Chapter B7

Atypical cadherins Celsr1–3 and planar cell polarity in vertebrates

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Abstract

Cadherin EGF LAG seven-pass G-type receptors 1, 2 and 3 (Celsr1-3) form a family of three atypical cadherins with multiple functions in epithelia and in the nervous system. During the past decade, evidence has accumulated for important and distinct roles of Celsr1-3 in planar cell polarity (PCP) during the development of the brain and some other organs. Although Celsr function in PCP is conserved from flies to mammals, other functions may be more distantly related, with Celsr working only with one or a subset of the classical core PCP partners. Here, we review the literature on Celsr, focusing on PCP and particularly on brain development.

Keywords: axon guidance, neural tube closure, neuronal migration, cell polarity

I. Introduction to Celsr proteins and PCP.

Celsr genes were first identified in the mouse [1-3]. They form a highly conserved family with orthologs in ascidians, worms, flies, and vertebrates. In mammals, the *Celsr* family is composed of three members, whereas the chick apparently lacks *Celsr2* [4], and the zebrafish has four members, including *Celsr1a* and *1b* [5]. In the mouse, *Celsr1-3* genes have similar genomic organization, with 35 exons (*Celsr1* & *Celsr3*) or 34 exons (*Celsr2*). Apart from studies on 3' alternative exons in *Celsr2*, alternative splicing has not been investigated in detail. *Celsr1-3* genes encode atypical cadherins of more than 3000 amino acids (Fig. 1). The large ectodomain is composed of nine N-terminal cadherin repeats (typical cadherins have five repeats), six epidermal growth factor (EGF)-like domains, two laminin G repeats, one hormone receptor (HRM) motif, and a G-protein-coupled receptor proteolytic site (GPS). This is followed by seven transmembrane domains (classic cadherins are single-pass type I proteins) and a cytoplasmic tail (CT) that varies in size and, in contrast to the ectodomain, is poorly conserved among the three members. Celsr1-3 belong to the "adhesion receptor" family of G-protein-coupled receptors (GPCR), also referred to as long N-terminal group B (LNB) GPCRs. These receptors are thought to be natural chimeras of cell adhesion proteins and signaling receptors that can convert cell-cell communication cues into intracellular signals. Studies of the latrotoxin receptor latrophilin, a member of LNB-GPCR, showed that cleavage at the GPS proceeds intracellularly and, despite cleavage, the N-terminal cell adhesion domain and C-terminal (GPCR) fragments remain associated by noncovalent bonds at the plasma membrane [6, 7]. There is

clear evidence that Celsr3 is not processed at the GPS, but whether Celsr1 or Celsr2 undergo cleavage remains unclear. In addition to cadherin and EGF-like repeats, which confer adhesive properties, the extracellular domain of Celsr1-3 contains a hormone receptor motif that may bind putative ligands, although none has been identified thus far.

The fruit fly Celsr ortholog is known as *Flamingo* (*fmi*) or *Starry night* (*stan*) [8, 9]. Genetic studies have established a critical role for *fmi*/*stan* in PCP, particularly in the stereotypic organization of wing hairs (trichomes) and sensory bristles [8, 9], the orientation of ommatidia in the eye [10], and the asymmetric division of sensory organ precursors, SOP [11, 12]. Fly PCP has been reviewed extensively [11, 13-15]. Therefore, we will summarize only some data that are likely to help a better understanding of PCP in vertebrates. In *Drosophila*, fmi/stan interacts genetically and functionally with other "core PCP proteins", which include serpentine receptors Frizzled (among four fz receptors in flies, fz and fz2 are partially redundant in PCP), the tetraspannin Van Gogh (vang, also named strabismus), the three cytoplasmic proteins Dishevelled (dsh), Prickle (pk) and Diego (dgo) [16-21], and probably other less clearly identified members [22]. In the wing, the distribution of core PCP proteins is tightly and dynamically regulated. Initially distributed in all subapical junctions, they adopt a transient polarized partition along the proximal-distal axis shortly before the growth of trichomes, and this partition is essential for the establishment of polarity and the proper orientation of hairs. Fmi/stan, fzd, dsh and dgo are enriched at the distal border of wing cells, whereas fmi/stan, vang and pk are

enriched at the proximal border [9, 23, 24]. Since fmi/stan localizes to both proximal and distal junctions, it was considered merely a permissive molecule: fmi/stan homodimers would act as a scaffold, promoting cell adhesion and bridging the distal (fz-expressing) side of a cell and the proximal (vang-expressing) side of an adjacent cell. However, there is evidence that fmi/stan plays an instructive role: its central portion containing the HRM and TM domains interacts physically with fz. Furthermore, fmi/stan recruits fz and vang to opposite cell boundaries, thereby initiating bidirectional polarity signals [13, 25]. In line with this, genetic analyses of flies demonstrated a mutual requirement for fmi/stan, fz and dsh for achieving a correct partition of polarity complexes [26]. In the *Drosophila* eye, polarity results from the differentiation of the initially equipotent R3/R4 photoreceptor progenitors into an equatorial R3 and a polar R4. The key distinction is not between opposite sides of the same cell, but between adjacent sides of this pair of progenitor cells. Competition for Notch signaling activation between the R3/R4 pair becomes biased by the PCP signal so that the equatorial cell always expresses low Notch levels and becomes R3. PCP mutations lead either to incorrect R3/R4 fate decisions, or in some cases to indistinctly differentiated pairs of R3/R4 cells [13-15]. On the *Drosophila* notum (dorsal thorax), PCP controls the orientation of an asymmetric division. pI cells differentiate within the epithelium and divide asymmetrically to produce an anterior pIIb daughter cell and a posterior pIIa daughter cell. The PCP pathway distinguishes anterior and posterior sides of the pI cell through asymmetric interactions with its anterior and posterior neighbors. The pI cell therefore seems to

become polarized much like the surrounding epithelial cells, but it uses this polarity to position cell fate determinants and the mitotic spindle prior to an asymmetric division. In PCP mutants, this division is not correctly orientated [11, 27, 28]. Asymmetric localization of polarity complexes requires differential membrane addressing of at least one component. In wing cells, there is indeed a vectorial vesicular transport of Fz along microtubules to the distal membrane [29]. All together, these data point to the importance of polarization of the apical microtubule network [13, 14], a process that still requires further investigation.

