RESEARCH ARTICLE

Syndecan defines precise spindle orientation by modulating Wnt signaling in C. elegans
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ABSTRACT
Wnt signals orient mitotic spindles in development, but it remains unclear how Wnt signaling is spatially controlled to achieve precise spindle orientation. Here, we show that C. elegans syndecan (SDN-1) is required for precise orientation of a mitotic spindle in response to a Wnt cue. We find that SDN-1 is the predominant heparan sulfate (HS) proteoglycan in the early C. elegans embryo, and that loss of HS biosynthesis or of the SDN-1 core protein results in misorientation of the spindle of the ABar blastomere. The ABar and EMS spindles both reorient in response to Wnt signals, but only ABar spindle reorientation is dependent on a new cell contact and on HS and SDN-1. SDN-1 transiently accumulates on the ABar surface as it contacts C, and is required for local concentration of Dishevelled (MIG-5) in the ABar cortex adjacent to C. These findings establish a new role for syndecan in Wnt-dependent spindle orientation.

KEY WORDS: Heparan sulfate, Proteoglycan, Embryo, Endocytosis, Dishevelled, C. elegans

INTRODUCTION
Mitotic spindle orientation defines cell division orientation and plays crucial roles in animal development and tissue homeostasis (Gillies and Cabernard, 2011; Inaba and Yamashita, 2012; Lu and Johnston, 2013; Morin and Bélair, 2011; Siller and Doe, 2009). Aberrant cell division orientation is associated with neurological diseases and cancer (Noatynska et al., 2012; Pease and Timmerman, 2011). Cells can orient their spindles according to default intrinsic rules, or in response to external cues. Wnt/Frizzled signaling is a widespread extrinsic cue for mitotic spindle orientation (Segalov and Bellaiche, 2009). In the C. elegans early embryo, mitotic spindle orientation in the EMS and ABar blastomeres is regulated by directional Wnt/MOM-2 signals, acting in parallel to a Src-dependent pathway (Hardin and King, 2008; Park and Press, 2003). EMS cell fate determination is also dependent on these pathways, in which a Wnt signal pathway regulates transcription in a β-catenin/WRM-1-dependent manner (Sawa and Kurosawa, 2013). In contrast to cell fate determination of the EMS blastomere, spindle orientation of the EMS and ABar blastomeres is regulated in a β-catenin/WRM-1-dependent but transcription-independent manner (Cabello et al., 2010; Kim et al., 2013; Walston et al., 2004).

Although the above studies have shown the involvement of Wnt signaling in spindle regulation, it is not fully understood how Wnt signaling is spatially regulated to ensure precise spindle orientation. In vitro, a localized Wnt signal is sufficient to orient embryonic stem cell divisions (Habib et al., 2013). In C. elegans and vertebrates, Wnt signaling can be regulated at the subcellular level by controlling localization of downstream components (Lancaster et al., 2011; Mizumoto and Sawa, 2007; Taelman et al., 2010). However, how local transduction of a Wnt cue is established, maintained and terminated during mitosis is poorly understood.

Heparan sulfate proteoglycans (HSPGs) influence Wnt signaling and Wnt gradient formation in many systems (Liu, 2004). HSPGs are composed of negatively charged linear polysaccharides, composed of heparan sulfate (HS), which are attached to a core protein (Bishop et al., 2007). Interaction of HSPGs with Frizzled (Fz) receptors and Wnt ligands is thought to promote internalization of receptor-ligand complexes, which in turn either positively or negatively regulate extracellular Wnt ligand distribution and Wnt signaling (Gagliardi et al., 2008; Ohkawara et al., 2011). This biphasic activity of HSPGs is influenced by the ratio of ligand to receptor and co-receptors (Yan et al., 2009), the stability of free ligand (Kleinschmidt et al., 2013) and possibly by specific HS structures (Ai et al., 2003). Additionally, the HSPG core protein can modulate signaling independently of or in conjunction with its HS side chains.

In C. elegans, HS synthesis is essential for embryonic morphogenesis (Kitagawa et al., 2007). However, the cellular role of HSPGs in embryonic morphogenesis has remained unclear. The membrane-spanning HSPG syndecan (SDN-1) has been identified as a negative regulator of Wnt/e-gl-20 in distal tip cell migration (Schwabiuk et al., 2009), but it is not known whether HSPGs are involved in other Wnt-dependent processes. Here, we show that C. elegans embryos express the HSPG syndecan/SDN-1 from the one-cell stage onwards. We show that SDN-1 is required for a specific Wnt-dependent spindle orientation signal in the context of a newly formed cell-cell contact. Our results indicate that HSPGs can regulate precise spindle orientation by modulating Wnt signaling.

