubules from 5 WT and 7 mutant ovaries.

G. H&E stained ovary sections reveal CGs movement toward the cortex, structure of the vitelline envelope (VE) and the two layers of somatic follicle cells surrounding stage III oocytes of WT and *Mdchs1b* mutants.

H. F-actin labeling of polar bodies in unfertilized eggs fixed at 0 mpa with completion of meiosis indicated by the appearance of the polar body and the pronucleus from WT and *Mdchs1b* mutant eggs.
Figure 2.4 Survival, Morphology, And Histology Of WT And dchs Mutants

A. Survival and fertility for WT (n=587) and MZdchs1b^h275/h275 (n= 1034) mutant embryos.
B. Survival of MZdchs1b^h275/h275 embryos based on severity of yolk inclusions and delayed epiboly phenotypes.
C. Morphology of WT, MZdchs1b^h275/h275, and MZdchs1b^stl1/stl1 embryos at 24 hpf.
D. Bright field images of WT and MZdchs1b^h275/h274 time matched embryos at 1, 3, 6, 10, and 17 hpf.
E. Hematoxylin and Eosin (H&E) stained ovary sections of (a,c,e) WT and (b,d,f) maternal dachsous mutant ovaries reveal a normal composition of oocytes. The primary oocytes of (a) WT and (b) Mdcch mutant ovaries are polarized as indicated by the presence of the Balbiani body (Bb) in stage Ib (IB) oocytes. Cortical granules begin to accumulate in stage II (II) oocytes of (c,e) WT and (d,f) Mdcch mutants and localize to the cortex in stage III (III) oocytes, which are distinguishable by the presence of yolk granules (Ygs). The structure of the vitelline envelope (VE) surrounding the oocyte is indistinguishable between WT and mutants. Images are representative of oocytes from 3 WT and 3 mutant females examined.
were comparable. By 5 mpa, WT eggs had largely completed CGE, whereas CGs persisted in mutant eggs until ∼15 mpa (Fig. 2.3A). Consistent with delayed CGE, chorion expansion in mutants was delayed relative to WT (Movie S1). As histological analysis of ovaries revealed no overt differences between CG formation or distribution in WT and Mdhc1b oocytes (Fig. 2.3H and 2.4F), we conclude that maternal dchs1b function promotes CGE during egg activation.

2.3.5 dchs1b Function Is Required For Actin-Dependent Separation Of Yolk And Cytoplasm

Several lines of evidence implicate maternal Dchs1b function in cytoplasmic streaming. First, in the blastodisc of cleavage stage MZdhc1b mutants, we observed amorphous yolk masses (Fig. 2.1F), which were apparent by yolk autofluorescence (Fig. 2.5B). Second, visualizing F-actin using Tg[β-actin:utrophin-GFP] (Behrndt et al., 2012), revealed cytoplasm persisting within the yolk of mutants at 75 mpf, when most of the actin containing cytoplasm had segregated into the blastodisc of WT embryos (Fig. 2.5C and D). Third, spinning disk confocal time-lapse microscopy and particle image velocimetry (PIV) analysis (Prasad and Jensen, 1995; Yin et al., 2008) of internal movements of the cytoplasm during egg activation in WT revealed periods of robust animal-ward movements at the blastodisc yolk cell interface that were punctuated by smaller fluctuations towards the vegetal pole, reminiscent of ebb and flow motion (Fig. 2.5D and Movie S2). The initial surge of animal-ward movement in central regions of WT embryos at 30 mpf averaged 7 µm/min and was mirrored by smaller 3 µm/min vegetal-ward movements at the cortex followed by smaller ebb and flow movements (Fig. 2.5E). The vigorous movements appeared well organized with two centers of circular motion, which we interpret as a circular, toroidal movement within the yolk (Fig. 2.5D). All movements were of smaller amplitude in MZdhc1b mutants, with the maximum being 4 µm/min, the organized toroidal movements were lost, and coordination of central movements and cortical flow was impaired (Fig. 2.5D, E and
Figure 2.5 Egg Activation Defects In MZdchs1b And Cytochalasin D Treated WT Embryos

A. Max z-projection of phalloidin (green) and MPA (red) staining of activated WT and M or MZdchs1b eggs fixed at 1, 5, and 15 mpa.

B. Overlay of bright field and auto-fluorescent max z-projections of WT, MZdchs1b eggs at 40 mpa.

C. Single z-plane images from time-lapse movies of single embryos in grayscale, and overlay of five pseudo colored WT Tg[β-actin:Utrophin-GFP], MZdchs1b;Tg[β-actin:Utrophin-GFP], and WT Tg[β-actin:Utrophin-GFP] +3μg/mL cytochalasinD treated embryos at 15 mpf and 75 mpf.

D. Max z-projection images from time-lapse movies of WT, and MZdchs1b embryos in bright field at frames 14, 22, and 44 with PIV analysis overlaid. PIV analysis: red arrows=towards the animal pole; blue arrows=towards the vegetal pole; arrow length indicates movement magnitude. Left most panels: pseudo colored first frame (magenta) and last frame (green) overlaid.

E. Quantification of cytoplasmic movement with PIV for WT (n=8), and MZdchs1b (n=9) embryos. Blue lines represent center of embryos marked by blue boxes in left panels in A and orange lines represent both edges of embryos demarcated by orange crescent boxes in A. Left graphs show magnitude of motion and right graphs show magnitude of motion with respect to embryonic A/V axis. Graphs are plotted with standard deviation bars.
Movie S3). The ooplasmic streaming abnormalities and delayed CGE as well as bundled actin later in development (Fig. 2.11E) imply actin cytoskeleton deficits in MZdchs1b mutants, as both processes rely on F-actin dynamics (Becker and Hart, 1999).

### 2.3.6 Delayed And Abnormal Cleavages With Normal Aspects Of MBT In MZdchs1b Mutants

Time-lapse analyses also revealed delayed and non-uniform cleavages in MZdchs1b mutants. Quantification of cleavage cycle length from anaphase to anaphase revealed an average of 15 minutes in WT embryos, compared to on average 19 minutes and up to 40 minutes in MZdchs1b mutants (Fig. 2.6A and B). In addition to longer cleavage cycles, MZdchs1b mutants displayed abnormal cleavage patterns such that cells divided into three or more daughters, yielding differently sized blastomeres (Fig. 2.6C and Movie 4).

To determine whether subsequent developmental processes were delayed in MZdchs1 mutants, we analyzed MBT onset, marked by activation of zygotic transcription, appearance of YSL nuclei (YSN), and loss of cell division synchrony (Kane and Kimmel, 1993). Surprisingly, qRT-PCR revealed comparable expression onset for several zygotic genes, including boz/dharma, bmp2b, sqt, cyc, and chordin (Schulte-Merker et al., 1997; Sirotkin et al., 2000; Solnica-Krezel and Driever, 2001; Yamanaka et al., 1998) between WT and MZdchs1b mutants (Figs. 2.6D, 2.9B, 2.7A, B, and E). Upon YSL formation, the YSN undergo several divisions and spread towards the animal and vegetal poles (D'Amico and Cooper, 2001; Solnica-Krezel and Driever, 1994). YSN appeared on time in MZdchs1b mutants, although their distribution revealed by mxtx2 (Hong et al., 2011) or H2B-GFP labeling was abnormal, compared to uniformly spaced YSN in WT (Fig. 2.6E and F). Hence, although MZdchs1b mutants appeared morphologically younger than age-matched WT embryos, aspects of MBT occurred on time.
Figure 2.6

A

B

C

D

E

F

47
Figure 2.6 Uncoupling Of Cell Division And MBT In MZdchs1b Mutants

A. One cell division from early anaphase to the next early anaphase in WT and MZdchs1b blastula stage embryos.
B. Quantification of length of cell divisions in WT and MZdchs1b embryos.
C. Quantification of length of the longest cell axis in 128-cell stage WT and MZdchs1b embryos.
D. Zygotic expression of boz in MZdchs1b, MZdchs2^att/stl, and MZdchs1b^{h275/h275}, MZdchs2^att/stl mutants compared to WT relative to MBT.
E. mxtx2 WISH in time-matched WT and MZdchs1b embryos, labeling YSN at 4 hpf; animal pole view.
F. H2B-GFP labeling of YSN in WT and MZdchs1b embryos.
Figure 2.7

A) bmp2b gene expression

B) chd gene expression

C) sox17

D) cyc expression

E) 30% epiboly
Figure 2.7 Levels And Patterns Of Zygotic Gene Expression
A. Expression of zygotic gene \textit{bmp2b} in WT and MZ\textit{dchs1b} embryos during MBT by qRT-PCR.
B. Expression of zygotic gene \textit{chd} in WT and MZ\textit{dchs1b} embryos during MBT by qRT-PCR.
C. Expression of \textit{sox17} revealed by WISH in stage matched WT and MZ\textit{dchs1b} embryos at 30\% and 90\% epiboly. Animal pole view for 30\% epiboly embryos. Dorsal and later views for 90\% epiboly embryos.
D. \textit{cyc} WISH of stage matched WT and MZ\textit{dchs1b} embryos; animal pole view.
E. Quantitative RT-PCR of \textit{cyc} for time matched WT and MZ\textit{dchs1b} embryos during MBT.
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2.3.7 Abnormal Nodal Signaling In Mzdchs1b Mutant Blastulae

The YSL is a source of signals that induce and pattern germ layers (Carvalho et al., 2009; Chen and Kimelman, 2000; Fekany et al., 1999; Mizuno et al., 1999; Yamanaka et al., 1998). Given the abnormal YSN distribution in MZdchs1b mutants, we investigated YSL-mediated inductive events. The pan-mesodermal marker ntl (Schulte-Merker et al., 1994) was detected at 30% epiboly in a continuous ring around the blastoderm margin in WT, but the ntl domain was punctuated by gaps in about 30% of Mdchs1b and MZdchs1b embryos (Fig. 2.9A and B). The gastrula organizer markers chordin (chd) and goosecoid (gsc) were expressed in an arc of 60° and 75° respectively in WT, whereas both domains were significantly reduced or interrupted in MZdchs1b mutants (Fig. 2.8D-G). In WT gastrulae, sox17 expression marks endodermal precursors and dorsal forerunner cells (Alexander and Stainier, 1999; Engleka et al., 2001; Hudson et al., 1997). Mutants had fewer sox17 expressing endodermal cells, and the forerunner cell domain was vegetally displaced relative to the blastoderm margin and fragmented (Fig. 2.7C). As both mesendoderm and the Nieuwkoop center were aberrant in MZdchs1b mutants (Fig. 2.8A-G), we investigated the signals inducing them. Nodal morphogens induce mesodermal and endodermal tissues in a concentration dependent manner (Agius et al., 2000; Chen and Schier, 2001; Erter et al., 1998; Gritsman et al., 2000; Jones et al., 1995). Transcripts of Nodal signaling ligands, cyclops (cyc) and squint (sqt) were expressed in a continuous ring at the blastoderm margin of 30% epiboly WT blastulae (Chen and Schier, 2001; Erter et al., 1998; Feldman et al., 1998; Sampath et al., 1998), and discontinuous domains in MZdchs1b mutants (Fig. 2.9A and 2.7D). Consistently, qRT-PCR revealed significantly reduced abundance of cyc and sqt transcripts in MZdchs1b compared to WT (Fig. 2.9B and 2.7E). To functionally assess
Figure 2.8 Mesoderm Specification Is Deficient In MZdchs1b Embryos And WT Embryos With Impaired Cytoskeleton

A. *ntl* expression in MZdchs1b, Mdcchs1b, and MZdchs1b^{h275f:h275};MZdchs2^{at1/at1} stage-matched embryos at 30% epiboly; animal pole view.

B. Quantification of gaps in *ntl* expression in WT, Mdcchs1b, and MZdchs1b embryos.

C. *ntl* expression in WT embryos treated with DMSO, 5μM taxol, 3μg/mL cytochalasinD, and 0.01μg/mL nocodazole at 30% epiboly; animal pole view.

D. *chd* expression domain in WT, Mdcchs1b, and MZdchs1b embryos. Insets show embryos with gap in expression domain.

E. *gsc* expression in WT, MZdchs1b, MZdchs2^{at1/at1}, and MZdchs1b^{h275f:h275};MZdchs2^{at1/at1} stage-matched embryos at 30% epiboly; animal pole view. Inset in MZdchs1b panel shows representative image of disrupted *gsc* domain.

