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Dkk1 Controls Cell-Cell Interaction through Regulation of Non-nuclear β-Catenin Pools

Graphical Abstract

Highlights
- Dkk1 localizes to adhesion complexes in vivo
- Dkk1 signaling controls cell-cell connectivity and polarity
- Cell interaction control is independent of β-catenin transcriptional output and Wnt/PCP
- Dkk1 sequesters β-catenin at the plasma membrane

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In Brief
In this study, Johansson and colleagues show that Dkk1 controls cell migration, polarity, and adhesion independently of its known function in fate specification and modulation of Wnt/PCP signaling. Dkk1 localizes to cell adhesion complexes and polarized actomyosin in vivo and sequesters β-catenin to a distinct submembrane pool.
Dkk1 Controls Cell-Cell Interaction through Regulation of Non-nuclear β-Catenin Pools

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SUMMARY

Dickkopf-1 (Dkk1) is a secreted Wnt antagonist with a well-established role in head induction during development. Numerous studies have emerged implicating Dkk1 in various malignancies and neurodegenerative diseases through an unknown mechanism. Using zebrafish gastrulation as a model for collective cell migration, we unveil such a mechanism, identifying a role for Dkk1 in control of cell connectivity and polarity in vivo, independent of its known function. We find that Dkk1 localizes to adhesion complexes at the plasma membrane and regions of concentrated actomyosin, suggesting a direct involvement in regulation of local cell adhesion. Our results show that Dkk1 represses cell polarization and integrity of cell-cell adhesion, independently of its impact on β-catenin protein degradation. Concurrently, Dkk1 prevents nuclear localization of β-catenin by restricting its distribution to a discrete submembrane pool. We propose that redistribution of cytosolic β-catenin by Dkk1 concomitantly drives repression of cell adhesion and inhibits β-catenin-dependent transcriptional output.

INTRODUCTION

Signaling by Wnt proteins occurs through several receptor complexes, which trigger intracellular signaling cascades affecting cell fate, polarity, and proliferation. Activation of the canonical Wnt pathway occurs by formation of a ternary complex between the Wnt ligand, Frizzled receptor, and co-receptor LRP6, which ultimately protects cytosolic β-catenin from proteosomal degradation. The accumulated β-catenin translocates to the nucleus where it turns on transcription of target genes by associating with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. Secreted Dickkopf-1 (Dkk1) is thought to inhibit canonical Wnt signaling through its binding to the LRP6 co-receptor, preventing formation of an active signaling complex, consequently leading to degradation of cytosolic β-catenin and reduction of nuclear β-catenin-dependent gene transcription (Ahn et al., 2011; Cheng et al., 2011; Matoba et al., 2017).

β-catenin functions both as a structural component of adhesive complexes and as a regulator of transcription. The different functions of β-catenin necessitate different subcellular localizations, creating several pools of β-catenin inside the cell. Intracellular trafficking of β-catenin is seemingly regulated by phosphorylation and localized interaction partners, which themselves shuttle between compartments (Daugherty and Gottardi, 2007; Krieghoff et al., 2006).

Dkk1 is essential for formation of anterior structures and morphogenesis of the limbs in the developing embryo but is also expressed in the adult where it regulates formation, regeneration, and repair of several tissues (Florio et al., 2016; Himburg et al., 2017; Kang et al., 2013). Dereguation of Dkk1 expression is associated with metastasis of numerous cancers, with elevated levels of Dkk1 correlating with a poor prognosis in multiple myeloma, prostate cancer, and hepatocellular carcinoma (Rachner et al., 2014; Tian et al., 2003; Yu et al., 2009). Dkk1 has been mostly investigated in the context of canonical Wnt signaling, but it has become evident that alternative signaling pathways are activated by Dkk1 (Caneparo et al., 2007; Killick et al., 2014; Marzo et al., 2016). What these pathways are and how they relate to disease mechanisms is unresolved.

We used gastrulation in zebrafish as a model to study the role of Dkk1 in cell-cell interaction. Gastrulation is a highly dynamic process during which cells at the margin of the embryo internalize and migrate to form the mesendoderm germ layer. Axial mesoderm cells display a collective mesenchymal mode of migration during this process. A key feature of this type of migration is that cells within a group remain connected while moving. Mechanosensing within the group occurs through cadherin-mediated intercellular adhesions, which affect polarized protrusive activity (Dumortier et al., 2012; Tada and Heisenberg, 2012). Formation of appropriate dynamic adhesions is therefore essential for transmitting directional information within the group (Weber et al., 2012). Catenins, namely α-catenin, β-catenin, and p120-catenin, are core components of adherens junction adaptors complexes, which link the cytoplasmic ends of cadherins to actin filaments (Takeichi, 2014).

Here, we report that, in addition to its known function, Dkk1 coordinates cell polarity and cell-cell adhesion by controlling the subcellular distribution of β-catenin, independently of its regulation of the Axin/Gsk3β degradation pathway.

RESULTS

Dkk1 Controls Collective Cell Migration Independently of β-Catenin Transcription Regulation

dkk1 transcripts are first detected in the nascent mesoderm, prior to gastrulation. During gastrulation, dkk1’s dynamic expression...
is restricted to a small subset of the anterior axial mesoderm (Figure 1A). By the end of epiboly (EB), dkk1 is solely detected around the border of the prechordal plate.