RESULTS
SDN-1 is the predominant HSPG core protein in the early embryo
To understand the roles of HSPGs in early C. elegans embryogenesis, we first examined the expression of total HSPGs by immunostaining. The antibody 3G10 (David et al., 1992; Minniti et al., 2004) recognizes stubs of HS chains formed by heparitinase cleavage, allowing the detection of all HS-modified proteins. We detected total HS (3G10) in one-cell stage embryos, which displayed patchy but specific 3G10 staining on the cell surface (Fig. 1Aa); at later stages, HS was concentrated at cell contacts (Fig. 1Ab-f; supplementary material Fig. S1A). We did not detect 3G10 staining in the absence of heparitinase treatment (Fig. 1B; data not shown). We noted that in two- and four-cell stage embryos, expression of total HS was higher in anterior cells (AB and ABA/
ABp) than in posterior cells (P₁ and P₂/EMS) (Fig. 1Ab,c). At the eight-cell stage (~30 min post first cleavage, pfc), we detected HS on the surfaces of all blastomeres. HS was distributed at most cell-cell interfaces, but was highly concentrated at the contact site between ABar and C (Figs 1Ad and 2). We noticed that during ABar mitosis, HS localized to an intracellular punctum in ABar, close to the ABar-C cell contact site (Fig. 1Ae). Expression of HS gradually increased during embryogenesis, and by comma stage (395 min pfc) was visible on the surface of almost all cells (supplementary material Fig. S1B).

C. elegans encodes multiple HSPG core proteins, of which syndecan/SDN-1 and glypican/GPN-1 are HS modified (Hudson et al., 2006; Minniti et al., 2004). To determine which core proteins are expressed in early embryos, we examined total HS expression in embryos lacking known HSPG core proteins using null or strong loss-of-function mutants. UNC-52/perlecan was not expressed in early embryos, as judged by MH3 antibody staining (not shown), confirming previous results (Mullen et al., 1999). 3G10 staining of gpn-1(ok377), lon-2(e678) or agrin/agr-1(tm2051) embryos was indistinguishable from that of wild type (not shown). By contrast, 3G10 staining was undetectable in sdn-1(zh20) or sdn-1(ok449) embryos prior to the AB16 (28-cell) stage (Fig. 1C,D). In sdn-1(zh20) embryos, we detected 3G10 staining in a few posterior cells at the AB32 stage, suggesting that expression of additional HSPGs begins between the AB 16 and AB32 stages. We generated a functional SDN-1::GFP translational reporter under the control of its own promoter and 3′UTR (juSi119); using anti-GFP immunostaining, we detected SDN-1::GFP expression by the eight-cell stage (Fig. 1E), similar to the pattern of 3G10 staining. These observations suggest SDN-1 is the predominant HSPG core protein in early C. elegans embryos.
protein expressed in early embryos, and that SDN-1::GFP reflects endogenous SDN-1/HS expression.

**HS synthesis and SDN-1 are required for proper ABar division orientation**

SDN-1 is required for numerous aspects of post-embryonic development and morphology (Rhiner et al., 2005), and in ventral cleft closure during mid-embryogenesis (Hudson et al., 2006); however, it had not previously been implicated in early embryonic development. To assess the role of SDN-1 expression in the early embryo, we examined sdn-1(zh20) null mutant embryos using time-lapse DIC microscopy. sdn-1 mutants developed normally until the eight-cell stage, and displayed normal orientation of the EMS division (Figs 2 and 3A,B; supplementary material Movies 1 and 2). However, the division axis of ABar was consistently misoriented in sdn-1(zh20). We observed similar ABar division orientation defects in the HS synthesis mutants rib-1(tm516), rib-2(tm710) and hst-1(ok1068) (Fig. 3C, supplementary material Movie 3; data not shown). In wild-type embryos, the ABar spindle undergoes a distinctive rotation so that ABar divides orthogonally to the axes of division of the other AB granddaughters: ABal, ABpl and ABpr. In HS synthesis and sdn-1 mutants, ABar typically divided parallel to ABpr (five out of five embryos each for rib-1(tm516), hst-1(ok1068) and rib-2(tm710); eight out of 10 for sdn-1(zh20)].

