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The name of author Mihoko Kajita was spelt incorrectly. The corrected author list appears above.

The authors apologise to readers for this mistake.

Flamingo regulates epiboly and convergence/extension movements through cell cohesive and signalling functions during zebrafish gastrulation

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During vertebrate gastrulation, the body axis is established by coordinated and directional movements of cells that include epiboly, involution, and convergence and extension (C&E). Recent work implicates a non-canonical Wnt/planar cell polarity (PCP) pathway in the regulation of C&E. The *Drosophila* atypical cadherin Flamingo (Fmi) and its vertebrate homologue Celsr, a 7-pass transmembrane protein with extracellular cadherin repeats, regulate several biological processes, including C&E, cochlear cell orientation, axonal pathfinding and neuronal migration. Fmi/Celsr can function together with molecules involved in PCP, such as Frizzled (Fz) and Dishevelled (Dsh), but there is also some evidence that it may act as a cell adhesion molecule in a PCP-pathway-independent manner. We show that abrogation of Celsr activity in zebrafish embryos results in epiboly defects that appear to be independent of the requirement for Celsr in PCP signalling during C&E. Using a C-terminal truncated form of Celsr that inhibits membrane presentation of wild-type Celsr through its putative pro-region, a hanging drop assay reveals that cells from embryos with compromised Celsr activity have different cohesive properties from wild-type cells. It is disruption of this ability of Celsr to affect cell cohesion that primarily leads to the in vivo epiboly defects. In addition, Lyn-Celsr, in which the intracellular domain of Celsr is fused to a membrane localisation signal (Lyn), inhibits Fz-Dsh complex formation during Wnt/PCP signalling without affecting epiboly. Fmi/Celsr therefore has a dual role in mediating two separate morphogenetic movements through its roles in mediating cell cohesion and Wnt/PCP signalling during zebrafish gastrulation.

KEY WORDS: Epiboly, Convergent extension, Planar cell polarity, Celsr, Flamingo, *Drosophila*, Zebrafish

INTRODUCTION

During gastrulation, the embryonic body axes are established by a variety of coordinated and directional cell movements. The first morphogenetic movement in teleost and amphibian embryos is epiboly, during which embryonic blastodermal cells cover the extra-embryonic yolk during gastrulation until the blastodermal margin closes. One of main cellular events underlying epiboly is radial cell intercalation in the epiblast/ectoderm, during which embryonic epiblast/ectodermal cells move from the interior to external layers, thereby contributing to spreading of cells along the animal-vegetal axis to cover the yolk cell and to close the blastopore (Warga and Kimmel, 1990; Wilson et al., 1995; Wilson and Keller, 1991). The other driving force of epiboly in teleosts is generated by modulation of microtubules and the actin cytoskeleton in the yolk syncytial layer (YSL) (Betchaku and Trinkaus, 1978; Solnica-Krezel and Driver, 1994; Wilkins et al., 2008). Although cells in the extra-embryonic enveloping layer (EVL) change their shapes as they extend towards the vegetal pole during epiboly, the coordination of the EVL and YSL is mainly mediated by constriction of the actin cable at the leading edge (Cheng et al., 2004; Koppen et al., 2006).

One of the major driving forces of the gastrulation movements that contribute to the elongation of the body axis in vertebrates is convergence and extension (C&E). Large populations of dorsolateral mesodermal cells move dorsally and intercalate between neighbouring cells, leading to dorsalwards narrowing of the mesodermal tissues (convergence) and subsequently their lengthening along the anteroposterior axis (extension) (reviewed by Keller, 2002; Solnica-Krezel, 2005). Although convergence and extension movements are largely interdependent in *Xenopus*, extension can be separated from convergence in zebrafish (Glickman et al., 2003). Cells undergoing mediolateral cell intercalations are orientated with polarised protrusive activities that are biased along the mediolateral axis (Shih and Keller, 1992). Recent work has implicated non-canonical Wnt/Frizzled (Fz)/Dishevelled (Dsh) signalling, which is related to the planar cell polarity (PCP) pathway in flies, in the regulation of C&E during vertebrate gastrulation (reviewed by Tada et al., 2002; Seifert and Mlodzik, 2007; Zallen, 2007). The core members of the non-canonical Wnt/PCP pathway that have been shown to regulate C&E include Wnt11 (Heisenberg et al., 2000; Tada and Smith, 2000), Wnt5 (Kilian et al., 2003; Schambony and Wedlich, 2007), Fz7 (Djiane et al., 2000), Fz2 (Kilian et al., 2003), Dsh (Tada and Smith, 2000; Wallingford et al., 2000), Strabismus (Stbm)/Vangl2/Trilobite (Tri) (Goto and Keller, 2002; Jessen et al., 2002), Knypeck (kny)/Glypican4/6 (Ohkawara et al., 2003; Topczewski et al., 2001), Prickle (Pk) (Carreira-Barbosa et al., 2003; Takeuchi et al., 2003; Veeman et al., 2003), Diego (Dgo)/Inversin (Schwarz-Romond et al., 2002) and Flamingo (Fmi)/Celsr (Formstone and Mason, 2005).

celsr, the vertebrate homologue of *Drosophila flamingo (fmi)*, encodes an atypical proto-cadherin that is implicated in the regulation of several biological processes along with other core PCP genes, including C&E, cochlear cell orientation, axonal path-finding and neuronal migration (Formstone and Mason, 2005; Curtin et al.,

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2003; Shima et al., 2004; Senti et al., 2003; Lee et al., 2003; Tissir et al., 2005; Wada et al., 2006). In addition, it appears that Fmi/Celsr regulates neurite/dendrite outgrowth independently of PCP function (Gao et al., 2000; Kimura et al., 2006; Shima et al., 2004; Shima et al., 2007). There is strong evidence that Fmi/Celsr functions as an adhesion molecule as homophilic interactions are required to mediate the establishment of PCP in the *Drosophila* wing (Usui et al., 1999), and expression of *fmi/celsr* in S2 cells confers cohesive properties (Shima et al., 2004; Kimura et al., 2006). In addition to the cadherin repeats, Fmi/Celsr contains a seven-pass transmembrane domain, reminiscent of members of the G-protein-coupled receptor (GPCR) family, and, indeed, purified cadherin-repeats of Celsr can induce Ca^{2+} influx through the GPCR domain, suggesting Fmi/Celsr has a potential signalling function (Shima et al., 2007). However, it is unknown how Fmi/Celsr mediates such a variety of morphogenetic movements; is it through its homophilic interactions, which underlie modulation of cell adhesion, and/or through signalling functions?

In this study, we show a novel role for Celsr in regulating epiboly independently of the PCP pathway. Using three mutant forms of Celsr, we demonstrate that the cell cohesive property of Celsr is closely associated with its ability to regulate epiboly and that the conserved intracellular SE/D domain of Celsr is indispensable for its ability to functionally interact with the PCP pathway mediating C&E. Moreover, we reveal that the potential pro-region of Celsr may mediate dimer formation during the course of secretion or membrane presentation. Together, these results suggest a novel mechanism to explain how Celsr regulates cell cohesion and signalling in the vertebrate embryo.

MATERIALS AND METHODS

Zebrafish lines

Alleles used were homozygous *ord*^{rw71} and homozygous *ord*^{rw135} (Wada et al., 2006). In terms of penetration of the gastrulation and neuronal migration phenotypes, both alleles were almost identical when injected with *celsr1a* and *celsr1b* morpholinos, and therefore we used the *ord*^{rw71} allele carrying the *isl1:gfp* transgene in all the experiments.

