Ancestral roles of atypical cadherins in planar cell polarity

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Ancestral roles of atypical cadherins in planar cell polarity

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Fat, Fat-like, and Dachsous family cadherins are giant proteins that regulate planar cell polarity (PCP) and cell adhesion in bilaterians. Their evolutionary origin can be traced back to prebilaterian species, but their ancestral function(s) are unknown. We identified Fat-like and Dachsous cadherins in Hydra, a member of phylum Cnidaria a sister group of bilaterian. We found Hydra does not possess a true Fat homolog, but has homologs of Fat-like (HyFatI) and Dachsous (HyDs) that localize at the apical membrane of ectodermal epithelial cells and are planar polarized perpendicular to the oral–aboral axis of the animal. Using a knockdown approach we found that HyFatI is involved in local cell alignment and cell-cell adhesion, and that reduction of HyFatI leads to defects in tissue organization in the body column. Overexpression and knockdown experiments indicate that the intracellular domain (ICD) of HyFatI affects actin organization through proline-rich repeats. Thus, planar polarization of Fat-like and Dachsous cadherins has ancient, prebilaterian origins, and Fat-like cadherins have ancient roles in cell adhesion, spindle orientation, and tissue organization.

Hydra | planar cell polarity | evolution | adhesion | cadherins

Planar cell polarity (PCP) is the coordinate polarization of cells within the plane of a tissue. PCP signaling regulates a wide range of tissue organization in animal development, from hairs on insect wings, to convergent extension movements during vertebrate gastrulation, and oriented cell divisions (1). The mechanisms controlling PCP were first deciphered in Drosophila and have been shown to be conserved in bilaterians. Several modules are known to regulate planar polarity. The most studied and best understood is the “core module,” consisting of a number of transmembrane proteins and associated cytoplasmic components, such as flamingo, Frizzled, Dishevelled, Strabismus, and Prickle (reviewed in ref. 1).

Another critical regulator of planar polarity is the Fat/Dachsous (Fat/Ds) complex, consisting of two giant atypical cadherins, Fat and Ds. Fat and Ds interact via extracellular cadherin domains, and use their intracellular domains to transduce planar polarity signals, as well as to control proliferation via the Hippo pathway and to mediate metabolic control (reviewed in refs. 2 and 3). In addition, a closely related family of Fat-like cadherins, similar in their extracellular domains but with a distinct intracellular domain, can planar polarize cells and influence actin and microtubule dynamics (4–8). Although the extracellular domains of Fat and Fat-like cadherins are similar, Ds binding has only been observed with the Fat/Fat4 family of cadherins.

Fundamental questions regarding the origin and ancestral functions of PCP genes remain unanswered. For example: How did PCP evolve, and what is the minimal machinery required to polarize a cell within tissues? Homologs of PCP proteins have been identified across the animal kingdom (9–12); however, only the core PCP protein Strabismus has been studied in nonbilaterians, where it is required for gastrulation in the cnidarians Nematostella and Clytia (13, 14). Core PCP proteins have also been identified in the cnidian Hydra (15); however, the functions of Fat or Fat-like cadherins in cnidarians have not been explored.

The freshwater cnidian polyp Hydra is radially symmetric and has only two cell layers, an ectoderm and an endoderm. Both layers are composed of epithelial cells with intermingled cells of the interstitial stem cell lineage (16). The Hydra body column is a cylinder, with a head at one (oral) end and a foot at the other (aboral) end (Fig. 1A and B). The head contains the mouth and a ring of tentacles. Epithelial cells of the body column divide continuously and are constantly displaced toward the oral and aboral ends of the polyp, where they differentiate into head- and foot-specific cells and are eventually sloughed off (17). The orientation of cell divisions in the body column along the oral–aboral axis is thought to be a form of planar polarization (18). The direction of cell displacement changes when cells enter the tentacles and when a bud, Hydra’s asexual form of reproduction, is formed (Fig. 1A and B) (19). Another planar polarized feature of the Hydra body column is the organization of basal contractile protrusions of epithelial cells, which are called myonemes. Myonemes are oriented along the oral–aboral axis in the ectoderm and circumferentially in the endoderm (Fig. 1C).

In Hydra, expression of the core PCP genes frizzled and dishevelled is strongly up-regulated at the bases of the tentacles and early in bud evagination (15), suggesting that the core PCP

Significance

Planar cell polarization, PCP, describes a form of organization where every cell within a group acquires the same planar characteristics, whether it is orientation of cell division, direction of migration, or localization of a cellular structure. PCP is essential for correct organization of cells into tissues and building a proper body plan. Here we use Hydra, an organism with a single axis of symmetry and a very simple body plan to investigate the function of the cell adhesion molecules Fat-like and Dachsous. We show that Hydra Fat-like and Dachsous are planar polarized, providing a demonstration of planar polarization of proteins in a nonbilateral organism. We also discover roles for Hydra Fat-like in cell adhesion, spindle orientation, and tissue organization.

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The authors declare no competing interest.

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module is acting in those locations. However, in the body column of Hydra, despite planar polarization of myonemes, oriented cell division and directional displacement of epithelial cells (Fig. 1A and C) (18), expression of core PCP genes is low (15), suggesting that another planar polarity mechanism could be acting there.