F. Measurement of the *chd* expression domain in degrees for embryos shown in D.

G. Measurement of the *gsc* expression domain in degrees for embryos shown in E.
Figure 2.9 Reduced Nodal Signaling In MZdchs1b Embryos
A. sqt expression in stage-matched WT and MZdchs1b embryos; animal pole view.
B. Quantitative RT-PCR of sqt RNA in time-matched WT and MZdchs1b embryos during MBT.
C. Max z-projection of time-matched WT Tg[smad2-GFP] and MZdchs1b;Tg[smad2-GFP] embryos at 3.5 hpf.
D. Quantification of nuclear to cytoplasm ratio for GFP intensity in WT Tg[smad2-GFP] and MZdchs1b;Tg[smad2-GFP] embryos shown in E.
Nodal signaling, we analyzed nuclear accumulation of the transcription factor Smad2 (Saka et al., 2007; Schier and Shen, 2000). Using the Tg[β-actin:smad2-GFP] transgene reporter of Nodal activity in vivo (Dubrulle and Schier, in press eLife), we observed a significantly reduced ratio of nuclear to cytoplasmic Smad2-GFP in MZdchs1b blastulae compared to WT (Fig. 2.9C, D). Together, these results indicate reduced Nodal signaling partially accounts for the mesendodermal and Nieuwkoop center deficits of MZdchs1b embryos.

2.3.8 Impaired wnt8a RNA Translocation In MZdchs1b Mutants

Nieuwkoop center formation requires microtubule-dependent asymmetric transport of DDs, such as wnt8a mRNA, from the vegetal pole to the future dorsal side of the embryo (Ge et al., 2014; Gore and Sampath, 2002; Jesuthasan and Stahle, 1997; Lu et al., 2011; Nojima et al., 2010; Shao et al., 2012; Tran et al., 2012). WISH revealed proper vegetal pole localization of wnt8a transcripts in unactivated Mdchs1b eggs (Fig. 2.11A). However, animal-ward translocation of wnt8a RNA following fertilization was reduced in MZdchs1b embryos compared WT (Fig. 2.10A, B). Moreover, in some embryos wnt8a expression expanded symmetrically (Fig. 2.11A).

wnt8a mRNA transport is mediated by microtubules, which after egg activation, form transient parallel arrays aligning with the future dorsal side of the embryo (Lu et al., 2011; Tran et al., 2012). Therefore, we hypothesized that impaired transport of wnt8a in MZdchs1b embryos was due to microtubule abnormalities. Visualizing microtubules with the DM1α antibody in immunofluorescence and using Tg[XIeef1a1:dclk2-GFP], we observed parallel arrays that appeared misoriented in mutants, crossing each other (Fig. 2.10C and 2.11C), and more bundled as evidenced by higher intensity in Mdchs1b mutants compared to WT (Fig. 2.10D). Progressive bundling resulted in dramatically different appearances of vegetal microtubules during
Figure 2.10 MZdchs1b Embryos Exhibit Abnormal Wnt8a Expression Domain Shift And Vegetal Microtubule Populations And dchs1b RNA Rescue Of Microtubule Phenotype

A. wnt8a expression in WT and MZdchs1b embryos at 4 and 32 cell stages. Black bars mark the angle between the edge of wnt8a expression domain from the vegetal pole.

B. Quantification of the angle between the edge of the wnt8a expression domain and the vegetal pole for WT and MZdchs1b embryos at 4 and 32 cell stages. *** = p<0.005.

C. DM1α labeling of vegetal pole microtubules for WT and MZdchs1b embryos at 20 mpa. Top panels show parallel microtubule arrays in tangential view of embryos. Bottom panels higher magnification with black arrowheads pointing to crossing microtubules.

D. Plot profile of WT and MZdchs1b parallel arrays for intensity.

E. DM1α labeling of microtubules for WT, MZdchs1b, and MZdchs1b embryos injected with dchs1b full length RNA and ICD RNA at 40% epiboly.

F. Quantification of rescue of yolk microtubule phenotype with different doses doses of dchs1b full length or ICD RNAs. MZdchs1b is statistically different from all other conditions. ns = not significant; *** -> p<0.0001.
**Figure 2.11**

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Figure 2.11 Dorsal Determinant Transport And Organizer Formation Depend On Microtubules

A. wnt8a transcripts detected by WISH in WT and MZdchs1b<sup>h275/h275</sup> embryos at 2 mpa, 15 mpa, 4 and 32 cell stages. Representative patterns of wnt8a RNA expression domain in MZdchs1b<sup>h275/h275</sup> embryos at 32-cell stage. Black bars mark the angle of the edge of the wnt8a RNA expression domain from the vegetal pole.

B. β-catenin labeling in 3 hpf WT and MZdchs1b<sup>h275/h275</sup> embryos.

C. Max z-projection of vegetal pole microtubule at 4 hpf in WT and MZdchs1b<sup>h275/h275</sup> embryos.
gastrulation (Fig. 2.10E and 2.11D). Notably, microtubule bundling could be partially rescued by injection at one cell stage of synthetic RNAs encoding either full-length Dchs1b-sfGFP or Dchs1b intracellular domain (Fig. 2.10E, F). We posit these vegetal microtubules abnormalities in MZdchs1b mutants could impede translocation of wnt8a transcripts, consequently resulting in Nieuwkoop center and gastrula organizer deficiencies (Fig. 2.8D-G).

2.3.9 Disrupting Cytoskeleton Dynamics In WT Embryos Phenocopies Mzdchs1b Mutant Defects

We employed a pharmacological approach to determine whether specifically targeting actin or microtubules could phenocopy MZdchs1b defects. Partial disruption of the F-actin network (Fig. 2.12A) of Tg[β-actin:utrophin-GFP] embryos using 3µg/mL of cytochalasinD in the medium from activation (Cooper, 1987; Leung et al., 2000; Schliwa, 1982) impeded yolk/cytoplasm segregation similar to MZdchs1b mutants. Globular yolk inclusions occupied the blastodisc, while cytoplasmic islands remained in the yolk at 75 mpf (Fig. 2.5C). At 10-15µg/mL of cytochalasinD, CGE was perturbed, chorions did not fully expand, cytoplasmic streaming was blocked (Fig. 2.12B), and development arrested. We then assessed the effect of 3µg/mL cytochalasinD treatment on mesodermal specification, and found that a fraction of treated embryos had uneven ntl marginal domains and reduced gsc expression domains, similar but milder phenotypes than those in MZdchs1b mutants (Fig. 2.8C and 2.12C).

Next, we perturbed microtubule dynamics using nocodazole to prevent tubulin polymerization, or taxol to stabilize microtubules (Heidemann et al., 1980). Culturing WT embryos in 0.001, 0.002, and 0.005µg/mL of nocodazole or 5µM taxol from 10 mpa did not affect cytoplasmic streaming during egg activation. Microtubule inhibiting drugs were added at 1 hpf to test the effect on mesoderm formation to avoid interference with initial wnt8a
Figure 2.12 WT Embryos Treated With Cytoskeleton Altering Agents
A. Phalloidin labeling of F-actin at 16-cell stage for WT, WT treated with DMSO, WT treated with 1μg/mL cytochalasinD, and WT treated with 3μg/mL cytochalasinD from activation.
B. Bright field images of WT in vitro fertilized embryos in egg water, DMSO, 5μg/mL cytochalasinD, 10μg/mL cytochalasinD, and 15μg/mL cytochalasinD from activation at 50mpf.
C. gsc transcripts revealed by WISH in WT embryos treated at 1 hpf with DMSO, 3μg/mL cytochalasinD, 0.01μg/mL nocodazole, and 5μM taxol at 8 hpf; dorsal view.
D. ntl and gsc transcripts revealed by WISH in WT embryos treated at 1 hpf with DMSO, 0.002μg/mL nocodazole, and 0.005μg/mL nocodazole; 6 hpf animal pole view.
translocation. The treated embryos had punctuated marginal *ntl* domains and smaller or fragmented *gsc* domains, phenocopying *MZdchs1b* mutant defects (Fig. 2.8C and 2.12D). In embryos cultured with 0.002µg/mL and 0.005µg/mL nocodazole, *ntl* expression was reduced to one side of the embryo and *gsc* expression was absent (Fig. 2.12D). Surprisingly, taxol caused similar defects in mesoderm formation in WT embryos, suggesting that changing the dynamics of microtubules, whether destabilizing or stabilizing, produced *MZdchs1b*-like phenotypes (Fig. 2.8C, 2.12C, and D). Based on the similar defects observed in *MZdchs1b* mutants and pharmacological disruption of microtubule and/or actin cytoskeletons in WT, the abnormal microtubule and actin networks in mutants (Fig. 2.5C and 2.10C), and rescue of microtubule bundling by expressing *Dchs1b* (Fig. 2.10E and F), we propose that *Dchs1b* regulates the dynamics of the actin and/or microtubule networks to promote egg activation and early patterning.
2.4 Discussion

We have identified novel roles for Dchs1b in early patterning and morphogenesis at the earliest stages of zebrafish development, the transcriptionally silent egg and blastula, without perceivable defects in oogenesis. *Drosophila* Dachsous functions as a Fat ligand to regulate growth through Hippo signaling, planar polarity, and cell-cell adhesion in epithelial tissues (Casal et al., 2006; Clark et al., 1995; Ishiuchi et al., 2009; Rawls et al., 2002; Strutt and Strutt, 2002; Yang et al., 2002). In PCP regulation, Dachsous is proposed to be instructive in promoting polarity of apical microtubule arrays that mediate asymmetric transport of core PCP proteins (Harumoto et al., 2010; Matis et al., 2014). The essential function of Dchs1b in vertebrate development was revealed by pleiotropic phenotypes and postnatal lethality of Dchs1 knockout mice (Mao et al., 2011; Zakaria et al., 2014). Furthermore, in humans, *DCHS1* mutations can lead to a recessive syndrome characterized by pleiotropic phenotypes including periventricular neuronal heterotopia (Cappello et al., 2013). However, the cellular mechanisms via which Dch affects vertebrate development are unknown. We propose that in zebrafish zygotes and early embryos, Dchs1b coordinates CGE, cytoplasmic segregation, and maternal mRNA translocation by regulating the organization and dynamics of the actin and microtubule cytoskeleton, likely via a Fat and PCP independent mechanism. Indeed, MZ PCP pathway mutants, *trilobite/vangl2* and *knypek/glypican4*, do not exhibit such early developmental defects (Ciruna et al., 2006; Topczewski et al., 2001).

Zebrafish MZdchs1b and MZdchs2 mutants afforded assessment of the earliest *dchs* functions in embryogenesis. Correlated with its strong maternal expression, only MZdchs1b embryos exhibited pre-MBT phenotypes (Fig. 2.1). That two independent nonsense alleles manifest the same spectrum of phenotypes and that dchs1b RNA rescued abnormal microtubule
organization in the YSL provides evidence that the observed phenotypes are due to loss of dchs1b function. The variable penetrance and expressivity of MZdchs1b phenotypes is typical of other zebrafish maternal and MZ mutants: ichabod/β-catenin2, bozozok, and squint (Fekany et al., 1999; Kelly et al., 2000; Sirotkin et al., 2000). Functional redundancy between the three dchs genes is supported by reduced phenotypic variability and increased phenotypic severity in MZdchs1bfh275/fh275;MZdchs2stl1/stl1 compound mutants (Fig. 2.1F, 2.8A, E, and G).

A striking finding is the essential role Dchs1b plays in the single celled egg and early zygote. Mdchs1b mutants showed delayed CGE and incomplete cytoplasmic segregation (Fig. 2.5). These egg activation processes occur minutes after an egg is laid, and are independent of fertilization, transcriptional activity, and cell-cell interactions. Based on our histological findings these defects are proximal to Dchs1b rather than reflecting abnormalities during oogenesis. First, hallmarks of oocyte/egg polarity were normal in Mdchs1b mutants (Fig. 2.3 and 2.4E-G): Balbiani bodies were present, a single cytoplasmic island and single micropyle occupied the animal pole, and wnt8 maternal mRNA was localized at the vegetal pole. Additionally, CGs translocated to the cortex, and polar bodies were extruded normally. These analyses indicate that egg activation processes, which are dependent on dynamics of actin or microtubule cytoskeleton become defective in M or MZdchs1b mutants after activation/fertilization of the egg. That injection of synthetic RNA encoding Dchs1b intracellular domain rescued abnormal organization of microtubule network in the YSL (Fig. 2.10E, F), further strengthens the notion that Dchs1b has activities independent of intercellular interactions with Fat. This contrasts all previous studies that implicated Dachsous in multicellular or tissue contexts, where Dachsous functions through heterophilic intercellular interactions with Fat (Casal et al., 2006; Clark et al., 1995; Ishiuchi et al., 2009; Rawls et al., 2002; Strutt and Strutt, 2002; Yang et al., 2002).
consequences of inactivating the maternal Dchs function in these systems remain to be investigated.