RNA and morpholino (MO) injection

RNAs encoding zebrafish Fz7 (El-Messaoudi and Renucci, 2001) and *Xenopus* Dsh-GFP (Rothbacher et al., 2000); the truncated versions of Celsr (Celsr-ΔC, Lyn-Celsr and Lyn-Celsr-Δ); and the Celsr-Activin fusions FmiP-Act and FmiP-Act-RA were all linearised with *NotI* then synthesised with SP6 RNA polymerase essentially as described (Smith, 1993). All injections were performed on one-cell stage embryos. MOs against *fmi1a* and *fmi1b* were designed over the splice-donor site of the exon 2: 5'-TAAGA-GAATGACTGACCTGTAAAAT-3' and 5'-CATTTAGCAAACCTCAC-CTGTGAAGT-3', respectively (Wada et al., 2006). We also made other MOs for *celsr1a* and *celsr1b* over the initiation methionine: 5'-CATGGTGTAAACTCCGCCAACAGG-3' and 5'-CATCCATATCA-CTGGTAATCCATG-3', respectively (Witzel et al., 2006). Injection of a mixture of *celsr1a/celsr1b* MOs for both the splice and 5' region in wild-type and *MZord* embryos leads to very similar phenotypes and therefore we used the splice MOs in all experiments. The *stbm* MO is designed according to Park and Moon (Park and Moon, 2002). The sequence of *dv12* MO is designed over the initiation methionine (5'-TAAATTATCTTGGTC-TCCGCCATGT-3') and the activity was assessed by the ability to block the generation of fluorescent fusion proteins from RNAs, including the morpholino target sequence but not from RNAs in which the target sequence had been mutated (Tawk et al., 2007).

In situ hybridisation and immunohistochemistry

In situ hybridisation was carried out as previously described (Barth and Wilson, 1995). The probes used were *celsr2* (pBS-celsr2C) and others as described (Carreira-Barbosa et al., 2003). Immunohistochemistry was performed as described previously (Shanmugalingam et al., 2000). The

antibodies used for detection were anti-GFP polyclonal antibody (AMS Biotechnology), anti-α- and β-catenin polyclonal antibodies (Sigma) and anti-GM130 monoclonal antibody (BD Bioscience). They were visualised with Alexa488- or Alexa568-conjugated secondary antibody (Invitrogen). For visualising F-actin, Phalloidin-FITC (Invitrogen) was incubated in PBS/0.5% Triton with embryos fixed at 4°C overnight and washed with PBS/0.5% Triton several times prior to confocal analysis.

Analyses for subcellular protein localisation

To examine subcellular localisation of Dsh, embryos were injected with 150 pg RNA encoding Dsh-GFP in the presence or absence of 100 pg *fz7* RNA essentially as described (Carreira-Barbosa et al., 2003). To test effects of Lyn-Celsr on the membrane localisation of Dsh, we co-injected several different RFP-tagged constructs (100 pg) (monomeric RFP was kindly provided by Roger Tsien). The Rab5-CFP construct was a kind gift from Carl-Philipp Heisenberg.

Cell culture, immunoprecipitation and western blotting

HEK293 cells were cultured and transfected in 9 cm dishes as previously described (Schaeper et al., 2000). After 24 hours of transfection, cells were washed once with PBS and lysed for 30 minutes in 1 ml of Triton X-100 lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 1% Triton X-100) containing 5 μg ml⁻¹ leupeptin, 50 mM phenylmethylsulfonylfluoride (PMSF) and 7.2 trypsin inhibitor units ml⁻¹ of aprotinin. After centrifugation at 14,000 g for 10 minutes, the supernatants (about 0.5 mg of protein) were subjected to immunoprecipitation for 1 hour with 5 μl of mouse anti-GFP antibody (Roche) bound to 10 μl of protein G-Sepharose 4B beads (GE Healthcare; Piscataway, NJ). Immunoprecipitated proteins were further incubated at 65°C for 5 minutes in Laemmli's buffer with or without 840 mM 2-mercaptoethanol, followed by SDS-PAGE and western blotting with rabbit anti-GFP (Invitrogen) or rat anti-HA antibody (Roche). Western blotting was performed as described previously (Hogan et al., 2004).

Cell movement analysis by UV-mediated photoactivation

Embryos were injected with 120 pg RNA encoding Kaede (Mizuno et al., 2003) with or without 100 pg RNA encoding Lyn-Celsr at the one-cell stage. At shield stage, UV was irradiated using a DAPI filter with the smallest pin-hole at 20× on a Zeiss Axioplan2. Images of embryos were taken at shield and tail-bud stages with a Hamamatsu Orca ER digital camera using a Volocity Acquisition software (Improvision). The images were then analysed for convergence or extension using ImageJ essentially as described previously (Sepich and Solnica-Krezel, 2005).

Hanging drop assay

Embryos were injected with fluorescein-dextran or rhodamin-dextran at the one-cell stage and were dissociated in embryonic medium in the presence of trypsin/EDTA (Sigma) at oblong stage. Dissociated cells were suspended in L15 medium/15% FCS and two differently labelled populations of cells were mixed in a minimum volume (25 μl). The mixed cells were kept with hanging over the lid of a Petri dish at 25°C overnight. The aggregates formed were analysed by confocal microscopy with a 20× lens as described (Carreira-Barbosa et al., 2003). Six to 14 aggregates were examined for each group at least from two separate experiments.

RESULTS

Abrogation of Celsr functions leads to a defective epiboly

Three zebrafish *fmi/celsr* genes (*celsr1a*, *celsr1b* and *celsr2*) are expressed maternally and subsequently ubiquitously during gastrulation (Formstone and Mason, 2005) (see Fig. S1 in the supplementary material). It has been suggested that Fmi/Celsr genes regulate the gastrulation movements of C&E (Formstone and Mason, 2005). However, maternal-zygotic *ord/celsr2* (*MZord*) null mutant embryos exhibit few defects early in development other than an occasional slight delay in epiboly (Fig. 1A-C) (Wada et al., 2006). To test whether this is due to redundancy and perdurance of maternally derived contribution of Celsr proteins, we injected