II. Celsr1-3 expression patterns

Celsr1-3 expression is finely regulated spatially and temporally, hinting at their importance during development. A striking feature is the complementary pattern of *Celsr1* and *Celsr3* expression in different systems [2, 30, 31]. In the mouse nervous system, *Celsr1* mRNA is strongly expressed in zones of neural stem cell (NSC) proliferation, namely all ventricular zones during embryonic and early postnatal development, and telencephalic ependymal zones as well as the subgranular layer of the dentate gyrus in the mature brain. In contrast, *Celsr3* mRNA is absent from NSC and is instead associated with most postmitotic neural cells, whereas *Celsr2* mRNA is found in both NSC and postmitotic cells. Expression of *Celsr1* abates during early postnatal development, in parallel to decreasing numbers of NSC. Expression of *Celsr3* is sharply downregulated postnatally and persists in the cerebellar granular layer, the hilus of the dentate gyrus, the rostral migratory stream (RMS) and the

central region of the olfactory bulb. By contrast, *Celsr2* expression remains quite stable throughout life. These expression patterns suggest that *Celsr1* functions in NSC regulation, *Celsr3* in neural cell maturation, and *Celsr2* in development and maintenance of the nervous system. *Celsr1-3* mRNAs are also variably expressed in non-neural tissue, such as the skin, lungs, kidney and digestive and reproductive systems. In the rodent testis, the spatiotemporal pattern of *Celsr1-3* expression is somewhat reminiscent of that in the brain. *Celsr3* is expressed exclusively in postmeoitic germ cells, *Celsr1* and *Celsr2* in Sertoli cells, with postnatal downregulation of *Celsr1* and persistence of *Celsr2* in the adult [32-34].

Data on protein localization remain scant. Polyclonal antibodies against the extra- and intracellular segments of Celsr1 allowed detection of two forms, full length (p400) and cleaved (p85 kD), the latter generated by as yet unidentified proteolysis events not involving the GPS [35]. In the hindbrain and spinal cord, Celsr1 protein immunoreactivity was detected in the floor and roof plates, as well as in radial neuro-epithelial progenitors. Intriguingly, Celsr1 is present not only in the apical junction belt as expected, but also in end-feet abutting the pial surface, which belongs to the basolateral domain [35]. In the embryonic skin, Celsr1 is asymmetrically expressed in hair germ cells and in basal layer epidermal cells, a pattern evocative of that of Fmi/stan in the *Drosophila* wing [36]. Unfortunately, thus far the cellular localization of Celsr2 and Celsr3 could not be investigated due to lack of antibodies suitable for immuno-histochemistry.

III. Celsr1: a major player in vertebrate PCP

The main phenotypic traits in *Celsr1-3* and some other PCP mutant mice are summarized in Table 1. Two *Celsr1* mutant alleles, *Crash* (*Celsr1*^{Crsh}) and *Spin Cycle (Celsr1*^{Scy}*)*, were identified in an ENU screen [37]. In *Celsr1*^{Crsh}, a G-to-A mutation at nucleotide 3126 results in an aspartate to glycine substitution in codon 1040, within the eighth cadherin repeat. In *Celsr1*^{Scy}, a T-to-A point mutation at nucleotide 3337 results in an asparagine to lysine substitution in codon 1110, in a region connecting cadherin repeats 8 and 9. Heterozygous animals show abnormal head-shaking behavior. Both heterozygous and homozygous mice have defective organization of stereocilia bundles in inner ear hair cells. Normally, the apical surface of each cochlear hair cell is decorated with actin-filled stereocilia arranged into a "V" centered on one microtubular "kinocilium", with all "Vs" pointing to the external aspect of the cochlear canal. This organization is altered in *Celsr*^{Crsh} and *Celsr1*^{Scy} mutants, where stereociliary bundles are oriented nearly randomly, displaying up to 180° rotation [37]. The stereotyped orientation of ear hair bundles is a hallmark of PCP and parallels the polarized distribution of core PCP proteins such as Fzd3, Fzd6, Vangl2, and Prickle2 [38-41]. PCP proteins localize asymmetrically to one edge of the apical cortex of the cells by mechanisms involving selective targeting and protein stabilization or degradation. Intriguingly, studies of the distribution of PCP proteins were carried out in late embryogenesis, starting from embryonic day 18 when the kinocilium had already migrated from the center to the lateral edge of the cell. This raises the question of whether the asymmetric partition of PCP proteins is the initial

event that sets up the polarity and organizes the epithelium, or simply a molecular readout of polarity signals induced by as yet unidentified cues.