Our data support a novel Dchs1b role in mediating and coordinating multiple processes during early development. During egg activation in M/MZdchs1b mutants, CGE and cytoplasmic streaming were both delayed and cytoplasmic streaming was uncoordinated (Fig. 2.5). During cytoplasmic streaming, actin dependent movement of cytoplasm between the central and peripheral yolk were uncoordinated and the organized centers of motion present in WT were lost in MZdchs1b mutants. Later events that together constitute MBT were disassociated in MZdchs1b, with YSL appearance and zygotic transcription initiation of several zygotic genes occurring on time in mutants despite delayed and uneven maternal cell divisions (Fig. 2.6). This lack of coordination in MZdchs1b mutants is interesting in light of the well-established role for Dachsous in Drosophila planar polarity, as after all, planar polarity entails coordination of cell polarity across a tissue (Goodrich and Strutt, 2011).

Unexpectedly, MZdchs1b mutants also displayed dorsal organizer and mesendoderm deficiencies. As the underlying cellular mechanism that leads to MZdchs1b phenotypes, we implicated defective cytoskeletal dynamics through four non-mutually exclusive models (Fig. 2.13). First, in the mutant blastodisc, defective cytoplasmic streaming produces ectopic yolk masses (Fig. 2.5B, C), which later present physical obstacles to cell migration and morphogen diffusion and could reduce Nodal signaling (Fig. 2.9), leading to mesendoderm deficiencies (Fig. 2.8). In the current model for Nodal morphogen gradient formation, ligand travels through tissue via diffusion and is hindered by binding and tortuosity created by cells in the tissue (Muller et al., 2013). Second, the transient parallel microtubule arrays were more bundled and misoriented in MZdchs1b mutants compared to WT, and maternally deposited wnt8a mRNA that is
Figure 2.13 Model For Dchs1b Function During Early Embryogenesis
Left panels: MZdchs1b mutants show uncoordinated movement of actin containing cytoplasm leading to incomplete yolk-cytoplasm segregation. Insets show vegetal views of microtubules. Middle panels: Microtubule dependent transport of dorsal determinant is abnormal in MZdchs1b mutants, mutant blastoderm retains yolk while cytoplasm is present in the yolk cell. Right panels: MZdchs1b mutant gastrula display defects in YSN organization, microtubule bundling and mesoderm, irregular b-catenin nuclear distribution and organizer gene expression.
translocated upon fertilization by the microtubule cytoskeleton (Lu et al., 2011; Tran et al., 2012), displayed abnormal distribution after fertilization (Fig. 2.10A and B). Therefore, abnormal organization and function of microtubules in MZdchs1b mutants could lead to impaired dorsal determinant translocation thereby affecting β-catenin nuclear localization (Fig. 2.11C), and consequently β-catenin-dependent zygotic gene expression in the Nieuwkoop center and dorsal mesoderm, such as sqt, gsc, and ntl (Fig. 5)(Schulte-Merker et al., 1994; Schier and Shen, 2000; Chen and Schier, 2001; Erter et al., 1998; Feldman et al., 1998). Third, as the dorsal YSL is the initial source of Nodal signaling at the onset of MBT (Chen and Kimelman, 2000), the disorganized microtubule cytoskeleton and YSN on the dorsal side of MZdchs1b blastulae, where nuclear β-catenin initially accumulates (Kelly et al., 2000), may compromise Nodal signaling as well. Lastly, a model for mechanical induction of mesoderm and phosphorylation of β-catenin due to physical stress caused by epiboly was recently proposed (Brunet et al., 2013). The hyperbundled cytoskeleton (Fig. 2.10E, 2.11D, and E) in MZdchs1b yolks could lead to differential stress at the blastoderm margin, contributing to mesoderm deficiencies. All these mechanisms could contribute to the variable defects seen in MZdchs1b mutants (Fig. 2.13).

The unifying cause of all the abnormalities seen in Mdchs1b and MZdchs1b embryos can be traced back to defects in cytoskeletal dynamics. We posit that Dchs1b regulates both the actin and microtubule cytoskeletal systems independently as perturbation of either in WT embryos phenocopied unique subsets of mutant defects: e.g. perturbing actin but not microtubules led to defects in cytoplasmic streaming. In Drosophila, Dachsous regulate the unconventional myosin Dachs (Cho and Irvine, 2004; Mao et al., 2006), however its vertebrate homolog remains to be identified. Additionally, Dachsous regulates dynamics of non-centrosomal microtubules in Drosophila, where both alignment and asymmetric distribution are affected in mutants
(Harumoto et al., 2010; Matis et al., 2014). However, how Dachsous interacts with and regulates microtubules remains unknown. Identification of molecular links between Dchs and the cytoskeleton in vertebrates is an important future goal.

We have discovered an essential role for Dchs1b during early vertebrate morphogenesis and cell fate specification through regulation of the actin and microtubule cytoskeleton. We found that both MZdchs1b and MZdchs2\textsuperscript{mtl/mtl} mutant embryos display C&E defects during gastrulation, but whether Dchs does this by influencing PCP in zebrafish gastrula is unclear. Additionally, we observed no overt growth defect in MZdchs1b mutants as in Drosophila, where Ds regulates the Hippo pathway (Cho et al., 2006). However, we have not ruled out tissue specific growth and morphogenesis defects later in development. Recent studies show an intriguing relationship between cell polarity and fate with Hippo signaling in the mouse blastocyst (Anani et al., 2014; Hirate et al., 2013; Kono et al., 2014). Moreover, Hippo signaling pathway components can modulate the Wnt/β-catenin pathway in multiple contexts including the Drosophila wing imaginal disc, and murine kidney and heart (Baena-Lopez et al., 2008; Heallen et al., 2011; Imajo et al., 2012; Varelas et al., 2010). Furthermore, the Hippo pathway can both regulate and respond to the actin cytoskeleton during collective cell migration and cell polarization (Bertrand et al., 2014; Low et al., 2014; Lucas et al., 2013). These studies pose a fascinating web of possible genetic and functional interactions for Dchs in other developmental processes. Answering how Dchs functions during development will reveal which roles are conserved from Drosophila to vertebrates and shed light on how it leads to pleiotropic phenotypes in patients with Van Maldergem syndrome.
2.5 Materials and methods

Zebrasfish lines

AB, Tg[XIeef1a1:dclk2-GFP], Tg[β-actin:utrophin-GFP], and Tg[β-actin:smad2-GFP] (Campinho et al., 2013; Tran et al., 2012) lines were used. TILLING to generate dchs1b<sup>fh274</sup> and dchs1b<sup>fh275</sup> and dchs2<sup>stl1</sup> mutations was performed as described (Draper et al., 2004).

Embryo staging and maintenance

In vitro fertilization was used to generate time-matched WT and mutant embryos, whose age is reported as hours post fertilization (hpf). Stage-matched mutant and WT embryos were collected from pair-wise crosses that spawned within 10 minutes of each other and matched by morphological landmarks at the time of the experiment (Kimmel et al., 1995). Embryos were kept in egg water (60 µg/mL of Instant Ocean in distilled water) at 28.5°C.

Live imaging

Cytoplasmic streaming: WT and M<sub>dchs1b</sub> eggs were fertilized in vitro, activated in egg water for 8 minutes at room temperature (RT), manually dechorionated, and mounted in 0.3-0.5% low melting temperature agarose (LMA, Seaplaque Cat. No. 50100) in 0.3x Danieau’s buffer on a round #1 coverglass bottom dish. Z-stack time lapses were collected using spinning disc confocal microscope (SDCM) (Olympus IX81, Quorum) in bright field with a 10X objective, from 14-59 mpf. Each step in the z-stack was 3 µm and the entire stack was 55 slices with stacks collected every minute.

Vegetal microtubules: Tg[XIeef1a1:dclk2-GFP] and M<sub>dchs1b</sub>; Tg[XIeef1a1:dclk2-GFP] embryos were collected within three minutes of each other, manually dechorionated and
mounted as above. Z-stack time lapses were collected using SDCM with 491nm wave length laser at 10X with z-slice of 3 μm and 51 z-slices from 0.5 to 6.5 hpf every 3 mins or at 40X with z-slice of 0.5 μm and 33 slices from 15 to 30 mpa every minute.

Autofluorescence of yolk: WT and Mdcsh1b 40 mpa embryos were mounted as above. Z-stacks were collected with SDCM with 491 nm wavelength laser and DIC at 10X with z-slices of 3 μm.

Cell division: Embryos were injected with 70pg of H2B-GFP RNA at one cell and counter-stained with CellTrace Bodipy (C34556) at 1:100. Z-stacks were collected at 1 hpf with SDCM with 491 and 561 nm wavelength lasers at 10X with z-slices of 3 μm.

Nuclear labeling of YSL: 70pg of H2B-GFP RNA was injected into the YSL around 3 hpf. Z-stacks were collected at 4 hpf with SDCM with 491 nm wavelength laser 10X with z-slices of 3 μm.

Immunohistochemistry (IHC)

DAB: Embryos were fixed in 4% paraformaldehyde at 3 hpf, washed in PBS, blocked in 10%FBS in PBSTween. Primary antibody: β-catenin Sigma C7207. Development using Vectastain ABC vector kit (PK-6102) and ImmPACT DAB kit (SK-4105). Microtubule staining with CALBIOCHEM DM1α antibody (#CP06) at 50% epiboly and AlexaFluor goat anti-mouse secondary antibody (#A11031) was performed according to (Gard, 1991) with modification by Solnica-Krezel and Driever, 1994.

In situ hybridization
Embryos were fixed at various stages in buffer containing 4% paraformaldehyde (PFA), 4% sucrose, and 120µM calcium chloride at 4°C overnight. WISH was performed according to (Thisse and Thisse, 2008).

Quantitative RT-PCR

Each RNA sample was isolated using Trizol (Life Technologies, #15596-026) from 30 WT or mutant embryos. 1 µg of RNA was used to synthesize cDNA with the iScript kit (Bio-Rad, #170-8891) following manufacturer’s protocol. qRT-PCR reactions were set up using SoAdvanced Sybr green (Bio-Rad, #172-5265). Primers used are listed in Table S1.

Velocity Field Generation and Analysis

Particle Image Velocimetry (PIV)-type analysis was applied to time-lapse images collected at a single confocal plane parallel to the animal-vegetal axis that passed through the center of the embryo. Three passes using subwindows of 64, 32, and 16 pixels with an overlap of 50% were used to compute the velocity fields. The region of the image outside of the embryo was masked from the analysis and did not contribute to any of the subwindow matching. Prior to analysis, the contribution of spurious vectors was reduced by time-averaging velocity fields with a boxcar moving average filter of width 2. Three regions were defined for the analysis: a center region and two symmetric side regions. A vector extending from the vegetal to animal pole was defined to adjust for different absolute orientations of the embryos. For each of the three flow regions, the overall average magnitude and average magnitude in the animal pole direction were calculated as a function of time.
Ovary histology and Confocal Immunofluorescence

Females were anesthetized in Tricaine as described (Westerfield, 1995). Ovaries were dissected and fixed in 4% PFA overnight. Sectioning and Hematoxylin & Eosin (H&E) staining were performed as in (Hartung et al., 2014). Images were acquired using an Axioskop2 microscope and Axiocam CCD camera (Zeiss).

Anesthetized WT of dchs1b<sup>fh275/fh275</sup> females were squeezed to obtain unfertilized eggs, which were fixed immediately or at 0 and 2 mpa. For β-catenin and γ-tubulin IHC, samples were fixed with 4% PFA and were performed using either anti-β-catenin (C2206, Sigma) or anti-γ-tubulin (T5326, Sigma) antibodies diluted 1:1000. For tubulin IHC, samples were fixed according to (Gard, 1991) and were performed using anti-acetylated α-tubulin (T6793, Sigma) diluted at 1:1000. AlexaFluor488 and AlexaFluor568 (Invitrogen) secondary antibodies were diluted 1:500. For F-actin labeling, samples were fixed for 4 hrs at 4°C in 3.7% formaldehyde in (ASB) as in (Becker and Hart, 1999) then staining of oocytes was performed as described in (Topczewski and Solnica-Krezel, 1999) using 66 nM Rhodamine Phalloidin (R415, Life Technologies) for 1 hr at RT. F-actin labeling of polar bodies was performed as described in (Dekens et al., 2003) using 33nM Rhodamine Phalloidin (R415, Life Technologies) overnight at 4°C. All fluorescently labeled samples were mounted in VECTASHIELD® with DAPI (H-1200, Vector Laboratories). Maximal z-projections of AcTub immunostaining were thresholded using ImageJ and the number of objects ≥3μm<sup>2</sup> was calculated with the Analyze Particles feature. Images of F-actin labeled polar bodies were acquired with a Zeiss LSM5 Live DuoScan line scanning confocal image using a 10X/0.45 air objective. All other samples were imaged with a Leica SP2 point-scanning confocal microscope using either the 40X/1.25 or 63X/1.4 oil objective.
Pharmacological treatments

Eggs were fertilized in vitro, activated, and cultured in egg water containing 3µg/mL of cytochalasinD (Sigma, #C8273), 0.05µM of nocodazole (Sigma, #M1404), or 1µM taxol (Sigma, #T7191), in DMSO until desired stages. For live imaging, embryos treated with 3µg/mL of cytochalasinD were dechorionated after 8 minutes and mounted in 0.3% LMA containing 3mg/mL cytochalasinD on a round #1 coverglass bottom dish. Control embryos were treated with equivalent amounts of DMSO.