Here we characterize Fat-like (HyFatl) and Ds (HyDs) cadherins in Hydra. We show that a Fat-like cadherin regulates tissue organization in a nonbilaterian animal. Both HyFatl and HyDs are apically localized in epithelial cells and are planar polarized along the oral–aboral axis. These data indicate that planar polarization of Fat-like and Dachsous cadherins evolved prior to the divergence of cnidarians and bilaterians. No Fat/Fat4 homolog exists in Hydra; despite planar polarization of myonemes, oriented cell division and directional displacement of epithelial cells (Fig. 1A and C) (18), expression of core PCP genes is low (15), suggesting that another planar polarity mechanism could be acting there.

We show polarization of HyFatl is dynamic and increases in areas where cell displacement rates are high. Reduction of HyFatl leads to disruption of cell contacts and morphology of actin cables in myonemes. Overexpression experiments indicate the intracellular domain of HyFatl is involved in actin organization and oriented cell division. Transgenic knockdown experiments indicate that HyFatl plays a role in tissue level organization in the body column.

**Results**

**Hydra Possesses Single Homologs of Fat-like, Ds, and CELSR Cadherins.** Cadherins of the Fat-like (Mm Fat1 and Fat3, Dm Fat2) and true Fat (Mm Fat4, Dm Fat) families have a characteristic domain composition (Fig. 1D). Our analysis of Hydra genomic, transcriptomic, and expressed sequence tag (EST) data identified only a single Fat-like cadherin gene (HyFatl) and a single Ds gene (HyDs), with no true Fat homolog. HyFatl and HyDs have a similar structure as their vertebrate orthologs Fat1 and Dchs1 (Fig. 1D). However, the intracellular domains (ICDs) of HyFatl and HyDs show only low similarity with Drosophila and vertebrate homologs (14% for HyFatl, 18% for HyDs) (SI Appendix, Fig. S1A and E), and higher similarity with orthologs from the medusozoan group of cnidarians (26% to 55% for HyFatl, 21.1 to 29.3% for HyDs) (SI Appendix, Fig. S1D and E).

The ICDs of cnidarian Fat-like proteins have three proline-rich motifs (SI Appendix, Fig. S1A and D), which have homology to EVH1-binding domains of MmFat1 (8, 20), suggesting a role for HyFatl in the regulation of actin organization. HyFatl ICD also shares a short three amino acid sequence motif —HWD— with MmFat1 and MmFat3 (SI Appendix, Fig. S1A). This sequence is part of a larger domain that contributes to the kinesin-mediated apical localization of Fat3 in Madin-Darby Canine Kidney (MDCK) cells (21). HWD is present in the ICDs of most Fat-like proteins, but not in ICDs of Fat, Ds, CELSR, or classical cadherin protein families.

Phylogenetic analysis of HyFatl using either the intracellular domain (Fig. 1E) or the extracellular domain (SI Appendix, Fig. S1F) reveal homology with Drosophila Fat2 (also known as Fatlike and Kugelei) and the mammalian Fat-like cadherins Fat1, Fat2, and Fat3. In contrast, alignment of HyFatl with true Fats (MmFat4 and DmFat) reveals a lack of conserved domains and low similarity (10.8%) (SI Appendix, Fig. S1B). Alignment of

**Fig. 1.** Cnidarian Hydra possesses single homologs of atypical cadherins Fat-like, Ds, and CELSR. (A) Schematic of the Hydra body plan, arrows indicate directions of cell displacement. (B) Budding adult Hydra polyp. (C) Schematic (lateral view) of ectodermal epithelial cells of Hydra with confocal images of ectodermal and endodermal myonemes stained with phalloidin. (D) Domain composition of representative atypical cadherins. cadherin domain; EGF repeat-like domain; laminin domain; hormone receptor domain; latrophilin/CL-1-like GPS domain; and cellular membrane. (E) Phylogenetic tree of Fat, Fat-like, and Ds subfamilies based on the ICD sequence, maximum likelihood analysis (1000 bootstrap replicates, bootstrap values are indicated for each node) supports the clustering of the predicted Hydra homologs (underlined) with the Fat-like and Ds cadherin subfamilies. Alignments were done using MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/). Mm, Mus musculus; Dm, Drosophila melanogaster.
HyFat1 with Fat from *Nematostella vectensis* (10), also shows low similarity (11.8%) (*SI Appendix*, Fig. S1C). Thus, while *Nematostella* has a Fat and a Fat-like cadherin, *Hydra* has only a single Fat-like protein, and no Fat/Fat4 homolog. *Hydra* Ds has regions of similarity in the ICD with bilaterian Ds orthologs, most strikingly in a region partially overlapping HR2 (*SI Appendix*, Fig. S1E, double underlined) (22, 23). However, although conservation of the Ds ICD is clearest among three homology regions (HR1 to HR3) in bilaterian Ds (10), these regions are not present in ICD of HyDs nor in Ds homologs of the medusozoans *Clytia*, *Alatina*, or *Cassiopea* (*SI Appendix*, Fig. S1E).