To gain insight into the mechanism of Dkk1-driven regulation of cell motility in vivo, we expressed Dkk1 in transgenic zebrafish embryos in which the axial mesoderm is labeled with green fluorescent protein (GFP) (Doitsidou et al., 2002). In the presence of exogenous Dkk1, axial cells fail to converge normally and their anterior progression is severely perturbed (Figure 1B; Video S1). Although individual cells have protrusive activity and are motile within the anteriormost axial population, the cells lost persistence in directionality as a group, making the population fail to collectively progress in embryos expressing Dkk1 (Figures 1C and 1D; Video S2).

We went on to test whether the changes in cell behavior relied on the inhibitory function of Dkk1 on β-catenin-dependent gene transcription. Nuclear β-catenin activates transcription of a series of target genes by displacing Tcf3, a strong transcriptional repressor. The absence of Tcf3 leads to expression of these genes, even in the absence of β-catenin. We thus assessed the effect of Dkk1 in embryos lacking Tcf3a (Sokol, 2011). Dkk1-induced expansion of anterior mesoderm fate is partly corrected by Tcf3 knock down (Figure S1A) and broadening of forebrain identity is reversed into a reduced or even absent forebrain neural plate (Figure S1B). Despite the severe posteriorization of the neural plate by activation of β-catenin gene targets, these embryos displayed disrupted axial cell migration and defective tail extension (Figure 1E). These results show that Dkk1 disrupts collective migration by a mechanism independent of cell fate and β-catenin transcriptional activity.

The presence of an axial defect induced by Dkk1, despite the Tcf3 knock down background strongly suggests that the Dkk1-dependent changes in axial mesoderm migratory behavior are not caused by expansion of the axial cell population. To confirm that Dkk1-induced migratory changes are independent of cell number, we either increased the axial population in wild-type embryos by downregulating the nodal antagonists Lefty1 and Lefty2 or decreased the axial population in Dkk1-overexpressing embryos by shield cell ablation or injection of Lefty1 RNA. Quantification of the anterior progression of axial cells shows no significant difference between wild-type and Lefty1/2 knock down embryos, while progression is still significantly perturbed in Dkk1-expressing embryos with reduced axial cell population (Figures S1C–S1E).

**Figure 1. Dkk1 Disrupts Collective Cell Migration Independently of Cell Fate**

(A) Expression of dkk1 (green) and goosecoid (gsc, red) from 30% to 70% epiboly (EB), using RNAscope. Confocal imaging, dorsal view with anterior to the top. Maximum projection in the left panels, 5 μm z stacks at higher magnification on the right. Single-plane image at 80% EB shows dkk1 transcripts overlapping with gsc transcripts at the boundary of the gsc-expressing region. Scale bar, 10 μm.

(B) Progression of axial cells. Confocal maximum projections from shield to 80% epiboly stages in transgenic Tg(gsc:GFP) embryos, with or without injection of Dkk1 RNA. Embryos are shown in dorsal view with anterior to the top. Scale bar, 100 μm. See also Video S1.

(C) Maximum projections of anterior axial cells from time-lapse confocal imaging of Tg(gsc:GFP) embryos showing a lack of progression of the prechordal plate (green) in Dkk1-expressing embryos. Embryos were imaged at 75% epiboly for a time (t) period of 48 min. The dashed line shows the position of the anteriormost prechordal plate cells at the first and last time point (t = 0 min and t = 48 min, respectively). Scale bar, 20 μm. See also Video S2.

(D) Tracking of migrating axial cells from Tg(gsc:GFP) embryo time-lapse videos from 70% to 90% epiboly at 2-min intervals (n = 3 for each condition). Tracks from individual cells from one representative embryo for each condition are shown. Axial cells in Dkk1-expressing embryos move slower and exhibit reduced persistence and displacement. *p < 0.05 and **p < 0.01; p values were calculated using linear mixed-effects models.

(E) Dkk1 cell migration behavior is independent of transcriptional regulation of β-catenin target genes. 24 h post-fertilization (hpf) embryos (left) and confocal maximum projections of Tg(gsc:GFP) embryos at 80% epiboly (EB) (right). The Dkk1-induced cell migration defect persists in embryos at 80% EB lacking Tcf3a (Tcf3 KD). See also Figures S1A and S1B.

Taken together, these observations show that the perturbation in axial cell migration imposed by Dkk1 is independent of cell number, fate changes, and β-catenin nuclear transcriptional activity.

**Dkk1 Controls Cell Polarity and Cell-Cell Adhesion Independent of Wnt/PCP Signaling**

Toward the end of gastrulation, axial cells align at the midline and undergo mediolateral intercalation. Highly polarized actin cables, delimiting the axis, form during this process (Figure 2A, control). When Dkk1 is ubiquitously expressed, cells display perturbed distribution of filamentous actin, (Figure 2A, Dkk1 RNA), indicating a loss of polarity. In contrast, the loss of Dkk1 expression results in an opposite phenotype, characterized by a compacted axis with hyperpolarized actin in the adjacent adaxial population (Figure 2A, Dkk1 KD).