These observations used visual estimation of the division axis of ABar spindle reorientation relative to the onset of anaphase, defined as the beginning of chromosome separation (0 min). The ABar centrosomes start to migrate ~8 min before the onset of anaphase by ~7 to ~6 min, the two centrosomes reached opposite positions the nucleus. ABar begins to contact C at about ~7 to ~6 min. We analyze the ABar spindle by tracking centrosomal asters (TBB-2::GFP) from ~5.5 min, or analyze the ABar division angle, as defined by the orientation of daughter nuclei (HIS-72::GFP) 1 min after the onset of ABar anaphase.

**SDN-1 is required for proper orientation of the mitotic spindle in ABar**

To analyze ABar spindle orientation directly in these mutants, we labeled microtubules using GFP::TBB-2 (ojIs1) (Strome et al., 2001) and quantitated spindle dynamics, using NucleiTracker 4D software to track centrosomal asters. Previous studies indicated that the wild-type ABar spindle initially aligns parallel to the ABpr spindle, after which the aster closest to the ABar contact rotates to adopt the proper orientation before mitosis (Fig. 2) (Walston et al., 2004). The ABar-C contact is partly dependent on Wnt/MOM-2 (Pohl and Bao, 2010) and on the cell adhesion molecules L1CAM/SAX-7 and cadherin/HMR-1 (Grana et al., 2010). Using DIC microscopy, we observed that the ABar and C blastomeres form normal contacts in sdn-1 mutant embryos, starting 5.6±0.4 min (mean±s.d., n=10) before the onset of anaphase in ABar, similar to the wild type (6.1±0.3 min, n=10). Likewise the ABar-C cell contact appeared normal in rib-1 mutant embryos (supplementary material Movie 3). We conclude that neither SDN-1 nor HS are required for ABar to contact C, suggesting that the spindle orientation defects in these mutants arise from a failure in signaling.

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et al., 2004). Under our imaging conditions, in which we use bead mounting to reduce embryo compression during imaging (Giurumescu et al., 2012), we found that in wild-type embryos ABar astral arrays were initially set up in variable orientations, and by metaphase become oriented towards the ABar-C contact independent of their initial orientation (Fig. 4A,B; supplementary material Movie 4).

To assess this observation quantitatively, we measured ABar spindle orientation relative to the AP axis of the embryo. In the wild type, the ABar spindle becomes oriented with the posterior aster dorsal and the anterior ventral. The ABar spindle axis also has a large left-right component, in that the posterior pole lies on the left-hand side of the embryo. In \textit{sdn-1} mutants, the ABar spindle is initially variable in the AP, DV and LR planes, and rotates to an orientation that is consistent with respect to the AP-DV axis, but variable in the LR axis. Thus, ABar angles measured relative to the AP axis are initially variable but converge on an angle that is smaller than in the wild type (Fig. 4C-F). We also measured ABar orientation relative to the other AB granddaughters; ABar angles measured relative to the ABpr spindles were more variable in \textit{sdn-1} mutants because of the larger LR component of these angles (data not shown). The mean orientation of the ABar spindle in \textit{sdn-1} mutants was affected throughout the ABar cell cycle (Fig. 4E; supplementary material Movie 5). The abnormal spindle dynamics in \textit{sdn-1(zh20)} were rescued by overexpression of SDN-1::GFP driven by the germline-specific \textit{mex-5} promoter and the germline-permissive \textit{tbb-2} 3’UTR (Merritt et al., 2008) (Fig. 4E). We also observed that overexpression of SDN-1::GFP resulted in significantly lower variance in the orientation of ABar astral arrays throughout mitosis without affecting mean orientation (Fig. 4F). These observations suggest SDN-1 is not required for spindle rotation per se, but modulates a cue that orients the astral microtubule array.