Thus, analysis of domain compositions as well as phylogenetic analyses using either sequences comprising all cadherin domains or ICDs confirm that these cadherins belong to Fat-like and Ds families (Fig. 1D and E and *SI Appendix*, Fig. S1F). A true Fat homolog is present in the anthozoan cnidarians *Nematostella* and *Acropora* (24). However, we were unable to identify a homolog of Fat in *Hydra* or other medusozoan cnidarians. Analysis of proteins that were automatically annotated as *Hydra* Fat homologs in the National Center for Biotechnology Information (NCBI) GenBank revealed that their domain compositions differ from Fat family proteins (*SI Appendix*, Fig. S1G) and their intracellular domains are much shorter (69 to 157 amino acids) than Fat cadherins (456 to 542 amino acids), with no sequence similarity (*SI Appendix*, Fig. S1G). This suggests revealed a single Hydra homolog of Flamingo/Celsr, another atypical cadherin involved in PCP (25), that we call HyCelsr (*SI Appendix*, Fig. S1H). We conclude that *Hydra* has single orthologs of Fat-like, Ds, and Celsr cadherins, but does not have a Fat/Fat4 homolog.

HyFat1 and HyDs Are Planar Polarized in the Body Column. In situ hybridization shows that in adult *Hydra* polyps HyFat1 and HyDs are expressed in ecto- and endodermal epithelial cells throughout the body column, with weaker expression in the tentacles and hypostome (*SI Appendix*, Fig. S2). HyFat1 expression is strongest in the lower portion of the body column, just above the foot, and in the tentacle bases.

To examine HyFat1 cellular localization, we generated polyclonal antibodies against HyFat1 ICD (residues 4,191 to 4,393). Immunoblotting of *Hydra* lysates with anti-HyFat1 antisera identified a polypeptide of ~490 kDa, corresponding to the predicted molecular weight of full-length HyFat1. In addition, polypeptides of ~180 kDa and 100 kDa were specifically recognized by this antiserum (Fig. 2E). Because Fat and Fat-like cadherins have been shown to be posttranslationally cleaved (26–29), HyFat1 may also undergo proteolytic processing which could explain the lower molecular weight fragments. When HyFat1 antisera was preabsorbed with HyFat1-GST, but not GST (Fig. 2E), these bands were no longer recognized, supporting specificity.

Immunostaining of fixed *Hydra* polyps with HyFat1 antisera and affinity-purified anti-HyFat1 antibodies revealed strong expression of HyFat1 at apical ectodermal epithelial junctions (Fig. 2A–D). HyFat1 is also present at the basal membrane along the myonemes of ectodermal epithelial cells and along the lateral membranes of endodermal epithelial cells (*SI Appendix*, Fig. S3A, D, and G). In this study we focus on the expression of HyFat1 in the ectoderm, where expression is strongest.

HyFat1 staining accumulates strongly at tricellular junctions throughout the body column (Fig. 2A, D and F), similar to *Drosophila* Fat2/Fat-like (7, 30, 31). Significantly, HyFat1 staining is higher in membranes that are perpendicular to the oral–aboral axis of the animal (Fig. 2B, C, and H and *SI Appendix*, Fig. S3J and K), suggesting HyFat1 is planar polarized along the oral–aboral axis. To measure the degree of polarization, we calculated a polarization value (PV) of HyFat1, the ratio between intensity of staining of the apical membrane most perpendicular to the oral–aboral axis and the membrane most aligned with the oral–aboral axis (Fig. 2H). Interestingly, planar polarized localization of HyFat1 was seen in elongated cells of the body column, but not in the head or peduncle regions where the apical surfaces of ectodermal epithelial cell are rounded (Fig. 2A–D and H and *SI Appendix*, Fig. S3I). Both ecto- and endodermal HyFat1 staining were lost when the antisera was preabsorbed with HyFat1-GST, but not GST (Fig. 2F and G and *SI Appendix*, Fig. S3A–I), supporting antibody specificity. Interestingly, neither claudin, nor FGF2 (32) (membrane markers for *Hydra* ectodermal epithelial cells) are planar polarized in the body column (*SI Appendix*, Fig. S3 M–O), suggesting that oral–aboral planar polarization is not a general feature of *Hydra* membrane proteins.

We also generated antibodies against HyDs ICD (residues 2,958 to 3,113). Immunostaining with an affinity-purified HyDs antibody revealed a similar pattern as anti-HyFat1 at apical junctions of ectodermal epithelial cells (Fig. 2I–K). HyDs is also enriched at the tricellular junctions and at the apical membranes perpendicular to the oral–aboral axis. Similar to HyFat1, planar accumulation of HyDs along the apical membrane was observed only in the body column, not in the peduncle or hypostome (Fig. 2N and *SI Appendix*, Fig. S3L). The specificity of junctional staining of HyDs antibody was confirmed by preabsorbing with HyDs-GST antigen (Fig. 2L and M). Thus both HyFat1 and HyDs are planar polarized in the oral–aboral axis in the body column.

Planar Polarization of HyFat1 Is Highest in Ectodermal Epithelial Cells that Undergo Directional Displacement. Planar polarization of Fat-like proteins has been linked to their role in directional cell migration and asymmetric cell morphology in bilaterians (6, 8, 30, 31, 33). Interestingly, polarization of HyFat1 is observed only in the body column where cells undergo continuous oriented cell division and displacement toward the head and the foot (Figs. 1A and 2H). In the hypostome and peduncle, where the rate of epithelial cell displacement is much lower (17), the polarization of HyFat1 is lower as well. We hypothesized that HyFat1 becomes polarized when ectodermal epithelial cells are coordinate displacement.