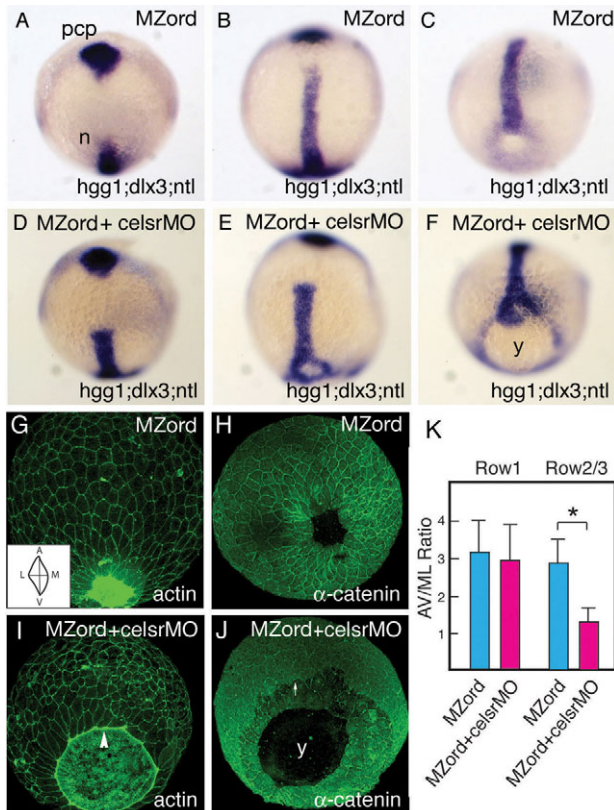


Fig. 1. Injection of *celsr1a* and *celsr1b* morpholinos in *MZord* embryos leads to a defective epiboly phenotype during zebrafish gastrulation. *MZord* embryos (A,B,C,G,H) or *MZord* embryos injected with 0.4 pmoles each of *celsr1a* and *celsr1b* morpholinos (D,E,F,I,J) were visualised at tail-bud stage with markers, as indicated in the bottom right-hand corner, by in situ hybridisation. *hgg1* was used to indicate the prechordal plate (pcp), *ntl* for the prospective notochord (n) and germ ring blastopore margin, and *dlx3* for the anterior edge of the neural plate (A–F). Embryos were also visualised at 90% epiboly with phalloidin for actin (G,I) or anti- α -catenin antibody (H,J). An arrowhead represents the leading edge of the EVL with the actin cable being formed (I). An arrow indicates the leading edge of deep cells (J). Note that the deep cells are delayed from the leading edge of the enveloping layer in the mutant/morphant embryos. y, yolk. (K) Cell elongation of the EVL along the animal-vegetal (AV) axis and the mediolateral (ML) axis at 90% epiboly is quantified as AV/ML ratio (see the inset of G), and the measurement is made using ImageJ from 80 cells (four embryos of each group). Cells that are attached with the actin cable are expressed as ‘Row1’, whereas cells that are not attached to that as ‘Row2/3’. Means and standard deviations (s.d.) are shown, and an asterisk indicates a statistically significant difference ($P < 0.05$; Student’s *t*-test).

MZord mutant embryos with *celsr1a* and *celsr1b* morpholinos. These embryos (*celsr* mutant/morphant embryos) failed to close the blastopore and exhibited a shorter notochord (Fig. 1D–F), whereas anterior migration of prechordal plate cells is slightly posteriorly positioned (Fig. 1D; see Table S1 in the supplementary material), but dorsoventral (DV) patterning remains largely unaffected (data not shown). These phenotypes are indicative of defects in epiboly (Kane et al., 2005; Shimizu et al., 2005).

To test the possibility that Celsr function is required for epiboly, we visualised phalloidin-labelled actin bundles and α -catenin in enveloping layer (EVL) and deep cells of wild-type and *celsr*

mutant/morphant embryos. The actin cable that reinforces the connection between the EVL and yolk syncytial layer (YSL) is thought to be part of driving forces of epiboly (Cheng et al., 2004; Koppen et al., 2006), and forms properly in *celsr* mutant/morphant embryos (Fig. 1I). Although other features of the EVL and YSL, including the organisation of YSL nuclei and the formation of dorsal forerunner cells are unaffected in *celsr* mutant/morphant embryos (see Fig. S2 in the supplementary material), the shapes and organisation of EVL cells are altered. Both EVL cells attached to the YSL (in the first row) and trailing cells not attached to the YSL (in rows 2 and 3) elongate along the animal-vegetal axis in a coordinated fashion in wild type (Fig. 1G). By contrast, although EVL cells in the first row can elongate along the animal-vegetal axis, cells distant from the germring are significantly less elongated in *celsr* mutant/morphant embryos (Fig. 1I,K). Moreover, the front line of deep cells becomes separated from the leading edge of the EVL cells or arrested in *celsr* mutant/morphant embryos, although the margin of the EVL advances to eventually cover the yolk cell (Fig. 1H,J; see Table S1 in the supplementary material). To prove a role for maternal contribution of Celsr2 protein in regulating epiboly, we injected with *celsr1a* and *celsr1b* morpholinos into embryos from crossing homozygous *ord* female and wild-type male (*Mord*). The *Mord* embryos injected with the *celsr1a/celsr1b* morpholinos show the epiboly defects in that deep cells are retracted from the leading edge of the EVL, but the phenotype is less strong than in *MZord* injected with the morpholinos (see Fig. S3 and Table S1 in the supplementary material). Taken together with the observation that injection of *celsr1a* and *celsr1b* morpholinos into wild-type embryos only leads to a weak C&E phenotype (see Table S1 in the supplementary material), these results suggest that maternal Celsr function is required for epiboly during zebrafish gastrulation.

A membrane-tethered extracellular domain of Celsr (Celsr- Δ C) inhibits epiboly

It has been suggested that Fmi/Celsr has two distinct functions: cell adhesion/cohesion, which is mediated by the extracellular cadherin repeats, and intracellular signalling mediated by the seven-transmembrane domain and cytoplasmic tail (Shima et al., 2004; Shima et al., 2007). This hypothesis prompted us to generate a mutant form of Celsr in which the extracellular domain and the first transmembrane domain remain intact while the remaining C-terminal regions are deleted (Celsr- Δ C). When wild-type embryos were injected with 300 pg Celsr- Δ C-HA RNA, it caused severe epiboly defects and occasionally severe C&E defects, reminiscent of *celsr* mutant/morphant embryos (Fig. 2A,D, see Fig. S4H,K and Table S1 in the supplementary material). To test whether this mutant Celsr acts as a dominant-negative form, we injected with a lower dose (150 pg) of Celsr- Δ C-HA RNA into *MZord* embryos as a sensitized background. Although this amount of Celsr- Δ C-HA RNA had little effect in wild-type embryos, it led to severe epiboly defects in *MZord* embryos comparable with injection of a high dose in wild-type embryos (Fig. 2B,C,E,F; see Table S1 in the supplementary material). This suggests that Celsr- Δ C acts as a dominant-negative form for mediating epiboly.

There is so far no evidence that PCP genes are required for epiboly in zebrafish, as even maternal zygotic *slb/wnt11;ppt/wnt5* double mutants and maternal zygotic *tri/stbm* mutants exhibit no epiboly phenotypes (Ciruna et al., 2006). This raises the issues of whether other PCP genes are also involved in the process of epiboly if maternal Celsr is compromised or whether PCP genes mediate C&E movements together with maternal Celsr. To address this, we analyzed epiboly and C&E in *MZord* embryos injected with doses

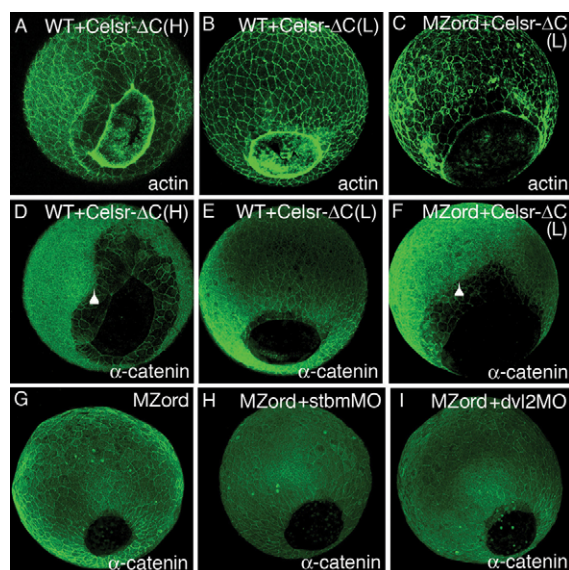


Fig. 2. A C-terminal truncated form of Celsr2 (Celsr-ΔC) is dominant negative and Celsr regulates epiboly independently of the PCP. Wild-type (A,B,D,E) or *MZord* embryos (C,F) were injected with 300 pg (H, high) (A,D) or 150 pg (L, low) (B,C,E,F) RNA encoding Celsr-ΔC-HA were visualised at 90% epiboly with phalloidin for actin (A-C) or anti-α-catenin (D-F). Arrowheads indicate the leading edge of deep cells, showing epiboly defects with deep cells retracted from the leading edge of the EVL. (G-I) *MZord* embryos (G) or *MZord* embryos injected with 0.4 pmoles *stbm*MO (H) or 0.75 pmoles *dvl2*MO (I) were visualised at 90% epiboly with anti-α-catenin. Dorso-posterior views of 90% epiboly embryos. Anterior is upwards and the antibodies used are indicated in the bottom right-hand corner. *MZord* embryos injected with *stbm*MO or *dvl2*MO show normal epiboly movements, as assessed by closure of the germ ring blastopore.

