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Role of Planar Polarity Proteins Celsr1-3 in Neuronal Migration and Ciliogenesis

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*PhD thesis in
Biomedical and Pharmaceutical Sciences*

2011

Acknowledgments

I am grateful to André GOFFINET for accepting me in his group. His brilliant scientific mind, availability and sense of humor created an excellent scientific and pleasant environment and influenced me deeply. I feel very lucky to be his student, and without his support, I would never have finished my project. Special thanks for all your help.

My sincere gratitude also to Fadel TISSIR. His generous help provided rigorous scientific training, invaluable advices and discussions that have been great supports to my work.

Members of my thesis committee, Professors Sonia GAREL, Frédéric CLOTMAN, Emmanuel HERMANS, Pascal KIENLEN-CAMPARD and Pierre VANDERHAEGHEN, accepted to review my work and helped me along the way. Thank you sincerely for your advice.

I thank all my colleagues in the lab: Atsushi BABA, Valérie BONTE, Camille BOUTIN, Guoliang CHAI, Rachid EL KADDOURI, Isabelle LAMBERMONT, Esther PAITRE, Yan PANG, Aurélia RAVNI, Yiqing XU, Libing ZHOU. Thank you all for your help and the friendly atmosphere.

During four years, I have been supported by grants from the Université Catholique de Louvain and the FRIA, and I appreciate that support highly.

My best wishes also to my great friend Yang LIU who was my classmate and became by my neighbor at UCL.

Last but not least, thanks to my parents for their unconditional love, the best visa to success.

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SUMMARY

Celsr1-3 (for Cadherin EGF Laminin Seven Pass Receptor), the mammalian orthologs of *Drosophila Flamingo/starry night*, encode proteins of the cadherin superfamily. *Celsr1-3* belong to a group named 'core PCP (Planar Cell Polarity) genes'. All three *Celsr* genes are expressed in the developing Central nervous system (CNS) in mice. Our lab is interested in studying their functions in mouse CNS development. In this program, I specifically addressed two issues, namely facial branchiomotor neuron (FBM neuron) migration and ependymal ciliogenesis.

During development, neurons are generated in ventricular zones, and postmitotic neurons usually need to migrate over variable distances to reach their final destination. Among the many neuronal migration events, that of FBM neuron in the hindbrain is unique and complex. In zebrafish, mutations in PCP genes such as *van gogh-like2*, *celsr2*, *frizzled3a*, *prickle1a* and *prickle1b* are known to affect normal FBM neuron caudal migration. This prompted us to investigate the role of *Celsr1-3*, and *Fzd3* in FBM neuron migration in mice. During mouse hindbrain development, FBM neurons migrate caudally from medial rhombomere (r) 4 to lateral r6. *Celsr1* is expressed in FBM neuron precursors and the floor plate, but not in FBM neurons themselves. In *Celsr1* constitutive mutants, caudal migration was compromised and neurons often migrated rostrally into r2 and r3, as well as laterally. Consistent with the expression profile, when we specially deleted *Celsr1* in FBM neurons using *Isl1-Cre*, no rostral migration stream was observed. In contrast, when *Celsr1* was deleted in FBM neuron precursors by *Nkx6.2-Cre*, abnormal rostral migration was seen in *Celsr1|Nkx6.2* mutants. Thus, *Celsr1* regulates FBM neuron migration direction in a non-FBM neuron-autonomous manner. In *Celsr2* mutants, FBM neurons initiated caudal migration but moved prematurely into lateral r4 and r5. This phenotype was enhanced by inactivation of *Celsr3* in FBM neurons and mimicked by inactivation of *Fzd3*. *Celsr2* and *Celsr3* act in a FBM neuron autonomous manner. Furthermore, *Celsr2* is epistatic to *Celsr1*. All these phenotypes were not caused by defects in hindbrain patterning or neuronal specification. These data indicate that *Celsr1-3* differentially regulate FBM neuron migration. *Celsr1* helps to specify the direction of FBM neuron migration, whereas *Celsr2* and 3 control its ability to migrate.

Studies in *Xenopus* indicate that there is a link between PCP signaling and ciliogenesis. In our mouse colony, I noticed that *Celsr2* and *Celsr3* double mutant mice developed huge hydrocephalus, and we decided to investigate the role of cilia in this phenotype. Ependymal cells form the epithelial lining of cerebral ventricles. Their apical surface is covered by cilia that beat in a coordinated fashion to facilitate circulation of the cerebrospinal fluid (CSF), and abnormal CSF circulation leads to hydrocephalus. In *Celsr2* mutant mice, the development and planar organization of ependymal cilia were compromised, leading to defective CSF dynamics and hydrocephalus. In *Celsr2* and *Celsr3* double mutant ependyma, ciliogenesis was markedly impaired, resulting in lethal hydrocephalus. The membrane distribution of Vangl2 and Fzd3, two key planar cell polarity proteins, was disturbed in *Celsr2* mutants, and even more so in *Celsr2* and *Celsr3* double mutants. Our findings suggest that planar cell polarity signaling is involved in ependymal cilia development and in the pathophysiology of hydrocephalus.

ABBREVIATIONS

AC	Anterior commissure
BM neuron	Branchiomotor neuron
CE	Convergent extension
CGE	Caudal ganglionic eminences
CNS	Central nervous system
CP	Cortical plate.
CSF	Cerebrospinal fluid
FBM neuron	Facial branchiomotor neuron
GE	Ganglionic eminences
IC	Internal capsule
IHC	Inner hair cells
IZ	Intermediate zone
LGE	Lateral ganglionic eminences
Mlf	Medial longitudinal fasciculus
MZ	Marginal zone
neo	neomycin
OCD	Oriented cell division
OHC	Outer hair cells
PCP	Planar cell polarity
r1-8	rhombomeres 1-8
SEM	Scanning electron microscopic
SM neuron	Somatic motor neuron
SOP	Sensory organ precursor
SVZ	Subventricular zone
TEM	Transmission Electron Microscopy
WT	Wild-type
VM neuron	Visceral motor neuron
VZ	Ventricular zone

1. INTRODUCTION

1.1. Planar cell polarity

Planar cell polarity (PCP), also known as tissue polarity, refers to the polarized organization of cells in the plane of an epithelium, perpendicular to the apical-basal axis of single cells. PCP is widespread in the animal kingdom and the correct establishment of PCP is an important feature of development. During development, PCP is not only seen in epithelial tissues, but also in mesenchymal cell types, such as the directed movement of mesenchymal cells during gastrulation (Klein and Mlodzik, 2005).