The generation of a new bud in *Hydra* provides a system for examining the effects of changes in the rates of cell displacement on HyFat1 (34). Cells that are being recruited into new bud tissue undergo extensive rearrangements, and their rate of displacement and junctional remodeling is significantly higher than in the maternal body column (35). During bud development, cells of the body column move coordinate into the growing bud, change their orientation, and align along a new oral–aboral axis, that is perpendicular to the maternal axis (35) (Fig. 3A). At budding stages 2 and 3, cells of the body column that are recruited into a new bud lose planar polarization of HyFat1 (Fig. 3B). As the bud develops, at stages 4 to 7, HyFat1 remains unpolarized at the tip of the bud, the future head cells. However, in the growing body column of the bud and in cells being displaced from the parent into the bud, HyFat1 is polarized along the bud’s oral–aboral axis (Fig. 3B and C). HyFat1 polarization in those cells was also higher than in the cells of a maternal body column (Fig. 3C and D). Thus HyFat1 polarization correlates with rate of cell displacement during budding.

In order to examine HyFat1 polarization in situations when the rate of displacement of epithelial cells is low we next examined the effects of starvation. Starvation slows down the cell cycle and the rate of epithelial cell displacement in the body column (36, 37). Significantly, starvation for 5 d resulted in a decrease in HyFat1 polarization (Fig. 3C and E).

We also examined HyFat1 staining during aggregation. *Hydra* can be dissociated into a suspension of single cells, which, when aggregated, give rise to new animals (Fig. 3F) (38). Forty-eight
hours after dissociation, aggregates form a hollow sphere with ecto- and endodermal layers; however, new axes are not yet present and there is no organized displacement of epithelial cells. In these conditions HyFat1 is visible at the apical junctions, but is not polarized (Fig. 3F). As the polyps forming from the aggregates elongate, HyFat1 becomes polarized again along the oral–aboral axis. Taken together, these data suggest that HyFat1 becomes planar polarized in the direction of epithelial cell displacement and this polarization increases with the rate of displacement.

Ectopic Expression of HyFat1 ICD Disrupts Organization of the Actin Cytoskeleton via EBM Motifs. MmFat1 and MmFat3 cadherins promote cell migration and polarization through regulation of actin polymerization and microtubule alignment via their intracellular domains (6, 8, 21, 33). Examination of the intracellular domain of HyFat1 revealed the presence of three EVH1 binding motifs (EBMs), similar to those found in mammalian Fat1 and Fat3, which have been suggested to regulate actin dynamics (6, 8, 33) (SI Appendix, Fig. S1A and D). To explore the role of the intracellular domain of HyFat1 and the EBM motifs, we generated two transgenic Hydra lines: ICD constitutively expressed GFP-tagged HyFat1 (residues 4,100 to 4,392) and dICD constitutively expressed an ICD constructs lacking all three EBMs (Fig. 4A). Transgenic Hydra were obtained by injecting plasmid DNA into one- to four-cell-stage embryos (39). Fully transgenic ICD-GFP and mosaic dICD-GFP lines (denoted ICD and dICD, respectively) were established. Mosaic dICD animals with the transgene expressed in at least 20% of ectodermal epithelial cells were used for experiments. Transgenic cells of interstitial lineage were not detected in either line.

Fig. 2. HyFat1 and HyDs are expressed at apical junctions of ectodermal epithelial cells in a planar polarized manner. White arrows indicate immunostaining of apical membranes perpendicular to the oral–aboral axis, yellow arrows indicate immunostaining at tricellular junctions, and asterisk indicates tip of the hypostome. (A–D) Apical views of ectoderm immunostained with anti-HyFat1 serum. (E) Immunoblot analysis of total protein lysate from a transgenic Hydra line constitutively expressing claudin-GFP; HyFat1 anti-serum was preabsorbed with either GST (lane 1) or GFT-HyFat1 antigen (lane 2); presented lanes are a selection from the original image. (F and G) Apical view of ectoderm immunostained with a HyFat1 anti-serum that was preabsorbed with (F) GST protein or (G) HyFat1-GST antigen. (H) Calculation of a polarization value (PV) for immunostained epithelial cell; gray arrow points to the apical membrane parallel to the oral–aboral axis; graph shows PVS of HyFat1 in different parts of the Hydra body. (I–K) Apical view of the ectoderm immunostained with affinity-purified anti-HyDs antibodies. (L and M) Apical view of ectoderm immunostained with affinity-purified anti-HyDs antibodies preabsorbed with (L) HyFat1-GST or (M) HyDs-GST antigens. (N) PVS of HyDs in different parts of the Hydra body. In H and N data shown as mean with SD, two-tailed t test; for each measurement three to five animals were used; ***P < 0.0001, **P = 0.0074, *P = 0.0113; the spread of the data points is shown in SI Appendix, Fig. S3L.
The body shape of fully transgenic ICD-GFP polyps (denoted ICD) and polyps mosaic for dICD animals (20 to 60% mosaic) was normal. Costaining with antibodies to GFP and HyFat1 showed that ICD-GFP and dICD-GFP are enriched along apical junctions, while in controls GFP is not (Fig. 4 B–D'). This suggests that sequences in the ICD aside from the EBMs mediate recruitment to apical junctions. The shape of both ICD- and dICD-expressing cells in the body column were more circular compared to more elongated control cells (Fig. 4 B–E). Interestingly, there was also a clear correlation between the circularity of the cell and polarization of HyFat1 in the control animals, with more elongated cells having more polarized HyFat1 (SI Appendix, Fig. S4A).