**SDN-1 acts in a Wnt-dependent spindle orientation pathway**

ABar spindle orientation requires two partly redundant signaling pathways: a Wnt pathway involving Wnt/MOM-2, Fz/MOM-5 and the Dishevelled proteins DSH-2 and MIG-5; and a receptor tyrosine kinase MES-1 that acts via Src/SRC-1 (Fig. 5A) (Walston et al., 2004). HSPGs, including syndecans, regulate Wnt signaling in many contexts (Lin, 2004; Munoz et al., 2006; Ohkawara et al., 2011), yet the involvement of HSPGs in Wnt-dependent spindle orientation has not been reported. Syndecan has also been implicated in Src-dependent stabilization of focal adhesions in fibroblasts (Morgan et al., 2013). To determine whether SDN-1...
Fig. 4. SDN-1 constrains the orientation of the ABar spindle but is not essential for its rotation. (A–D) In the wild type and in sdn-1 mutants, ABar spindles are initially set up with variable orientations. Two representative examples are shown from each genotype, in which centrosomes of ABar initially set up close to their final axis (‘less rotated’, A,C) or perpendicular to the final division axis (‘rotated’, B,D). ‘ar’ and ‘pr’ indicate the astral arrays of ABar and ABpr, respectively. Time-lapse images of GFP-labeled β-tubulin (TBB-2::GFP) in embryo (upper panels) or ABar (lower panels) of wild type (AB) and sdn-1(zh20) (C,D). Time in minutes relative to onset of anaphase. Lower images show higher magnification views of TBB-2::GFP in ABar from the same embryos. Scale bars: 10 μm (upper panels) and 5 μm (lower panels). (E) To quantify the orientation of the ABar spindle in the anteroposterior/dorsoventral (AP/DV) plane, we calculated the angle θ between the axis of the ABar asters and the overall AP axis of the embryo. θABar mean is plotted over time relative to the onset of anaphase. The θABar mean in sdn-1(−) is significantly different at t=−5 min (early prophase) and 0 min (onset of anaphase), which is rescued by SDN-1::GFP. Data were analyzed using ANOVA followed by Dunnett’s test: **P<0.01. sdn-1(−) and sdn-1(++) indicate sdn-1(zh20) and an integrated single-copy transgene juSi98[Pmex-5-SDN-1::GFP-tbb-2 3' UTR], which overexpresses SDN-1::GFP in the early embryo, respectively. (F) Bar graphs showing θABar standard deviation of indicated genotypes at t=−5 min (early prophase) and 0 min (onset of anaphase). The variance of ABar spindle orientation in the AP/DV plane is significantly higher in sdn-1 than in wild type at the onset of anaphase, whereas it is smaller in sdn-1(++) (F-test: *P<0.05). The ABar spindle angle is calculated by tracking centrosomal asters (TBB-2::GFP) from −5.5 min prior to onset of ABar anaphase.

SDN-1 accumulates on the ABar protrusion and then at the ABar-C contact, and is then bi-directionally internalized into ABar or C

To address how SDN-1 modulates Wnt-dependent spindle orientation, we analyzed the dynamics of SDN-1 subcellular localization. The SDN-1::GFP transgene expressed under endogenous control elements (Fig. 1E) rescued the ABar division orientation defects of sdn-1(zh20) (supplementary material Fig. S5C,D), but its fluorescence level was too low for live imaging. For live imaging, we overexpressed SDN-1::GFP using the germline-specific mex-3 promoter. Pmex-5-SDN-1::GFP (juSi99) colocalized with the membrane marker pleckstrin homology domain PH::mCherry (Kachur et al., 2008) and appeared uniform on the surface of ABar and C at the six-cell stage (supplementary material Movie 6). To correlate SDN-1::GFP localization with cell division dynamics, we also expressed SDN-1::GFP with HIS-48::mCherry (supplementary material Fig. S3). When ABar was about to contact C, SDN-1::GFP began to accumulate on the tip of ABar closest to C (~1.6±0.3 min from cell contact, n=18, Fig. 6A); this SDN-1::GFP accumulation persisted 6.9±1.1 min after contact with C (n=18, Fig. 6B,D; supplementary material Fig. S5B). Three-dimensional reconstruction from orthographic views revealed...
that SDN-1 accumulation on ABar has ABpr and/or ABpl immediately underneath it (Fig. 6A, right). Subsequently, SDN-1::GFP formed a ∼0.5 µm diameter punctum either in ABar (13 out of 18, Fig. 6B) or in C (four out of 18, Fig. 6C, supplementary material Movie 7). These puncta may reflect internalization of the entire SDN-1 protein, as we observed similar structures using 3G10 immunostaining, which reflects endogenous SDN-1 HS chains (Fig. 1Ae). As the behavior of SDN-1::GFP-enriched puncta on ABar resembled that of midbody remnants (Singh and Pohl, 2014), we tested whether endogenous HS localizes to midbody remnants using the midbody marker ZEN-4::GFP. At the eight-cell stage, although the midbody remnant in P2 (originating from P1) did not contain HS, the midbody remnant localizing to the ABar-C interface showed strong HS expression (Fig. 6E), suggesting SDN-1 associates with a subset of midbody remnants.