Molecular cloning

To generate the dchs1b-sfGFP and dchs1b intracellular domain constructs, the full-length dchs1b ORF or intracellular domain exon was obtained by multi-step PCR and subcloning from zebrafish cDNA prepared by SuperScript III RT(Invitrogen). The full-length dchs1b ORF was further fused in frame with a 9aa linker and the sfGFP sequences by annealing extend PCR.

Statistical analyses

Statistical analysis was performed using GraphPad Prism 6. Statistical significance was estimated using a two-tailed unpaired Student’s t test to compare two populations.
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Chapter 3: Overlapping Functions of Dachsous1b and Dachsous2 Cadherins in Zebrafish Gastrulation

3.1 Introduction

In Chapter 2 of this thesis, I described pleiotropic defects in early embryogenesis due to loss of maternal and zygotic dchs1b mutants. Whereas inactivation of dchs2 alone did not cause such defects, it increased penetrance and expressivity when combined with inactivation of dchs1b in double mutants. The mutant phenotypes include defects in egg activation, cell division, and cell fate specification due at least in part to defects in the actin and microtubule cytoskeleton. These initial analyses also revealed defects in gastrulation cell movements of epiboly and C&E in both MZdchs1b and MZdchs2 mutants. Here, we extend studies of these gastrulation defects caused by loss of dchs1b and/or dchs2 functions.

3.1.1 Zebrafish Gastrulation – Definition And Morphogenetic Movements

Vertebrate gastrulation entails the formation and shaping of the three embryonic germ layers from a morphologically uniform population of cells and the concurrent patterning of the germ layers to specify many cell fates, to establish the basic animal body plan. By the onset of gastrulation, the zygote proliferated to generated thousands of cells forming a blastoderm perched on the large yolk cell, the zygotic genome became activated and the earlier inductive processes specified the dorsal gastrula organizer and germ layers (Kimmel et al., 1995). Gastrulation is marked by massive morphogenetic cell movements that place the embryonic germ layers: ectoderm, mesoderm, and endoderm in their characteristic arrangement and subsequently shape them into the embryonic body. During this process, dorsal/ventral, anterior/posterior, and left/right body axes become defined and specific cell lineages within each of the germ layers are born (Roszko et al., 2009; Schier and Talbot, 2005; Munoz-Sanjuan and
Brivanlou, 2004). While some of the cell movements are organism specific, four morphogenetic movements occur during gastrulation in all vertebrates: epiboly, internalization, convergence and extension (C&E) (Keller et al., 2003; Solnica-Krezel, 2005).

*Morphogenetic movements*

Epiboly cell movements drive the thinning and isometric spreading of tissues. During zebrafish epiboly, which is the first morphogenetic cell movement, cells spread from the animal pole to the vegetal pole thinning the germ layers of the embryo, and by its completion, the embryo proper covers the entire yolk. The completion of epiboly and the formation of the tailbud signal the end of the gastrula period (Warga and Kimmel, 1990). Internalization marks the beginning of the gastrula period and is the process by which in different vertebrates cells ingress, involute, or invaginate from the exterior into the interior of the embryo and give rise to the prospective endoderm and mesoderm. Internalization commonly ends by midgastrulation (Keller et al., 2008).

Convergence starts soon after internalization begins and continues throughout gastrulation into segmentation, concurrently with extension. C&E is the process by which cells of all germ layers move and rearrange, by directed migration, radial and planar mediolateral intercalation, to narrow mediolaterally and to elongate anteroposteriorly the animal body axis (Keller et al., 2003; Solnica-Krezel, 2005). In zebrafish, all three embryonic germ layers undergo C&E during gastrulation (Warga and Kimmel, 1990; Concha and Adams, 1998; Pezeron et al., 2008; Jessen et al., 2002; Sepich et al., 2005). While the cellular and molecular mechanisms that underlie C&E of endodermal and ectodermal cells remain less studied, the cell behaviors of mesoderm cells undergoing C&E are much better understood.

*Mesodermal cell behaviors underlying C&E*
The rate and type of movement of mesoderm cells varies depending on the stage of gastrulation and the position of cells along the dorsoventral and anteroposterior gastrula axis (Myers et al., 2002; Sepich et al., 2005; Keller et al., 2008). At midgastrulation, most mesodermal cells initiate C&E and begin their dorsal migration, however, cells in the most ventral gastrula regions never undergo C&E, rather they migrate towards the vegetal pole and contribute to the tail (Kanki and Ho, 1997; Kimmel et al., 1990; Myers et al., 2002). Initially, lateral mesodermal cells slowly migrate towards the dorsal side, and then increase their rate of migration as they approach the dorsal side (Jessen et al., 2002; Myers et al., 2002). Medial presomitic mesodermal cells undergo C&E largely driven by mediolateral and radial intercalation of adjacent cells (Keller et al., 2003; Kimmel et al., 1994; Yin et al., 2008). In the dorsal axial domain, anterior prechordal mesoderm cells undergo anterior directed migration and more posterior chordamesoderm cells undergo mediolateral intercalation movements resulting in fast extension and modest convergence (Myers et al., 2002; Yin et al., 2008; Glickman et al., 2003). In addition to these cell behaviors, polarized cell divisions along the animal-vegetal axis also contribute to extension during gastrulation (Concha and Adams, 1998; Gong et al., 2004; Sepich et al., 2005), however whether stereotypical orientation of cell division contributes to or is dispensable for elongation of the body axis during gastrulation remains unresolved (Gong et al., 2004; Quesada-Hernandez et al., 2010).

3.1.2 Cell Properties Underlying Gastrulation Movements

Cell polarity

Some migratory mesenchymal cells display planar polarity with a front and back or other features polarized in the tissue plane (Abercrombie et al., 1971; Ridley et al., 2003; Hay, 2005). During C&E movements, cellular migration behaviors described above rely on and manifest
different cell polarity (Myers et al., 2002; Weiser et al., 2007; Keller et al., 2000; Yin et al., 2009). Prior to the initiation of C&E, mesodermal cells display rounded and not highly polarized morphology and move with a slight dorsal bias along complex trajectories (Sepich et al., 2006; Solnica-Krezel 2006). As gastrulation progresses, lateral mesoderm cells become mediolaterally elongated and migrate efficiently with a strong dorsal bias (Myers et al., 2002; Concha and Adams, 1998; Jessen et al., 2002). Once these cells reach the dorsal region of the embryo, they undergo both radial intercalation and mediolateral intercalation in the paraxial presomitic mesoderm and largely mediolateral in the axial mesoderm (Yin et al., 2008; Glickman et al., 2003; Lin et al., 2005; Roszko et al., 2009). Defective mediolateral elongation of cells and less polarized cell migration and intercalation leads to shorter and wider embryonic axis (Heisenberg et al., 2000; Wallingford et al., 2000; Jessen et al., 2002; Topczewski et al., 2001; Yin et al., 2008).

**Cell-cell adhesion**

The morphogenic movements that occur during gastrulation necessitate dynamic regulation of cell adhesion to allow for cell migration and intercalation that drive these movements. The cadherin superfamily regulates cell-cell adhesion among other processes. Cadherins were first identified as calcium dependent cell-cell adhesion proteins (Yoshida and Takeichi, 1982; Gallin et al., 1983; Peyreras et al., 1983). Since the identification of the first cadherin, over a hundred family members have been identified, all of which contain the characteristic cadherin repeats in the extracellular domain, a single transmembrane and intracellular domain (Nollet et al., 2000). Cadherin proteins are divided into three subfamilies: classical cadherins, protocadherins, and atypical cadherins. Cadherins from each subfamily are involved in regulating tissue movements.
In zebrafish, the most prominently expressed cadherins during gastrulation are E-cadherin (Cdh1), in the blastomeres, and N-cadherin (Cdh2), in the yolk cell and in the nascent neuroectoderm (Tay et al., 2010). Mutations in E-cadherin lead to decreased adhesion and delayed epiboly, affecting the enveloping layer more than deep cells, which still expressed β-catenin at the membrane, suggesting interaction with other cadherins, likely N-cadherin (Shimizu et al., 2005; Kane et al., 2005). Radial intercalation of deep cells contributes to epiboly progression by thinning out cell layers (Keller, 1980; Warga and Kimmel, 1990; Bensch et al., 2013). In the half-baked (hab) mutant, loss of E-cadherin led to both reduced and unstable radial intercalation such that cells would interclate and subsequently de-intercalate bi-directionally (Kane et al., 2005; Song et al., 2013; Morita and Heisenberg, 2013). Other proteins that affect adhesion during epiboly progression include: hetero-trimeric G-proteins, which bind to the intracellular domain of E-cadherin and may compete for β-catenin binding; other cadherin family members such as Protocadherin; endocytosis of E-cadherin regulated by Wnt11; and stabilization of E-cadherin at adherence junction in the enveloping layer regulated by atypical RhoGTPase Chp (Tay et al., 2010; Lin et al., 2009; Aamar and Dawid, 2008; Carreira-Barbosa et al., 2009; Song et al., 2013). Cell-cell adhesion during gastrulation is also regulated transcriptionally by Snail proteins, which themselves are regulated both at transcriptional and posttranscriptional levels (Yamashita et al., 2004; Blanco et al., 2007; Speirs et al., 2011; Ma et al., 2014).

The C&E cell movement behaviors such as directed migration, mediolateral intercalation, and radial intercalation, also require precise dynamic regulation of adhesion (Solnica-Krezel, 2005; Warga and Kimmel, 1990; Sepich et al., 2005; Shimizu et al., 2005; Concha and Adams, 1998; Kimmel et al., 1994). In addition to epiboly defects, E-cadherin mutants also show mediolaterally wider somites, notochord and neuroectoderm, and impaired anterior migration of
the axial mesoderm (Shimizu et al., 2005). Moreover, N-cadherin is essential to convergence of lateral plate mesoderm as loss of N-cadherin in *bibber* mutants led to broader paraxial mesoderm due to slower and less directed migration (Warga and Kane, 2007). Protocadherins Flamingo and PAPC affect C&E by regulating anterior migration of axial mesoderm to promote extension; and convergence through RhoA and Rac1 as well as reducing C-cadherin mediated adhesion, respectively (Formstone and Mason, 2005; Unterseher et al., 2004; Chen and Gumbiner, 2006; Chen et al., 2007). Additionally, transcriptional regulation of E-cadherin downstream of Prostaglandin E2 led to increased cell-cell adhesion and affected all gastrulation movements (Speirs et al., 2010).

*Cortical tension*

Along with cell-cell adhesion, cortical tension also plays an important role in controlling formation of cell-cell contacts and thereby cell sorting (Krieg et al., 2008; Manning et al., 2010). Contrasting to adhesion, which increases cell-cell contacts, cortical tension reduces contact area causing cells to round up, much like surface tension of liquid droplets (Manning et al., 2010; Bertet et al., 2004; stewart et al., 2011). Cadherins modulate cortical tension through adhesion tension by recruiting catenins that regulate actomyosin cytoskeleton at the cell-cell contacts (Maître and Heisenberg, 2011; Yamada and Nelson, 2007; Miyake et al., 2006). The dynamic making and breaking of cell-cell contacts and changing of cell shape by modulating adhesion and actin and microtubule cytoskeleton are essential in driving morphogenetic movements such as those during gastrulation (Adams and Nelson, 1998; Schoetz, 2008; Lecuit and Lenne, 2007; Krieg et al., 2008).

3.1.3 Molecular Regulators Of Cell Behaviors
The molecular mechanisms that drive spatiotemporally dynamic C&E cell movements are complex and involve many components. For example cadherins, cytoskeleton, non-canonical Wnt/PCP pathway, and the Bone Morphogenetic Protein (BMP) signaling pathway all regulate the complex migratory cell behaviors during C&E. In general, these pathways are required in different domains of the embryo to control these movements, and crosstalk between these pathways ensures proper spatiotemporal coordination between all the cell movements.