PCP in *Drosophila*

Historically, the study of PCP began more than 30 years ago in *Drosophila*, where most adult cuticle structures show a planar organization (Gubb and Garcia-Bellido, 1982; Lawrence and Shelton, 1975). In *Drosophila*, the main readouts of PCP, illustrated in Fig. 1, are the organization of hairs on the wing (Adler, 2002), ommatidia rotation in the eye (Zheng et al., 1995), the orientation of body appendages (Fabre et al., 2008) and the division of somatosensory organ precursors (Bellaiche et al., 2004; Segalen and Bellaiche, 2009). Systematic genetic screens and elegant genetic analyses were carried out to identify PCP genes and understand PCP processes. This led to identification of a set of PCP genes named ‘core PCP group’ composed of the following components: *Frizzled (Fz)*, *Flamingo/Starry night (Fmi/Stan)*, *Strabismus/Van Gogh (Stbm/Vang)*, *Dishevelled (Dsh)*, *Prickle (Pk)* and *Diego (Dgo)*. These core PCP proteins form membrane-associated signaling complexes whose subcellular localization, stoichiometry and distribution are crucial for normal PCP signaling (Adler, 2002; Seifert and Mlodzik, 2007).

In *Drosophila*, the wing is covered by an epithelium of hexagonal cells oriented parallel to the proximal-distal axis. A single actin-rich wing hair (trichome) grows at the distal side of each hexagonal cell and points distally. In PCP defective wings, both the orientation of wing hairs and the hair initiation site in the hexagonal cell are affected (Adler and Lee, 2001). As illustrated in Fig. 2a, whereas wild-type (WT)

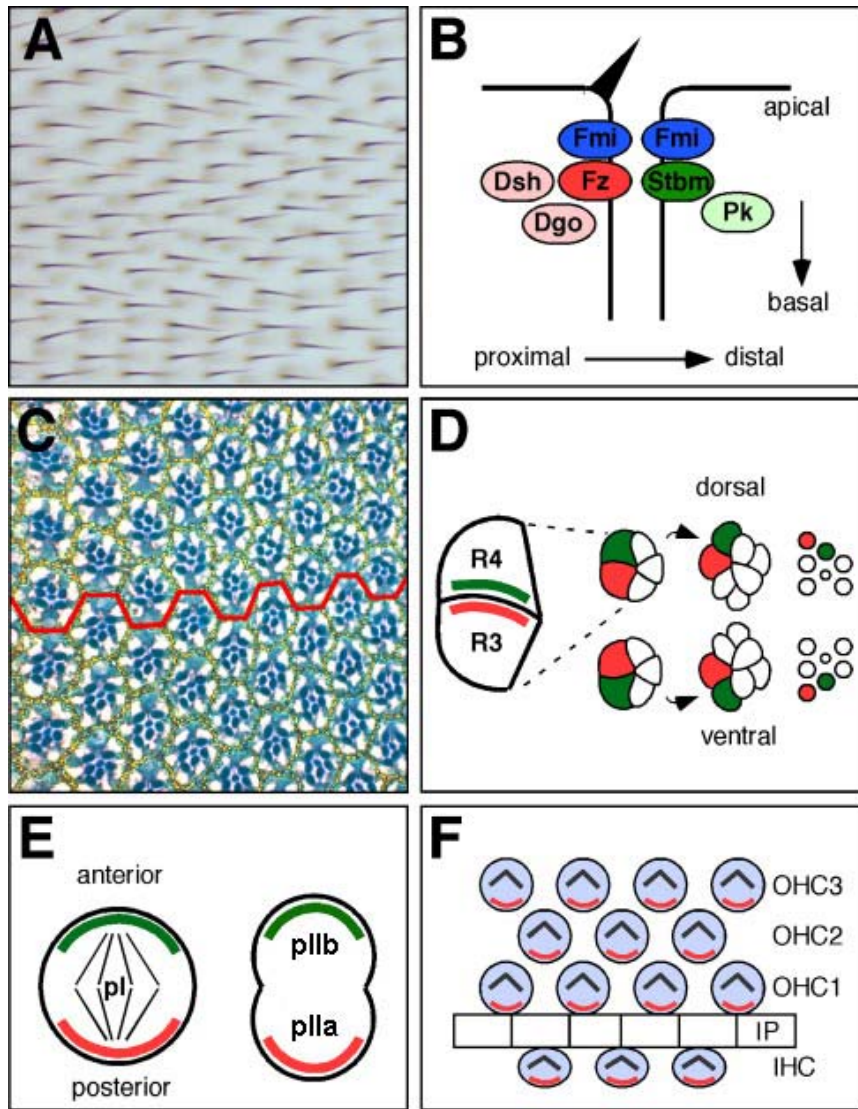


Fig. 1. Asymmetric localisation of core planar polarity proteins. (A) Wildtype adult wing, in which each cell produces a distally pointing hair or trichome. (B) Prior to trichome emergence, core planar polarity proteins localize to the apicolateral junctional region, forming an asymmetric complex as illustrated. (C) Ommatidia in the adult eye point in opposite directions on either side of the equatorial midline (red line). (D) Fz (red) and Stbm (green) localize asymmetrically on either side of the R3-R4 photoreceptor cell boundary, with Fz localizing on the side of the boundary closest to the dorsoventral midline. Ommatidia then rotate such that the cell localizing Fz moves away from the midline. (E) In the sensory organ precursor cell pI, Stbm (green) localizes at the anterior cortex and Fz (red) localizes to the posterior cortex. This orients the axis of cell division and determines the correct segregation into daughter cells of anterior and posteriorly localized cell fate determinants. (F) Inner hair cells (IHCs) and outer hairs cells (OHCs) in the vertebrate organ of Corti contain a chevron-shaped arrangement of stereocilia with a kinocilium at its apex. Core planar polarity protein homologs localize asymmetrically, for example Fz (red) localizes to the cell cortex opposite the kinocilium. (Strutt and Strutt, 2009)

wings display a parallel alignment of distally pointing hairs, wings in PCP mutants have globally aberrant wing hair patterning (compare Fig. 2a and 2b). An asymmetric distribution of core PCP proteins was observed in normal wing cells. Fmi is present symmetrically, whereas Fz and Vang are targeted to opposing cell edges, Fz to the distal side where the hair is located, and Vang to the proximal side (Fig. 1B). Interactions mediated by PCP complexes propagate asymmetric signals from cell to cell, a distal signal via the Fz/Dsh complex, and a proximal one via the Vang/Pk complex (Strutt and Strutt, 2009; Tissir and Goffinet, 2010). Contrary to vertebrates (see below), no Wnt factor seems to be involved in the regulation of PCP in flies.