of *tri/stbm* or *dishevelled* (*dvl2*) morpholinos that give little or no obvious phenotypes in wild-type embryos (termed subthreshold doses throughout the remaining text). *MZord* embryos injected with a sub-threshold dose of *stbm* or *dvl2* morpholino underwent normal epiboly as assayed by closure of the germ ring blastopore (Fig. 2H,I; see Table S1 in the supplementary material) but exhibited defects during C&E (see Fig. S4B,C,E,F and Table S1 in the supplementary material). Consistent with the previous observation that *fmi/celsr* genetically interacts with other PCP genes in regulating C&E (Formstone and Mason, 2005), maternal deposition of Celsr also contributes to the regulation of C&E, together with core PCP components such as *Stbm/Tri* and *Dsh*. These results confirm that *Ord/Celsr2* does play a role in C&E movements in conjunction with *Dvl2* and *Tri/Stbm*, but also suggest that Celsr regulates epiboly independently of Wnt/PCP signalling.

Celsr has a putative pro-region and may function as a dimer

We further investigated how Celsr-ΔC works at the cellular level. Surprisingly, when Celsr-ΔC-HA is expressed in the embryo, the protein remains in the golgi ($n=7$) but not in endocytic vesicles ($n=13$), and is never presented at the plasma membrane (Fig. 4A-F). This led us to hypothesize that Celsr/Fmi can be post-translationally modified during the course of membrane presentation. One obvious feature in the extra-cellular domain of Celsr is a cluster of Arg residues, in front of the Cadherin repeats, that is highly conserved

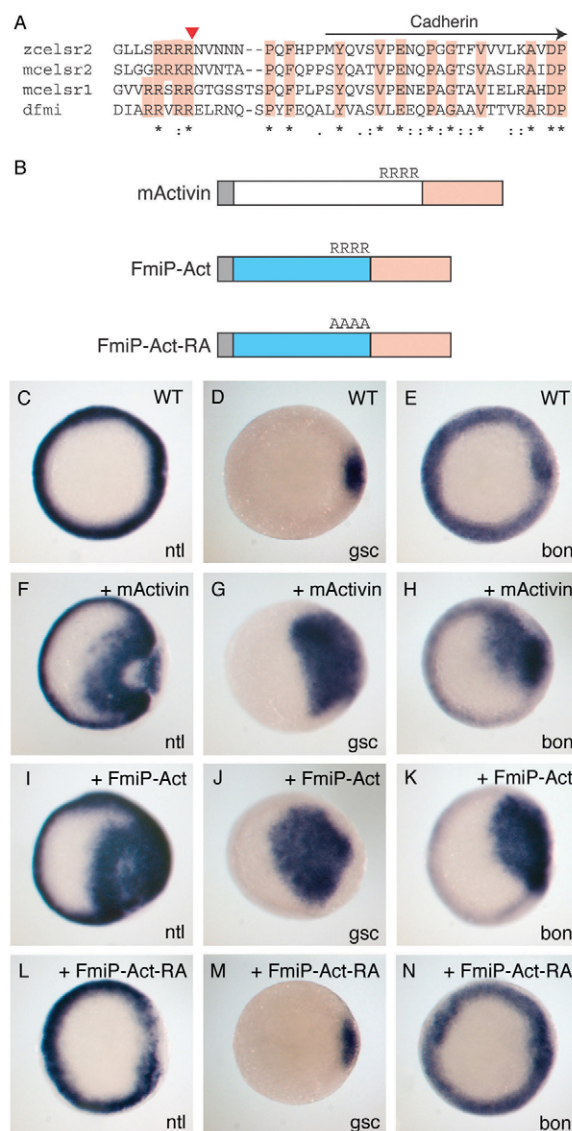


Fig. 3. Celsr/Flamingo has a potential pro-region mediating dimer formation. (A) The comparison of the region N-terminal to the Cadherin repeats indicates a Furin-like protease cleavage site (arrowhead) conserved amongst *Drosophila* Fmi and vertebrate Celsr. (B) The design for Celsr-Activin fusion proteins. A potential pro-region of zebrafish Celsr2 (blue), including the Furin-like cleavage site, is fused to the mature region of mouse Activin A (pink) to generate FmiP-Act (see Materials and methods for details). The potential cleavage site is mutated in alanine. (C-N) Wild-type embryos were injected with 5 pg mouse activin RNA (F-H), 50 pg FmiP-Act RNA (I-K), 100 pg FmiP-Act-RA RNA (L-N) or left uninjected (C-E), and fixed at 50% epiboly to examine expression of the mesoderm or endoderm markers *ntl* (C,F,I,L), *gsc* (D,G,J,M) or *bon* (E,H,K,N).

between vertebrates and *Drosophila* (Fig. 3A), and is reminiscent of the Furin-like serine protease processing site (RXR/KR). This is characteristic of members of the TGF-β family with a pro-region in front of the mature region that serves for dimer formation of the mature region followed by cleavage at the processing site during the course of secretion (reviewed by Shen, 2007).

To test the hypothesis that the region of Celsr/Fmi in front of the Cadherin repeats serves as a pro-region, we generated a fusion protein in which the putative pro-domain of Celsr2 is fused to the

mature region of Activin (FmiP-Act) (Fig. 3B), as Activin activity is well defined as a mesendoderm inducer in the embryo (Jones et al., 1996; Gritsman et al., 1999) (Fig. 3F-H). When FmiP-Act is expressed in the early embryo, it ectopically induced mesodermal markers *gsc* and *ntl* and the endodermal marker *bon* (Fig. 3I-K) (*gsc*, 50%, *n*=22; *ntl*, 48%, *n*=46; *bon*, 61%, *n*=23). If it is a bona fide pro-region, it must be cleaved to become an active form. To test this, we generated a mutation on the putative processing sites of FmiP-Act (FmiP-Act-RA) (Fig. 3B). As would be expected, FmiP-Act-RA no longer induced mesodermal markers in the early embryo (Fig. 3L-N) (*gsc*, 0%, *n*=32; *ntl*, 0%, *n*=41; *bon*, 0%, *n*=29). To test whether Celsr/Fmi forms a homo-dimer, Celsr-Venus and Celsr-ΔC-HA were co-transfected in HEK293 cells, and were subjected to immunoprecipitation with anti-GFP antibody followed by detection with anti-HA antibody. Celsr-ΔC-HA was co-precipitated with wild-type Celsr (Fig. 4M), indicating formation of Celsr homodimer. However, the size of co-precipitates is the same in the presence or absence of reducing reagents (Fig. 4M), suggesting that Celsr forms a homo-dimer non-covalently, unlike that of TGF-β family members, which form dimers covalently through disulphide bonds. Thus, it appears that Celsr/Fmi possesses a potential pro-region prior to its cadherin repeats that presumably mediates homo-dimer formation.