**Cytoskeleton**

At the onset of zebrafish gastrulation, the yolk cell domes into the overlying blastoderm and pushes deep cells outward (Warga and Kimmel, 1990; Wilson et al., 1995). At this developmental stage, two populations of microtubules are present in the yolk cell: a network of short, intercrossed microtubule arrays associated with yolk syncytial nuclei, and feather-like arrays of microtubules along the animal/vegetal axis in the yolk cytoplasmic layer (Solnica-Krezel and Driever, 1994). These microtubule networks are essential to epiboly progression as disruption by stabilizing or destabilizing microtubules result in epiboly defects (Strahle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994). Actin cytoskeleton dynamics also play an important role in epiboly progression. Soon after the onset of epiboly, around the shield stage, a wide actomyosin band forms around the EVL and decreases in width with progression of epiboly until it forms a ring around 70% epiboly (Köppen et al., 2006; Cheng et al., 2004). Disruption of the actomyosin band locally through UV laser cutting leads to epiboly delay around the disruption site and over all disruption of actin in the yolk cytoplasmic layer results in overall epiboly delay (Köppen et al., 2006; Lin et al., 2009; Cheng et al., 2004 Behrndt et al., 2012).

**BMP signaling pathway**
In developing vertebrate embryos, BMPs functions as a morphogen in a gradient dependent manner from ventral to dorsal to specify ventral cell fates and pattern C&E movement domains (Whitman, 1998; Hogan, 1996; De Robertis and Kurodo, 2004; Myers et al., 2002). BMPs are part of the TGFβ superfamily and signal through a complex of type I and type II TGFβ receptors to regulate activity of downstream Smad transcription factors. BMP signaling is required for ventral ectoderm and mesoderm cell fates and loss of BMP signaling results in increased formation of dorsal at the expense of ventral cell fates (Hawley et al., 1995; Sasai et al., 1995; Schmidt et al., 1995). In zebrafish, reduction of Bmp signaling results in dorsalized embryos that lack ventral and posterior features such as tail structures in part due to transformation of ventral cell fates into more dorsal ones. In addition, in dorsalized gastrulae, ectopic and excessive C&E cell movements occur on the ventral gastrula region called the “no convergence no extension zone” where cells only migrate vegetally to form tail structure and fail to do so (Hammerschmidt et al., 1996; Hammerschmidt and Mullins, 2002; Solnica-Krezel et al., 1996; Myers et al., 2002). The Bpm gradient has also been found to play a role in establishing a reverse gradient of cell-cell adhesion that mediates dorsal migration of lateral mesodermal cells in a cadherin dependent and PCP pathway independent manner (von der Hardt et al., 2007).

**Wnt/PCP pathway**

Among the non-canonical Wnt pathways, the best studied is the Wnt/PCP pathway, which coordinates cell polarity across a tissue plane. This pathway does not directly regulate β-catenin, instead it signals via the activation of small GTPases, which regulate the cytoskeleton and planar polarity (Park et al., 1996; Collier and Gubb 1997; Strutt and Warrington 2008; Strutt et al., 1997). The effect of PCP signaling can be easily visualized in cells that form hairs in mammalian skin or hairs and bristles in *Drosophila* epithelia. In WT tissues the hairs all point in
the same direction, but when PCP signaling is disrupted the orientation of hairs becomes less regular, random, or forms a characteristic swirling pattern (Wong and Adler, 1993; Strutt and Strutt, 1999; Zheng et al., 1995; Wang et al., 2006; Guo et al., 2004). The PCP pathway regulates a subset of cell behaviors to converge tissues dorsally and extend them anteroposteriorly during C&E (Sepich et al., 2005; Warga and Kimmel, 1990; Yin et al., 2008; Sepich et al., 2000; Myers et al., 2002). C&E cell movement behaviors that are regulated by the PCP pathway include the anterior migration of prechordal mesoderm cells towards the animal pole; mediolateral cell intercalation in the chordamesoderm domain; the combination of polarized radial and planar mediolateral intercalations in the paraxial domain; dorsally directed fast migration of the lateral mesodermal cells; and oriented cell division of the dorso-lateral ectodermal cells (Rohde and Heisenberg, 2007; Yin et al., 2008; Glickman and Kimmel, 2003; Myers et al., 2002; Concha and Adams, 1998; Gong et al., 2004). Mutants in the core PCP pathway components can have defects in several of these cell behaviors; tri and kny mutants both have defects in mediolateral intercalation and fast dorsal migration (Jessen et al., 2002; Topczewski et al., 2001). Perturbation of Wnt ligands also interfere with C&E movements: loss of Wnt11 results in slower and less directed migration of prechordal plate cells with abnormally orientated protrusions; and loss of Wnt5 leads to defects in C&E in posterior ectoderm and mesoderm (Ulrich et al., 2003; Heisenberg et al., 2000; Kilian et al., 2003).

Trilobite and Knypek PCP mutants
Trilobite/Vangl2 (Tri/Vangl2) is the homolog of Drosophila Van Gogh/Strabismus (Park and Moon, 2002; Jessen et al., 2002). As in Drosophila, Tri/Vangl2 in zebrafish is a core PCP component. Tri protein function is required for proper C&E movements (Hammerschmidt et al., 1996; Heisenberg and Nusslein-Volhard, 1997; Marlow et al., 1998; Solnica-Krezel et al., 1996).
In *tri* mutants, mediolateral cell elongation is reduced, as this process is critical for polarized intercalation cell behaviors and fast dorsal migration during C&E, this accounts for the short body mutant phenotype (Jessen et al., 2002; Yin et al., 2008). They also found that at midgastrulation, mesodermal *tri* cells undergoing slow dorsal migration behave like WT cells, however by late gastrulation, cells in *tri* mutants fail to elongate mediolaterally and move much slower towards the dorsal side of the embryo with less direct trajectories. This indicates that *tri* dependent cell polarization is required for these movements. The authors also found that *tri* functions cell-autonomously and non-autonomously to confer cell shape and orientation on neighboring cells, suggesting that Tri mediates cellular interactions underlying PCP signaling, another hallmark of the PCP pathway.

In 2001, Topczewski et al. showed that Knypek (Glypican 4) is the homolog of *Drosophila* Dally-like protein, which encodes for a heparan sulfate proteoglycan, of the glypican class. This cell surface GPI-anchored protein is part of the vertebrate but not *Drosophila* PCP pathway and is necessary for controlling cell polarity in C&E processes during gastrulation. The authors also found that Knypek protein localized to the membrane and based on epistatic experiments they proposed that Knypek promoted non-canonical Wnt11 signaling during C&E. Such a of Glypican4 in promoting Wnt/PCP signaling and C&E in part by binding to Wnt11 ligand has been demonstrated in *Xenopus* (Ohkawara et al., 2003).

3.1.4 Dachsous And Fat Atypical Cadherins

*Dachsous and Fat in adhesion*

Unlike most cadherins, neither Ds nor Ft atypical cadherins mediate homophilic cell-cell adhesion; rather Ds and Ft together appear to engage in cell-cell adhesion via heterophilic interactions (Takeichi, 1995; Matakatsu and Blair, 2004; Ishiuchi et al., 2009). For example,
Matakatsu and Blair showed that *Drosophila* S2 cells expressing either *ds* or *ft* do not form homophilic cell aggregates. However, when *ds* and *ft* were co-transfected, or when cells expressing *ds* or *ft* were mixed, cell aggregates formed efficiently. These aggregates were inhibited by EGTA, suggesting the ability of Ds and Ft to interact depends on calcium like in the case of classical cadherins. Ds and Ft also stabilize each other on the cell membrane both *in vitro* and *in vivo* (Takeichi, 1995; Matakatsu and Blair, 2004). In cells transfected with either Ds or Ft, antibody staining showed that most of the Ds and Ft protein localized to “internal vesicle-like structures” with very low protein levels detected at cell membranes. In contrast, in mixed cultures of cells transfected with *ds* or *ft*, Ds and Ft localization at the cell-cell interfaces was much stronger. In third instar wing discs, overexpression of *ds* heightened anti-Ft antibody staining and conversely, overexpression of *ft* heightened anti-Ds antibody staining. These results indicate that heterophilic interactions between Ds and Ft stabilize the proteins at the cell membrane, supporting the proposed ligand-receptor relationship between Ds and Ft by Ma et al., 2003.

Work in vertebrate systems supports the model that Dachsous and Fat mediate heterophilic interactions in an evolutionarily conserved manner. For example, Fat4 and Dachsous1 co-localize to the apical portion of neural progenitor cells in the cerebral cortices (Ishiuchi et al., 2009). Antibody staining of cerebral cortices from an E14.5 mouse embryo showed both Fat4 and Dachsous1 were distributed throughout the cortex but were more concentrated together at the apical cell surfaces. Paralleling results from *Drosophila* S2 cells described above, L cells, which are cadherin null fibroblasts, transfected with either Fat4 or Dachsous1 alone did not form cell aggregates, and each protein localized diffusely throughout the cells. However, once Fat4-expressing and Dachsous1-expressing cell populations were
mixed, cell aggregates formed and both proteins became concentrated at the “heterotypic cell boundaries”. siRNA mediated knockdown of Fat4 in the embryonic cortices causes a reduction in Dachsous1 expression, but also a modest upregulation of Fat4 expression. These data suggest that in mammals, Fat 4 stabilizes Dachsous1 at the cell membrane; Dachsous1 negatively regulates Fat4; and that Dachsous 1 and Fat 4 form heterophilic dimers to mediate cell-cell adhesion, as proposed for *Drosophila*.

Dachsous and Fat regulation of PCP

The Wnt/PCP pathway is a key regulator of C&E, but the factors that activate this signaling pathway to regulate polarity across a tissue have not been identified. Studies have linked the Ft and Ds signaling pathway to the regulation of planar polarity in *Drosophila*. Two context dependent models have been developed over the past fifteen years. One model places Ds and Ft signaling upstream of the PCP pathway in a gradient dependent manner that is dependent on the golgi-localized kinase, Four-jointed (Fj) to coordinate polarity. Another model suggests that Ds and Ft act in an independent pathway and confer polarity in parallel to the PCP pathway (Matis et al., 2014; Hale et al., 2015; Yang et al., 2002; Rawls et al., 2002; Casal et al., 2002; Casal et al., 2006; Donoughe and Dinardo, 2011; Simon et al., 2010).

Several lines of genetic evidence support that Ds acts upstream of the PCP pathway to control planar cell polarity. In particular, *ds* mutations result in tissue polarity defects similar to those of *fz* mutants, but with unique patterns in the wing hairs such as regions of reversed polarity (Adler et al., 1998). In genetic interaction studies between *ds* and other core PCP pathways genes, PCP signaling occurred in *ds*- mutants but in an abnormal way: the intracellular transduction of the Fz signal was not affected but the polarity of the cell was (Adler et al., 1998; Krasnow and Adler, 1994). Furthermore, large *ds*- mutant clones often altered the polarity of
surrounding WT cells, suggesting ds domineering non-autonomy as seen in vang and fz mutant clones (Taylor et al., 1998; Wolff et al., 1999; Adler et al., 1998; Vinson and Adler, 1987). These data imply that ds changes the interactions between the core PCP pathway proteins and alters the direction of PCP signaling, which results in altered tissue polarity, placing Ds upstream of the core PCP pathway. Additional studies in the Drosophila eye, abdomen, and wings support a model where Fj phosphorylates both Ds and Fat; but while phosphorylation of Ds by Fj inhibits Ft binding, phosphorylation of Ft by Fj promotes Ds binding, which sets up opposing gradients to regulate polarity across tissues. However, how Ds and Ft gradients affect polarity is different in different tissues (Yang et al., 2002; Rawls et al., 2002; Casal et al., 2002; Strutt and Strutt, 2002; Hale et al., 2015). Although these are strong arguments supporting the model that Ds/Ft system signals to regulate polarity through the PCP pathway, no physical interactions between Ds or Ft and any core PCP proteins have been identified, and there is no direct data showing levels of Ds and Ft affect distribution of the core PCP proteins at a subcellular level. However, Ft/Ds/Fj gradients do regulate polarization of apical acentrosomal microtubules prior to polarization of the core PCP pathway components. As Dsh containing vesicles are trafficked along these microtubules, microtubules provide a possible mechanistic link between Ft/Ds/Fj and the core PCP pathway (Matis et al., 2014; Harumoto et al., 2010).

The second model places Ds and Ft in a pathway acting in parallel to the core PCP pathway as the Ds/Ft system can still confer and propagate planar cell polarity when core PCP components Fm and Fz are absent (Casal et al., 2006). Furthermore, when Ds is absent, cells in the Drosophila abdomen seem to be more responsive to the core PCP signaling (Casal et al., 2006). Overexpression of Ds or Ft can repolarize bristles in a fmi and fz null background, indicating Ds and Ft can affect planar cell polarity independent of core PCP signaling (Casal et
al., 2006). They speculate that in specific tissues such as the eye the requirement for core PCP signaling may be so strong, it becomes impossible for the Ds system to reorganize planar polarity in their absence. More recently, evidence from studies of denticle patterning in the Drosophila larval ventral epidermis further supports the model that the core PCP pathway and the Ds/Ft pathway act in parallel to each other (Donoughe and DiNardo, 2011). In loss of Ds function background, the core PCP signaling still functions, and overexpression of the Ds extracellular domain in a fz null background can reorient adjacent cells. These observations argue that the core PCP pathway does not absolutely depend on signaling from the Ds/Ft pathway and Ds can signal in the absence of PCP signaling, which confirms the findings of Casal et al., 2002. Interestingly, the authors also found that in ds maternal zygotic (MZ) mutants as well as fz maternal zygotic mutants F-actin protrusion placement defects were present in the wing epithelium cells suggesting similar downstream effectors that regulate the cytoskeleton for both systems. Taken together, these observations support the notion that different tissues have different requirements for conferring planar cell polarity and Ds and Ft functions in a separate pathway from core PCP components at least in some tissues.