The *Drosophila* compound eye contains 750-800 ommatidia, and each ommatidium is composed of eight photoreceptors (R1-8), and several other accessory cells. The eight photoreceptors differentiate in a fixed sequence, with R8 differentiated first, followed sequentially by R2/5, R3/4, R1/6 and finally R7 (Tomlinson and Ready, 1987). During development, the immature ommatidia at the five photoreceptor stage are first symmetrically arranged and then rotate 90° towards the equator, with opposite rotation direction in the dorsal and ventral part. Thus, the mature ommatidia have two possible chiralities, with the R3 and R4 photoreceptors positioned asymmetrically at the tip of the ommatidial trapezoids (Mlodzik, 1999). In WT flies, ommatidia are orientated with respect to both the anterior-posterior and dorsal-ventral axes to form two mirror image zones on either side of the dorsal-ventral equator (Fig. 1C, Fig. 2e). In PCP mutants, ommatidia rotation is randomized and the mirror image zones are lost (Fig. 2f) (Wang and Nathans, 2007). In contrast to the asymmetric subcellular localization in the wing, the disposition of ommatidia is dictated by the R3/R4 cells. Accordingly, the distribution of core PCP proteins in the eye is cell-type specific: Fz is found on the R3 side of the R3/R4 photoreceptor cell boundary and Vang on the R4 side (Fig. 1D). The R3/R4 asymmetric interaction seems to dictate the direction of ommatidia rotation by mechanisms that remain poorly understood (Rawls and Wolff, 2003; Strutt et al., 2002; Strutt and Strutt, 2009).

PCP genes are also involved in asymmetric division of sensory organ precursor (SOP) cells in *Drosophila*. Each bristle mechanosensory organ (microchaete) in the *Drosophila* dorsal thorax (notum) is composed of two external support cells (the shaft and the socket cell) and two internal cells (the neuron and the sheath cell). These cells originate from a single SOP cell (or pI cell) by successive asymmetric divisions. Besides these four cells, there is a glial cell generated during the second asymmetric

division of the pI cell but undergoes apoptosis soon after birth. At each division, cell fate determinants such as Numb, Neuralized (Neur), and Partner of Numb (Pon) are asymmetrically segregated into daughter cells to which they confer distinct fates. The mitotic spindle of the pI precursor is orientated parallel to the anterior-posterior axis of the fly. Numb, Neur and Pon are localized at the anterior cortex of the pI cell and inherited by the anterior pIIb cell. Core PCP proteins are asymmetrically localized in the pI precursor, Fz, Dsh at the posterior side, and Vang and Pk at the anterior side, and this asymmetry determines the axis of cell division (Fig. 1E). In *dsh* and *fz* mutants, pI divisions are nearly randomly orientated in the epithelial plane. However, cell fate determinants are still distributed normally to daughter cells. As a result, sensory organs develop normally, but bristles assume aberrant orientations on the notum (Bellaiche et al., 2004; Gho and Schweisguth, 1998; Strutt and Strutt, 2009).

Core PCP complexes and their downstream effectors align individual cells and their neighbors, but how this alignment can be arranged in relation to the global direction of tissue axis remains to be understood. One possible model is that a set of upstream PCP factors link the global direction to single cells. This set of PCP factors may include atypical cadherins Fat (Ft), Dachshous (Ds) and the Golgi kinase Four-jointed (Fj) (Axelrod, 2009; Vladar et al., 2009). Ft and Ds interact in a heterophylic manner and form asymmetric dimers that - through some unknown mechanism - can translate the global cues to the core PCP complexes. Loss of Ft function in the fly wing and eye results in uncoupling of the individual cell direction from the tissue axis (Ma et al., 2003; Simon, 2004; Yang et al., 2002). Four-jointed is a protein kinase that phosphorylates serine or threonine residues within extracellular cadherin domains of Ft and Ds and influences their activity. Thus, Fj regulates Ft signaling by phosphorylating cadherin domains of Ft and Ds as they transit through the Golgi (Ishikawa et al., 2008).

PCP in vertebrates

All *Drosophila* core PCP genes have orthologs in human and mice. There are ten *Frizzled* (*Fzd1-10*), four *Prickle* (*Prickle1-4*), three *Dishevelled* (*Dvl1-3*), two *Van Gogh* (*Vangl1-2*) and three *Flamingo/starry night* (*Celsr1-3*, 'cadherin EGF laminin seven-pass receptors 1-3') in mammals. Orthologs are also described in zebrafish and *Xenopus*, sometimes under different names (Tissir and Goffinet, 2010). PCP processes that typically involve epithelia and received attention in mammals are: i) Neural tube closure (Copp and Greene, 2010); ii) Hair bundle orientation in inner ear sensory cells (Rida and Chen, 2009); and iii) Hair follicle orientation in the skin (Devenport and Fuchs, 2008; Ravni et al., 2009; Wang et al., 2006b).

The organ of Corti, the auditory receptor, contains four rows of receptor cells: a single row of inner hair cells (IHC) and three rows of outer hair cells (OHC). Actin stereocilia on the apical surface of cochlea hair cells form a V shaped, descending staircase, with a single primary kinocilium positioned near the tallest stereocilia at the apex of the V. Thus, the V-shape hair bundles are polarized and orientated with the vertices pointing in the lateral direction (Fig. 1F). All the IHCs and OHCs are arranged in the same orientation in WT mice (Fig. 2g). This organization is affected in mice with mutations of PCP genes (Fig. 2h) such as *Celsr1* (Curtin et al., 2003), *Vangl2* (Montcouquiol et al., 2003; Montcouquiol et al., 2006b), *Fzd3* and *6* (Wang et al., 2006c).

The arrangement of hairs over the mammalian body surface is a vertebrate PCP process that resembles the wing hair patterning in *Drosophila*. Each hair follicle and its associated hair are orientated with respect to the body's axis (Wang and Nathans, 2007). In WT mice, body hairs point caudally (Fig. 2c) and those on the legs point distally. In *Fzd6* mutant mice, this regular arrangement is disturbed, so that hairs form whorls, waves and tufts (Fig. 2d) (Guo et al., 2004; Wang et al., 2006b). As illustrated in Fig. 7 (see below), *Celsr1* knockout mice mimic the skin hair phenotype in *Fzd6* mutant mice, indicating that both proteins act in a common pathway (Ravni et al., 2009).