How is SDN-1 enriched in the protrusion of the ABar blastomere? Syndecans are clustered by extracellular ligand stimulation through their HS side chains (Tkachenko and Simons, 2002). SDN-1 contains three potential glycosaminoglycan (GAG) attachment sites, i.e. Ser-Gly motifs; based on sequence context, only the first two Ser-Gly motifs are likely to be modified (Minniti et al., 2004). We expressed mutant forms of SDN-1, in which the first two putative GAG attachment sites were mutated (S71A, S86A or 2×S>A). When expressed under the control of the mex-5 promoter, SDN-1::GFP induced strong expression of total HS in sdn-1(zh20) early embryos (supplementary material Fig. S4C). SDN-1(2×S>A)::GFP resulted in much weaker but detectable HS immunoreactivity in sdn-1(zh20) early embryos (supplementary material Fig. S4D), suggesting that although these GAG attachment sites are predominant, they may not account for all early embryonic HS. As the third potential GAG attachment site (S214) does not appear to contribute to early embryonic HS (supplementary material Fig. S4E), we conclude that S71 and S86 are the major HS modified sites in SDN-1, and that other sources of HS may account for the residual HS detected in these embryos (see Discussion). SDN-1(2×S>A)::GFP enrichment on the ABar protrusion before cell contact was delayed relative to wild type (−0.7±0.2 min before cell contact, n=10, supplementary material Fig. S5B). The SDN-1 (2×S>A)::GFP accumulation remained 12.2±1.7 min after contact with C (n=10, supplementary material Fig. S5B).

We next addressed whether the intracellular domain of SDN-1 is required for SDN-1 dynamics. To test this, we examined GFP tagged-
SDN-1 lacking its cytoplasmic domain (SDN-1ΔC). Although SDN-1ΔC::GFP accumulated on the tip of ABar before cell contact (supplementary material Fig. S5B), endocytosis of this mutant form was delayed and less frequent compared with wild-type SDN-1::GFP (supplementary material Fig. S5B). We attempted to express SDN-1 lacking both its cytoplasmic domain and GAG attachment sites (S71A, S86A). However, this mutant form of SDN-1::GFP was not correctly localized on the cell surface (supplementary material Fig. S5A).

To address the importance of GAG modification and cytoplasmic domain of SDN-1, we next examined whether SDN-1ΔC::GFP and SDN-1ΔC::GFP expressed under the control of the sdn-1 promoter and 3′ UTR could rescue abnormal ABar spindle dynamics in sdn-1(zh20). SDN-1::GFP (wild type) rescued both the variable initial spindle orientation and the continuously misoriented ABar spindle phenotypes of the sdn-1(zh20) mutant (supplementary material Fig. S5A, D). SDN-1ΔC::GFP rescued the variable initial spindle orientation, but failed to fully rescue the continuous misorientation of the ABar spindle, suggesting the SDN-1 cytoplasmic domain is required for precise spindle orientation regulated by Wnt. Correlating with its ability to restore low levels of HS expression, SDN-1ΔC::GFP rescued both sdn-1 phenotypes.

Wnt/MOM-2 defines the site of SDN-1 accumulation, which in turn is required for local accumulation of MIG-5/Dsh

To examine the effect of Wnt signaling on SDN-1 accumulation, we tested SDN-1::GFP dynamics in mom-2 RNAi and dsh-2 mig-5 double RNAi-treated embryos. Depletion of mom-2 by RNAi eliminated SDN-1::GFP accumulation on ABar; instead, we observed premature SDN-1::GFP accumulation on C or ABpl during early prophase of ABar (Fig. 7B,C). We did not observe premature accumulation of SDN-1::GFP in dsh-2 mig-5 double RNAi embryos (Fig. 7A,C), suggesting that MOM-2 engagement rather than downstream Wnt signal transduction defines the location of the site of SDN-1::GFP accumulation.
However, unlike SDN-1::GFP, MIG-5::GFP accumulation was not observed before cell contact. MIG-5::GFP accumulation on the ABar-C contact site was reduced in embryos treated with mom-2 RNAi, even in embryos where ABar-C contact occurred normally, suggesting that local accumulation of MIG-5::GFP might reflect activation of Wnt signaling rather than contact itself (Fig. 7E,G). Importantly, MIG-5::GFP accumulation on the ABar-C contact site was significantly reduced in sdn-1(zh20) (Fig. 7F,G), indicating that SDN-1 is required for MIG-5 accumulation. Dishevelled overexpression has been shown to rescue defective convergent extension caused by loss of glypicans 4 and syndecan 4 in Xenopus (Munoz et al., 2006; Ohkawara et al., 2003). However, overexpression of MIG-5::GFP (4- to 5-fold overexpression; data not shown) did not rescue the ABar misorientation phenotype in sdn-1(zh20) (Fig. 7H), suggesting that SDN-1 localization does not simply enhance Wnt signaling but provides a positional cue.