Celsr1^{Crsh} and *Celsr1*^{Scy} homozygotes as well as compound *Celsr1*$^{Crsh/Scy}$ heterozygotes exhibit craniorachischisis, a severe neural tube defect due to a failure to initiate neural tube closure in the cervical region [37]. The role of *Celsr1* in neural tube closure was recently confirmed by identification of six *Celsr1* mutations in human fetuses with craniorachischisis. In *in vitro* assays, all these mutations impaired trafficking of Celsr1 protein, reducing its membrane localization [42]. In the bending neural plates, Celsr1 is concentrated in adherens junctions (AJs) oriented toward the mediolateral axes of the plates, and this was proposed to explain its role during neural tube closure [43]. Neural tube closure defects are observed in *Vangl2*, double *Dvl1* and *-2*, and *Fzd3* and *-6* mutant mice [39, 44, 45]. Similarly, in zebrafish, inactivation of Vangl2 also leads to a neural tube closure defect [46], further confirming the importance of classical PCP signaling in this developmental event.

To palliate embryonic lethality of *Celsr1*^{Crsh} and *Celsr1*^{Scy} mutants, a conditional allele (*Celsr1*^{f}) was generated [47], from which a null allele *Celsr1*^{ko} was derived by crosses with PGK-Cre mice. Western blot with an antibody against the N-terminal region of Celsr1 confirmed the absence of Celsr1 protein in embryonic brain extracts. Unlike heterozygous *Celsr1*^{Crsh} and *Celsr1*^{Scy} mice, heterozygous *Celsr1*$^{ko/+}$ mice have no perceptible phenotypic abnormality. In contrast, homozygous *Celsr1*$^{ko/ko}$ mutants display abnormal behavior such as turning/circling, hyperactivity and abnormal

squeaking. About 20% die *in utero* with various degrees of neural tube closure defects, and many have a looping tail.

Some Celsr1 null mutants have striking skin hair patterning defects, with whorls and crests instead of regular caudal and distal hair orientation on the body and limbs [47]. This phenotype is identical to that reported in Fzd6 null mice [48]. When *Celsr1* is inactivated in crosses with *Emx1-Cre* mice, absence of the protein in the apical ectodermal ridge induces a whorl in distal hind limbs, showing that the action of Celsr1 is autonomous of ectodermal cells. The mechanism of Celsr1 action in the hair bulb has been studied in detail in the Fuchs lab [36]. Prior to hair growth, Celsr1 becomes asymmetrically localized along the anterior/posterior (A/P) axis in basal epidermal cells in hair follicles and in interfollicular epithelium. This asymmetric localization is essential for anterior-posterior (A/P) orientation of skin hairs. Hair follicles fail to adopt the A/P orientation in skin explants isolated from E13.5 embryos, before the polarization of Celsr1. In contrast, when explants are isolated from E14.5 embryos, when Celsr1 is fully polarized, epidermis keeps the Celsr1 polarization established *in vivo* and the A/P polarity. Consistent with this, hair follicles are misaligned in E18.5 embryos with homozygous *Crsh* mutation. In mutant embryos, Celsr1 protein is produced as in the wild type but is no longer asymmetrically distributed. Moreover, the membrane recruitment of Fzd6 and the asymmetric localization of Vangl2 along the A/P axis are compromised, with some Vangl2 immunoreactivity forming intracellular puncta. Taken together, these results demonstrate that Celsr1 plays a critical role for PCP establishment in the developing

skin and hair follicles [36]. During hair development, Vangl1 and Vangl2 act redundantly: whorls on hind limbs are invariably seen following double inactivation of Vangl1 and Vangl2, but inconsistently when one allele remains intact (Qu et al., unpublished observation).

These data provide strong evidence that Celsr1 is a key partner in classical PCP, where it works with Fzd3 and -6, Dvl1 and -2, Vangl1 and -2, and probably with other PCP proteins that have not yet been studied in that context.

IV. Celsr2 and -3 in ciliogenesis

Based on their structure and motility, cilia are classified into three types: motile, primary and nodal cilia [49]. In mice, motile cilia garnish the apical surface of epithelial cells lining airways, reproductive tracts and cerebral ventricles; in *Xenopus*, some skin cells bear multicilia, providing an accessible model system [50-52]. Motile cilia have a central pair of microtubules surrounded by 9 doublets, an organization known as 9+2, and are anchored to a basal body in the apical cortex. They form tufts and their concerted beats generate directional flow. In the airways, they are crucial for clearing mucus and debris, and in genital tracts they assist in the transit of sperm and eggs [53, 54]. In the mouse brain at late embryonic stages and during the first postnatal week, neuroepithelial cells that line the cerebral ventricles differentiate into a monolayer of ependymal cells. At birth, ependymal cells are not multiciliated yet. Motile cilia on these cells develop progressively and reach their mature shape around P10.

The basal body of each cilium has a lateral extension called "basal foot" that can be seen by transmission electron microscopy. Basal feet point in the effective beat direction and are used as a hallmark of cilia polarity [55-57]. To generate an efficient directional flow, cilia coordinate their beats within the same cell (each cell has dozens of motile cilia) and in all cells in the epithelial sheet. Thus, basal feet are aligned in the same orientation with respect to the tissue polarity axis, a process referred to as "rotational polarity" [58]. Elegant studies on explants of *Xenopus* ciliated epidermis, isolated at different developmental stages, showed that motile cilia acquire their