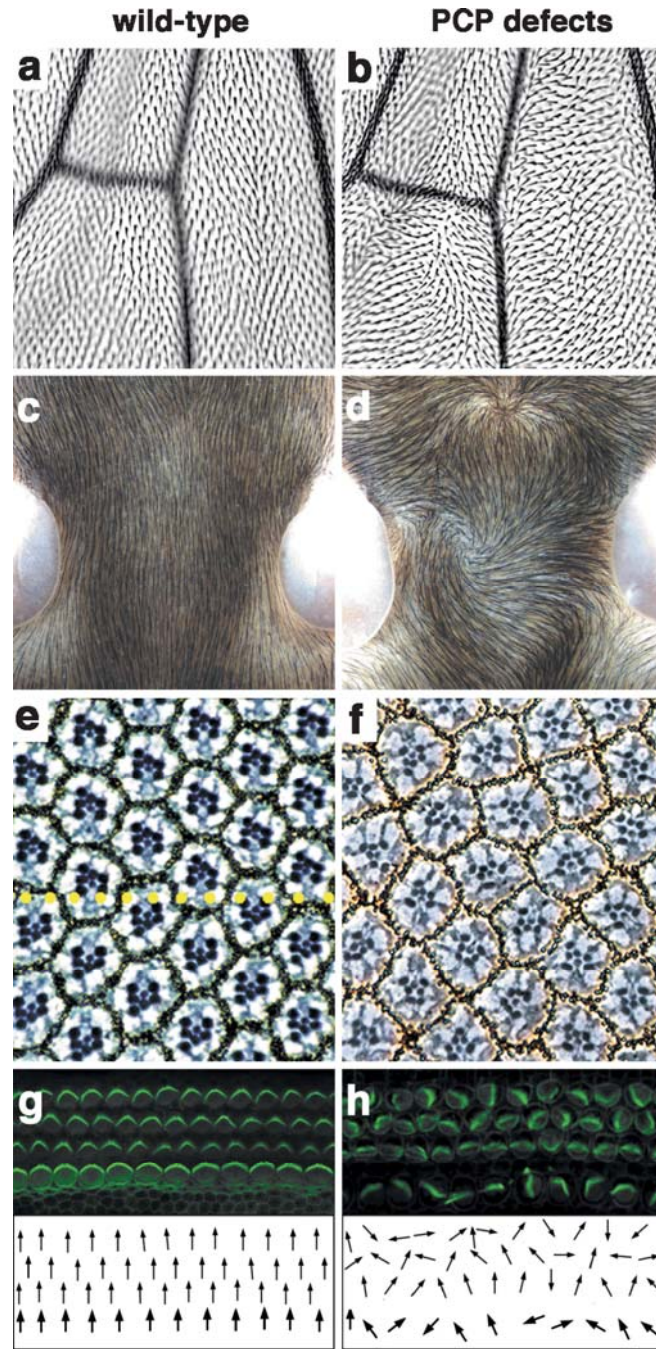


Fig. 2. Examples of PCP in *Drosophila* and mammals. Wild-type is shown in panels *a*, *c*, *e*, and *g* (note the very regular arrangements in all tissues), and mutant appearance of the same tissues is shown in *b*, *d*, *f*, and *h*. (*a,b*) *Drosophila* wing hair patterns; (*c,d*) mouse skin hairs (in dorsal view of neck); (*e,f*) *Drosophila* eye neuroepithelium; (*g,h*) mouse inner ear neuroepithelium. Orientation of inner ear sensory cells is highlighted with arrows below. A similar schematic could be shown for other tissues reflecting similar underlying defects in all tissues. (Klein and Mlodzik, 2005).

Intriguingly, in both invertebrates and vertebrates, some events during neural development, such as axon guidance and neurite growth, involve one or more of the core PCP genes/proteins, although they are not directly related to tangential epithelial organization. In *Drosophila*, *Fmi* is involved in photoreceptor axon guidance and dendritic morphogenesis (Gao et al., 2000; Kimura et al., 2006; Lee et al., 2003; Senti et al., 2003). In rat, *Celsr2* enhances neurite growth, whereas *Celsr3* suppresses it (Shima et al., 2007; Shima et al., 2004). In mice, *Fzd3* and *Celsr3* are crucial for development of several major axonal tracts in the central nervous system (CNS). As shown in Fig. 3, in both mutant mice, commissural spinal cord axons fail to turn rostrally after crossing the midline (Lyuksyutova et al., 2003; Price et al., 2006).

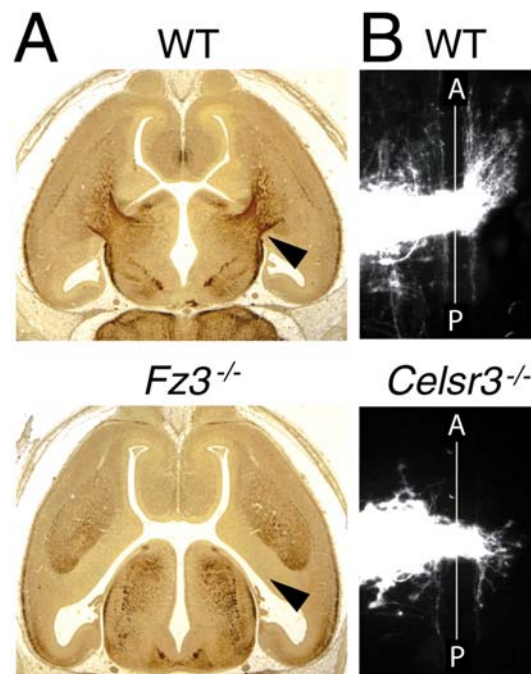


Fig. 3. Axonal growth and guidance defects in the developing *Fz3*^{-/-} and *Celsr3*^{-/-} central nervous system. (A) Anti-neurofilament staining of horizontal sections through E15 WT and *Fz3*^{-/-} mutant mouse brains shows a complete absence of thalamocortical and corticothalamic fiber bundles in an otherwise normal-appearing *Fz3*^{-/-} forebrain; the normal trajectory of these fibers is indicated by an arrowhead. (B) DiI tracing of commissural axons in E11 WT and *Celsr3*^{-/-} mouse spinal cords shows a failure of rostral turning by *Celsr3*^{-/-} axons after midline crossing. The spinal cord has been opened at the dorsal midline and flattened (so-called 'open book' preparation). DiI was placed in commissural cell bodies beyond the left edge of each image. The vertical white line indicates the midline. A, Anterior/rostral; P, posterior/caudal. (Wang and Nathans, 2007).

Thalamocortical, corticofugal and subcerebral axons cannot travel through the ventral telencephalon to reach their targets (Fig. 3, arrowheads), and the development of some major commissures, particularly the anterior commissure, is defective (Tissir et al., 2005; Wang et al., 2002). *Celsr3* and *Fzd3* mutants have almost identical phenotypes, suggesting strongly that both genes function together in a common axon guidance mechanism. Whether these processes share the same PCP signaling mechanism that regulates tissue polarity in epithelia is still unclear, but observations thus far indicate that the two kinds of cellular processes are related at the molecular level.

A link between PCP and neuronal migration was suggested only in the case of facial branchiomotor neurons (Wada et al., 2006). Thus far, PCP proteins were not shown to play any significant role in other neuronal migration events.