If Celsr/Fmi possesses the pro-region, it might mediate homo-dimer formation during the course of membrane presentation. If this is the case, a possible mechanism by which Celsr-ΔC acts as a dominant-negative form would be that it dimerizes with wild-type Celsr but remains in the golgi, thereby inhibiting the function of endogenous Celsr at the membrane. To test this, we co-injected with full-length Celsr-Venus and Celsr-ΔC-HA in the embryo to examine the localization of Celsr-Venus. Celsr-Venus is localized normally at the membrane (Witzel et al., 2006), but it was sequestered by the presence of high amounts of Celsr-ΔC-HA in the golgi (Fig. 4J-L), whereas it presented at the membrane if there is a low level of Celsr-ΔC-HA (Fig. 4G-I). Thus, it appears that Celsr-ΔC dimerizes with wild-type Celsr and prevents it from presenting at the membrane.

A membrane-tethered intracellular domain of Celsr (Lyn-Celsr) inhibits C&E without affecting epiboly

To further gain insight into the mechanism by which Celsr/Fmi functionally interacts with other core PCP components, as there is evidence for its genetic interaction with PCP genes during C&E (Formstone and Mason, 2005) (see Fig. S4 in the supplementary material), we generated a mutant form of Celsr in which the intracellular region (cytoplasmic tail) of Ord/Celsr2 is fused with the membrane-localisation signal from Lyn tyrosine kinase (Lyn-Celsr) to preferentially modulate its putative signalling function. We envisaged that it could either promote signalling in a manner independent of extracellular interactions [e.g. by analogy with Fz8 mediating apoptosis (Lisovsky et al., 2002)] or perhaps more likely interfere with function by titrating away proteins that would normally interact with full-length Fmi/Celsr [by analogy, see Hooper (Hooper, 2003) in the case of Smoothened for Hedgehog signalling].

First, we tested whether Lyn-Celsr can modulate C&E movements in the zebrafish embryo. Wild-type embryos expressing Lyn-Celsr exhibited a shorter body axis than wild type, occasionally being associated with cyclopia (Fig. 5A,E). In situ hybridisation visualising relative positions between the mesodermal tissues in relation to the anterior edge of the neural plate revealed that in these embryos prechordal plate cells are positioned posterior to the neural plate border, the notochord becomes shorter and the somitic

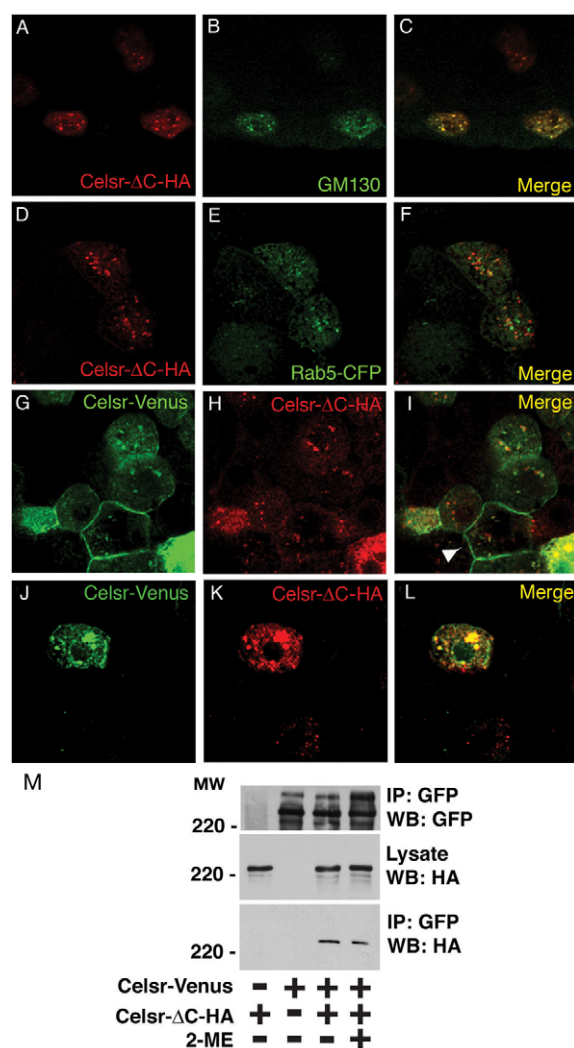


Fig. 4. Celsr-ΔC is retained in the golgi and inhibits membrane presentation of Celsr. (A-F) Wild-type embryos were injected with 30 pg Celsr-ΔC-HA DNA alone (A-C) or together with 30 pg Rab5-CFP RNA (D-F) and fixed at 40% epiboly to visualise with an HA antibody (A,D) or either a GM130 antibody for the golgi (B) or a GFP antibody for early endosomes (E). The merge images are shown in C and F. (G-L) Wild-type embryos were co-injected with 30 pg Celsr-Venus DNA and 30 pg Celsr-ΔC-HA DNA, and fixed at 40% epiboly to visualise with a GFP antibody (G,J) or an HA antibody (H,K). The merge images are shown in I and L. When the level of Celsr-ΔC is low, Celsr-Venus is presented at the membrane (shown by arrowhead) (62 cells out of 15 embryos examined) but the level of Celsr-ΔC is high, Celsr-Venus is prevented from presenting at the membrane (15 cells out of 15 embryos examined). (M) Non-covalent dimer formation of Celsr. Celsr-Venus and/or Celsr-ΔC-HA were transiently expressed in HEK293 cells. Cell lysates were immunoprecipitated with anti-GFP antibody, and immunoprecipitated proteins were further incubated in Laemmli's buffer with or without 2-mercaptoethanol (2-ME), followed by western blotting with anti-GFP or anti-HA antibody.

mesoderm is compressed along the anteroposterior (AP) axis (compare Fig. 5F,G with 5B,C), whereas dorsoventral (DV) patterning is unaffected (Fig. 5D,H). This phenotype is reminiscent of that of *slb/wnt11;ppt/wnt5* double mutants (Kilian et al., 2003). To further analyse this phenotype, we examined the ability of axial cells or lateral cells in the mesoderm to undergo anterior migration