Since the HyFat1 antibody recognizes both endogenous and ectopic HyFat1, we could not determine if overexpression of HyFat1-ICD altered polarization of endogenous HyFat1. We could, however, test if planar polarization of HyDs is affected in ICD cells. HyDs shows planar polarization in the absence or presence of ectopic HyFat1, indicating that ICD of HyFat1 does not alter HyDs polarization. There were occasional increases of HyDs staining in ICD expressing cells, but those differences were not significant (SI Appendix, Fig. S4B).

To examine the junctional actin cytoskeleton, control and transgenic animals were stained with phalloidin. The intensity of phalloidin staining at the apical junctions was not significantly different between control polyps and ICD and dICD transgenics (SI Appendix, Fig. S4 C and D). Thus, overexpressed HyFat1 ICD does not alter junctional actin distribution.

The apical surface of ectodermal cells has thin phalloidin-positive microvilli. Strikingly, multiple, elongated microvilli were observed on the apical surface of the ICD ectoderm (Fig. 4F), and both their length and number were significantly higher than in control (Fig. 4 F, H, and I). In contrast, dICD cells had significantly fewer and shorter apical microvilli (Fig. 4 H and I) than animals expressing ICD-GFP. These data suggest that overexpression of HyFat1 can alter actin rich microvilli in an EBM-dependent manner.

We also noticed that nuclei of ectodermal epithelial cells, that are normally located in the basal half of the cell, had an abnormal apical position in ICD cells (Fig. 4 G and J), but not in GFP or dICD cells.

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Fig. 3. Planar polarization of HyFat1 increases in ectodermal epithelial cells that undergo displacement. (A) Schematic of bud development is adapted from ref. 34. (B) Dynamic of HyFat1 localization during bud development; asterisk indicates the tip of the bud. At stage 2 (st. 2), HyFat1 polarization is lost and it increases at the following stages. (C) PV of HyFat1 of the body column during budding and after 5 d of starvation; the “maternal body column” bar is duplicated for better visualization. Stages 4 and 5 were combined; during these stages, cells of the maternal body column continue to move into the growing bud (34), therefore, from the point of tissue dynamics these stages are similar and can be combined. Mean with SD, one-way ANOVA (***P < 0.0001) and two-tailed t tests (**P = 0.0009), three to four animals for each group; data for individual animals used in the analysis are presented in SI Appendix, Fig. S3P. (D) Schematic of polarization values of HyFat1 determined for st. 5 bud, PV of the body column cells (1.48) increases as they are being recruited into the bud tissue (2.01), stays high in the body column of a bud (2.1), and drops at the tip of the bud (1.18). (E) Apical view of ectoderm of Hydra starved for 5 d immunostained with anti-HyFat1; HyFat1 is not polarized. (F) Schematic of Hydra aggregate development and apical view of 48-h and 7-d-old aggregate immunostained with anti-HyFat1; in 48-h-old aggregate no polarization of HyFat1 is visible; statistical analysis is provided in SI Appendix, Fig. S3Q.
Ectopic Expression of HyFat ICD Affects Oriented Cell Division. Because oriented cell division can be regulated by PCP, we examined orientation of the mitotic spindle in Hydra that overexpress HyFat-ICD. Anti-Phospho-Histone H3 staining was used to visualize cells in metaphase and anaphase and to determine orientation of the mitotic spindle (Fig. 5 A and B). Hydra ectodermal epithelial cells divide in the direction of the oral–aboral axis (18). The mean angle between the mitotic spindle of ectodermal cells in GFP controls (32°) was not significantly different from the wild-type AEP strain (29°) (Fig. 5F) and similar to published data (33°) (18). Importantly, however, in Hydra that overexpress HyFat-ICD, the spindle angle of ICD cells was less tightly linked with the oral–aboral axis (49°) (Fig. 5 C, D, and F). The mean spindle angle of dICD cells (38°), however, did not differ significantly from controls (Fig. 5 E and F). Thus, ectopic expression of HyFat ICD affects orientation of the mitotic spindle, and this effect is diminished if the EBMs are deleted.

Down-Regulation of HyFat Affects Adhesion. Next, we sought to inactivate HyFat using an RNA hairpin approach (40). Two different hairpin-expressing sequences, shFat9 (nucleotides 12,573 to 13,178) and shFat4 (nucleotides 11,493 to 11,792) were inserted downstream of GFP under control of an actin gene promoter that is active in all epithelial cells (41) (Fig. 6 A, Top). We could not obtain stable transgenic lines expressing the hairpins under control of the actin promoter because hatchlings rapidly lost transgenic cells. These data suggest that the down-regulation of HyFat caused by constitutive expression of the hairpins led to loss of cells. Only one line bearing the GFP-shFat4 hairpin survived for a month. To overcome the lethality of shFat knockdown, we cloned shFat9 hairpin into the doxycycline-inducible vector pIndGFP (Fig. 6 A, Bottom). We were able to establish one Hydra line that expressed low levels of the shFat9 hairpin, constitutively, without induction by doxycycline (SI Appendix, Fig. S5). We made multiple attempts to establish additional lines; however, expression of the marker of the transgenic vector was consistently lost. These data suggest that strong inhibition of HyFat leads to cell loss and is lethal to the animal.