A relationship between ciliogenesis and PCP molecules was initially suggested in studies of frog multiciliated epidermal cells ((Park et al., 2006; Park et al., 2008), as will be discussed below.

PCP and Wnt signaling

Whereas PCP was shown to be independent from Wnt signaling in flies, several studies implicate Wnt signaling in vertebrate developmental events that resemble PCP. In man and mouse, there are 19 Wnt genes that activate so called canonical and noncanonical pathways, some of which are known to be involved in PCP signaling through noncanonical pathways. In canonical pathway, Wnts activate Fzd and LRP5/6 (lipoprotein receptor-related protein 5/6) receptor complexes, recruit Dvl to the plasma membrane and then disrupt the β -catenin degradation complex through a number of factors including Axin and GSK3. β -catenin is thereby stabilized and translocates to the nucleus where it interacts with members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family to regulate the transcription of target genes (Fig. 4a). In noncanonical pathway, Wnt factors still bind to Fzd and activate Dvl, but, contrary to the classical pathway, neither Gsk-3 β nor β -catenin are involved (Fig. 4b). Distinction of canonical versus non canonical Wnt signaling is partly attributed to the ligand, with Wnt4, Wnt5a and Wnt11 activating preferentially noncanonical signals.

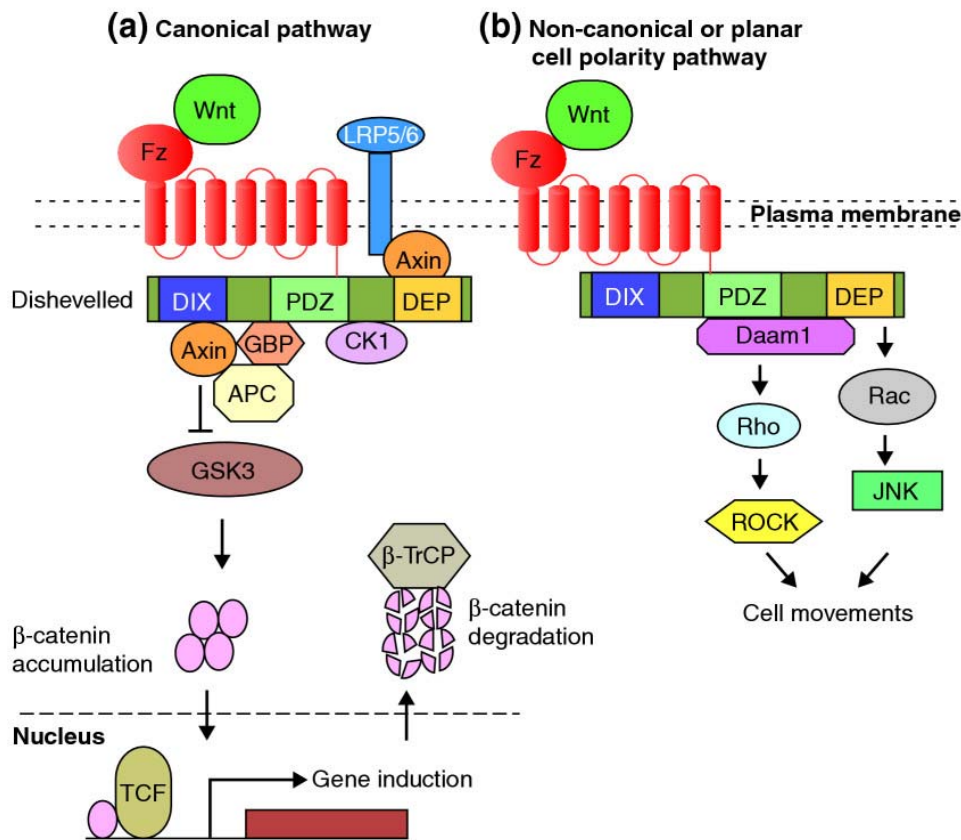


Fig. 4. A schematic representation of the Wnt signal transduction cascade. (a) For the canonical pathway, signaling through the Frizzled (Fz) and LRP5/6 receptor complex induces the stabilization of β -catenin via the DIX and PDZ domains of Dishevelled (Dsh) and a number of factors including Axin, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). β -catenin translocates into the nucleus where it complexes with members of the LEF/TCF family of transcription factors to mediate transcriptional induction of target genes. β -catenin is then exported from the nucleus and degraded via the proteosomal machinery. **(b)** For non-canonical or planar cell polarity (PCP) signaling, Wnt signaling is transduced through Frizzled independent of LRP5/6. Utilizing the PDZ and DEP domains of Dsh, this pathway mediates cytoskeletal changes through activation of the small GTPases Rho and Rac. (Habas and Dawid, 2005)

Several studies show that noncanonical pathways are involved in classical PCP events such as convergent extension (CE) during gastrulation. Loss of function and overexpression experiments in zebrafish and *Xenopus* showed that *Wnt11* and *Wnt5a* regulate CE movements (Heisenberg et al., 2000; Kilian et al., 2003; Rauch et al., 1997; Ulrich et al., 2003; Wallingford et al., 2001). Defective Wnt11 signaling leads to abnormal axial extension with fusion of the eyes and other midline defects in the head. Wnt11 binds to Fzd7 and induces accumulation of Dvl at the membrane, fostering its interaction with the formin homology protein Daam1 (Dvl associated activator of morphogenesis 1) and WGEF (weak-similarity guanine nucleotide-exchange factor), leading to formation of the Rho-GTP complex, activation of ROCK kinase and cytoskeletal remodeling (Gao and Chen, 2010; Montcouquiol et al., 2006a; Vladar et al., 2009). Wnt5a regulate CE in a different manner, by activating Dvl-Rac and the downstream effector c-jun N-terminal kinase (JNK) (Gao and Chen, 2010; Vladar et al., 2009).

Some studies show that Wnts participate in other PCP processes such as hair bundle orientation in inner ear sensory cells. In vitro application of Wnt7a or inhibitors of Wnt signaling disrupts hair bundle orientation (Dabdoub et al., 2003). *Wnt5a* mutant mice have shortened and widened cochlea, and *Wnt5a* interacts genetically with *Vangl2* in inner ear sensory cell stereocilia orientation, cochlear extension and neural tube closure (Qian et al., 2007). Recent studies in mouse limb development show that a Wnt5a gradient can establish PCP by inducing Vangl2 phosphorylation through Ror2 (Gao et al., 2011).