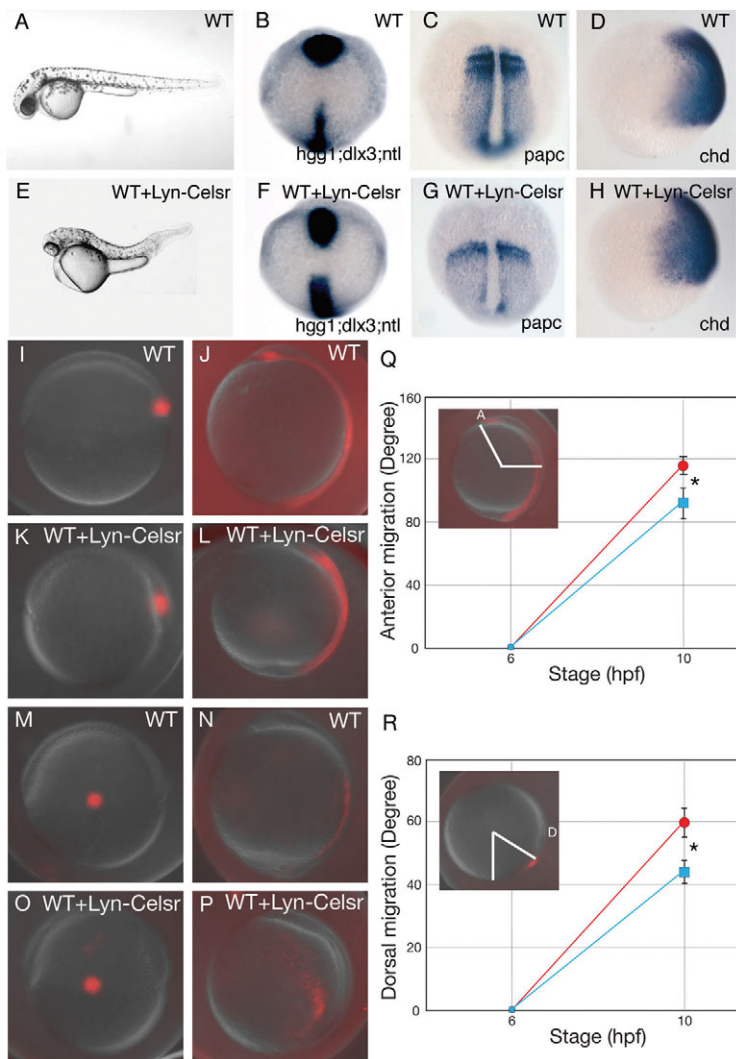


Fig. 5. Overexpression of a membrane-targeted intracellular domain of Celsr (Lyn-Celsr) causes a convergence extension defect during zebrafish gastrulation. (A-H) Wild-type (WT) embryos (A-D) or wild-type embryos injected with 100 pg RNA encoding Lyn-Celsr (E-H). (A,E) Lateral views of pharyngula stage living WT (A) and Lyn-Fmi-expressing (E) embryos. The Lyn-Fmi-expressing embryo shows a shorter body axis in this case associated with cyclopia. Dorsal views (B,C,F,G) of tail-bud stage wild-type embryos (B,C) and Lyn-Celsr-expressing embryos (F,G). Lateral views of 80% epiboly WT (D) and Lyn-Celsr-expressing (H) embryos. Anterior is upwards and genes analysed are indicated in the bottom right-hand corner. (I-R) Analysis of axial and lateral mesendodermal cells using a photo-conversion strategy. Labelled axial mesendodermal cells at shield stage (6 hpf) in control (I) and Lyn-Celsr embryos (K) were analysed at tailbud stage (10 hpf) (J,L, respectively). Labelled lateral mesendodermal cells at shield stage (6 hpf) in control (M) and Lyn-Celsr embryos (O) were analysed at tailbud stage (10 hpf) (N,P, respectively). Lateral views (I-P). Quantification of anterior migration of axial cells (Q) and dorsal migration of lateral cells (R). Blue, Lyn-Celsr-expressing embryos; red, WT embryos. Means and s.d. are shown. Asterisks indicate statistically significant differences ($P < 0.05$; Student's t -test). Note that Lyn-Celsr-expressing embryos show both convergence and extension defects.

or dorsal convergence, respectively, using a Kaede-mediated photo-conversion strategy. Anterior migration of axial cells in the embryo expressing Lyn-Celsr was significantly reduced when compared with control embryos (Fig. 5I-L,Q). Similarly, lateral cells of Lyn-Celsr embryos dorsally moved less than those in control embryos (Fig. 5M-P,R). Together with the fact that the Lyn-Celsr-expressing wild-type embryos do not exhibit any epiboly defects even at high doses (see Fig. S3A in the supplementary material), these results suggest that overexpression of Lyn-Celsr inhibits both convergence and extension movements rather than epiboly.

Lyn-Celsr inhibits membrane localisation of Dishevelled through a highly conserved SE/D domain

To investigate mechanisms by which Lyn-Celsr modulates the Wnt/PCP pathway, we examined whether Lyn-Celsr affects membrane localisation of Dsh as this event is closely associated with the activation of the Wnt/PCP pathway (Park et al., 2005). First, we tested the ability of Lyn-Celsr tagged with RFP to target Dsh-GFP to the membrane in pre-gastrulae embryos. The punctate cytoplasmic localisation of Dsh is unaffected by the presence of Lyn-Celsr (Fig. 6B-C; 100%, $n=6$), implying that Lyn-Celsr is unlikely to bind to Dsh directly. Next, we tested whether Lyn-Celsr modulates Fz-induced membrane localisation of Dsh (e.g.

Rothbacher et al., 2000; Carreira-Barbosa et al., 2003). Dsh-GFP localises to the membrane in response to Fz7 (100%, $n=5$) but this is inhibited by Lyn-Celsr-RFP (compare Fig. 6H-J with 6E-G; 80%, $n=25$), indicating that Lyn-Celsr acts upon the Fz/Dsh complex either directly or indirectly.

The observation that zebrafish Lyn-Celsr induces a PCP mutant phenotype in the *Drosophila* wing (see Fig. S6 in the supplementary material) and that only one region of the intracellular region of Celsr protein (the serine and acidic amino acid-rich domain: SE/D) is highly conserved between vertebrates and *Drosophila* (Fig. 6A) suggests that this region might be responsible for the ability of Lyn-Celsr to modulate the PCP activity. To test this, we generated a deleted version of Lyn-Celsr-RFP lacking the conserved SE/D domain (Lyn-Celsr- Δ -RFP). Lyn-Celsr- Δ -RFP no longer disturbed Fz7-induced membrane localisation of Dsh-GFP (Fig. 6K-M; 94%, $n=17$). Furthermore, when injected with a Venus-tagged Lyn-Celsr- Δ , wild-type embryos showed little or no CE defects during gastrulation (see Table S1 in the supplementary material). Together with the observation that Lyn-Celsr1a has the same activity as Lyn-Celsr2 in wild-type embryos (data not shown) despite the fact that only two domains of the intracellular region are conserved between Celsr1a and Celsr2 (Fig. 6A), these results suggest that the conserved SE/D domain is required for Fmi/Celsr to directly or indirectly interact with core components of the Wnt/PCP pathway.

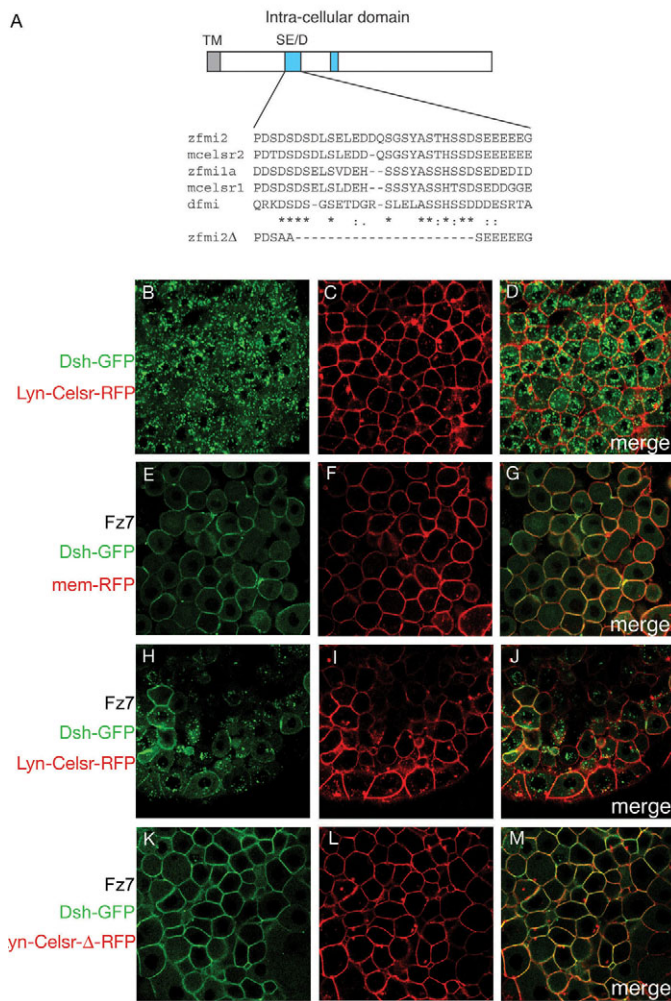


Fig. 6. Lyn-Celsr inhibits Frizzled-induced membrane localisation of Dishevelled through a highly conserved SE/D domain.