Expansion of the axial population or activation of Wnt target gene transcription has no effect on polarity and distribution of filamentous actin (Figure 2A, Lefty KD and Tcf3 KD, respectively). We have previously shown that Wnt/PCP (planar cell polarity) signaling is upregulated as a consequence of Dkk1 interaction with LRP5/6 (Caneparo et al., 2007). We therefore tested whether this cellular phenotype was due to increased PCP signaling activity. Using loss of function of Vangl2 (Williams et al., 2012), we found that disrupting the PCP pathway does not perturb actin distribution or the shape of the notochord boundary along the axis. In fact, the notochord boundary is straighter than the control in Vangl2 morphants (Figures 2B and 2C). Moreover, Dkk1-induced actin and boundary phenotypes are not rescued by lowering PCP signaling (Figures 2B and 2C), excluding PCP pathway involvement in Dkk1’s influence on cell polarity and actin redistribution. In zebrafish, BMP (bone morphogenetic protein) signaling has been shown to modulate cell-cell interaction during gastrulation (Myers et al., 2002). However, we did not detect any statistically significant change in BMP activity at the early gastrula stage in Dkk1-expressing embryos (Figure S2). Dkk1’s impact on axial cell movement and polarity is therefore independent of the known signaling pathways involved in this process.

To further our understanding of the loss of polarity induced by Dkk1, we assessed Myosin II distribution using a transgenic line expressing an EGFP-tagged myosin light chain subunit (Behrndt et al., 2012). Myosin II is enriched along the axis in control embryos, but this planar polarized distribution of actomyosin is markedly disrupted in Dkk1-expressing embryos (Figure S3). By the end of gastrulation, the distribution of E-cadherin...
between axial cells is also profoundly modified (Figure 3B). Since collective migration relies on the formation of appropriate cell-cell contacts, we next went on to study cell connectivity by labeling adhesion complexes in migrating cells. In the presence of ectopic Dkk1, axial cells display a striking loss of adhesion complex integrity and a diffuse plasma membrane (PM) morphology (Figure 3C). Quantitation of the relative distribution of E-cadherin at the PM shows a significantly broader distribution of E-cadherin laterally along the boundaries between neighboring cells in Dkk1-expressing embryos (Figures 3D and 3E).

Quantitative assessment of cell orientation, shape, and size in the presence or absence of exogenous Dkk1 demonstrated significant changes in angle, elongation, and cell area, with cells in Dkk1 embryos being more rounded and having a larger area than control (Figures 4A and 4B). Dkk1 thus controls cell orientation, shape, and size in the axial mesoderm.

To understand the extent to which the loss of Dkk1 impacts cell-cell interaction, we examined the organization of the actin cytoskeleton in gastrula embryos lacking Dkk1. Embryos display a significant increase in filamentous actin around the anterior prechordal mesoderm (Figure 5A). Moreover, they show a distinct pattern of actin-enriched puncta in the PM, both around the prechordal plate and alongside more posterior axial cells (arrowheads in Figure 5B). These puncta suggest increased actin organization at adhesion complexes.

Dkk1 KD embryos injected with low levels of Dkk1 RNA display an additive phenotype (n = 11). Scale bar, 20 μm.

Dkk1 gain- and loss-of-function phenotypes therefore demonstrate that Dkk1 is controlling cell polarity and adhesion through a yet unknown molecular mechanism.

**Dkk1 Localizes to Adhesion Complexes and Actomyosin-Rich Membranes In Vivo**

To assess whether Dkk1 is tuning cell-cell adhesion through direct interaction with adhesion complexes, we sought to detect
Dkk1 protein localization in vivo. Dkk1 is expressed in discrete cell populations dynamically changing along the axis (Figure 1A), but nothing is known about Dkk1 protein localization in vivo, due to the lack of an antibody that is able to detect the secreted protein in complex tissues.

To address this question, we performed in vivo localization studies using transgenic embryos carrying a heat-shock-inducible Dkk1GFP locus (Stoick-Cooper et al., 2007). We reasoned that, when induced, the transgenic embryos will express Dkk1 across all tissues, global expression would reveal Dkk1 protein subcellular localization and any possible variation in molecular interaction across embryonic tissues. The distribution of Dkk1GFP in the heat-induced live embryo shows striking heterogeneity in distribution, with dramatically low levels in axial cells compared to surrounding paraxial cells and an accumulation of unbound extracellular protein at the animal pole (Figure 6A). To minimize phenotypic effects caused by Dkk1 overexpression, heterozygous embryos were fixed one hour after a brief heat shock at 70% EB. Dkk1 was detected at actin-enriched PM regions in adjacent cells and colocalized with concentrated actomyosin along the lateral borders of the axis (Figure 6B). Discrete patches of Dkk1GFP at the PM also colocalized with endogenous membrane-associated β-catenin (Figure 6C). Dkk1 is therefore able to very closely interact with adhesion complexes. Moreover, its normal localization at the boundary between the axial and paraxial mesoderm in late gastrula embryos further support a direct involvement in modulation of polarity at the axial border itself.