While clear evidence exists that the atypical cadherins Ds and Fat influence tissue polarity in Drosophila, relatively little is known about the functions of Dchs and Fat homologs in vertebrates. The first insights into the vertebrate Dchs1, Fat1, and Fat4 proteins come from studies in the mouse (Ciani et al., 2003; Saburi et al., 2008; Mao et al., 2011), where analysis of Dchs and Fat mutant mice revealed a plethora of developmental defects. Phenotypes characteristic of PCP signaling defect, however, were not seen. Mouse mutants for core PCP genes have phenotypes such as craniorachischisis, defects in orientation of coat hair, stereocilia polarity disruption in the inner ear, and abnormal cell polarization during gastrulation (Kibar et
Fat1 mutant mice are perinatally lethal with partially penetrant holoprosencephaly and anophthalmia phenotypes (Ciani et al., 2003). In 2011, Mao et al. found that unlike in Drosophila, where Ds and Ft are expressed in epithelia, in mice Dchs1 and Fat4 are also expressed in mesenchymal tissues. Some possibly PCP related phenotypes exhibited by these mutant mice include shorter cochleae in Dchs1, Fat4, and Dchs1;Fat4, but without differences in stereocilia orientation and smaller cystic kidneys with less branching. These modest phenotypes may be due to the existence of multiple copies of Dchs and Fat with possible overlapping functions. The authors also show that Dchs1 mutants have stronger staining in the kidney for Fat4 and vice versa, consistent with the ligand/receptor relationship between Dchs and Fat shown in previous Drosophila and mammalian studies. These data establish function for Dchs and Fat during vertebrate development; however, characterizations of these mutant mice have been concentrated on postnatal development, giving little information on the effects of these mutations during embryogenesis. Studies in early development may provide to us the elusive link between Dchs, Fat, polarity, and the PCP pathway in vertebrates.

Here, we show that Dchs atypical cadherins regulate epiboly and convergence and extension during zebrafish gastrulation. Dchs1b functions in adhesion of deep cells and the EVL during epiboly possibly through regulation of actin and microtubule cytoskeletons. While both MZdchs1b and MZdchs2 mutants show convergence and extension defects. Studies using MZdchs2 mutants show that Dchs2 regulates both cell orientation and cell shape in several C&E movement domains. Furthermore genetic studies using MZdchs1b, kny, and tri mutants show that dchs1b interacts with the PCP pathway components in zebrafish.
3.2 Results

3.2.1 Mzdcshs1b And Mzdcshs2 Mutants Display Delayed Epiboly Progression

In Chapter 2, we have previously described that zygotic mutants for dcsh1b and dcsh2 undergo development to form morphologically normal adults with defects seen only in maternal zygotic (MZ) mutants. Both MZdcshs1b and MZdcshs2 mutants showed delayed epiboly progression compared to WT (Fig. 3.1A). MZdcshs1b mutants were more severely affected and with higher variability in penetrance and expressivity (Fig 3.1A and C). During epiboly progression, MZdcshs1b mutants could be sorted into three categories based on the severity of epiboly delay: mildly affected, moderately affected, and severely affected (Fig. 3.1B). The severity of epiboly delay significantly affected embryonic mortality in MZdcshs1b mutants, as less than 2% of severely affected embryos survived until 24 hpf while more than 95% of mildly affected embryos survived to 24 hpf (Fig. 3.1B and C). MZdcshs1b mutants injected at one cell stage with synthetic RNA encoding either full-length Dchsh1b-sfGFP or Dchsh1b intracellular domain (ICD) showed a mild rescue of epiboly progression although this result was not significant (Fig. 3.1D).

3.2.2 Dchsh1b Regulates Cell Adhesion During Gastrulation

During epiboly, once the blastoderm covers 50% of the embryo, the EVL migrates past the deep cells and form contacts with the YSL. For the remainder of the epiboly progression, the deep cell margin lags slightly behind the EVL and YSL margins (Solnica-Krezel and Driever, 1994; Warga and Kimmel, 1990; Koppen et al., 2006). In addition to delayed epiboly, MZdcshs1b mutants showed a larger gap between the deep cell layer and the EVL compared to WT gastrulae (Fig. 3.2A and 2B). This enlarged gap between the deep cells and EVL could be rescued by injection at one cell stage of RNA encoding full-length Dchsh1b-sfGFP at all doses tested but not by RNA encoding Dchsh1b ICD at any dose into MZdcshs1b embryos (Fig. 3.2B). Based on these
Figure 3.1: Epiboly Progression Is Delayed In MZdchs1b And MZdchs2 Mutants

A. Time-matched WT, MZdchs1b, MZdchs2, and MZdchs1b;MZdchs2 embryos at 7 hpf.

B. Representatives of mildly affected, moderately affected, and severely affected epiboly progression in MZdchs1b embryos.

C. Quantification of MZdchs1b mutant sorted based on severity of epiboly progression phenotype survival to 24 hpf.

D. Rescue in epiboly progression of MZdchs1b mutant deep cell layer and enveloping layer by full-length dchs1b-sfGFP and dchs1b ICD
Figure 3.2: EVL and Deep Cell Gap Is Dchs1b Dependent
A. Stage-matched WT and MZdchs1b embryos at 75% epiboly. Black arrow heads point to deep cell layer and EVL.
B. Rescue in gap size between deep cell layer and EVL in MZdchs1b mutant by full-length dchs1b-sfGFP and dchs1b ICD
data taken together with known functions of Dchs, we hypothesize that Dchs1b plays a role in regulating cell adhesion during zebrafish epiboly.

We tested whether MZdchs1b mutants and WT cells exhibit differential adhesion by employing an *ex vivo* cell-cell adhesion assay (Speirs et al., 2010; Ulrich et al., 2005). We coinjected both WT and mutant embryos at one cell stage with RNA encoding membrane RFP or GFP to label all cells and cyc RNA encoding a Nodal ligand to induce mesendodermal fates in all cells of the embryo and eliminate differential adhesion between germ layers (Krieg et al., 2008). The injected embryos were physically dissociated into single cells at 4.5 hpf, their mixtures were seeded onto fibronectin coated wells, and formation of cell aggregates was assessed after six hours of incubation. We hypothesized that loss of *dchs1b* would result in cells with lower adhesion and sort to the outside of cell aggregates while more adhesive WT cells would sort to interior positions in cell aggregates. Surprisingly, aggregates from the mixtures of RFP labeled MZdchs1b and GFP labeled WT cells (or vice versa) did not show differential sorting by color compared to WT mixtures (Fig. 3.3A).

We next asked whether E-cadherin expression is normal in MZdchs1b mutants compared to WT as E-cadherin plays a crucial role in cell adhesion during zebrafish gastrulation. We used quantitative RT-PCR to compare *cdh1* transcript levels in WT and MZdchs1b embryos at 8-cell, 1K, 30% epiboly, shield, and 70% epiboly stages. *cdh1* transcript levels were comparable in WT and mutant embryos at early stages (Fig. 3.3B). Unexpectedly, *cdh1* levels were significantly increased at 70% epiboly in mutants compared to WT (Fig. 3.3B). Accordingly, using western blotting, we found that Cdh1 protein levels were increased at 70% epiboly but not at 30% epiboly in MZdchs1b mutants (Fig. 3.3C). Furthermore, Cdh1 protein exhibited a slightly increased molecular weight in MZdchs1b mutants compared to WT.
Figure 3.3: Dchs1b and Dchs2 Regulate Cell-Cell Adhesion

A. *Ex vivo* cell aggregation assay 6 hours post seeding.
B. *cdh1* levels in WT and MZdchs1b mutants at 8 cell, 1K, 30% epiboly, shield, and 70% epiboly stages.
C. Western blot with Cdh1 antibody against 1, 2 or 3 WT and MZdchs1b embryos.
D. Example of *in vivo* cell scatter area trace.
E. Quantification of cell scatter trace area for WT and MZdchs2 mutants.
To test *in vivo* whether loss of Dchs leads to decreased adhesion, we used a cell tracing experiment to track scattering of cells in the blastoderm (Warga and Kane, 2003; Lin et al., 2009). We employed MZdchs2 mutants for this experiment as the yolk inclusion noted (Chapter 2) in MZdchs1b mutants could act as physical barriers for cell scattering. We injected H2B-RFP synthetic RNA into one cell at the animal pole of 128-cell stage MZdchs2 and WT embryos. The injected embryos were incubated until 4.5 hpf and imaged to analyze cell scattering, by tracing the total area covered by the resulting cellular clone (Fig. 3.3D). We hypothesized that the cell scattering area trace would be greater in MZdchs2 mutants compared to WT indicative of lower adhesion between blastomeres. However, we found that the scatter area trace in MZdchs2 mutants were smaller in mutants compared to WT (Fig. 3.3E). These results suggest increased adhesion between blastomeres in MZdchs2 mutants.

3.2.3 Both MZdchs1b And MZdchs2 Show Cytoskeletal Defects In The Yolk Cytoplasmic Layer

The cytoskeleton in the yolk cytoplasmic layer of the zebrafish embryo plays a large role in gastrulation and perturbing either actin or microtubule cytoskeleton affect all gastrulation movements (Solnica-Krezel and Driever, 1994; Cheng et al., 2004). During epiboly stages, there are two populations of microtubules, a mesh of microtubules in the YSL and long arrays radiating into the yolk cytoplasmic layer oriented along the animal vegetal axis (Solnica-Krezel and Driever, 1994). We’ve previously used the Tg[XIEef1a1:dclk2-GFP] transgenic line, which highlights microtubules, to show that the radiating arrays of microtubules in the yolk cytoplasmic layer were bundled in MZdchs1b mutants compared to WT (Chapter 2). Here, we found that the radiating microtubule arrays in the yolk cytoplasmic layer were also bundled in MZdchs2 mutants using Tg[XIEef1a1:dclk2-GFP] similar to MZdchs1b mutants although to a lesser degree of severity (Fig. 3.4A).
Figure 3.4: Dchs1b and Dchs2 Regulate Yolk Cytoplasmic Layer Cytoskeleton
A. WT and MZdchs2 mutant embryos at early gastrula stage. Microtubules labeled by Tg[XIeef1a:dclk2-GFP].
B. WT and MZdchs1b mutant embryos at early gastrula stage. Actin labeled by Tg[β-actin:utrophin-GFP].
Next, we wanted to assess whether Dchs cadherins affect actin cytoskeleton during gastrulation. Three populations of actin have been described: an actin band in the YSL during early epiboly that transforms into a F-actin ring at the blastoderm margin during late epiboly, and an actin cap in the vegetal portion of the yolk cell (Behrndt et al., 2012; Cheng et al., 2004). Using the Tg[$\beta$-actin:utrophin-GFP] transgene, we observed all three actin populations in MZdchs1b mutant gastrulae. However, the vegetal cap actin became increasingly bundled as epiboly progressed and the F-actin ring at the margin as well as the margin itself were uneven compared to WT (Fig. 3.4B). We have provided evidence previously that Dchs1b regulates the actin cytoskeleton during egg activation. Based on these data we propose Dchs1b continues to regulate actin dynamics during epiboly.

### 3.2.4 Stiffer Yolk Cell In MZdchs1b Mutants

As both actin and microtubule networks in the yolk cell are bundled in MZdchs1b mutants, we hypothesized that the mutant yolk cell is under more tension compared to WT yolk cell. To test this hypothesis, we performed yolk deformation experiments. We drew parallels from studies using a cylindrical indenter on fluid filled rubber shells of different thicknesses and enucleated pig eyeballs to test rigidity through deformation (Taber, 1983; Taber, 1984). We used sphere stage MZdchs1b and WT embryos, the stage that has fully manifest yolk and cytoskeleton bundling in the mutants. We placed individual embryos in a corner formed in high percentage agarose gel with the yolk facing out towards the glass indenter beam (Fig. 3.5A). The indenter beam was mounted on a motorized stage that moved with a known velocity, which in addition to using a glass indenter with a known stiffness allowed us to calculate the force exerted on the yolk cell to cause a certain deflection. We found that it took more force from the glass beam to
Figure 3.5: MZdchs1b Mutant Yolk Is Under More Tension Than WT Yolk

A. Example of sphere stage embryo mounted in the corner of agarose mode with cylindrical beam mounted on motor.

B. Quantification of tissue deflection resulting from various forces. Averages are graphed. WT n=9; MZdchs1 = 12.
cause the same amount of tissue deflection in MZdchs1b mutant yolks as in WT, which supports our hypothesis that the mutant yolk cell is under more tension than WT yolk cell (Fig. 3.5B).