(A) Schematic illustration of the intracellular region of Fmi/Celsr. Two domains are conserved among vertebrates as highlighted in blue. Only one of these, the SD/E domain is conserved between vertebrates and *Drosophila*. The partial sequence of a construct lacking this domain is shown. (B–M) Wild-type embryos injected with 150 pg Dsh-GFP RNA in the absence (B–D) or presence (E–M) of 100 pg Fz7 RNA, together with 100 pg membrane-RFP (E–G), Lyn-Celsr-RFP (B–D, H–J) or Lyn-Celsr-Δ-RFP (K–M), as indicated and analysed at 40% epiboly by confocal microscopy. Scanning for GFP and RFP was carried out simultaneously and merged (D, G, J, M). Fz7-mediated membrane localisation of Dsh is inhibited by Lyn-Celsr (H, J) but not by Lyn-Celsr-Δ (K, M), which lacks the SD/E domain conserved between vertebrates and *Drosophila* (A).

Celsr regulates epiboly through its cell cohesive property

The SE/D domain appears indispensable for the function of Celsr in Wnt/PCP signalling to regulate C&E movements; however, it may be dispensable for its function in cell adhesion as both a full-length version and a splice-variant form of mouse Celsr2 which lacks the exon encoding this region, possess the ability to induce cohesive aggregates when transfected in *Drosophila* S2 cells (Shima et al., 2004). The epiboly defects of *celsr* mutant/morphant embryos might therefore be a consequence of Celsr-dependent cell adhesion rather than due to loss of intracellular Celsr signalling. Indeed, the epiboly

phenotype of *celsr* mutant/morphants is similar to that observed when the activity of another adhesion protein Half-baked (hab)/E-cadherin is partially compromised (Kane et al., 2005; Shimizu et al., 2005). We speculated, therefore, that the epiboly phenotypes in *celsr* mutant/morphant embryos would result primarily from defects in Celsr-dependent adhesion/cohesion, whereas, by contrast, the defects in C&E in embryos expressing Lyn-Celsr would be associated with the signalling function of Celsr protein.

To test this hypothesis, we compared the cohesive properties of *celsr* mutant/morphant cells in a hanging drop assay (Fig. 7A). When dissociated cells from differentially labelled wild-type embryos (red and green) are allowed to aggregate, red and green cells become well intermingled (Fig. 7B). By contrast, when cells from *celsr* mutant/morphant embryos (green) are mixed with wild-type cells (red), the two populations segregate from each other (Fig. 7C), indicating that *celsr* mutant/morphant cells possess different cell cohesive properties from those of wild-type cells. To see whether epiboly defects in the embryo expressing Celsr-ΔC-HA are closely associated with altered cohesive property of cells, we mixed wild-type cells (red) and cells expressing Celsr-ΔC-HA (green) in the same assay. Consistent with this idea, cells expressing Celsr-ΔC-HA are segregated from wild-type cells (Fig. 7D). Furthermore, we investigated whether cells from wild-type embryos expressing Lyn-Celsr are distinct in cell cohesion when compared with those from wild-type embryos. Both wild-type cells (red) and wild-type cells expressing Lyn-Celsr (100 pg RNA) (green) are relatively well intermingled, although the behaviour of Lyn-Celsr cells is slightly different from that of wild-type cells (Fig. 7E), but a high dose (300 pg RNA) does not lead to severe segregation phenotypes as observed with Celsr-ΔC-HA (not shown). To further link the possession of the cadherin repeats of Celsr with its cell cohesive property, we tried to rescue the altered property of *celsr* mutant/morphant cells with Celsr-ΔN, which lacks the cadherin repeats and most of the extracellular region but contains the pro-region, transmembrane and intracellular domains. Despite the ability of Celsr-ΔN to substantially recruit Dsh to the membrane without exogenous Fz (see Fig. S5A–C in the supplementary material) and to cause CE defects in wild-type embryos (see Fig. S5D, E in the supplementary material), Celsr-ΔN was unable to restore altered cohesive property of *celsr* mutant/morphant cells (see Fig. S5I in the supplementary material) as well as the epiboly phenotype of *celsr* mutant/morphants (see Table S2 in the supplementary material). Taken together, these results suggest that the ability of Celsr to regulate epiboly is closely associated with its ability to modulate cell cohesive property, whereas its ability to interact with the PCP pathway to regulate CE may not require cell cohesive property mediated by the cadherin repeats.

DISCUSSION

Possible mechanisms by which Celsr regulates epiboly independently of the PCP pathway

In this study, we have demonstrated that, in zebrafish, Celsr proteins are required for the regulation of epiboly and that this process is mediated primarily by a cell cohesive function that is different from that associated with PCP. This raises the possibility that several processes requiring Flamingo proteins that do not appear to require PCP gene activity; e.g. target selection of retinal axons in the *Drosophila* visual system (Lee et al., 2003; Senti et al., 2003) are also mediated by this adhesive/cohesive activity. Interestingly, it has been demonstrated that, in addition to Fmi, N-cadherin plays a role in retinal target selection in flies (Lee et al., 2001). This is reminiscent of the roles for Celsr and E-cadherin during epiboly in

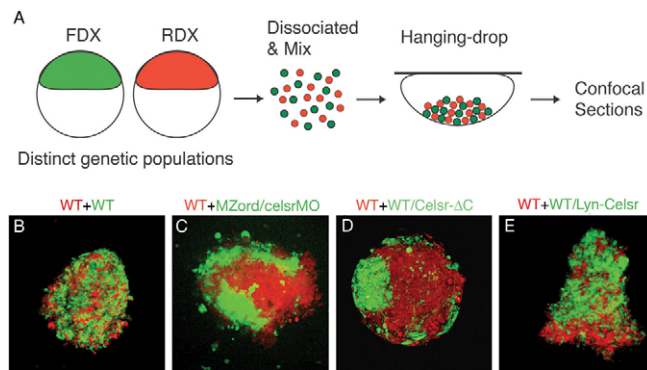


Fig. 7. Differential cell cohesive properties of wild-type cells compared with cells from *celsr* mutant/morphants and cells from embryos expressing *Celsr-ΔC*. Wild-type and *MZord* embryos were injected with RNA/morpholinos together with fluorescein-dextran (FDX, green) or rhodamine-dextran (RDX, red), as indicated. (A) 30–40% epiboly embryos were dissociated and dissociated cells from different populations were mixed in a minimal volume of a hanging drop and kept overnight to analyse aggregates by confocal microscopy. (B) Cells from wild-type embryos (green) and wild-type embryos (red). (C) Cells from *MZord* embryos injected with 0.4 pmol each of *celsr1a* and *celsr1b* morpholinos (green) and wild-type embryos (red). (D) Cells from wild-type embryos injected with 300 pg *Celsr-ΔC*-HA RNA (green) and wild-type embryos (red). (E) Cells from wild-type embryos injected with 100 pg *Lyn-Celsr* RNA (green) and wild-type embryos (red). *Celsr* mutant/morphant cells (C) or *Celsr-ΔC*-expressing cells (D) are strongly segregated from wild-type cells, whereas *Lyn-Celsr*-expressing cells are only weakly segregated from wild-type cells (E).