shFat9 polyps show loss of HyFat transcript by in situ hybridization (Fig. 6 B and C). Immunoblotting showed consistent decreases in HyFat protein in shFat9 polyps (Fig. 6D). Immunostaining of shFat9 and shFat4 animals with HyFat antiserum also showed reduced levels of HyFat along the membranes of ectodermal epithelial cells and at tricellular junctions (Fig. 6 G I), further validating the HyFat antibody. However, residual staining indicated that knockdown of HyFat in shFat9 and shFat4 polyps was incomplete. Analysis of shFat9 partial knockdown animals revealed several phenotypic features. Transgenic animals bearing an empty vector were used as controls. The body column of shFat9 was notably elongated compared with controls and had a “lumpy
Down-Regulation of HyFatl Alters the Actin Cytoskeleton. Hydra myonemes are contractile processes located on the basal side of epithelial cells, attached to the mesoglea (Fig. 1C) (42). A manifestation of PCP in Hydra is the parallel arrangement of ectodermal myonemes along the oral–aboral axis. HyFatl is weakly detected along the ectodermal myonemes (SI Appendix, Fig. S3 A, B, D, and E). We examined whether down-regulation of HyFatl or ectopic expression of HyFatl ICD affects the alignment of ectodermal myonemes. Myonemes in shFatl9 Hydra were still oriented in the oral–aboral direction. However, actin cables in the myonemes were less straight than in controls and had a “frizzled” appearance (Fig. 8 A and B). Actin cables in ICD cells looked somewhat thicker but otherwise normal (Fig. 8C). We also examined if abundance of polymerized actin is changed in shFatl9 cells. Staining of control and shFatl9 animals with phalloidin did not reveal any significant changes (SI Appendix, Fig. S6B). Thus, down-regulation of HyFatl affects the morphology of actin bundles, but does not have an effect on the actin at apical junctions.

Discussion

Our results indicate that the function of Fat-like cadherins in organizing cells and tissues was established early in animal evolution. Both gain-of-function and loss-of-function studies implicate Hydra Fat-like in cell–cell adhesion and actin organization. Remarkably, we also find that both HyFatl and HyDs are planar polarized in the body column of Hydra. This is a demonstration of planar polarization of a protein in a nonbilateralian. Fat and Ds proteins in bilaterians have been intensively investigated, and their asymmetric localization is thought to be crucial for their role in planar polarization of tissues (reviewed in ref. 43). Planar polarization of Fat-like proteins has also been observed and is linked to their role in directional cell migration and asymmetric cell morphology (8, 30, 31, 33, 44).

HyFatl is planar polarized at epithelial membranes that are perpendicular to the oral–aboral axis. It is also enriched at tri-cellular junctions, similar to Drosophila Fat2 (7, 30, 31). However, we note that the planar polarization of HyFatl is not as strong as Drosophila Fat2. We find that HyFatl is localized along the total length of apical junctions, although clearly enriched at oral–aboral membranes. This might reflect its additional function in cell adhesion: partial knockdown of HyFatl leads to weakening of the cell–cell contacts. Cells are also rapidly lost from transgenic lines with strong knockdown of HyFatl, also consistent with a role of HyFatl in cell adhesion. Importantly, where cells are...

sock” appearance (Fig. 6 E and F). We did not observe an altered body shape with shFatl4, likely due to the smaller number of transgenic cells. The apical contacts between ectodermal epithelial cells of shFatl9 and shFatl4 often become separated upon fixation (Fig. 6 H and I). The number of such broken cell–cell contacts was significantly higher in shFatl9 animals in comparison with both controls and ICD animals (Fig. 6J). This suggests that loss of HyFatl weakens cell–cell adhesion. Although the shFatl9 line was more stable than shFatl4, elimination of transgenic cells occurred in shFatl9 polyps as well: we often observed multicellular junctions resembling healing wounds along the body column of shFatl9 animals (SI Appendix, Fig. S6). We propose that they are the result of healing at sites where cells have been lost. Over time, the shFatl9 line had reduced viability and was also lost. Taken together, these data suggest that HyFatl is required for cell–cell adhesion.

Down-Regulation of HyFatl Affects Cell Alignment and Apical Surface Area of Ectodermal Epithelial Cells. Epithelial cells in the Hydra body column are a sheet of similar-sized cells with the axes of their longest dimensions oriented in the same direction. However, in shFatl9, shFatl4, ICD, and dICD animals, epithelial ectodermal cells were misaligned (Fig. 7 A–D). This misalignment was also observed in the endoderm of shFatl9 (Fig. 7 E and F), where HyFatl was partially inactivated (see in situ hybridization and immunoblot analyses) (Fig. 6 B–D).