**DISCUSSION**

This study reveals a highly specific requirement for syndecan in Wnt-dependent mitotic spindle regulation. Our results support a model in which SDN-1 functions in the Wnt signaling pathway, either at the level of Wnt/MOM-2 or Fz/MOM-5, to promote ABar spindle reorientation. Localization of SDN-1 on ABar requires MOM-2, and SDN-1 is required for localization of Dsh/MIG-5. Moreover, the difference between the sdn-1 phenotype and the dsh-2 mig-5 phenotype can be explained if SDN-1 restricts Wnt signaling. Speculatively, syndecan/SDN-1 on the ABar surface might concentrate either Wnt/MOM-2 or its receptor Fz/MOM-5. The HS side chains of SDN-1 might allow this process to begin prior to physical contact with the C blastomere, triggering SDN-1 clustering (Fig. 8). SDN-1 accumulation could concentrate Wnt/MOM-2 onto the ABar protrusion, which in turn weakly orients the mitotic spindle in ABar towards C. In addition, we have analyzed ABar spindle dynamics in sdn-1(zh20) dsh-2 mig-5 double RNAi, and find that they resemble dsh-2 mig-5 double RNAi, i.e. no spindle rotation. This epistasis test shows that the variable rotation in sdn-1 mutants requires Dishevelled activity, consistent with our model. At present, reagents to visualize Wnts or their receptors in the early embryo are not available. Our attempts to generate MOM-2 transgenic animals have so far been unsuccessful, and antibodies to MOM-2 have not been generated. MOM-5::GFP expression is not detectable in the early embryo (Park et al., 2004). Wnt ligands and receptors may be expressed transiently or at low levels in the early embryo, necessitating the involvement of accessory proteins such as SDN-1. We do not yet know whether SDN-1 function is required in the Wnt-sending or Wnt-receiving cell, or both, and we cannot yet exclude models in which HSPG/SDN-1 are required in both cells. Our results support a role for HS or SDN-1 in the orientation of the EMS spindle towards P2 (supplementary material Fig. S6). An explanation for the differential requirement for HSPGs in EMS versus ABar is that in the case of EMS/P2, the interacting cells are sisters and are in direct contact throughout their cell cycle. By contrast, reorientation of ABar towards C undergoes spindle reorientation. Localization of SDN-1 on ABar requires HSPG activity, consistent with previous studies (Walston et al., 2004). However, we find (at most) a very minor role for HS or SDN-1 in the orientation of the EMS spindle towards P2 (supplementary material Fig. S6). An explanation for the differential requirement for HSPGs in EMS versus ABar is that in the case of EMS/P2, the interacting cells are sisters and are in direct contact throughout their cell cycle. By contrast, reorientation of ABar towards its non-sister cell C requires formation of a new cell-cell contact, and therefore involves additional signal-concentrating or amplifying proteins such as SDN-1. The more elongated cell shape of EMS compared with ABar also suggests that EMS may be intrinsically...
more able to orient its spindle along its long axis (Hertwig’s rule) even in the absence of positional cues (Goldstein, 1995).

Cytokinetic midbody remnants have been recently shown to contribute to orientation of mitotic spindles in the early embryo (Singh and Pohl, 2014). We found that total HS colocalized with midbody remnants on the ABar-C contact site. Some, but not all, midbody remnants contain total HS, suggesting that HSPGs may be selectively recruited into midbodies. Based on its location, the midbody remnant containing HS at the 8-cell stage seems to be that generated from the division of ABp. A previous study demonstrated that the midbody remnant from ABp is inherited by MS (Singh and Pohl, 2014), different from the dynamics of SDN-1. Possibly, SDN-1 is dissociated from the midbody remnant and is internalized into ABar or C during or after ABar mitosis.