**Dkk1 Is Able to Repress β-Catenin Nuclear Localization through Sequestration at the PM**

Inhibition of β-catenin nuclear localization is thought to be achieved by proteosomal degradation of the protein, which is
mediated by the Axin-Gsk3β1 complex. However, cell culture experiments have shown that β-catenin, released from dissociated adherens junctions, can translocate into the nucleus, indicating the existence of two interconnected subcellular β-catenin pools (Kam and Quaranta, 2009). This apparent paradox led us to investigate whether there was an alternative mechanism, unrelated to β-catenin protein level, by which Dkk1 affects β-catenin function. For this purpose, we analyzed endogenous β-catenin distribution in axin1 null mutants, in which proteosomal degradation of β-catenin fails to occur (Heisenberg et al., 2001; van de Water et al., 2001).

At 24 h post-fertilization (hpf), axin1 mutants show a typical strong reduction in anterior fate and normal body length (Figure 7C). According to the current Wnt signaling model, the phenotype induced by loss of the intracellular Axin protein should be insensitive to the level of extracellular Dkk1 protein as the latter acts upstream of Axin. However, we observe that Dkk1-expressing axin1 mutant embryos exhibited enlarged heads and severe shortening of the axis (Figure 7D). Increased stabilization of β-catenin in the axin1 mutant was therefore not sufficient to prevent the overall anteriorization of the CNS or to correct the axial extension defect caused by Dkk1, showing that these are likely to be insensitive to the β-catenin protein level.

To better understand the protein degradation-independent response of β-catenin to Dkk1, we investigated β-catenin distribution in the axin1+/− background. In sibling (Sib) embryos, β-catenin showed the expected differential subcellular distribution in different regions of the embryo, with no nuclear localization anteriorly (where endogenous Wnt inhibitors are expressed), and an intermediate more variable level in the axis (where endogenous Wnt inhibitors are expressed), and an intermediate more variable level in the axis. Ubiquitous Dkk1 expression is expected to lower the level of β-catenin across all compartments through β-catenin degradation. We observe the expected substantial reduction of β-catenin protein in all nuclei but little change in levels at the PM (Figures 7B, 7E, and 7F). This result strongly indicates that Dkk1 controls β-catenin localization, in addition to its Frizzled-dependent effect on the β-catenin protein level.

In the absence of axin1 expression, the expectation is an increase of the β-catenin protein level across all three embryonic areas, with a stronger effect in anterior and axial levels. Intriguingly, although β-catenin protein levels are indeed clearly increased across the embryo (Figure 7C), the levels are still differential across the three areas, recapitulating the distribution seen in wildtype siblings (Figures 7C and 7E). This suggests that the degradation pathway is still partly active and/or endogenous Dkk1 expression in the mutant influences the distribution of the protein. The obvious increase of the protein level in the non-nuclear compartment in anterior cells, where endogenous Dkk1 resides, without much increase in nuclear localization, argues in support of the second possibility.

Crucially, whether or not axin1−/− embryos are null or hypomorphic for β-catenin protein degradation, the substantial increase in β-catenin protein across the whole embryo does not modify the differential effect of Dkk1 on nuclear versus membrane located β-catenin proteins. Indeed, the axial cell population in embryos.
injected with Dkk1 RNA showed a markedly different distribution of β-catenin in both Sibs and mutants, with significantly higher levels of β-catenin staining at the PM (Figures 7B, 7D, and 7E). In both Dkk1-expressing Sibs and axin1 mutants, abnormally diffuse PM morphology is evident in the anterior and lateral regions and is particularly pronounced in the axial population. This observation reveals that the loss of cell connectivity, induced by Dkk1, is not restored by increasing the level of β-catenin protein. These results therefore show that Dkk1 prevents nuclear accumulation of β-catenin independently of its degradation, probably by retaining the protein at the PM while concomitantly downregulating cell-cell adhesion.

DISCUSSION

Our findings identify a new signaling function of Dkk1, regulating axial-paraxial interaction during formation of the notochord in vertebrates. Formation of correctly oriented adhesions underlies coordinated migration of axial cells, and may also contribute to successful mediolateral intercalation between neighboring cells during convergence and extension. We show that Dkk1 reduces cell-cell adhesions and cell polarity by a mechanism unrelated to cell fate specification and independent of both Wnt/PCP and β-catenin transcriptional regulation.

Dkk1 expression is spatially and temporally very dynamic and associated to areas of the embryo undergoing reshaping and/or movement, such as the lateral line and the tail fin fold (McGraw et al., 2014; Venero Galanternik et al., 2016). High-resolution detection of dkk1 transcripts at gastrula stages reveals exclusive expression in a subpopulation of axial cells of Dkk1 KD embryos (arrowheads) (n = 10–15 for each condition). Scale bar, 20 μm.

Figure 5. Loss of Dkk1 Increases Cell Adhesion and Polarization

(A and B) Single-plane confocal images of Tg(gsc:GFP) embryos (green axis) at 80% epiboly (EB) stained with phalloidin (red). (A) Prechordal plate leading edge. A stitch-like pattern of actin distribution in cells surrounding the prechordal plate is evident in Dkk1 knock down (KD) embryos (arrowheads). (B) Filamentous actin along the anterior half of the axis. Dashed rectangles indicate the area of higher magnification of the single-plane confocal images shown on the right panels. Filamentous actin is highly concentrated in discrete puncta at the plasma membrane in the paraxial cells of Dkk1 KD embryos (arrowheads) (n = 10–15 for each condition). Scale bar, 20 μm.

