Dachsous1b cadherin regulates actin and microtubule cytoskeleton during early zebrafish embryogenesis

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Abstract

Dachsous (Dchs), an atypical cadherin, is an evolutionarily conserved regulator of planar cell polarity, tissue size, and cell adhesion. In humans, \textit{DCHS1} mutations cause pleiotropic Van Maldergem syndrome. Here, we report that mutations in zebrafish \textit{dchs1b} and \textit{dchs2} disrupt several aspects of embryogenesis, including gastrulation. Unexpectedly, maternal zygotic (MZ) \textit{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, MZ\textit{dchs1b} mutants exhibit altered dorsal organizer and mesendodermal gene expression, due to impaired dorsal determinant transport and Nodal signaling. Mechanistically, MZ\textit{dchs1b} 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 MZ\textit{dchs1b} 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 indicate novel roles for vertebrate Dchs in actin and microtubule cytoskeleton regulation in the unanticipated context of the single-celled embryo.
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
activity is uniquely required for egg activation, focusing our investigation on early 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 maternal (MZ) \textit{dchs1b} and MZ\textit{dchs2} mutants and found they exhibit epiboly and convergence and extension (C&E) defects during gastrulation, while only MZ\textit{dchs1b} mutants display egg activation and cell fate specification defects. Signifying that MZ\textit{dchs1b} phenotypes are due to cytoskeletal abnormalities, actin and microtubule networks in MZ\textit{dchs1b} 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.
Results

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. 1A). Notably, *dchs1b* transcripts were more abundant maternally, whereas expression of both *dchs1a* and *dchs2* peaked during zygotic stages (Fig. 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. 1B and S1A). 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* \(fh274\) (C11527T; Q924) and *dchs1b* \(fh275\) (C11683T; Q976) mutations both generated amber stop codons, whereas *dchs2* \(stl1\) (T6528A; Y201) mutation yielded the ochre stop codon, with all three mutations predicted to truncate the proteins early in the extracellular domains (Fig. 1C and 1D). qRT-PCR analysis revealed significant reduction of *dchs1b* and *dchs2* mRNA levels in both alleles of MZ*dchs1b* and MZ*dchs2* mutants respectively compared to WT (Fig. 1E and S1B). Notably, *dchs1a* and *dchs2* transcript abundance in MZ*dchs1b* mutants and *dchs1a* and *dchs1b* transcript in MZ*dchs2* mutants were unchanged (Fig. S1C-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.

MZ*dchs1b* 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. 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. 1G and 1H). 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. 1I, J).

Next we investigated whether delayed gastrulation was due to earlier defects. Whereas
MZ*dchs2*<sup>stl1/stl1</sup> mutants progressed through cleavage and blastula stages normally, compared to
time-matched WT embryos, MZ*dchs1b*<sup>fh275/fh275</sup> mutants displayed defects beginning from
fertilization including smaller blastodiscs with non-uniform cleavages, producing variably sized
blastomeres (Fig. 1F and 4C). We detected globular yolk-like inclusions in the blastodiscs of
MZ*dchs1b*<sup>fh275/fh275</sup>, hereafter called MZ*dchs1b* mutants, but not in WT blastodiscs (Fig. 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. S2A). Images in Figure 1F represent moderate mutant phenotypes. MZ*dchs1b*<sup>fh274/fh274</sup>
mutants showed a similar array of abnormalities, indicating these defects are specific to loss of
maternal and zygotic *dchs1b* function (Fig. S2D). MZ*dchs1b*<sup>fh275/fh275;MZ*dchs2*<sup>stl1/stl1</sup></sup>
compound mutant phenotypes resembled those of single MZ*dchs1b* embryos, albeit occurring with higher
penetrance and more uniform expressivity (Fig. 1F, 5A, E, G and data not shown). Detailed
analyses of *dchs* functions during gastrulation will be described elsewhere. Hereafter, we further
investigate the early developmental roles of MZ*dchs1b*.