rotational polarity in two steps. Early in development, genetic cues specify a rough planar axis that allows cells to produce a directional flow, thereby inducing a positive feedback loop that tunes basal feet polarity [59]. In mice, *Celsr1*, *2* and *3* are expressed in the developing ependymal layer. Inactivation of *Celsr2* alone or in combination with *Celsr3* ablation impairs ependymal ciliogenesis [60]. Although differentiation of ependymal cells occurs normally, their motile cilia never develop in normal numbers, and those that develop display abnormalities in position and planar organization. Basal feet are misoriented, a rotational polarity defect [58], and some basal bodies assemble deep in the cytoplasm. At the tissue level, ciliary tufts from neighboring cells point aberrantly in divergent directions, generating a "translational polarity" defect [58]. Residual mutant cilia display the typical "9+2" structure and are still able to beat with the same frequency as the controls. Yet, because of their abnormal orientation, they fail to generate a robust and directed flow. The membrane localization of Vangl2 and Fzd3 is disrupted in mutant ependymal cells, providing strong evidence that Celsr2 and Celsr3 regulate ciliogenesis via PCP signaling. In accord with this, downregulation of core PCP genes *Disheveled1–3*, and PCP effectors *Inturned* and *Fuzzy* affects the orientation of multicilia in *Xenopus* [51, 52]. Furthermore, mouse ependymal cells with the *"loop-tail"* Vangl2 mutation fail to align their motile cilia in response to hydrodynamic forces *in vitro* [61].

V. Celsr1-3 in neuronal migration.

The migration of facial branchiomotor (FBM) neurons in the developing rhombencephalon is an intriguing case that combines tangential and radial migration modes. FBM neurons, which innervate muscles responsible for facial expression [62-64], are generated in medial rhombomere 4 (r4) at E9.5–10.5 and immediately extend their axons laterally towards muscle targets. At E11.5, their cell bodies initiate a tangential caudal migration from r4 to r6. They migrate in the subventricular region, pass medial to the nucleus abducens (nVI) in r5 [65], and then move laterally and dorsally in r6. Finally, they undergo a radial migration in r6 to reach their subpial location, where they form the motor nucleus of the facial nerve (nVII) (Fig. 2A) [62]. The caudal soma translocation of FBM neurons, with looping of their axons (so-called *genu* of facial nerve), is conserved from fish to mammals, with important species differences; for example, it is blunted in chick [66]. The first indication that *Celsr* genes are involved in FBM neuron migration came from an ENU mutagenesis screen in zebrafish, which identified four point mutations in the *Celsr2*/*off road* locus [5]. In these mutants, FBM neurons fail to migrate caudally to r6, moving instead prematurely into lateral r4-r5. Morpholino knock-down experiments showed that *Celsr1a* and *1b* have adjunct functions in FBM neuron migration. Whereas combined downregulation of *Celsr1a* and *Celsr1b* has little effect on its own, it worsens the *off road* mutant phenotype, with more cells stacked in r4 in *Celsr1a*; *Celsr1b; Celsr2* triple mutants than in *Celsr2* mutants. In normal mice, migrating FBM neurons form streams from medial r4 to lateral r6, with a sharp rostral edge. In *Celsr1*$^{ko/ko}$ mice, FBM neurons are still able to move out of r4, but a subset migrates rostrally into r3 and r2

rather than caudally, a phenotype never seen in any other vertebrate (Fig. 2B). This rostral migration phenotype is fully penetrant, although with variable expressivity. Caudally directed *Celsr1*$^{ko/ko}$ neurons move through r5, medially to the abducens nucleus (nVI), before moving laterally in r6 like their wild type counterparts. Hence, a facial (nVII) nucleus forms in its normal location in lateral r6 by E13.5. In addition, rostrally migrating FBM neurons form an ectopic nucleus adjacent or even rostral to the trigeminal (nV) nucleus, but send their axons normally in the facial nerve [67]. In the hindbrain, *Celsr1* is expressed in progenitors and in the floor plate, but not in postmitotic FBM neurons. Consistent with this, conditional inactivation of *Celsr1* in FBM neuron progenitors upon *Nk6.2-Cre*-mediated recombination induces abnormal rostral migration, whereas its deletion in FBM neurons using *Isl1-Cre* does not.

In *Celsr2* knock-out mice, like in the *off road* mutant fish [5], the caudal FBM neuron migration stream is severely truncated [67]. Cells turn laterally, rostral to the abducens nucleus, so that axons do not loop around nVI, and an elongated facial nucleus forms in r4 and r5 instead of r6 (Fig. 2C). A similar phenotype is generated when *Celsr2* is specifically inactivated in FBM neurons using *Isl1-Cre* mice, suggesting that *Celsr2* is required cell autonomously in FBM neurons for their caudal migration. This does not rule out the possibility that *Celsr2* may also be necessary in neuroepithelial cells along the migration path, as suggested for zebrafish [5]. The aberrant migration of FBM neurons in *Celsr2* mutant mice is reminiscent of the situation in birds, which have no facial nerve *genu* and, intriguingly, lack the *Celsr2* gene [4]. Whether there is a causal link between the absence of *Celsr2* and blunted

FBM neuron migration in chick remains an open question. In zebrafish, *Celsr2* and *Celsr1a* and *-1b* regulate FBM neuron migration redundantly. In mouse, double inactivation of *Celsr1* and *Celsr2* phenocopies single *Celsr2* mutation, with no obvious rostral migration of FBM neurons, suggesting that *Celsr2* is epistatic to *Celsr1*. It looks as if *Celsr2* deficiency hampers or slows down neuronal migration, thus masking the *Celsr1* mutant phenotype.