(C) Single-plane confocal images of β-catenin antibody-stained Tg(gsc:GFP) embryos at the tail bud (TB) stage show strong patches of endogenous β-catenin (red) along the plasma membrane between connected cells. Dashed squares indicate the area of higher magnification shown on the right side of single-plane confocal images. Control (Ctrl) n = 5 and Dkk1 KD n = 6. Scale bar, 20 μm.
protein throughout the embryo. We however find that Dkk1 becomes specifically concentrated in certain embryonic regions, coinciding with highly polarized actomyosin-enriched areas. We show that Dkk1 has profound effects on the organization of the actin cytoskeleton in these regions, suggesting that it locally regulates the filamentous actin network. Spatiotemporal regulation of this network is essential for adhesion complex formation and maturation (Cavey and Lecuit, 2009). Since appropriate tension is required for formation of stable bonds between cadherin-catenin and actin (Buckley et al., 2014), Dkk1 could exert its destabilizing effect on cell-cell adhesion by affecting actin tension. The colocalization of Dkk1 with β-catenin-positive puncta at the PM suggests that Dkk1 may destabilize cell-cell junctions by perturbation of the actin cytoskeleton either at the point of cell-cell contact initiation or junctional maturation, possibly by interfering with Rac1 or Rho small GTPase activation.

Dkk1 perturbs notochord boundary formation independently of PCP and BMP signaling, which indicates that alternative pathways are involved. A PCP-independent pathway controlling notochord boundary formation has been recently identified in zebrafish (Williams et al., 2018). The nature of alternative signaling pathways regulated by Dkk1 are, however, unknown and will be a topic for future investigations.

The function of Dkk1 reported here demonstrates that β-catenin-dependent transcription is not only inhibited by β-catenin protein degradation but also by Dkk1 preventing nuclear accumulation of β-catenin, irrespective of degradation. Numerous studies on the different cellular pools of β-catenin have demonstrated crosstalk between adhesion complex- and nuclear-associated β-catenin. These studies have shown that disassembly of adhesion complexes releases signaling competent β-catenin, which can translocate to the nucleus (Eger et al., 2000; Gayrard et al., 2018; Kam and Quaranta, 2009; Mao et al., 2013; Orsulic et al., 1999; Wang et al., 2010). Our study demonstrates that in the presence of Dkk1, β-catenin is retained at the PM despite the loss of adhesion complex integrity. This PM retention effectively sequesters β-catenin away from the nucleus, thereby preventing activation of target gene transcription.

Our results also shed a new light on how Dkk1 could affect processes such as synapse maintenance and cancer
metastasis. Activation of Wnt signaling has been well documented in various cancers (Zhan et al., 2017). One would therefore expect Dkk1 to act as a tumor suppressor, yet increased levels of Dkk1 correlate with metastases and a poor outcome in numerous malignancies (Kagey and He, 2017). Notably, the poor prognosis in some malignancies where Dkk1 is elevated persists in the presence of accumulated β-catenin (Xu et al., 2012; Yu et al., 2009). Downregulation of Dkk1 by antibody treatment has been shown to decrease tumor spreading in xenograft models, making Dkk1 a potential molecular target for cancer therapy (Goldstein et al., 2016; Mazon et al., 2016; Sato et al., 2010). Dkk1 has been associated with several aspects of tumor biology, including increased tumor growth, migration and metastasis, and more recently organotropism (Zhuang et al., 2017). Here, we show that Dkk1 increases mesenchymal characteristics in receptive cells, the hallmark of invasive cell behavior. Supporting a role for Dkk1 in inducing invasiveness in vivo, we found that downregulation of Hef1 (human enhancer of filamentation), an activator of epithelial mesenchymal transition and pro-invasive cell behavior (O’Neill et al., 2007; Tornillo et al., 2014), rescued the Dkk1 migratory phenotype (data not shown). In Dkk1-expressing embryos, cell adhesion is compromised leading to loss of contact-dependent mechanosensing, and as a consequence coordinated progression fails (Theneau et al., 2010; Weber et al., 2012). Our findings therefore suggest that Dkk1 imposes an invasive migratory phenotype by inducing the loss of cell-cell adhesion, independently of nuclear β-catenin target gene transcription and overall β-catenin protein level.

Dkk1 has been found at neuron synapses during development and in adults. Elevation of Dkk1 expression has been observed in human brain tissue from patients with Alzheimer disease, and inducible expression of Dkk1 has been shown to lead to a loss of synapses in the adult hippocampus (Caricasole et al., 2004; Marzo et al., 2016). Cell adhesion molecules between pre- and post-synaptic membranes confer structural stability and plasticity to synapses, and patients with Alzheimer’s disease displaying loss of synapses have been shown to have reduced synaptic adhesion (Leschyns’Ka et al., 2015). Based on our results, it is conceivable that Dkk1 may contribute to synaptic loss in neurodegenerative disease by downregulating synaptic adhesion.

Resolving the molecular mechanism by which Dkk1 modulates adhesion may therefore have a key impact on deciphering cancer as well as neurodegenerative processes.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.devcel.2019.10.026.