Largely normal progression of oogenesis in *Mdchs1b* mutants

As defects were already apparent in MZ*dchs* eggs upon fertilization, we investigated potential *dchs*
roles in oogenesis. Zebrafish oogenesis consists of five stages with characteristic features that
appeared largely normal in *dchs1b* mutant oocytes. Microtubule organizing centers (MTOCs) were present at stage Ia and lost by stage Ib in WT and mutant oocytes (Fig. 2A and 2B). 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. 2C and S2E). Moreover, the presence of a single Balbiani body in stage Ib and II oocytes of WT and mutants indicated *dchs* mutant oocytes are polarized (Fig. 2D and S2F). 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. 2E, 2F and S2G). In unactivated and unfertilized WT and *Mdchs1b* mutant eggs, vitelline envelope morphology, CG size, number, and distribution at the cortex were comparable (Fig. 2G). Lastly, as in WT, polar bodies were extruded from mutant eggs (Fig. 2H). Taken together, these data indicate Dchs1b is dispensable for zebrafish oogenesis.

**Delayed CGE in *Mdchs1b* mutants**

Upon activation, delayed CGE in *Mdchs1b* mutant eggs was evident from stereomicroscopic analysis (data not shown). We next labeled CGs in fixed eggs using fluorescent dye-conjugated MPA (*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 *Mdchs1b* eggs were comparable. By 5 mpa, WT eggs had largely completed CGE, whereas CGs persisted in mutant eggs until ~15 mpa (Fig. 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 *Mdchs1b* oocytes (Fig. 2H and S2F), we conclude that maternal *dchs1b* function promotes CGE during egg activation.

*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 MZ*dchs1b* mutants, we observed amorphous yolk masses (Fig. 1F),
which were apparent by yolk autofluorescence (Fig. 3B). Second, visualizing F-actin using \( Tg[\beta-actin:utrophin-GFP] \) (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. 3C, 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. 3D 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. 3E). The vigorous movements appeared well organized with two centers of circular motion, which we interpret as a circular, toroidal movement within the yolk (Fig. 3D). All movements were of smaller amplitude in MZdchs1b 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. 3D, E and Movie S3). The ooplasmic streaming abnormalities and delayed CGE as well as bundled actin later in development (Fig. S4E) imply actin cytoskeleton deficits in MZdchs1b mutants, as both processes rely on F-actin dynamics (Becker and Hart, 1999).

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. 4A, 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. 4C 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. 4D, 6B, S3A, S3B, and S3E). 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. 4E, F). Hence, although MZdchs1b mutants appeared morphologically younger than age-matched WT embryos, aspects of MBT occurred on time.

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. 6A, 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. 5D-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. S3C).

As both mesendoderm and the Nieuwkoop center were aberrant in MZdchs1b mutants (Fig. 5A-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 MZ*dchs1b* mutants (Fig. 6A and S3D).

Consistently, qRT-PCR revealed significantly reduced abundance of both *cyc* and *sqt* transcripts in MZ*dchs1b* compared to WT (Fig. 6B and S3E). To functionally assess Nodal signaling, we analyzed nuclear accumulation of the transcription factor Smad2 (Saka et al., 2007; Schier and Shen, 2000). Using the *Tg[ß-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 MZ*dchs1b* blastulae compared to WT (Fig. 6C, D). Together, these results indicate reduced Nodal signaling partially accounts for the mesendodermal and Nieuwkoop center deficits of MZ*dchs1b* embryos.

**Impaired wnt8a RNA translocation in MZ*dchs1b* 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 M*dcsh1b* eggs (Fig. S4A). However, animal-ward translocation of *wnt8a* RNA following fertilization was reduced in MZ*dchs1b* embryos compared WT (Fig. 7A, B). Moreover, in some embryos *wnt8a* expression expanded symmetrically (Fig. S4A).