Although *Celsr3* seems to be required for tangential migration of calretinin-positive interneurons and radial migration of calbindin-positive interneurons in the forebrain [68], its deficiency does not affect the migration of FBM neurons. It does, however, exacerbate the *Celsr2* phenotype: the facial nucleus is greatly reduced in size in the double knockouts *Celsr2*$^{ko/ko}$; *Celsr3*$^{ko/ko}$ and *Celsr2*$^{ko/ko}$; *Celsr3*^{Isl1cko} as compared to *Celsr2*$^{ko/ko}$ mutants, and Celsr3 deficiency increases FBM neuron apoptosis (Fig. 2D) [67]. The phenotype of *Celsr2*$^{ko/ko}$; *Celsr3*$^{ko/ko}$ double mutants is similar to that of *Fzd3* mutants, and other PCP related genes such as *Vangl2* and *Wnt5a* have been implicated in FBM neuron migration, suggesting that *Celsr1-3* regulate neuronal migration along the rostrocaudal axis by PCP-dependent mechanisms [67, 69]. In line with this, normal functionality of PCP genes such as *van gogh-like 2* (*vangl2*), *fzd3a*, *celsr2*, *prickle1a*, and *prickle1b* is required for caudal migration of FBM neurons in fish [5, 70-74].

VI. Celsr2 and Celsr3 in brain wiring

Functional neuronal networks are crucial for brain function. Network formation is finely orchestrated at the cellular and molecular levels by genetic programs and interactions with the environment. After reaching their location, postmitotic neurons extend axons that are guided to their targets by intrinsic programs, guidepost cells, and attractive or repulsive molecular cues. They ramify receptive dendritic fields according to tiling and self-avoidance rules. In addition to their role in neuronal migration, *Celsrs* are implicated in dendrite development and axon guidance, from *C. elegans* and *Drosophila* to mammals [75-77].

In flies, sensory neurons extend dendrites dorsally towards the midline. Dendrites from homologous neurons in two opposite hemisegments avoid each other, leading to a dendrite-free zone near the dorsal midline. This reciprocal inhibition of dendrite growth is defective in *Fmi/stan* mutants, where dendrites do grow across the midline to occupy fields overlapping those of homologous neurons [76]. A role for Fmi/stan in the tiling of dendrites is further supported by the identification of Fmi/stan mutants in genetic screens for aberrant dendritic extension of mushroom body neurons [78]. The function of Fmi/stan in dendrite growth does not rely solely on adhesion mediated by the extracellular domain, because overexpressing a N-terminally truncated Fmi that lacks cadherin, EGF-like and LaminiG motifs rescues the dendritic phenotype partially [79]. Intriguingly, neither loss nor gain of function of *frizzled*, nor overexpression of *dsh* phenocopy the *Fmi/stan* dendritic phenotype. Moreover, to our knowledge, no dendrite phenotype has been described in other fly PCP mutants. In the mammalian nervous system, *Celsr2* is

required for the maintenance of dendritic arbors: RNAi-induced downregulation of *Celsr2* in rat brain slices reduces the length of dendrites in cortical pyramidal neurons and the complexity of dendritic trees of Purkinje cells [80]. By contrast, silencing of *Celsr3* leads to dendritic overgrowth. The opposite effects of Celsr2 and Celsr3 are related to a single amino acid change in the first intracellular loop: like Fmi/stan, the mammalian Celsr3 has a histidine at position 2413, and both repress growth and/or induce retraction of dendrites. That histidine residue is replaced with arginine in Celsr2, which promotes dendrite growth and maintenance [81].

Early observations showed that *Fmi/stan* is essential for the development of axonal tracts. Null mutations fmi^{E45} and fmi^{E59} are embryonic lethal, and, in addition to displaying PCP defects, mutant embryos display abnormalities in longitudinal axonal tracts. Rescue of axonal, but not PCP defects, by brain specific expression of *fmi* cDNA restores viability [9]. Subsequent elegant studies in the visual system showed that fmi/stan mediates axon-axon and axon-target interactions required for guidance of photoreceptor axons [82, 83]. It looks as if growth cones "compare" their fmi/stan levels with that of their neighbors to "decide" whether to establish contact or not [84].

In mice, *Celsr3* is crucial for axon guidance. *Celsr3* mutant mice display marked defects in major tracts such as the anterior commissure, internal capsule, medial lemniscus and corticospinal tract. *Celsr3* is also essential for the anterior-posterior organization of monoaminergic axons in the brainstem [85], and for the rostral turning of commissural axons after midline crossing in the spinal cord [86,

87]. Celsr3 deficiency does not affect axonal growth *per se*, but rather guidance, resulting in stalling at intermediate targets, or misrouting of axons [88]. For instance, during development of reciprocal corticothalamic projections, mutant corticofugal axons travel normally in the subcortical layer but stall at the pallial-subpallial boundary. Reciprocally, thalamic axons never reach their cortical targets in layer 4; instead, they course ventrally along the hypothalamus and then turn externally towards the cortical marginal zone. Conditional removal of *Celsr3* in a stream that extends in the basal forebrain and ventral diencephalon precludes the entry of corticosubcortical and thalamocortical fibers in the basal telencephalic "corridor" and results in a defective internal capsule. This result demonstrates that *Celsr3* is required in intermediate targets to connect the cortex with sub-cortical structures. Targets cells are molecularly defined by expression of Dlx5/6 and qualify as "guidepost cells" [89]. To test whether guidance of cortifugal axons is mediated by homotypic interactions of *Celsr3* in, respectively, navigating growth cones and guidepost cells, *Celsr3* was specifically deleted in corticofugal axons by crossing the $Celsr3^{fl}$ allele with *Emx1-Cre*. Celsr3-defective corticothalamic axons develop normally, strongly suggesting that those fibers use guidance mechanisms independently of Celsr3-Celsr3 homophilic interactions.