Noncanonical Wnt4 and Wnt5a have been implicated in axonal guidance in mice, in collaboration with the core PCP effector Fzd3 (Lyuksyutova et al., 2003). Dvl1 can inhibit PCP signaling by increasing phosphorylation and preventing internalization of Fzd3, and this is antagonized by Vangl2. In commissural axon growth cones, Vangl2 is predominantly localized on the membrane and enriched on the tips of filopodia. This led to suggestion that PCP complexes could sense directional cues elicited by gradients of non canonical Wnts, thereby assisting in growth cones steering (Shafer et al., 2011).

Thus far, phenotypes of noncanonical Wnt knock-out mice do not provide clear arguments, possibly due to genetic redundancy. Thus, the role of noncanonical Wnt signaling in mammalian PCP remains controversial.

1.2. Mammalian *Celsr1-3*

Celsr1-3 (Cadherin EGF Laminin Seven Pass Receptor), the mammalian orthologs of *Drosophila Flamingo/starry night*, encode proteins of the cadherin superfamily. Celsr proteins have nine N-terminal cadherin repeats, followed by several EGF and laminin G repeats, seven transmembrane segments and an intracellular C-terminus. Contrary to other cadherins that have one transmembrane domain, Celsr1-3 are related to G-protein coupled receptors of the secretin receptor family. The three Celsr protein sequences are more than 50% identical in their extracellular and transmembrane regions but differ in their cytoplasmic domains. The C-terminal intracellular domains of Celsr1 and 2 are about 300 amino acids long, whereas that of Celsr3 contains about 590 amino acids (Tissir et al., 2002). The genomic organization and corresponding protein domains of *Celsr3* are shown in Fig. 5. The genomic structures of *Celsr1* and 2 are similar to that of *Celsr3*, with 35 exons. Quite unusually, exon1 is larger than 4000 nucleotides and encodes all nine cadherin repeats. This is followed by a region that contains several EGF (epidermal growth factor, cd00054) and Laminin G-like (cd00110) domains, both of which are often implicated in protein-protein interactions. There is also a “H” domain present in other G-protein coupled receptors of the

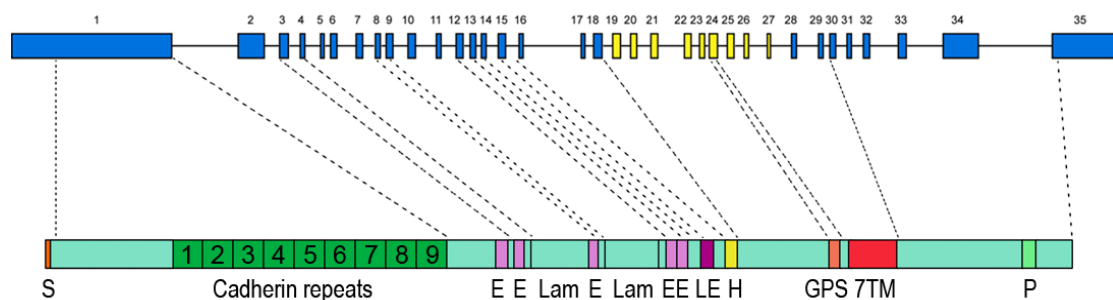


Fig. 5. Genomic organization of the mouse *Celsr3* gene and corresponding protein. Exons are indicated with the encoded protein shown below; the transcription start site was not mapped. The protein domains were defined used the Pfam database (Bateman et al., 2000). S, signal sequence; E, EGF-like motif; Lam, laminin G domain; LE, laminin-type EGF motif; H, domain present in hormone receptors; GPS, latrophilin-CL1-like G protein coupled receptor proteolytic site; 7TM, seven-pass transmembrane domain (secretin family); P, proline-rich region. The genomic structures of *Celsr1* and 2 are similar to *Celsr3*, except that the cytoplasmic tails are shorter. (Tissir et al., 2002)

secretin receptor type. A poorly conserved putative proteolytic cleavage site (named GPS domain) is found between the ecto domains and the transmembrane region; this site is not used in *Celsr3*, but processing of *Celsr1* and *Celsr2* has not been examined thus far.

Celsr1-3 expression patterns in mice

The three *Celsr* genes are predominantly expressed in the developing CNS in mice. In addition, *Celsr3* is also moderately expressed in the kidney and lungs, whereas *Celsr1* and 2 are also found at low levels in the fetal lungs, kidney and epithelia (Tissir et al., 2002). In the brain, *Celsr1-3* have different expression patterns. *Celsr1* is mainly expressed in neural stem/precursor cells in the ventricular zone (VZ) (Fig. 6A, D). By contrast, *Celsr3* is expressed in postmitotic cells, but not in precursors (Fig. 6C, F). The expression of *Celsr2* overlaps that of *Celsr1* and 3: it is expressed both in neural precursors and posmitotic cells (Fig. 6B, E). After birth, the expression of *Celsr1* and 3 are sharply downregulated, whereas that of *Celsr2* persists in the adult brain. Altogether, the expression data may indicate a role for *Celsr1* in stem cell biology, *Celsr2* in neuron maintenance, and *Celsr3* in neuronal maturation.

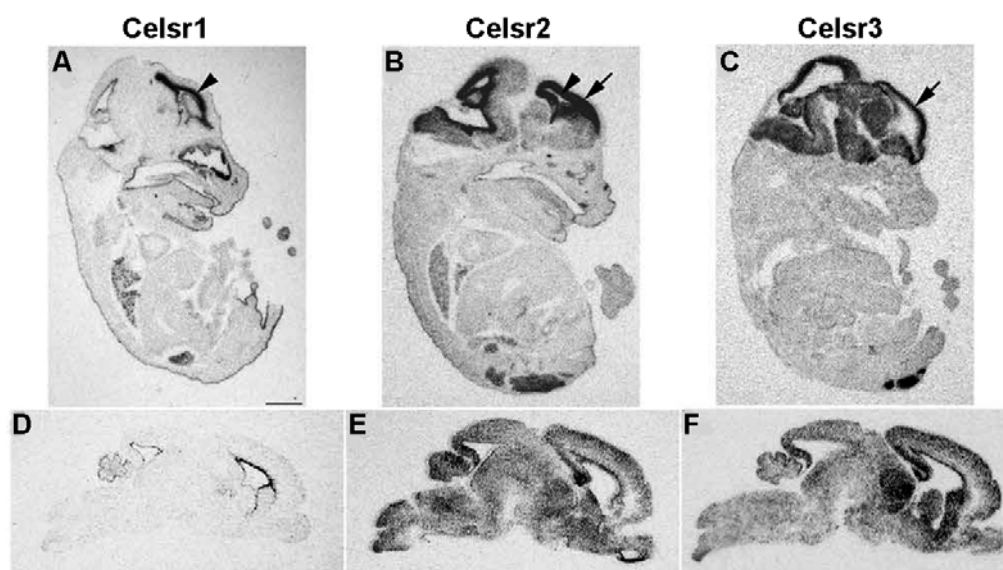


Fig. 6. *Celsr1-3* mRNA expression patterns using [^{33}P]-labeled riboprobes. A-C: sagittal sections through whole embryos at E15.5. D-F: sagittal brain sections at birth (P0). Black arrowheads point to signal in ventricular zones, where stem/progenitor cells are located. Black arrows point to signal in the cortical plate, populated by postmitotic, end-migration neurons. A, D: *Celsr1*; B, E: *Celsr2* and C, F: *Celsr3*. Bar=1 mm. (Tissir et al., 2002)