*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 MZ*dchs1b* embryos was due to microtubule abnormalities. Visualizing microtubules with the DM1α antibody in immunofluorescence and using *Tg[XlEef1a1:delk2-GFP]*, we observed parallel arrays that appeared misoriented in mutants, crossing each other (Fig. 7C and S4C), and more bundled as
evidenced by higher intensity in $M_{dchs1b}$ mutants compared to WT (Fig. 7D). Progressive bundling resulted in dramatically different appearances of vegetal microtubules during gastrulation (Fig. 7E and S4D). 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. 7E, F). We posit these vegetal microtubules abnormalities in $MZ_{dchs1b}$ mutants could impede translocation of $wnt8a$ transcripts, consequently resulting in Nieuwkoop center and gastrula organizer deficiencies (Fig. 5D-G).

**Disrupting cytoskeleton dynamics in WT embryos phenocopies $MZ_{dchs1b}$ mutant defects**

We employed a pharmacological approach to determine whether specifically targeting actin or microtubules could phenocopy $MZ_{dchs1b}$ defects. Partial disruption of the F-actin network (Fig. S5A) of $Tg[\beta\text{-actin:utrophin-GFP}]$ 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 $MZ_{dchs1b}$ mutants. Globular yolk inclusions occupied the blastodisc, while cytoplasmic islands remained in the yolk at 75 mpf (Fig. 3C). At 10-15μg/mL of cytochalasinD, CGE was perturbed, chorions did not fully expand, cytoplasmic streaming was blocked (Fig. S5B), 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 $MZ_{dchs1b}$ mutants (Fig. 5C and S5C).

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 $wnt8a$ translocation. The treated embryos had punctuated marginal $ntl$ domains and smaller or fragmented $gsc$ domains, phenocopying $MZ_{dchs1b}$ mutant defects (Fig. 5C and S5D). 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. S5D). 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. 5C, S5C, and S5D). 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. 3C and 7C), and rescue of microtubule bundling by expressing Dchs1b (Fig. 7E, F), we propose that Dchs1b regulates the dynamics of the actin and/or microtubule networks to promote egg activation and early patterning.
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 MZ*dchs1b* and MZ*dchs2* mutants afforded assessment of the earliest *dchs* functions in embryogenesis. Correlated with its strong maternal expression, only MZ*dchs1b* embryos exhibited pre-MBT phenotypes (Fig. 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 MZ*dchs1b* 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 \(\text{MZdchs1b}^{fh275/fh275};\text{MZdchs2}^{stl1/stl1}\) compound mutants (Fig. 1F, 5A, 5E, and 5G).

A striking finding is the essential role Dchs1b plays in the single celled egg and early zygote. \(\text{Mdchs1b}\) mutants showed delayed CGE and incomplete cytoplasmic segregation (Fig. 3). 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 examined were normal in \(\text{Mdchs1b}\) mutants (Fig. 2 and S2E-G): Balbiani bodies were present, a single cytoplasmic island and single micropyle occupied the animal pole, and \(\text{wnt8a}\) maternal mRNA was localized at the vegetal pole. Additionally, CGs translocated to the cortex, and polar bodies were extruded normally. However, as we did not examine every aspect of oogenesis, the possibility of Dchs-mediated intercellular interactions during early oogenesis cannot be fully ruled out. These analyses indicate that egg activation processes, which are dependent on dynamics of actin or microtubule cytoskeleton become defective in \(\text{M}\) or \(\text{MZdchs1b}\) mutants after activation/fertilization of the egg. That injection of synthetic RNA encoding Dchs1b intracellular domain rescued abnormal organization of microtubule network in the syncytial yolk cell (Fig. E, 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). The 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 \(\text{M/MZdchs1b}\) mutants, CGE and cytoplasmic streaming were both delayed and cytoplasmic streaming was uncoordinated (Fig. 3). 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. 4). 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. 8). First, in the mutant blastodisc, defective cytoplasmic streaming produces ectopic yolk masses (Fig. 3B and 3C), which later present physical obstacles to cell migration and morphogen diffusion and could reduce Nodal signaling (Fig. 6), leading to mesendoderm deficiencies (Fig. 5). 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 translocated upon fertilization by the microtubule cytoskeleton (Lu et al., 2011; Tran et al., 2012), displayed abnormal distribution after fertilization (Fig. 7A and 7B). Therefore, abnormal organization and function of microtubules in MZdchs1b mutants could lead to impaired dorsal determinant translocation thereby affecting β-catenin nuclear localization (Fig. S4C), and consequently β-catenin-dependent zygotic gene expression in the Nieuwkoop center and dorsal mesoderm, such as sqrt, 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. 7E, S4D, and S4E) in MZ\textit{dchs1b} 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 MZ\textit{dchs1b} mutants (Fig. 8).