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Figure 7. Dkk1 Sequesters β-Catenin at the Plasma Membrane

(A–D) Sibling and axin1 mutant embryos at 24 h post-fertilization (hpf) (left column) and 80% epiboly (EB) with (B) and (D) or without (A) and (C) Dkk1 mis-expression. 80% EB embryos were stained with an antibody against β-catenin (green) to assess endogenous β-catenin distribution in different subcellular compartments in various regions of the embryo. White-bordered squares in the second column indicate the location of single-plane confocal images in three regions of the embryo shown at a higher magnification in the three last columns. Scale bar, 20 μm. (A) Sibling embryos show patterned distribution of β-catenin (green) in different regions of the embryo at tissue and subcellular levels. In the anterior region β-catenin is detected exclusively at the plasma membrane, while high levels are seen in both nuclei and the plasma membrane, laterally. Axial cells show a low level of β-catenin in both nuclei and plasma membrane. (B) Dkk1-expressing sibling embryos display large heads and extension defects at 24 hpf and show a loss of nuclear β-catenin (green) in anterior, lateral, and axial regions of the embryo but maintain β-catenin at the plasma membrane despite the loss of cell-cell adhesion. Arrowheads point to diffuse membrane morphology. (C) axin1 null mutants show a loss of anterior fate at 24 hpf and β-catenin-positive (green) nuclei in the anterior region at 80% EB. Contrary to the current model, these gastrula embryos display the same relative subcellular distribution of β-catenin found in normal siblings across the three areas measured. (D) Despite the expected lack of telencephalon and eyes, the Dkk1-expressing axin1 mutants show an overall AP organization similar to Dkk1-expressing embryos at 24 hpf. Similar to Dkk1-RNA-injected siblings, β-catenin was retained at the plasma membrane, and cells displayed a loss of cell-cell adhesion. Arrowheads point to diffuse membrane morphology.

(E) Nucleus to plasma membrane (PM) β-catenin (green) fluorescence intensity ratios quantified in anterior, lateral, and axial cells in sibling (Sib) and axin1 mutant (axin1−/−) embryos, with or without Dkk1 expression. A significantly higher proportion of β-catenin is present at the PM in Dkk1-expressing Sibs and mutants in the lateral and axial regions than control embryos. Fluorescence intensity ratios were measured in 10 cells per region in each embryo (Sib, n = 2; Sib + Dkk1 RNA, n = 2; axin1−/−, n = 1; and axin1−/− + Dkk1 RNA, n = 1). p > 0.05 ns, **p < 0.05, and ***p < 0.001.

(F) Absolute levels of β-catenin nuclear expression, calculated by fluorescence intensity in 10 cells in each of the three regions per embryo, A, anterior; L, lateral; and Ax, axial. p > 0.05 ns, **p < 0.01, ***p < 0.001.
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**AUTHOR CONTRIBUTIONS**

C.H. designed the research project and supervised and secured funding. M.J. and C.H. wrote the manuscript, with input from F.A.G. M.J. carried out all of the experiments. F.A.G. developed the program codes and carried out the cell tracking and analysis and quantification of the E-cadherin boundary distribution. T.F. carried out the rx3 and emx3 and dkk1 RNAscope in situ hybridization assays.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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### STAR Methods

#### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse Anti-beta-Catenin Monoclonal Antibody Clone 15B8 | Sigma-Aldrich | Cat# C7207: RRID:AB_476865 |
| Purified Mouse Anti-E-cadherin antibody | BD Biosciences | Cat# 610181: RRID:AB_397580 |
| Phospho-Smad1/5 (Ser463/465) (41D10) Rabbit mAb antibody | Cell Signaling Technology | Cat# 9516: RRID:AB_491015 |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-11001: RRID:AB_2534069 |
| **Bacterial and Virus Strains** |        |            |
| NEB® 5-alpha Competent E. coli (High Efficiency) | New England Biolabs | Cat# C2987H |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Alexa Fluor™ 568 Phalloidin | Life Technologies | Cat# A12380 |
| Blocking Reagent | Roche | Cat# 11096176001 |
| NBT | Roche | Cat# 11585029001 |
| BCIP | Roche | Cat# 11383221001 |
| **Critical Commercial Assays** |        |            |
| mMESSAGE mMACHINE™ SP6 Transcription Kit | Ambion | Cat# AM1340 |
| MinElute PCR Purification Kit | Qiagen | Cat# 28004 |
| RNeasy Mini Kit | Qiagen | Cat# 74104 |
| RNAse/™ Multiplex Fluorescent Reagent Kit | Advanced Cell Diagnostics | Cat# 320850 |
| T3 RNA Polymerase | Roche | Cat# 11031163001 |
| T7 RNA Polymerase | Roche | Cat# 10881767001 |
| DIG RNA Labelling Mix | Roche | Cat# 11277073910 |
| Fluorescein RNA Labelling Mix | Roche | Cat# 11685619910 |
| SIGMAFAST Fast Red TR/Naphthol AS-MX Tablets | Sigma | Cat# F4523-5SET |
| Mini Quick Spin RNA Columns | Roche | Cat# 11814427001 |
| **Experimental Models: Organisms/Strains** |        |            |
| Zebrafish: AB Wild type Danio rerio | Zebrafish International Resource Center (ZIRC) | N/A |
| Zebrafish: Tg(-1.8gsc:GFP);ml1 | Doitsidou et al., 2002 | ZFIN: ZDB-TGCONSTRCT-070117-4 |
| Zebrafish: Tg(actb2:myl2.1-EGFP) | Behrndt et al., 2012 | ZFIN: ZDB-TGCONSTRCT-130108-2 |
| Zebrafish: Tg(hsp70:dkk1b-GFP) | Stoick-Cooper et al., 2007 | ZFIN: ZDB-TGCONSTRCT-070403-1 |
| Zebrafish: axin1tm213 | Heisenberg et al., 2001 | ZFIN: ZDB-FISH-150901-23659 |
| **Oligonucleotides** |        |            |
| Morpholino: MO-dkk1 TAGAGAAGCATGCGATGTGACATCAT | GeneTools | ZFIN: ZDB-MRPHLNO-070604-2 |
| Morpholino: MO-lefty1 CGCGGACGAAAGTACATCTTTTTCAAG | GeneTools | ZFIN: ZDB-MRPHLNO-070410-3 |
| Morpholino: MO-lefty2 AGCTGAGATAGAACAGAGGACATGCT | GeneTools | ZFIN: ZDB-MRPHLNO-070410-4 |
| Morpholino: MO-tcf3 CTCGGTTAATGAGCAGGTGTG | GeneTools | ZFIN: ZDB-MRPHLNO-060705-5 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marie Johansson (marie.johansson@kcl.ac.uk). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish (Danio rerio) were maintained at 28°C on a 14 hr light/10 hr dark cycle (Brand et al., 2002). Embryos collected were cultured in fish water. The zebrafish lines used in this study were wild type (wt) AB from the Zebrafish International Resource Center (ZIRC), Tg(-1.8gsc:GFP)ml1 (Doitsidou et al., 2002), Tg(actb2:myl12.1-EGFP) (Behrndt et al., 2012), Tg(hsp70l:dkk1b-GFP) (Stoick-Cooper et al., 2007) and axin1tm213 (Heisenberg et al., 2001). Homozygous Tg(-1.8gsc:GFP)ml1 and homozygous Tg(actb2:myl12.1-EGFP), respectively, were incrossed for experiments. Homozygous Tg(hsp70l:dkk1b-GFP) were outcrossed with wt AB fish to generate heterozygotic embryos. Tg(hsp70l:dkk1b-GFP) embryos were heat shocked at 37°C for 25 minutes, 1 hr before either live-imaging or fixation. The animal experimentations were authorized by the KCL Ethic Review Committee under the HO license P70880F4C.

METHOD DETAILS

Plasmids and Constructs

The plasmid encoding the actin binding domain of utrophin fused to monomeric red fluorescent protein (mRFP-utrophin) (Burkel et al., 2007) was kindly provided by John Wallingford, University of California Berkeley, USA. The plasmid encoding full-length zebrafish Dkk1 was kindly provided by Makoto Furutani-Seiki, University of Bath, UK.

RNA and Morpholino Injections

Capped RNAs were transcribed using the mMESSAGE mMACHINE™ SP6 Transcription Kit (Ambion). Embryos were injected at one-cell stage with 7.5 pg full-length dkk1 RNA, or 50 pg histone2B-RFP RNA, or 50 pg mRFP-utrophin RNA (Burkel et al., 2007) RNA, or 4 pg lefty1 RNA (Thisset and Thisset, 1999). For knockdown experiments, embryos at one-cell stage were injected with 0.1 pmol of Lefty1 morpholino together with 0.1 pmol of Lefty2 morpholino (Agathon et al., 2001), 0.2 pmol Tcf3a morpholino (Dorsky et al., 2003), 0.4 pmol of Vangl2 morpholino (Williams et al., 2012), or 0.4 pmol Dkk1 morpholino (Caneparo et al., 2007) (GeneTools).

In Situ Hybridization and RNAscope

In situ hybridization was carried out as described in Xu et al. (1994). Plasmids containing emx3 and rx3 constructs were linearized and the DNA was purified using Minelute PCR Purification kit (Qiagen). In vitro transcription was carried out according to manufacturer’s instructions using DIG Labelling Mix (Roche) or fluorescein Labelling Mix (Roche). Probes were purified using mini Quick Spin columns (Roche). Embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and washed with PBS/0.1% Tween-20 (PBST) and dehydrated with methanol before storage at -20°C. Embryos were progressively rehydrated on ice with PBST. Embryos were incubated in 5xSSC, 1% Tween-20, 0.5 mg/ml torula RNA, 50μg/ml heparin, 0.1% CHAPS, 50% formamide (hybridization buffer) at 65°C for several hours before incubation with probes overnight. Embryos were washed with hybridization buffer followed by several washes with pre-warmed 2xSSC/0.1% CHAPS over several hours, followed by several washes with 0.2xSSC/0.1% CHAPS. Embryos were then washed 4× with MAB/0.1% Tween-20 (MABT) at room temperature and blocked for at least an hour with 2% Blocking Reagent (Roche). Embryos were incubated at 4°C with 1:4000 anti-digoxigenin-AO (Roche) in blocking solution and washed with MABT several times NBT/BCIP (Roche). Embryos were washed with PBST and fixed for an hour at room temperature with 4% PFA, washed with MABT and incubated with 1:1000 anti-Fluorescin-AP (Roche) in blocking solution. Fast Red (Sigma) staining was carried out according to manufacturer’s instructions. Embryos were then fixed for one hours in 4% PFA and stored in 70% glycerol.