An unexpected finding in this study is that SDN-1::GFP is apparently endocytosed into the signaling or receiving cells after spindle reorientation is complete. Endocytosis can either positively or negatively regulate Wnt signaling (Gagliardi et al., 2008). In Xenopus, syndecan 4 promotes Wnt/PCP signaling by inducing clathrin-mediated endocytosis of Rspo3, a positive Wnt modulator (Ohkawara et al., 2011). SDN-1 endocytosis in ABar might promote establishment or maintenance of the external cue for mitotic spindle orientation by recruiting the signaling complex to the acidic environment where the signal is activated (Niehrs and Boutros, 2010) or by sequestering unidentified negative regulators (Gagliardi et al., 2008). Alternatively, SDN-1 endocytosis by C may be involved in signal termination, as it was observed after ABar was in anaphase (Fig. 6C and supplementary material Fig. S5B). As SDN-1 is expressed at high levels in ABar and at lower levels in C, it is unclear whether the endocytosis of SDN-1 into C reflects endocytosis of SDN-1 on the C cell surface or bidirectional endocytosis of ABar-expressed SDN-1. In any case, the variable SDN-1 dynamics after accumulation may reflect a flexible or a context-sensitive signal modulation by SDN-1, which provides robustness in oriented cell division. Our experiments demonstrated the requirement of the SDN-1 cytoplasmic domain for regulation of ABar spindle orientation and SDN-1 internalization during mitosis. However, overexpression of SDN-1 lacking its cytoplasmic domain can rescue the ABar spindle orientation phenotype (supplementary material Fig. SSC,D). This suggests that the cytoplasmic domain modulates Wnt signaling rather than playing an essential role in signal transduction. Despite our findings that HS synthesis mutants display strong ABar spindle orientation defects, overexpression of a mutant form of SDN-1 predicted to lack GAG attachments was able to rescue sdn-1 spindle orientation defects. This is reminiscent of previous findings where HSPG core proteins have been shown to

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**Fig. 8. Model for SDN-1 function in Wnt-dependent spindle orientation.**

(A) Timing of SDN-1 accumulation and endocytosis relative to ABar-C contact, MIG-5 accumulation and onset of anaphase. (B) In response to C-derived Wnt/MOM-2, SDN-1 (green) accumulates on the tip of ABar closest to C prior to cell-cell contact. The local accumulation of SDN-1 on the midbody remnant derived from ABp forms a Wnt ‘signaling platform’ that recruits downstream signaling molecules, such as Dsh/MIG-5, that then orient the ABar spindle towards the Wnt signaling platform. Alternatively, HS/SDN-1 may enhance availability of Wnt to facilitate signaling. (C) In the absence of SDN-1 the ABar spindle initially has a more variable orientation (blue bar), potentially reflecting a wider distribution of Wnt. In the absence of SDN-1, Wnt signaling platforms arise at ectopic locations and the ABar spindle orients toward these ectopically formed Wnt signaling platforms (magenta arrows), but Wnt signaling per se is not completely impaired in the absence of syndecan. We analyze the ABar spindle by tracking centrosomal asters (TBB-2::GFP) from −5.5 min, or analyze the ABar division angle, as defined by the orientation of daughter nuclei (HIS-72::GFP) 1 min after the onset of ABar anaphase.
function independently of their HS side chains (Chanana et al., 2009; Kirkpatrick et al., 2006; Williams et al., 2010; Yan et al., 2009). However, we observed weak restoration of HS expression by overexpression of these SDN-1 mutants. There are several possible explanations for this unexpected result: SDN-1 itself might be modified at additional non-canonical sites; SDN-1 overexpression may induce the expression of other HSPG core-protein(s); or overexpression of an unmodifiable SDN-1 may result in inappropriate modification of other proteins not normally HS modified. The relationship of the GAG-dependent and core-protein functions of syndecans is complex (Eriksson and Spillmann, 2012) and an important avenue for future investigation.

Syndecans might be involved in mitotic spindle orientation in other situations when the signal is transiently transmitted from nascent cell contacts, e.g. in stem cell competition for niche occupancy (Johnston, 2009; Zhao and Xi, 2010). Syndecan 1 has been shown to promote proliferation of neural progenitor cells via canonical Wnt signaling (Wang et al., 2012). Because syndecans play major roles in wound healing and cancer progression (Alexander et al., 2000; Echtermeyer et al., 2001), such a context-specific mechanism may be involved in mitotic regulation of pathology in mammals, in addition to the well-established role of syndecans in cell migration.

**MATERIALS AND METHODS**

**C. elegans strains**

Strains used are summarized in supplementary material Table S1.

**Plasmid construction and transgene generation**

Plasmids were made by Gibson isothermal assembly (Gibson et al., 2009). To make the mutated nucleotides, site-directed mutagenesis was performed with Phusion polymerase (NEB). Mos-SCI was performed as described, using strains EG4322 and EG6699 (Frøkjær-Jensen et al., 2008). Plasmids used are summarized in supplementary material Table S2.