3.2.5 MZdchs1b And MZdchs2 Mutants Show Cell Polarity Defects During C&E

Ds in Drosophila has been shown to regulate planar cell polarity both upstream and in parallel to the core PCP pathway (Matis et al., 2014; Hale et al., 2015; Yang et al., 2002; Rawls et al., 2002; Casal et al., 2002; Casal et al., 2006; Donoughe and Dinardo, 2011; Simon et al., 2010). There is little direct evidence that Dchs regulates cell polarity during vertebrate development and the relationship between Dchs and the PCP pathway in vertebrates remains elusive. We have shown in Chapter 2 that both MZdchs1b and MZdchs2 mutants show C&E defects during gastrulation: shorter and broader axial domain at 70% epiboly; broader neural plate at 2-somites; and prechordal plate migration defects at 2-somites. We next asked whether Dchs affects cell polarity during zebrafish gastrulation. We analyzed cell polarity in MZdchs2 mutant gastrulae so to avoid potential secondary defects in MZdchs1b mutants resulting from primary defects in early development we documented in Chapter 2.

We analyzed cell elongation and mediolateral orientation as cell polarity metrics and found that cells in MZdchs2 mutants exhibited less polarization in several tissues and domains during gastrulation (Fig. 3.6). First, we analyzed lateral mesoderm cells located 90° from dorsal at 90% epiboly and 90 min later, as by these stages, clear differences in cell polarity could be seen in PCP mutants (Jessen et al., 2002; Topczewski et al., 2001). We found that in MZdchs2 mutants, cells were less elongated and less mediolaterally aligned than equivalent WT cells (Fig. 3.6A and B). Additionally, we found that there were more cells across the axial mesoderm (mediolateral dimension) in mutants at both stages analyzed (Fig. 3.6C and D), revealing convergence defect. We then looked earlier during gastrulation at 80% epiboly in different
Figure 3.6: Dchs2 Regulates Cell Polarity In Mesoderm And Ectoderm During Gastrulation

A. Mediolateral alignment of mesodermal cells located 90° from dorsal at 90% epiboly and 90 minutes later in WT and MZdchs2 mutants.
B. Quantification cell elongation for mesodermal cells located 90° from dorsal at 90% epiboly and 90 minutes later in WT and MZdchs2 mutants.
C. Axial mesoder at 90% epiboly and 90 minutes later in WT and MZdchs2 mutants. Ten random lines were drawn and cells touched by lines were counted.
D. Quantification of number of cells across the axial mesoderm in WT (n=6) and MZdchs2 mutants (n=8).
E. Mediolateral alignment of mesodermal and ectodermal cells in different domains at 80% epiboly in WT and MZdchs2 mutants.
F. Cell elongation of mesodermal and ectodermal cells in different domains at 80% epiboly in WT and MZdchs2 mutants.
domains of mesoderm and ectoderm. We found that at this stage of gastrulation Dchs2 regulates cell polarity in axial mesoderm, lateral, lateral mesoderm, and neural ectoderm but not in paraxial mesoderm or lateral ectoderm (Fig. 3.6E and F).

As Dchs regulates cell polarity in cell populations where the PCP pathway functions, we then wanted to ask whether Dchs interacts with the PCP pathway by using genetic studies between Dchs and PCP pathway components (Jessen et al., 2002; Topczewski et al., 2001; Marlow et al., 1998). We used cyclopia and synophthalmia as readouts for genetic interaction between dchs1b and tri, as tri mutants have been shown to display degrees of synophthalmia and cyclopia (Marlow et al., 1998). We hypothesized that if dchs1b genetically interacts with tri, compound mutants would exhibit increased incidences of synophthalmia and cyclopia. We found that indeed, in a tri mutant background, loss of maternal and zygotic function of dchs1b increased penetrance and expressivity of the synophthalmia and cyclopia phenotype; with the highest incidence in MZdchs1b;Ztri^vu67 compound mutants (Fig. 3.7A and B). Additionally, we found that loss of dchs1b in kny mutant background led to enhancement of the short embryonic body axis phenotype (Fig. 3.7C and D). These data suggest Dchs interaction with the PCP pathway.
Figure 3.7: Dchs1b Genetically Interacts With Components Of The PCP Pathway

A. *kny* single and *kny;dchs1b* compound mutant short body axis phenotypes.
B. Quantification short body axis in correlation to *dch1b* genotype.
C. Modified from Marlow et al., 1998. Degrees of synalphtemia and cyclopia.
D. Penetrance and expressivity of synalphtemia and cyclopia phenotype correlated to *dchs1b* genotype.
3.3 Discussion

We have identified essential and overlapping functions for Dchs1b and Dchs2 in regulating epiboly and C&E cell movements during zebrafish gastrulation. We propose that Dchs1b and Dchs2 regulate epiboly through regulation of cell adhesion and of actin and microtubule cytoskeletal networks in a PCP pathway independent manner, as PCP pathway mutants, tri/vangl2 and kny/gpc4, do not show defects in epiboly movements during gastrulation (Solnica-Krezel et al., 1996; Hammerschmidt et al., 1996; Ciruna et al., 2006; Jessen et al., 2002; Topczewski et al., 2001). Because our analyses of transient C&E defects in MZdchs2 mutants revealed impairment of mediolateral cell elongation, polarized dorsal cell migration, and mediolateral cell intercalation, similar to those observed fish and frog embryos deficient in PCP signaling (Wallingford et al., 2000; Heisenberg et al., 2000; Jessen et al., 2002; Topczewski et al., 2001; Yin et al., 2008), we posit that Dchs cadherins regulate C&E at least in part by regulating PCP signaling.

Here, as in Chapter 2 we used strong loss of function/null mutations in Dchs1b and Dchs2 to determine Dchs function during zebrafish development. Although MZdchs1b mutants exhibit stronger phenotypes, we employ MZdchs2 mutants for some cell behavior studies to avoid confounding effects of the early phenotypes observed in MZdchs1b mutants. In order to assess the full function of Dchs cadherins, we will focus future analyses on MZdchs2:Zdchsb mutants as well as dchs1a single and triple mutants in combination with dchs1b and dchs2.

We have observed an enlarged gap between EVL and deep cells during epiboly in MZdchs1b embryos compared to WT (Fig. 3.2A and B). Interestingly, loss of Dchs1b in MZdchs1b mutants leads to up regulation of E-cadherin both at the transcript and protein levels (Fig. 3.3C and 3D). How a membrane protein such as Dchs1 can regulate transcription of another
gene poses an intriguing question. Although there have been precedence for Ds intracellular domain regulation of cell polarity and the Hippo pathway (Matakatsu and Blair, 2012; Sharma and McNeill, 2013). Moreover, in Drosophila, the cytoplasmic domain of Fat atypical cadherin has been shown to bind mitochondrial proteins (Sing et al., 2014). This interaction is made possible through post-translational processing to generate three fragments: a large extracellular fragment, a small transmembrane fragment, and an intracellular cytoplasmic fragment that binds to the mitochondrial protein (Sing et al., 2014). It is conceivable that the largely unknown intracellular domain of Dchs1b can also undergo post-translational modification and have unexpected functions such as regulating transcription of cdh1. It will be also important to determine whether Dchs cadherins regulate epiboly at least in part by their heterophilic interactions with Fat, which in Drosophila have been shown to be regulated by Fj kinase (Matis et al., 2014; Hale et al., 2015; Yang et al., 2002; Rawls et al., 2002; Casal et al., 2002; Casal et al., 2006; Donoughe and Dinardo, 2011; Simon et al., 2010). Our laboratory generated mutations in several of the six Fat homologues and in the only Fj homolog, Fjx1. It will be interesting to assess if any of the single or compound Fat mutants affect epiboly, and whether mutations in these genes exacerbate epiboly defects of embryos lacking Dchs cadherin function.

In Chapter 2, we have shown that only Dchs1b regulates actin and microtubule cytoskeletons during early developmental processes such as egg activation (actin), and dorsal determinant transport (microtubules). We observed that during gastrulation, both MZdchs1b and MZdchs2 mutants show bundling of actin and microtubule cytoskeleton in the yolk cytoplasm suggesting overlapping of function between dchs1b and dchs2. This is congruent with high expression of dchs1b during maternal stages and expression of both during gastrulation stages (Chapter 2 Fig. 1). It has been shown that stabilizing/bundling of the microtubule cytoskeleton in
drug treated embryos led to epiboly delays in WT (Solnica-Krezel and Driever, 1994). Although both MZdchs1b and MZdchs2 mutants show bundled microtubules, the phenotype appears to be less severe in MZdchs2 mutant consistent with less delayed epiboly movements. Our initial attempt to rescue these epiboly defects by injecting RNAs encoding either full-length Dchs1b-sfGFP or Dchs1b ICD into MZdchs1b mutants led to a mild although not significant amelioration of epiboly defects in MZdchs1b mutants (Fig. 3.1D). This could be due to staging, as epiboly delays are more exaggerated during 70-80% epiboly and our rescue embryos were analyzed at 60% epiboly. It is also conceivable, that epiboly defect of MZdchs1b mutants are in part secondary to the earlier defects in egg activation and their rescue will require expression of Dchs1b protein in the early zygote or even during oogenesis. Therefore, it would be important to carry out such rescue experiments in MZdchs2 mutants, which do not show strong egg activation phenotypes. It would also be interesting to perform rescue experiments in the yolk cell only by injecting full-length dchs1b-sfGFP and dchs1b ICD into the yolk cell of MZdchs1b (or dchs2-sfGFP RNA into yolk cell of MZdchs2 mutants) when the blastoderm and yolk cell are complete separate rather than injecting synthetic RNA into the whole embryo at one cell stage. Additionally, if expression of Dchs2-GFP can rescue epiboly and microtubule bundling phenotype in MZdchs2 mutants, expression of Dchs1b-GFP in MZdchs2 mutant embryos would reveal whether Dchs1b and Dchs2 have truly overlapping functions in regulation of epiboly and yolk cytoplasmic microtubules.

In addition to bundling microtubules, MZdchs1b mutants also show abnormal actin bundling in the yolk cell (Fig. 3.4B). In cell culture, stress fibers, composed of bundled F-actin filaments and other proteins such as myosins, can cause contraction and tension that can be measured using tools such as atomic force microscopy (Kassianidou and Kumar, 2015; Burridge
and Wittchen, 2013; Falkin et al., 2012). As bundled actin cytoskeleton in MZdchs1b mutant yolk cell can be analogous to stress fibers in cells, we performed a macro scaled experiment similar to atomic force microscopy to probe the tension/stiffness of yolk cells and found that mutant yolk cells are stiffer than WT yolk cells. Cells can sense forces in their environment including the stiffness of their substrate and propagate signals from mechanical force to affect cellular behaviors such as locomotion/migration, morphology, and adhesion (Geiger and Bershadsky, 2002; Georges and Janmey, 2004). It is interesting to consider how changes in the stiffness of the yolk cell in MZdchs1b mutants can affect cell behaviors of the overlaying blastoderm.

Contrasting to our hypothesis that loss of function of a cadherin family gene would lead to decreased cell-cell adhesion in mutant embryos, we found that there is no difference in adhesion between MZdchs1b cells and WT cell in cell aggregation assay. Furthermore, from cell scattering assay, we found that MZdchs2 blastomeres appeared to be more adhesive to each other than WT blastomeres. One possibility is up regulation of E-cadherin in MZdchs1b mutants and perhaps also in MZdchs2 mutants compensate for reduced adhesion caused by loss of Dchs1b and Dchs2 function. On the other hand, we have observed dramatic bundling of actin and microtubule cytoskeleton in the yolk cell; it stands to reason to hypothesize similar abnormalities in mutant blastomeres as well, which could change cell-cell interactions leading to increased adhesiveness possibly through reduced cortical tension.