In shFatl knockdowns, epithelial cells also differed from each other in size. This difference was especially obvious in the basal disk (Fig. 7 G and H) where HyFatl is highly expressed but unpolarized. We used a “neighbor” method (Materials and Methods) to measure the degree of cell misalignment and cell size difference. In the body column in control polyps, neighboring cells have a similar size and orientation. The orientation of shFatl knockdowns, ICD, and dICD cells is irregular, although the phenotype is less penetrant in ICD and dICD cells (Fig. 7I). Cell size is significantly different in shFatl knockdowns, but not in ICD or dICD cells (Fig. 7I). Interestingly, shFatl9 cells are more rounded than controls, resembling the phenotype of ICD and dICD cells (Figs. 4E and 7K). Since shape is altered in both shFatl knockdowns and ICD and dICD cells, the observed misalignment could be due to altered shape. Shape, orientation, and circularity of cells expressing a control hairpin for FoxO (40) are not altered (Fig. 7 I–K).

Fig. 5. Ectopic expression of HyFatl ICD interferes with mitotic spindle orientation. (A and B) Confocal images of ectodermal epithelial cells in anaphase immunostained with anti-phospho-histone H3 antibodies. Dashed lines indicate the orientation of mitotic spindles. (C–E) Distribution of angles between the mitotic spindles and oral–aboral axis shown as a fraction. (F) Graph showing the angles between the mitotic spindles and oral–aboral axis. Mean with SD, two-tailed t test, (***P < 0.0001), number of animals used for analysis: GFP, 12; ICD, 17; dICD, 8; and AEP, 2.

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not polarized, such as the foot, HyFat1 still functions, as seen as increased size variability in knockdown conditions.

Planar polarization of HyFat1 is most notable in epithelial cells undergoing displacement, but not in the foot or hypostome, where the speed of an epithelial cell’s displacement is lower than in the body column (17). HyFat1 polarization increases further in the growing bud and is reduced in starved animals and in early stage aggregates, where cell displacement is slowed down or stopped. This dynamic nature of HyFat1 localization is also reminiscent of Drosophila Fat2 during egg chamber rotation: normally planar polarized along the migrating epithelium, Fat2 loses its polarization in eggs with migration defects (30).

Oriented cell division can be regulated by planar polarity signaling in bilaterians (1). Our studies raise the possibility that planar polarization of HyFat1 may have a role in oriented cell division in Hydra because we find that ectopic expression of the intracellular domain of HyFat1 results in less precise orientation of the mitotic spindle. Because our studies indicate that loss and gain of function of HyFat1 affects cell adhesion and the cytoskeleton, we cannot be sure if this PCP phenotype is due to a role for HyFat1 in PCP, or indirectly due to alterations in cell adhesion and the actin cytoskeleton. Both reduction of HyFat1 and overexpression of HyFat1 ICD led to rounding of cells, suggesting that overexpression of ICD might have some dominant negative effects.

Mammalian Fat1 and Fat3 promote cell migration and polarization through regulation of actin and microtubule alignment via interactions of their intracellular domains with Ena/VASP (6, 8, 33) binding EBM motifs. Interestingly, despite low overall identity with bilaterian homologs, knockdown or overexpression of HyFat1 alters the actin cytoskeleton, and this effect is reduced by deletion of EBMs. Down-regulation of HyFat1 overexpression of HyFat1 ICD led to rounding of cells, suggesting that overexpression of ICD might have some dominant negative effects.

Fig. 6. Down-regulation of HyFat1 affects the adhesion of ectodermal epithelial cells. (A) Schematic of constitutive (Top) and doxycycline-inducible (Bottom) shFat1 constructs in pIndGFP vector. (B and C) In situ hybridization with HyFat1 antisense and sense probes. (D) Immunoblot analysis with affinity-purified anti-HyFat1 antibodies of lysate from control and shFat1 polyps. Arrows indicate the full length and 100 kDa HyFat1; asterisk indicates a nonspecific 35 kDa band. (E and F) shFat1 mutant has an altered body column shape. (G–I) Immunostaining of the ectoderm with anti-HyFat1 antibodies reveals weaker adhesive contacts and irregularly shaped epithelial cells in shFat1 and shFat4 polyps. (J) Graph showing the number of broken cell–cell contacts per 10 cells. n, number of animals used for analysis; mean with SEM (**P = 0.0073). The total number of cell pairs scored was 1,294 for the control, 356 for shFat1, and 996 for ICD.
detected on the apical surface of ectodermal epithelial cells; however, in overexpression conditions, ICD-GFP and dICD-GFP are found at apical membranes (Fig. 4G), where it can induce microvilli. Similarly, ectopic expression of the intracellular domain of MmFat1 can induce actin polymerization at ectopic sites (6). In contrast, overexpression of HyFatl ICD did not alter the amount of actin at adherens junctions, as assessed by phalloidin staining, suggesting that HyFatl may have spatially restricted effects on the cytoskeleton.

Interestingly, planar polarization of Hydra Ds occurs without the presence of a classical Fat cadherin. This indicates that Ds homologs can function independently of Fat/Fat4 cadherins in Hydra. Although Fat and Ds cadherins often function together, work in Drosophila and zebrafish has suggested that Ds cadherins may have Fat-independent functions (45–47). In addition, interactions between Ds and Fat-like cadherins have not been reported in other organisms. We cannot exclude the possibility that HyDs and HyFat1 interact; however, ectopic expression of HyFat1 ICD does not change the polarization of HyDs.