Celsr1

Two *Celsr1* mutant alleles, *Crash* (*Crsh*) and *Spin Cycle* (*Scy*), were identified in an ENU screen in mice. In *Crsh*, a G-to-A point mutation at nucleotide 3126 results in an aspartate to glycine substitution at codon 1040, which lies within the eighth cadherin repeat. In *Scy*, a T-to-A point mutation at nucleotide 3337 results in an asparagine to lysine substitution at codon 1110, which lays in the linker region connecting adjacent cadherin domains. Heterozygous animals show abnormal head-shaking behavior. Both heterozygous and homozygous mice have defects in the arrangement of sensory hair cells in the organ of Corti, which is a typical PCP phenotype. *Crsh* and *Scy* homozygotes and double heterozygotes (*Crsh*/+; *Scy*/+) suffer from severe neural tube closure defects, a defect that could be due to abnormal CE in the neuroepithelium (Curtin et al., 2003). Mutations of other PCP genes, such as *Vangl2*, double *Fzd3* and 6, *Dvl1* and 2, have defective CE, with looping tail and open neural tube (Kibar et al., 2001; Wang et al., 2006a; Wang et al., 2006c; Ybot-Gonzalez et al., 2007).

Celsr1 Crsh and *Scy* homozygous mutants are embryonic lethal. To study the function of *Celsr1* further, our lab has produced a conditional *Celsr1* allele (*Celsr1^F*) that allows region/tissue-specific inactivation under expression of promoter specific Cre (Fig. 7A) (Ravni et al., 2009). To produce a conditional *Celsr1* allele, loxP sites were introduced into introns 25 and 29. Upon expression of Cre recombinase, sequences between loxP sites are deleted, which include exons 26-29 that encode transmembrane domains 5-7. The deletion also causes a reading frame shift, with a premature stop codon in the mutant mRNA. Western blot with antiCelsr1 antibodies (provided by C. Formstone) confirms absence of protein in embryonic mouse brain extracts. A constitutive *Celsr1* mutant allele (*Celsr1^{KO}*) was generated by crossing the conditional allele with PGK-Cre mice which express Cre recombinase ubiquitously in somatic and germ-line cells.

Heterozygous *Celsr1^{KO}* mice are fertile and look normal. Homozygous *Celsr1^{KO}* mutants are sterile and have abnormal behavioral traits, such as turning and moving almost continuously. About 20% of homozygotes die at embryonic stages with various degrees of neural tube closure defects, and many of homozygous mutants have a kinky or looping tail (Fig. 7B). Some of the homozygotes have abnormal skin hair patterning that develops independently of the looptail trait. Instead of regular caudal-pointing hairs, *Celsr1^{KO}* homozygous mice develop whorls or crests (Fig. 7C) (Ravni et al., 2009). This hair patterning phenotype is similar to that found in *Fzd6*

mutant mice (Guo et al., 2004), indicating that *Celsr1* and *Fzd6* cooperatively regulate hair patterning in mice.

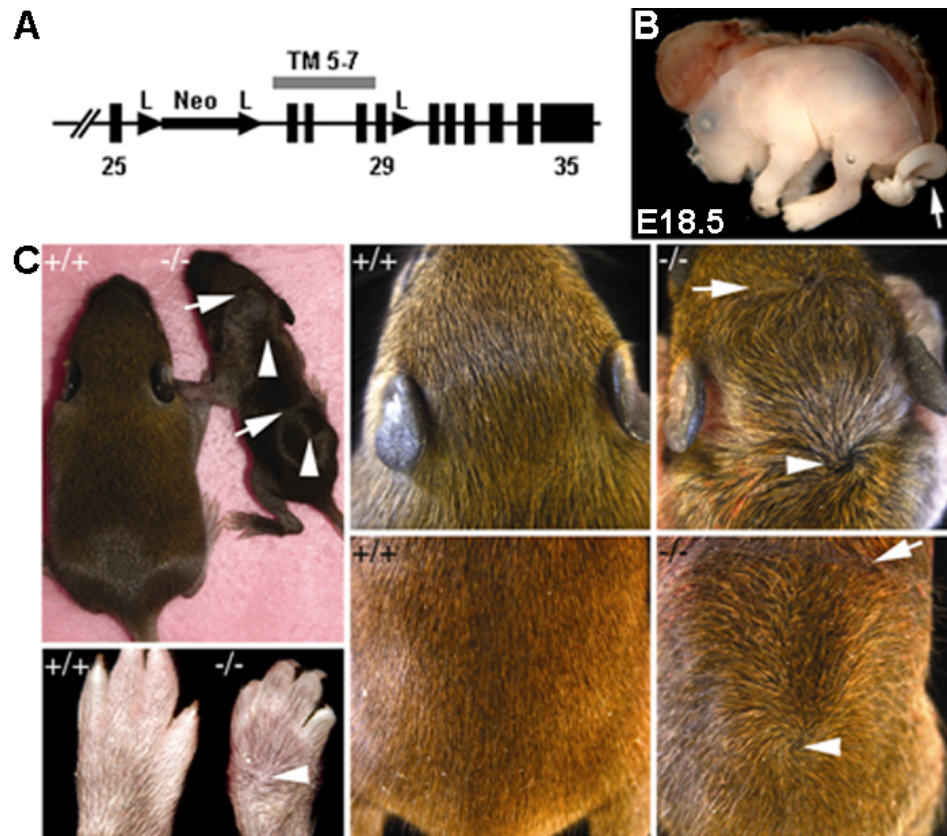


Fig. 7. *Celsr1* targeting strategy and abnormalities in *Celsr1*^{KO} homozygous mice. (A) Exons 26–29, which encode transmembrane segments 5 to 7 (TM 5–7), were flanked by LoxP sites (L) to allow their deletion by the Cre recombinase. Neo, neomycin cassette. (B) *Celsr1*^{KO} homozygous embryo at E18.5 with open neural tube and looping tail. (C) *Celsr1* mutation disrupts skin hair patterning. Whorls (arrowheads) and crests (arrows) are observed on the body and feet in mutant (-/-) but not wild-type (+/+) mice (Ravni et al., 2009).