Dachsous atypical cadherin has been shown in Drosophila to regulate planar cell polarity both upstream and in parallel to the core PCP pathway in a tissue context dependent manner. We show here that Dchs2 regulates cell polarity in several mesodermal and ectodermal regions during gastrulation where PCP signaling also functions (Fig. 3.6). In addition to cell polarity, it
Figure 3.8: Cell Divisions During Blastula Stage Are Misoriented In MZdchs1b Mutants
Cartoon shows orientation of embryos at 8 cell state. Rose diagrams depict direction of cell divisions using karyokinesis in WT and MZdchs1b mutants.
would be interesting to analyze cell divisions during gastrulation in MZdchs1b and MZdchs2 to test whether Dchs regulates polarized cell division, as early cleavages during blastula stage are misoriented in MZdchs1b mutants (Fig. 3.8). More detailed analyses are needed to determine whether Dchs regulates cell polarity in a region independent of the PCP pathway (Jessen et al., 2002), where these movements are regulated by small G proteins (Lin et al., 2009) and BMP (von der Hardt et al., 2008). Additionally, we have found that dchs1b genetically interacts with tri and kny, as compound mutants produce more severe PCP mutant phenotypes, consistent with these genes actin in the same or parallel pathways to mediate C&E. Although we have shown genetic interaction between Dchslb and components of the vertebrate PCP pathway, the nature of this interaction is to be determined. Whether Dchs acts upstream and/or in parallel to the core PCP pathway to regulate cell polarity; how are Dchs cadherins distributed in cells, asymmetrically, in a gradient, ect.; if and how Dchs regulate asymmetrical distribution of core PCP proteins such as Vangl2 and Pk; in which tissues and processes and what PCP independent functions do dchs genes have in vertebrates are all to be determined. Better understand of Dchs functions in zebrafish can contribute to better understanding of underlying mechanisms that contribute to phenotypes seen in Van Maldergem patients.
3.4 Materials And Methods

Zebrafish lines

AB, Tg[XlEef1a1:dclk2-GFP] and Tg[β-actin:utrophin-GFP] (Campinho et al., 2013; Tran et al., 2012) lines were used. TILLING generated mutant lines dchs1bfh275 and dchs2stl1 mutations were used.

Embryo staging and maintenance

In vitro fertilization was used to generate time-matched WT and mutant embryos, whose age is reported as hours post fertilization (hpf). Stage-matched mutant and WT embryos were collected from pair-wise crosses that spawned within 10 minutes of each other and matched by morphological landmarks at the time of the experiment (Kimmel et al., 1995). Embryos were kept in egg water (60µg/mL of Instant Ocean in distilled water) at 28.5°C.

Live imaging

Cell polarity: WT and Mdchs2 embryos were fertilized in vitro, injected with 100pg of mEGFP, manually dechorionated, and mounted in 0.3-0.5% low melting temperature agarose (LMA, Seaplaque Cat. No. 50100) in 0.3x Danieau’s buffer at 75% or 85% epiboly oriented dorsally or 90 degrees from dorsal on a round #1 coverglass bottom dish. Z-stack time lapses were collected using spinning disc confocal microscope (SDCM) (Olympus IX81, Quorum) using 481nm laser, 10X objective, from 80% epiboly plus 90 min or 90% epiboly plus 90 min. Each step in the z-stack was 3 µm and the entire stack was 55 slices with stacks collected every three minutes.

Yolk actin and microtubules: Tg[XlEef1a1:dclk2-GFP] and MZdchs2; Tg[XlEef1a1:dclk2-GFP]; Tg[β-actin:utrophin-GFP] and MZdchs1b; Tg[β-actin:utrophin-
*GFP*1 embryos were collected within three minutes of each other, manually dechorionated and mounted as above at 4 hpf. Z-stack time lapses were collected using SDCM with 491nm wavelength laser at 10X with z-slice of 3 μm and 51 z-slices every 3 mins.

Cell division: Embryos were injected with 70pg of *H2B-GFP* RNA at one cell and counter-stained with CellTrace Bodipy (C34556) at 1:100. Z-stacks were collected at 1 hpf with SDCM with 491 and 561 nm wavelength lasers at 10X with z-slices of 3 μm.

Cell scatter: one cell in 128 cell embryos was injected with 70pg of *H2B-GFP* RNA Z-stacks were collected using SDCM. Each step in the z-stack was 3 μm and the entire stack was 55 slices. ImageJ was used to max z-project z-stacks and outline area.

Cell aggregation: one cell embryos were injected with 100pg of mEGFP or mRFP RNA. Cells were harvested by physically dissociating embryos and seeded on to fibronectin coated wells. Cells were incubated at 28C and Z-stacks were collected using SDCM. Each step in the z-stack was 3 μm and the entire stack was 10 slices. ImageJ was used to max z-project z-stacks.

Quantitative RT-PCR

Each RNA sample was isolated using Trizol (Life Technologies, #15596-026) from 30 WT or mutant embryos. 1 μg of RNA was used to synthesize cDNA with the iScript kit (Bio-Rad, #170-8891) following manufacturer’s protocol. qRT-PCR reactions were set up using SoAdvanced Sybr green (Bio-Rad, #172-5265).

Statistical analyses

Statistical analysis was performed using GraphPad Prism 6. Statistical significance was estimated using a two-tailed unpaired Student’s *t* test to compare two populations.
Chapter 4: Perspective

Dachsous atypical cadherin is conserved from *Drosophila* to human. In *Drosophila*, it has established roles in regulating planar cell polarity across tissues, growth control, and cell adhesion largely through its heterophilic intercellular interactions with Fat atypical cadherin (Clark et al., 1995; Takeichi, 1995; Matakatsu and Blair, 2004; Adler et al., 1998; Casal et al., 2006; Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006)). However, functional analyses of vertebrate Dachsous cadherins are just beginning. Studies in mice and human implicate one of two mammalian Dachsous homologs, Dchs1, in development of many organs including brain, and *DCHS1* mutations in humans cause pleiotropic Van Maldergem syndrome (Mao et al., 2011; Cappello et al., 2012).

Here, by investigating mutant phenotypes of two zebrafish homologs, *dchs1b* and *dchs2*, we uncovered their novel essential independent and overlapping roles in mediating egg activation, embryonic axis and germ layers specification, and gastrulation movements in part through regulation of actin and microtubule cytoskeleton. *MZdchs1b* embryos show delayed CGE and delayed and uncoordinated cytoplasmic streaming during egg activation without perceivable oogenesis defects. Additionally early cleavages are delayed and misoriented in *MZdchs1b* mutants. *MZdchs1b* mutants also exhibit abnormal expression of the gastrula organizer and mesodermal genes, which are in part due to impaired translocation of dorsal determinants and reduced Nodal signaling. Our studies attribute these phenotypes to actin and microtubule cytoskeletal defects. Indeed, both the actin and the microtubule networks appeared excessively bundled and disorganized in *MZdchs1b* mutants both during blastula and gastrula stages. Interference with either actin or microtubules by pharmacological agents in WT embryos could phenocopy subsets of *MZdchs1b* phenotypes. Furthermore, providing full-length Dchs1b-
sfGFP or Dchs1b ICD could rescue microtubule bundling defects. These findings are novel and significant, as in contrast to other known functions of Dachsous in Drosophila and mammals in multicellular context via its intercellular interaction with Fat, our studies imply that Dachsous can function in a single cell and likely independent of Fat. My work motivates future investigations to identify molecular links between Dachsous and cytoskeleton to delineate these new roles in early embryogenesis.

During gastrulation, both MZdchs1b and MZdchs2 mutants show abnormal epiboly and C&E movements. The elaborate zebrafish yolk cell is easily accessible to imaging in the process of epiboly, which affords a new and fascinating system in which to study the effects of Dachsous cadherins on microtubule and actin cytoskeleton dynamics and function. We have found that defects in actin and microtubule cytoskeleton in the yolk cytoplasmic layer could contribute to delayed epiboly. As Dchs1b and Dchs2 are cadherins, we expected reduced adhesion in mutant embryos. Surprisingly, we did not find significant differences in cell adhesion between MZdchs1b and WT cells in ex vivo cell aggregation assay. Moreover, through in vivo cell scattering experiments, we found that cells in MZdchs2 embryos showed less scattering than in WT embryos suggesting increased adhesion in mutants. Intriguingly, E-cadherin is up regulated in MZdchs1b mutants suggesting an unexpected role for Dchs1b in regulating expression of E-cadherin and possibly other cadherins. As in Drosophila and during cartilage development in zebrafish, the Dachsous/Fat system can regulate gene expression via their interaction with Atrophin, it will be interesting to analyze the maternal zygotic phenotype of Atrophin2a mutants (Le Pabic et al., 2015) during early development and gastrulation. Moreover, cell-sorting behaviors are influenced not only by cell-cell adhesion but also by cortical tension (Maître et al., 2012; Maître and Heisenberg, 2013; Smutny et al., 2015; Krieg et al., 2008; Schötz et al., 2008).
Given our finding that actin cytoskeleton is abnormal and tension is increased in MZdchs1b mutants, studies of cortical tension in the mutant cells are warranted.

Our initial interest in Dchs cadherins stemmed from its roles in regulating planar cell polarity of different tissues in Drosophila and interactions with the Wnt/PCP pathway. Acquiring planar cell polarity is essential for tissues undergoing massive morphogenetic movements such as those during gastrulation and organogenesis. Indeed recent antisense morpholino interference with Dchs2 and Fat3 implicate these atypical cadherin in cell polarity underlying chondrocyte intercalation and cartilage morphogenesis (Le Pabic et al., 2015). We found that both MZdchs1b and MZdchs2 mutants showed morphological phenotypes characteristic of C&E cell movement defects due to cell polarity abnormalities. Both mutants exhibited shorter and wider chordamesoderm, wider neural plate, and impaired migration of prechordal plate. We correlated these morphogenetic defects in MZdchs2 embryos during late gastrulation with impaired mediolateral cell elongation in several domains of mesoderm and ectoderm. Furthermore, as the PCP pathway regulates mediolateral cell polarity in the same domains (Jessen et al., 2002; Topczewski et al., 2001; Yin et al., 2008), we used genetic interaction to test whether Dchs interact with components of the PCP pathway. We found that compound mutants between dchs1b and tri or kny showed increased severity in cyclopia and body length phenotypes suggesting Dchs does interact with the PCP pathway. In order to establish the nature of this interaction, upstream and/or in parallel, we need to completely deplete maternal and zygotic function of not only dchs but the components of the PCP pathway as well. Moreover, additional analyses are necessary to determine whether Dchs regulates cell polarity in a region/tissue that is not under regulation by the PCP pathway.
Figure 4.1: Novel and conserved functions of Dachsous
Schematic of
In summary, we have established novel roles for Dchs in regulation of cytoskeleton at different stages of zebrafish embryogenesis to regulate different processes from egg activation to gastrulation movements. In addition, we have found conservation of Dchs function in regulating mediolateral planar cell polarity underlying C&E gastrulation movements. However, many questions remain to be explored. Where are Dchs proteins localized? Through homologous recombination, we have generated sfGFP knockins in the endogenous \textit{dchs1a, dchs1b, and dchs2} genes, which will allow us to image membrane and possibly intracellular distribution of these proteins in live embryos during different stages of development. We have also acquired and generated mutations in multiple Fat homologs and the single Fj homolog, Fjx1. Careful analysis of single and compound mutants in the Dchs/Fat/Fjx1 pathway will be necessary to begin to reach the depth of understanding for this signaling pathway in planar cell polarity, adhesion, and other functions as achieved in \textit{Drosophila}. Another important future direction is to identify proteins that interact with Dchs cadherins in order to understand how they regulate actin and microtubule cytoskeleton and other possible downstream effectors. Better understanding of Dchs functions in vertebrates will contribute to understanding the basis of phenotypes presented by patients with Van Maldergen syndrome and likely other diseases such as cancer in which these atypical cadherins are increasingly implicated.
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Movie S1
Chorion expansion in WT and MZdchs1b^{h275/jh275} mutants
Time lapse imaging of WT and MZdchs1b^{h275/jh275} mutants beginning at 40 seconds post activation with interval of 10 seconds for 4 minutes.

Movie S2
Representative of PIV analysis of WT embryo
Time lapse imaging of WT embryo beginning at 8 mpf with interval of 1 minute for 45 minutes. PIV analysis overlay bright field imaging. Red->animal pole ward. Blue->vegetal pole ward.

Movie S3
Representative of PIV analysis of MZdchs1b^{h275/jh275} embryo
Time lapse imaging of MZdchs1b^{h275/jh275} embryo beginning at 8 mpf with interval of 1 minute for 45 minutes. PIV analysis overlay bright field imaging. Red->animal pole ward. Blue->vegetal pole ward.

Movie S4
Cell division in MZdchs1b^{h275/jh275} embryo
Time lapse imaging of MZdchs1b^{h275/jh275};Tg[β-actin:Utrophin-GFP] embryo beginning at 8 cells with interval of 2 minute for 31 minutes. Lineage trace indicated by color of dots. Abnormal cell divisions indicated with outlining.

Movie S5
Single abnormal cell division in MZdchs1b^{h275/jh275} embryo
Time lapse imaging of MZdchs1b^{h275/jh275} embryo injected with H2B-GFP beginning at 32 cells with interval of 1 minute for 22 minutes. Lineage trace indicated by color of dots.