All of the proteins making up the core PCP module in bilaterians have been found in anthozoan and medusozoan cnidarians (11, 15) as well as in Trichoplax and some sponge species (11). This suggests the basic PCP machinery was established before the appearance of bilaterians. Cadherin molecules designated as Fat homologs have been identified in lineages that diverged prior to Cnidaria (9, 24). However, the molecular structure of these putative Fat proteins, in particular the number of cadherin repeats, varies dramatically: 14 in Amphimedon queenslandica, 37 in Monosiga brevicollis, and 9 in Trichoplax adhaerens. In contrast, the anthozoan cnidarian Nematostella vectensis has a complete repertoire of atypical cadherins involved in PCP with a clear Fat homolog (24). Therefore, the molecular structure of these putative Fat proteins, in particular the number of cadherin repeats, varies dramatically: 14 in Amphimedon queenslandica, 37 in Monosiga brevicollis, and 9 in Trichoplax adhaerens. In contrast, the anthozoan cnidarian Nematostella vectensis has a complete repertoire of atypical cadherins involved in PCP with a clear Fat homolog (24). Hydra and other medusozoan cnidarians have only a single Fat-like cadherin, but lack a true Fat homolog, which may have been lost during evolution. Thus, the anthozoans are the only nonbilaterian with a Fat homolog. Interestingly, anthozoans are distinct from other cnidarians in having bilaterality displayed by a pair of directive mesenteries and an asymmetric siphonoglyph (reviewed in ref. 48). It is interesting to speculate that the addition of a Fat/Ds module to the
core complex could have led to a new level of planar cell polarization and appearance of a second axis of symmetry. This intriguing scenario calls for functional studies of Fat and Ds in anthozoans, e.g., Nematostella.

In summary, our studies indicate that planar polarization of Fat-like and Ds cadherins was established before the divergence of cnidarians and bilaterians, and that Fat-like cadherins have roles in tissue organization, cell adhesion, and the cytoskeleton in Hydra.

Materials and Methods
See SI Appendix for the full description of Materials and Methods.

Animals, Culture Conditions, and Transplantation Experiments. The AEP strain of Hydra vulgaris was cultured at 18 °C in hydra medium (1.0 mM CaCl₂, 1 mM NaHCO₃, 0.1 mM MgCl₂, 0.03 mM KNO₃, 1 mM Tris HCl, pH 7.8, and fed with Artemia nauplii every 2 d.

In Situ Hybridization. Briefly: samples were fixed in 4% paraformaldehyde (PFA) and hybridized with antisense dioxygenin-labeled RNA probe at 0.1 μL/mL nitroblue tetrazolium chloride (NBT) and 3.75 μL/mL 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in the dark.

Production of Antibodies. Peptides corresponding to the C-terminal portions of HyFat (residues 4,191 to 4,393) and HyDs (residues 9,144 to 9,799) were expressed as GST fusions using the GEXKt-1 vector (Millipore) in Escherichia coli strain BL21 and purified on glutathione-agarose (Thermo Scientific). Purified peptides were used to immunize guinea pigs (Cocalico Biologic, Inc.) and rats (Covance). Polyclonal antibodies were purified by binding to anti-germ immobilized on glutathione-agarose beads and elution with 100 mM glycine (pH 2.5) into 1 M Tris, pH 8.0.

Immunoblots and Phenylogentic Analysis. To perform immunocytochemistry on whole mounts, animals were relaxed in 2% urethane for 2 min, and then fixed in either Lavadovskii’s fixative (ethanol/formaldehyde/acetic acid)H₂O 50:10:436 for HyFat, HyDs, GFP, phospho-Ena H3, and FGF2 antibodies, or in 4% paraformaldehyde for phalloidin staining overnight at 4 °C. Primary antibody dilutions were as follows: anti-HyFat total serum, 1:1,000; affinity-purified anti-HyFat, 1:100; anti-HyDs total serum, 1:400; affinity-purified anti-HyDs, 1:100; anti-GFP (Abcam, ab13970), 1:400; anti-phospho Histone H3 (Millipore, 05-806), 1:400; anti-FGF2 (Santi Cruz, sc7911), 1:100. Fluor-conjugated secondary antibodies (The Jackson Laboratory) were used at 1:400 dilution. Alexa555-phalloidin (Abcam) was used at 1:1,000 dilution.

Database Search and Phylogenetic Analysis. To identify cnidarian homologs of Fat-like, Ds, and CELSR proteins we searched the NCBI (https://www.ncbi.nlm.nih.gov) and National Human Genome Research Institute (NHGRI) (https://research.nhgri.nih.gov) databases. As queries, we have used sequences of the intracellular portions of N. vectensis Fat-like, Ds, and CELSR proteins (10). We have also used H. vulgaris homologs of Fat-like (XM_001270814.1, inner XM_002167158.2), Ds (PT_com.25284.C0_seq.01), and CELSR (XM_002163205), Alatina alata partial sequences of Fat-like (GEU01029859.1) and Ds (GEU01029600.1) homologs, Cassiopea xamachana partial sequences of Fat-like (OLMO01020275.1) and Ds (OLMO01079121.1), Aurelia aurita Fat-like homolog (GHA01167428.1), and Hydractinia symbioticargus partial Fat-like sequence (GAWH01043253.1). For generation of the phylogenetic tree, the sequences were aligned using MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/).

Data Availability.
All data are contained in the manuscript text and SI Appendix.

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