The axonal Celsr3 phenotype is very similar to that generated by constitutive inactivation of Fzd3 [90, 91], indicating that a PCP-like mechanism might be involved. However, the role of other PCP genes such *Vangl1* and -2 remains to be investigated. Consistent with the role of Celsr3 in axon guidance, a phenotype of

axons stalled at intermediate target cells was reported for fmi-defective sensory neurons in flies [92]. This phenotype can be rescued by fmi constructs that lack most of the extracellular domains, indicating that the advance of sensory axons in flies does not depend on fmi-fmi homophilic interactions [92]. Contrary to corticothalamic axons, corticospinal axons do not develop in *Celsr3*^{Emx1cKO} mice, suggesting that *Celsr3* mediates homophilic interactions between corticospinal growth cones and guidepost cells. In support of this, in the fly visual system, fmi mediates interactions between the growth cones of photoreceptor axons and their targets in the medulla [93]. Like in flies and mammals, FMI-1, the sole *C. elegans* fmi/stan ortholog, enables navigation of both pioneer and follower axons in the worm ventral nerve cord. Rescue of the mutant phenotype with different portions of FMI-1 revealed that the C- and N-termini are required for guidance of, respectively, pioneer and follower axons [94]. Taken together, these data show that flamingo (Fmi) and its mammalian ortholog *Celsr3* are major players in axon guidance, and that their mechanisms of action are context-dependent and involve both homophilic and heterophilic interactions.

VII. Structure-function data on Celsr

As mentioned in the introduction, Celsr proteins contain a large extracellular N-terminus with 9 cadherin repeats, several EGF-like and laminin G-like motifs, a HRM, and a G-proteolysis site, followed by seven transmembrane domains and a variable intracellular C-terminal tail (Fig. 1). Although the functional relevance of these motifs remains poorly understood, some studies reveal a few hints. In

Drosophila S2 cells, which have no self aggregation properties, expression of full length fmi induces cell aggregation, whereas expression of a form lacking most of the ectodomain does not, showing that the extracellular domain indeed promotes homophilic cell adhesion [9]. Expression of mutant forms of mammalian Celsr2 in S2 cells demonstrated cadherin domain-mediated homophilic interactions compatible with adhesive properties of the cadherin repeats [80].

Dendritic overgrowth and tiling defects of *fmi* mutants, as well as fly viability, can be rescued by expression of full length fmi, but the rescue can be ascribed to two different functions of the protein. In transgenic rescue experiments, ΔNfmi —lacking all extracellular domains except the HRM— can rescue the dendrite overgrowth but not the tiling phenotype. Unlike full length fmi, ΔNfmi does not promote S2 cell aggregation *in vitro*. By contrast, C-terminally deleted fmi (ΔCfmi) retains the ability to mediate homotypic binding *in vitro* and recues the tiling phenotype but neither dendritic overgrowth nor fly viability, pointing to a key role of the C terminus. Thus, a dual molecular function of fmi plays pivotal roles in dendrite morphogenesis. In the initial growth phase, fmi might function as a receptor for an unidentified ligand and this hypothetical heterophilic interaction would be responsible for limiting branch elongation. At a later stage, homophilic fmi binding at dendro-dendritic interfaces would elicit avoidance between dendritic terminals from opposing neurons [79]. Likewise, studies on worms, flies and mice show that the function of fmi/Celsr in axon guidance does not rely solely on its ability to mediate homophilic

binding via cadherin repeats, but requires interactions of the C-terminus with unknown molecules [89, 92, 94].

The concept of distinct roles of the extra- and intra-cellular domains of fmi/Celsr is supported by mutation analysis in vertebrates. In rodents, Celsr2 and Celsr3 have opposing roles on dendrites in brain slice in culture, with Celsr2 promoting and Celsr3 restricting dendritic growth [80, 81]. Studies of chimeric constructs in which the ectodomains of Celsr2 and Celsr3 are swapped show that, whereas homophilic interactions (Celsr2-Celsr2 and Celsr3-Celsr3) are important, the transmembrane domains and C-terminus determine the dendrite enhancing or suppressing action. Celsr2 and Celsr3 have different effects on calcium release and activate, respectively, CamKII and Calcineurin signaling, and an amino acid change (R2413H) in the first intracellular loop is crucial for these distinct functions [81].

Other evidence for the importance of the C-terminus comes from a study on zebrafish [95]. A serine acidic amino acid-rich domain (SE/D) in the cytoplasmic tail of Celsr is required for membrane localization of the Frizzled-Dishevelled complex, PCP signaling and convergent extension [95]. Injection of a C-terminally truncated form of Celsr (ΔC-Celsr; laking 6 TM and tail) in wild type embryos generates epiboly defects. This is accompanied by sequestration of ΔC-Celsr in the Golgi, where the protein behaves as a dominant negative molecule, dimerizing with and precluding trafficking of the wild-type protein. Dimerization is thought to involve a conserved arginine-rich sequence N-terminal to the first cadherin repeat. Injection of the cytoplasmic tail of Celsr2 fused to the membrane localization signal from the Lyn

tyrosine kinase (Lyn-Celsr) generates convergent extension defects without affecting epiboly. Lyn-Celsr perturbs the Frizzled-induced membrane localization of Disheveled and PCP signaling. Interestingly, when the conserved SE/D domain is deleted from Lyn-Celsr, the membrane localization of Disheveled is restored [95]. Whereas the extracellular and TM domains are important for the distribution of Frizzled-Disheveled complexes at the membrane, this process depends critically on the C-terminal SE/D domain. The ability of Celsr to regulate epiboly is closely associated with its ability to modulate cell cohesive properties, whereas its ability to interact with the PCP pathway to regulate convergent extension may not require cell adhesion mediated by the cadherin repeats.