zebrafish (Kane et al., 2005) (this study). The ability of both proteins to regulate epiboly is closely associated with their cell adhesion/cohesion functions as cells with compromised *Celsr* or E-cadherin activity segregate from wild-type cells in adhesion/cohesion assays (Montero et al., 2005) (this study). Considering the fact that the protocadherin PAPC regulates cell adhesion by modulating cell adhesive/cohesive properties of the classical E-cadherin (Chen and Gumbiner, 2006), it will be intriguing to test the possibility that *Celsr* regulates epiboly together with E-cadherin in a similar scenario.

Recent work using atomic force microscopy demonstrates that regulation of cortical tension contributes to cell sorting as well as to cell adhesion (Krieg et al., 2008). Notably, *Rok2*, a key factor that regulates cortical tension, is also a downstream mediator of the Wnt/PCP pathway (Marlow et al., 2002). This raises the possibility that *Celsr* also regulates cortical tension underlying cell sorting through the Wnt/PCP pathway. However, we do not favour this possibility as *Lyn-Celsr* has a little effect on cell sorting behaviour in the hanging drop assay, whereas it strongly affects PCP-dependent processes during C&E, indicating that the regulation of cell cohesion is independent of the Wnt/PCP pathway in this context.

Possible PCP-dependent mechanisms by which *Celsr* regulates C&E

In contrast to its role during epiboly, *Celsr* regulates C&E together with other elements of the Wnt/PCP pathway (Formstone and Mason, 2005) (this study). An interaction with the Fz/Dsh complex through the conserved SE/D domain of *Fmi/Celsr* appears to be essential for this function. However, it is very unlikely that *Celsr* directly binds to Dsh to regulate C&E, as *Lyn-Celsr* fails to recruit

Dsh to the membrane in the absence of Fz. Consistent with the idea that the SE/D domain is required for signalling, the ability of *Celsr2* to regulate neurite outgrowth is diminished when the region including the SE/D domain is deleted from *Celsr2* (Shima et al., 2007). One possible candidate for a molecule that binds to the SE/D domain is Fz, as has been reported in *Drosophila* (Chen et al., 2008). However, this is unlikely as *Lyn-Celsr* does not bind to Fz7 in co-precipitation assay (data not shown). Hence, it will be of great importance to search for a factor that binds to the SE/D domain of *Celsr* to regulate C&E, allowing *Celsr* to switch its activities preferentially towards PCP signalling.

On the contrary, the SE/D domain is dispensable for the cell cohesive properties of *Celsr* in aggregates of S2 cells (Shima et al., 2004), supporting the notion that *Celsr* regulates C&E primarily through its putative signalling function, by which *Celsr* would be capable of associating with other PCP proteins. Although *Celsr* does not require PCP-signalling activity to elicit its role in the regulation of cell cohesion underlying epiboly, it is involved in modulation of cell-cell contact persistency along with Wnt11 (Witzel et al., 2006). Similarly, Wnt11 and its receptors mediate processes underlying tissue separation and co-ordinated movements of cells, which apparently require modulation of cell cohesion (Winklbaier et al., 2001; Cavodeassi et al., 2005; Ulrich et al., 2005). Thus, to understand how the two distinct modes of action of *Celsr* in modulating cell cohesion in Wnt/PCP-dependent and -independent manners require further investigations.

Multifunctionality of Flamingo as a ligand, homophilic/heterophilic adhesion molecule and signalling receptor

Celsr possesses a potential pro-region, N-terminal to the Cadherin repeats, that allows the mature region of *Activin* to confer its activity as a mesendoderm inducer, and, furthermore, in co-immunoprecipitation experiments, *Celsr* forms a dimer non-covalently. Together with the observation that *Celsr-ΔC* retains wild-type *Celsr* in the golgi, these raise the interesting possibility that *Celsr/Fmi* forms a dimer in the golgi during the course of membrane presentation rather than making homo-dimers after membrane presentation, as happens with classical cadherins (Patel et al., 2003). Recently, it has been shown that the golgi kinase Four-jointed phosphorylates cadherin domains of the atypical cadherins *Dachsous* and *Fat* as they transit through the golgi, and these phosphorylation sites are required for their proper activities during PCP (Ishikawa et al., 2008). This prompts us to speculate that the pro-region might also be required for potential post-translational modifications in the golgi in relation to the ability of *Celsr* to regulate cell adhesion/cohesion. However, the biological significance of *Celsr* dimer formation and roles of its pro-region in relation to cell adhesion/cohesion remain to be investigated.

Celsr2 and *Celsr3* have opposing roles in regulating neurite outgrowth of pyramidal neurons in rat cortical-slice cultures through activation of distinct intracellular signals by *Celsr2-Celsr2* and *Celsr3-Celsr3* homophilic interactions (Shima et al., 2007). There is, so far, no evidence that *Celsr2/Ord* regulates epiboly by counteracting the activities of *Celsr1a* and *Celsr1b*, but rather they appear to act together during this process as the epiboly phenotype is more prominent when all the *Celsr* activities are knocked down. However, a caveat to the simple dose model for the effect of *Celsr* on epiboly is that we cannot rule out the possibility that maternal *Celsr2* more preferably contributes to epiboly, whereas maternal *Celsr1a/1b* might rather regulate CE.

Shima et al. (Shima et al., 2007) propose that the extracellular domain of Celsr, including the Cadherin repeats, acts as a ligand. Together with our results, this raises the possibility that the formation of active ligand may require dimerization during the course of secretion or membrane presentation, as in the case of TGF β super-family members. Interestingly, the pro-region mediates a range of functions such that the pro-region of Activin confers long-range signalling ability (Jones et al., 1996). This prompts us to speculate the intriguing scenario that a form of Celsr containing the Cadherin repeats may be secreted and act as a ligand at a distance and mediate the cell-non-autonomous functions of PCP (reviewed by Seifert and Mlodzik, 2007). Furthermore, this raises several questions: which part of the extracellular domain could be cleaved and how does Celsr play a role – as a cell adhesion molecule or as a ligand?

In the future, it will be of interest to dissect how Fmi/Celsr acts either together with, or separately from, the Wnt/PCP pathway by studying possible downstream mediators of epiboly and C&E. In order to achieve this, using *MZord* as a sensitised background for eliciting both C&E and epiboly phenotypes will hopefully allow us to genetically dissect out both Wnt/PCP-dependent and -independent processes. Moreover, Lyn-Celsr, which specifically inhibits Fmi/Celsr function in Wnt/PCP-mediated processes, will facilitate further investigation of the cellular mechanisms by which Fmi/Celsr functions in different biological contexts.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/3/383/DC1>

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