**Immunofluorescence**

Embryos and gonads dissected from gravid adult worms were put on slides coated with poly-L-Lysine (Sigma-Aldrich), fixed in −20°C chilled methanol for 3 min, and treated with Heparan lyase II (Sigma-Aldrich) in buffer A [50 mM sodium acetate, 5 mM CaCl₂, 0.05% Tween-20 (pH 6.0)] for 2-3 h at 37°C, then blocked with TBS containing 0.2% Tween-20 and 5% BSA. After blocking, primary antibody was added and incubated overnight at 4°C. After washing twice with TBST, secondary antibody was added and incubated at room temperature (23-25°C) for 2 h. 3G10 antibody overnight at 4°C. After washing twice with TBST, secondary antibody was added and incubated with Phusion polymerase (NEB). Mos-SCI was performed as described, using strains EG4322 and EG6699 (Frøkjær-Jensen et al., 2008). Plasmids used are summarized in supplementary material Table S2.

**Measurement of total HS, SDN-1::GFP and MIG-5::GFP**

For all fluorescence intensity measurement, we used maximum intensity projections of three z-sections. The first peak obtained by a line scan (perpendicular to the cell edge) was defined as a cell border and was used to select an ROI (3×3 pixels) on the cell border, then the mean intensity was acquired. To measure the intensity of SDN-1::GFP and MIG-5::GFP, fluorescence intensity ratios were calculated by dividing the mean intensity of GFP by that of PH::mCherry. Three-dimensional projections and measurements used ImageJ.

**Confocal microscopy**

Imaging was performed as previously reported (Giurumescu et al., 2012). Briefly, living embryos were observed on LSM510 or LSM710 confocal microscopes with 100× NA 1.46 oil immersion objectives. One to three embryos obtained from four or five gravid adults were imaged in each experiment. Each data set is derived from at least six experiments. Three-dimensional stacks were acquired every minute (for HIS-72::GFP) or every 30 s (other backgrounds). Thirty-five z-sections were collected at 0.85 μm intervals for HIS-72::GFP and TBB-2::GFP imaging. To avoid photobleaching and phototoxicity, only the dorsal one-third of the embryo (10 slices, 0.85 μm intervals for SDN-1::GFP and MIG-5::GFP) was scanned. Each embryo was imaged for ~5-30 min, and we confirmed that this was not toxic to wild-type embryos. When calculating the ABxx division angle from HIS-72::GFP tracking, we compared cells 1 min after the chromosomes segregate, as this was the first time point when daughter nuclei positions are automatically determined by NucleiTracker4D.

**RNAi**

PCR was performed with primer containing T7 promoter sequence on the 5’ end using N2 (Bristol) total cDNA as a template. Primers used are listed below. Double stranded RNA (dsRNA) was synthesized using Megascript kit (Ambion), and then purified through a PCR Purification kit (QIAGEN). The purified dsRNA was injected into 1 mg/ml into young adult worms 22-28 h before analysis. Primers used for synthesis are summarized in supplementary material Table S3.

**Data analysis**

Semi-automated tracking of histone-labeled nuclei (transgene zulu178) using NucleiTracker4D was performed as previously described (Giurumescu et al., 2012). We found that the NucleiTracker4D program could also track TBB-2::GFP-labeled centrosomal asters without further modifications. The angle between two vectors in 3D space is given by the inner product of two vectors:

\[
\cos \theta = \frac{A \cdot B}{\|A\|\|B\|}
\]

After selecting four nuclei to generate two vectors, the formula is used to measure the angle between two selected vectors. The resulting angle θ ranges from 0 to 180°. We also want to measure the relative angle between the vector of two sister nuclei and the entire embryonic axis. The anterior-posterior axis (AP vector) was manually estimated from two projection images in xy plane and xz plane. The x and y points of the AP vector were decided by selecting two points in xy projection image. z points of the AP vector are decided by an identical method in an xz projection image. The angle between the AP vector and the vector of two sister nuclei is computed using the equation above.

To visualize the angles on a circular plot, the MATLAB toolbox CircStat (http://www.jstatsoft.org/v31/i10) is used. Codes are available upon request. As the range of angles used in this study was 0-180°, the angles were statistically treated as linear data. Statistical analysis used GraphPad Prism. We used the F-test for comparison of variance, Student’s t-test and Fisher’s exact test for comparison of two independent data sets. For multiple comparisons, we used one-way analysis of variance (ANOVA) followed by a Tukey or Dunnett post-hoc test for multiple comparison.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

K.D. and A.D.C. designed the experiments, analyzed data and wrote the manuscript. K.D. performed the experiments. S.K. contributed analytical tools. A.D.C., S.M. and P.C.C. supervised the project.

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