For RNAscope™ (Advanced Cell Diagnostics) in situ hybridization, embryos were fixed, dehydrated and rehydrated in the same manner as described for wholemount in situ hybridization and subsequently treated as described (Gross-Thebing et al., 2014),
with minor modifications. Probes for gsc and dkk1 (Advanced Cell Diagnostics) were mixed and pre-warmed to 40°C before cooling to room temperature. Embryos were incubated with the probe mixture overnight at 45°C. Embryos were washed three times with 0.2x SSC/0.01% Tween-20 (SSCT) and fixed with 4% PFA for ten minutes at room temperature. Embryos were incubated at 45°C with AMP1 (ACD) for 30 minutes, AMP2 (ACD) for 15 minutes and Amp3 for 30 minutes with washes between each incubation. Detection was carried out with the Amp4 AltA Colour Module. Embryos were washed with 0.2xSSCT and stored in PBS at 4°C.

**Immunofluorescence and Confocal Microscopy**

For immunostaining, paraformaldehyde-fixed embryos were washed with PBS, permeabilized with 0.5% Triton X-100/1%DMSO/PBS and blocked with 1% bovine serum albumin/PBS at room temperature for 1 hours. Embryos were incubated with anti-E-cadherin 1:300 (BD Biosciences), anti-β-catenin 1:500 (Sigma), or anti-phospho-Smad 1/5 1:200 (Cell Signaling Technology) at +4°C overnight, washed with PBS and incubated with Alexa Fluor 488/568 secondary antibody (Life Technologies) at +4°C overnight. Alexa Fluor 568 phalloidin (Life Technologies) was used at 1:2000. Confocal imaging was carried out with a Nikon Eclipse C1 microscope. Images were processed using ImageJ.

**Cell Track Analysis**

For global analysis of axial cell migration, dechorionated embryos were mounted in 0.7% low melting point agarose in fish water. Z-stacks were collected from 70% to 90% epiboly at 2-min intervals on a thermostated Leica SP5 confocal microscope. Nuclei were automatically tracked by using Imaris software (Bitplane). Tracks were manually corrected and validated. Speed and persistence analyses were performed in MATLAB. For each tracked embryo, instant speed was computed for every cell on every time interval. The average velocity vector was then calculated, and all 3-d tracks were rotated to align the velocity vector with the x axis. For each embryo, tracks were then registered relative to the position of the front of the prechordal plate (for x axis) and, between embryos, tracks were registered relative to the center of the plate (y and z axes). Instant speed and persistence were computed for each cell. Instant speed was computed over 2-min intervals. Persistence was defined as the ratio between net displacement and total displacement, computed over 10-min intervals (Dumontier et al., 2012).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Axial Cell Progression**

Axial progression was determined by calculating the anteriormost position of gsc-positive cells assessed by in situ hybridization. The radius (r') of the embryo (see schematic in Figure S2B) was used to calculate the perimeter of the embryo. Angles were measured between the margin (m') to the anteriormost position of the prechordal plate (g') and from the margin (m') to the animal pole (a'). The perimeter was used to convert the measured angles to distance (perimeter/360 x measured angle degree). The relative progression of the prechordal plate was calculated as a fraction of the distances between these two points. The correction value for epiboly was calculated as a fraction of the distance between the margin (m') and the animal pole (a') and the distance between the animal and vegetal pole (v', A-V angle = 180 deg). The relative prechordal plate distance was then divided by the obtained epiboly value to get a final corrected progression value for each embryo.

**Protein Distribution and Colocalization**

For quantitation of relative E-cadherin distribution, 12 μm confocal z-stacks spanning the axial cell population depth were acquired. For each embryo, an area of six neighboring axial cells was selected at 2–3 μm from the optical section at the highest position. Fluorescence intensity profiles were measured with ImageJ across boundaries between neighboring cells, and normalized per embryo. Data were processed with R. Pearson’s R value was determined using the Coloc 2 plugin in ImageJ. Randomized regions of interest were created by vertically flipping the single plane image on one channel. Distribution of anti-phospho-Smad 1/5 was quantified using the Plot Profile plugin in ImageJ.

**Cell Shape Analysis**

Cell angle, area and aspect ratios were quantified in dorsally mounted embryos using fit ellipse in ImageJ. For quantification of β-catenin and Dkk1 GFP colocalization, ten regions of interest spanning the boundary plasma membrane between interconnected cells were specified on a single optical section in each embryo.

**Statistics**

The number of embryos (n) and samples (n) and definition of statistical significance are indicated in the figure legends. Mean values were calculated and plotted using GraphPad Prism (GraphPad Software, San Diego, United States of America). Unpaired t-tests were used to compare means. Kolmogorov-Smirnov test was used to compare distributions. When multiple measurements were carried out on the same embryo, linear mixed-effects models were used to take into account resampling of the same statistical unit.

**DATA AND CODE AVAILABILITY**

No large-scale datasets or new code were generated in this study.