In the mouse skin, Celsr1 is internalized during epidermal basal progenitor division, a process that is crucial for balanced distribution of PCP proteins Fzd6 and Vangl2 in daughter cells and requires a cytoplasmic dileucine motif [96]. Whereas E-Cadherin is normally not internalized, its fusion with the Celsr1 cytoplasmic domain induces internalization during mitosis and enables the chimeric protein to recruit Fzd6 and Vangl2. When two consecutive leucines (2748-2749) are mutated to alanines, chimeric E-Cad-Celsr1, like mutated Celsr1, no longer translocates to endosomes [96].

The similarity between the cytoplasmic tails of Celsr1, 2 and 3 is much less than the similarity between their extracellular and TM regions. Since Celsr1 is directly implicated in PCP, and Celsr2-3 is implicated in more distantly related processes, could differences in the C-terminus account for functional differences? In

Drosophila, fmi, fz and dsh depend on each other for their membrane localization [26]. Fmi interacts physically with fz via a region encompassing its HRM and TM domains, and selectively recruits fz and vang (strabismus) to opposing cell boundaries [25]. In mice, the membrane localization of Fzd3 and Vangl2 in ependymal cells, and localization of Fzd6 and Vangl2 in skin epithelial cells, depend on Celsr2 and Celsr1, respectively [36, 60]. In *Celsr1*$^{Crsh/Crsh}$ mutant mice, a single amino acid substitution results in failure of the Celsr1 protein to reach the apical membrane [35, 36, 47]. Furthermore, all six Celsr1 mutations recently identified in human fetuses with craniorachischisis impair membrane trafficking of Celsr1 in *in vitro* assays [42]. Although not directly useful for characterization of structure-function relationships, this exquisite sensitivity to minor sequence changes underscores the importance of the fmi/Celsr conformations for their membrane localization as well as for the correct targeting of partner proteins. This raises the question of the role of putative proteins assisting in folding, like receptor activating protein RAP does for the lipoprotein receptor family [97], or the beta-2 microglobulin for major histocompatibility complexes [98]. Do Celsr, Fzd and Vangl help each other's folding and complex formation in the endoplasmic reticulum and traffic as a complex to the Golgi and to the membrane? Or are they assisted by other unidentified partners? In the latter case, what molecules would act as chaperonins?

Clearly, we are still a long way from integrating mechanistically the action of Celsr proteins, particularly their interactions with other core PCP components and downstream signaling pathways and terminal cell effectors. Elucidating those

questions will further our understanding of PCP during normal development, in pathological conditions such as ciliopathies, and in stem cell biology and regeneration.

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Table 1

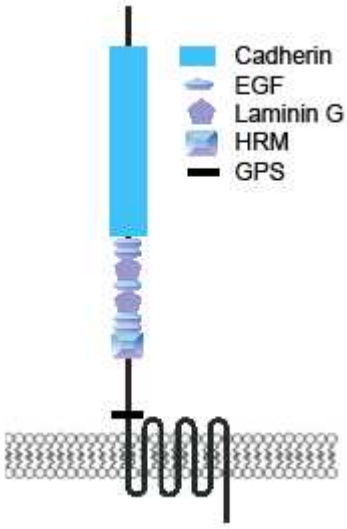
Summary of phenotypes of *Celsr1-3* and some other core PCP mutant mice

Gene	Allele	Phenotype[#]	References
Celsr1	*Celsr1 Crsh* and *Scy*	Heterozygotes have inner ear PCP abnormalities, head shaking, loop-tail, abnormal FBM neuron migration and lung alveologenesis defects. Homozygotes are embryonic lethal with open neural tube.	[37, 99]
	Celsr1 KO	Heterozygotes are normal and fertile. Homozygotes viable but mostly sterile. About 20% die *in utero* with neural tube closure defects. Adults have abnormal behavioral traits, a looping tail, and abnormal skin hair patterning. The direction of FBM neuron migration is affected.	[36, 47, 67]
Celsr2	*Celsr2gt*	Heterozygotes are normal and fertile. Homozygotes fertile, except for some females with vaginal atresia. There is abnormal trajectory of FBM neuron migration and hydrocephalus due to defective ependymal ciliogenesis.	[60, 67]
	Celsr2 KO and *Celsr2 F*	Heterozygotes are normal and fertile. Homozygotes are fertile and have abnormal FBM neuron migration.	[67]
Celsr3	*Celsr3 KO* and *Celsr3 F*	Heterozygotes are normal and fertile. Homozygotes die in a few hours after birth and have severe axonal defects.	[88, 89]
Fzd3	*Fzd3 KO*	Heterozygotes are normal and fertile. Homozygotes die in a few hours after birth and have axonal defects similar to those in *Celsr3* mutants. Some have loop tail or open neural tube and PCP phenotype in inner ear. The trajectory of FBM neuron migration is affected and mimics *Celsr2* and *3* double KO.	[39, 67, 91]
Fzd6	*Fzd6 KO*	Homozygous mutants survive and breed, and have abnormal skin hair patterning.	[48]
Vangl2	*Vangl2 Lp (Looptail)*	Some heterozygotes have kinked or looped tails. Homozygotes do not survive due to open neural tube, loop tail, inner ear PCP defects, FBM neuron migration defects and other defects in	[41, 69, 100]

		cardiovascular, skeletal and respiratory systems.	
	Vangl2 KO and Vangl2 F	Heterozygotes are normal and fertile. Homozygote nulls have open neural tube and other PCP defects in inner ear. Regulates nodal cilia beats and laterality.	[101]
Vangl1	*Vangl1 gt*	Homozygotes normal and fertile	[101]