The abnormalities seen in \textit{M{\textit{dchs1b}}} and MZ\textit{dchs1b} 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 \textit{Drosophila}, Dachsous regulates 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 \textit{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. However, it remains to be determined whether all MZ\textit{dchs1b} mutant defects can be explained by the loss of Dchs1b regulation of the cytoskeleton in the affected processes, or some are secondary to earlier abnormalities. We found that both MZ\textit{dchs1b} and MZ\textit{dchs2}\textit{stl1/stl1} 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 MZ\textit{dchs1b} mutants as in \textit{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; as zebrafish \textit{dchs2} morphants
have been shown to display craniofacial defects (Le Pabic et al., 2014). 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.
**Materials and methods**

Zebrafish lines

AB, Tg[XlEef1a1: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\( \text{fh274} \) and dchs1b\( \text{fh275} \) and dchs2\( \text{stl1} \) 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 (60μg/mL of Instant Ocean in distilled water) at 28.5°C.

Live imaging

Cytoplasmic streaming: WT and M\( \text{dchs1b} \) 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[XlEef1a1:dclk2-GFP] and M\( \text{dchs1b} \); Tg[XlEef1a1: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 *Mdchs1b* 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/h275/fh275 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
AlexaFlor488 and AlexaFlor568 (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 ≥3μm² 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 3μg/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. The intracellular
domain sequence spans from amino acid 2398 to amino acid 2756.

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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References


Main Figures

Figure 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<sup>attl/satl</sup>, and MZdchs1b<sup>29275/29275</sup>;MZdchs2<sup>attl/satl</sup> 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<sup>attl/satl</sup> 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<sup>attl/satl</sup> (n=486) embryos.
I. hgg1 and dlx3 WISH analysis of WT, MZdchs1b, and MZdchs2<sup>attl/satl</sup> 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<sup>attl/satl</sup> (n=18), shown by black line.
Figure 2

Mdcbs1b 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 Mdcbs1b ovaries reveal normal polarization of stage IB oocytes as indicated by the presence of the Balbiani body (black arrowhead).

E. WT and Mdcbs1b 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 Mdcbs1b 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 Mdcbs1b mutant eggs.
Figure 3
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.
Figure 4
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^{st11/st11}, and MZdchs1b^{h275/h275};MZdchs2^{st11/st11} mutants compared to WT relative to MBT.
E. mxt2 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 5
Mesoderm specification is deficient in MZdchs1b embryos and WT embryos with impaired cytoskeleton.

A. *ntl* expression in MZdchs1b, Mdchs1b, and MZdchs1b<sup>h275/h275</sup>;MZdchs2<sup>stl1/stl1</sup> stage-matched embryos at 30% epiboly; animal pole view.
B. Quantification of gaps in *ntl* expression in WT, Mdchs1b, 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, Mdchs1b, and MZdchs1b embryos. Insets show embryos with gap in expression domain.
E. *gsc* expression in WT, MZdchs1b, MZdchs2<sup>stl1/stl1</sup>, and MZdchs1b<sup>h275/h275</sup>;MZdchs2<sup>stl1/stl1</sup> 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 6
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.
Figure 7

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 8.
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 parallel array microtubules.
Middle panels: Dorsal view, 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 β-catenin nuclear distribution and organizer gene expression.