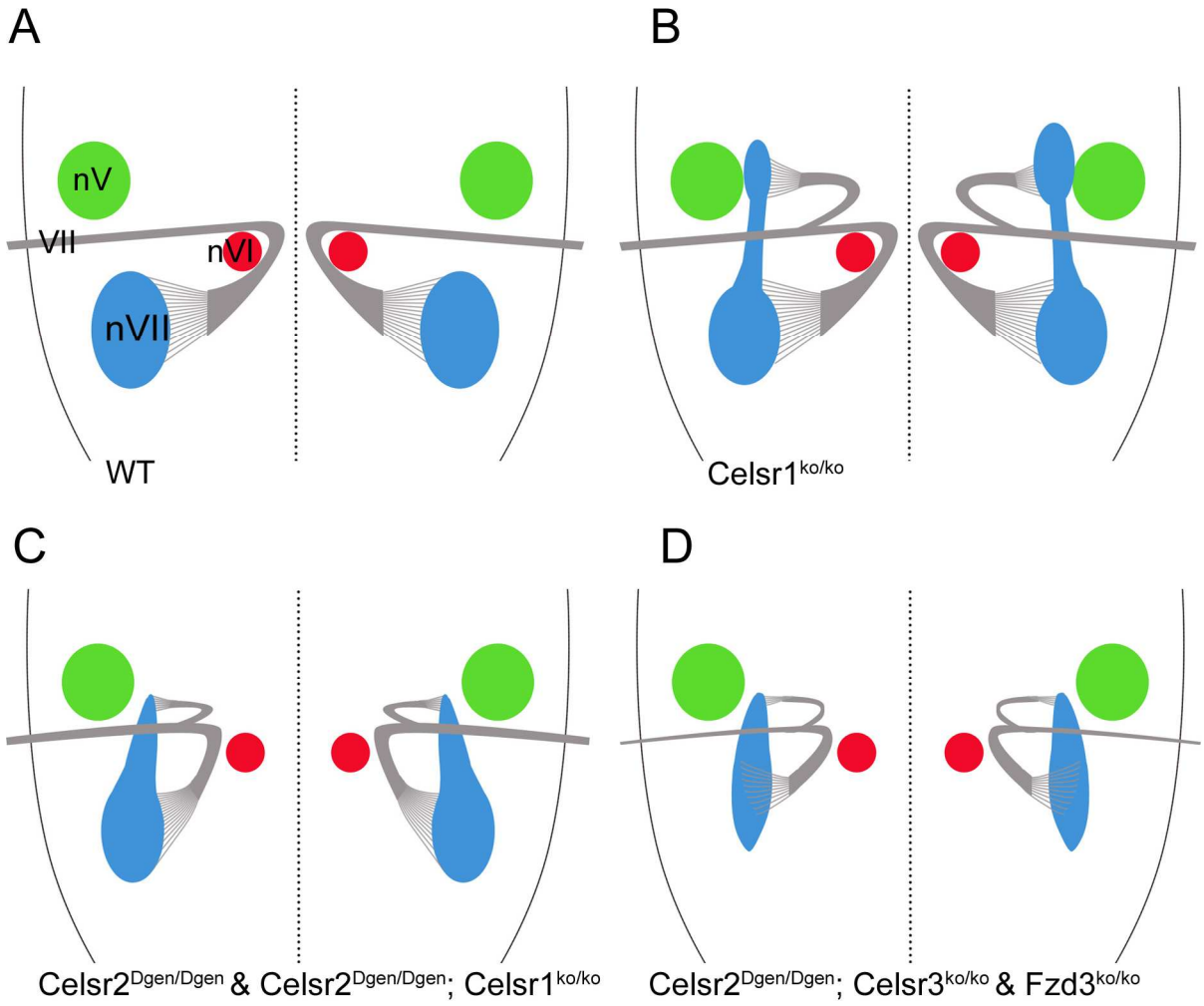
#: FBM, facial branchiomotor; KO, knockout; PCP, planar cell polarity

Fig. 1



Legend to figure 1: Schematic representation of the Celsr1-3 proteins. The extracellular domain includes nine cadherin repeats, six epidermal growth factor (EGF)-like domains, two laminin G repeats, one hormone receptor (HRM) motif, and a G-protein-coupled receptor proteolytic site (GPS). In contrast to typical cadherins, which are single-pass proteins, Celsr1, 2 and 3 cadherins are anchored to the plasma membrane by seven transmembrane domains. The cytoplasmic tail varies in size and is poorly conserved among the three Celsrs.

Fig 2.



Legend to figure 2: Migration of Facial Branchiomotor (FBM) neurons in normal and *Celsr1, 2, 3* knockout mice. Drawings summarize the phenotypes at P0. In wild type animals (A), FBM neurons form a single nucleus (nVII, blue) in rhombomere (r) r6. Their axons loop around the abducens nucleus (nVI, red)) before exiting the rhombencephalon caudally to the trigeminal nucleus (nV, green). In *Celsr1*$^{ko/ko}$ mutants (B), in addition to the normal nVII, FBM neurons form another ectopic nucleus at the level of nV, but their axons leave the rhombencephalon at the right position. This is due to aberrant migration of FBM neurons in r2 during embryogenesis. In *Celsr2*$^{Dgen/Dgen}$ and *Celsr2*$^{Dgen/Dgen}$; *Celsr1*$^{ko/ko}$ (C), the facial nerve *genu* is abnormal and axons do not loop around nVI, because FBM neurons migrate prematurely in lateral r4-r5, forming lateral ectopias. In *Celsr2*$^{Dgen/Dgen}$; *Celsr3*$^{ko/ko}$ and in *Fzd3* $^{ko/ko}$, in addition to the absence of the *genu* of the facial nerve, the size of nVII is reduced because of cell death (D). "r": rhombomere; nV: motor trigeminal nucleus; nVI: abducens nucleus; nVII: facial nerve nucleus.