Celsr2

The Celsr2 protein is distributed in both dendrites and axons of neurons such as hippocampal pyramidal cells, Purkinje cells, and olfactory neurons (Shima et al., 2002). Using an RNAi system and organotypic brain slice cultures, *Celsr2* was shown to regulate dendritic maintenance and growth (Shima et al., 2004). Knocking down *Celsr2* caused a marked decrease in dendritic complexity, which included reductions in the length of apical dendrites and in the number of basal dendrites of pyramidal neurons, a phenotype that is probably due to dendritic retraction. In my work, I used a gene trap generated Celsr2 Δ allele that behaves as a recessive. We also generated a novel floxed Celsr2F mutant (see Results).

Celsr3

Celsr3 plays a critical role in development of major axon tracts, particularly the anterior commissure (AC) and the internal capsule (IC). *Celsr3* and *Fzd3* mutant mice have similar axonal guidance defects that cause prominent brain wiring abnormalities in the spinal cord, brainstem, mid- and forebrain (Lyuksyutova et al., 2003; Tissir et al., 2005; Wang et al., 2002). To study the mechanism whereby *Celsr3* regulates axon guidance, our lab produced conditional *Celsr3* mutants, which were then crossed with several Cre-expressing mice to inactivate *Celsr3* in different specific brain regions (Zhou et al., 2008). By analyzing *Celsr3*|*Emx1* mice, *Celsr3* was shown to be required in the neurons of origin of the AC and corticospinal tract. The same strategy was used to identify guidepost cells in the basal forebrain, which are positive for *Dlx5/6* and a subset of which expresses *Islet1*, and are derived from *Nkx2.1* and *Gsh2* negative precursors. Expression of *Celsr3* in these guidepost cells is indispensable for development of all three components of the IC (thalamocortical, corticothalamic and subcerebral tracts). When *Celsr3* was inactivated in cortex, subcerebral tracts failed to grow, but corticothalamic axons developed normally, indicating that besides guidepost cells, additional Celsr3-independent cues can assist in their progression (Zhou et al., 2008). Cortical afferent and efferent fibers are absent in *Celsr3*|*Foxg1* and *Celsr3*|*Dlx5/6* mutant mice, generating an ‘isolated cortex’ *in vivo*. These animals can survive sometimes up to P20, when cortical maturation is complete, and they can walk, bite, climb and swim. Analysis of this *in vivo* “neocortex isolé” model showed that early cortical lamination and arealization (so-called “protomap”) develop

normally in absence of extrinsic connections, but survival of projection neurons and acquisition of mature morphological and some electrophysiological features depend on the establishment of normal cortical-subcortical relationships (Zhou et al., 2010).

Summary of phenotypes

A table with summary of the phenotypes in *Celsr1-3*, *Fzd3*, *6* and *Vangl2* mutant mice (including those described in my work) is provided below.

Gene	Mutant allele	Phenotype
<i>Celsr1</i>	<i>Celsr1 Crsh</i> and <i>Scy</i>	Heterozygotes have inner ear abnormalities with head shaking, looptail and abnormal FBM neuron migration. Homozygotes are embryonic lethal with open neural tube.
	<i>Celsr1 KO</i>	Heterozygotes are normal and fertile. Most homozygous mutants can survive but are sterile. About 20% die at embryonic stages with various degrees of neural tube closure defects. Many homozygous adults have abnormal behavioral traits, a looping tail, and abnormal skin hair patterning. The direction of FBM neuron migration is affected.
<i>Celsr2</i>	<i>Celsr2 Δ</i>	Heterozygotes are normal and fertile. Homozygotes are fertile, except for some females with vaginal atresia. The trajectory of FBM neuron migration is affected. They develop hydrocephalus due to defective endymal ciliogenesis.
	<i>Celsr2 KO</i> and <i>Celsr2 F</i>	Heterozygotes are normal and fertile. Homozygotes are fertile and have abnormal FBM neuron migration.
<i>Celsr3</i>	<i>Celsr3 KO</i> and <i>Celsr3 F</i>	Heterozygotes are normal and fertile. Homozygotes die in few hours after birth and have severe axonal defects.
<i>Fzd3</i>	<i>Fzd3 KO</i>	Heterozygotes are normal and fertile. Homozygotes die in few hours after birth and have axonal defects similar to those in <i>Celsr3</i> mutants. Some have loop tail or open neural tube. The trajectory of FBM neuron migration is affected and the phenotype mimics <i>Celsr2 & 3</i> double KO.
<i>Fzd6</i>	<i>Fzd6 KO</i>	Homozygous mutants can survive and breed, and have abnormal skin hair patterning.
<i>Vangl2</i>	<i>Vangl2 Lp</i> (<i>Looptail</i>)	Some heterozygotes have kinked or looped tails. Homozygotes do not survive past birth. They have an open neural tube, loop tail, inner ear defects, FBM neuron migration defects and other defects in cardiovascular, skeletal and respiratory systems.

1.3. Neuronal migration

During development, neurons are generated in proliferative zones around cerebral ventricles, named ventricular zones (VZ). Postmitotic neurons then migrate over variable distances from VZ to their final destination, where they differentiate and integrate into neural networks. Defects in neuronal migration cause various developmental abnormalities in both human and mouse.

Two major types of neuronal migration take place in the mammalian brain: radial migration and tangential migration. Radial, also named gliophilic migration, is quantitatively the main mode of migration, in which neurons migrate from VZ towards the surface of the brain along radial glial fibers that extend from the VZ to the marginal zone (MZ) where they terminate by forming end-feet abutting the basal lamina (Fig. 8B) (Marin and Rubenstein, 2003). A well-known example of radial

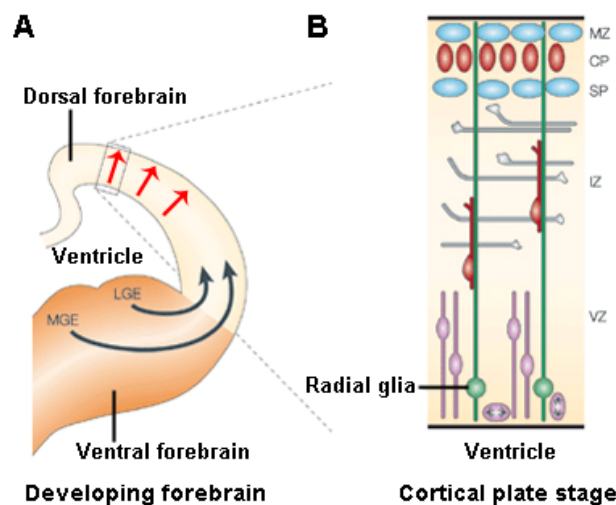


Fig. 8. Radial and tangential migrations in the cerebral cortex. (A) Schematic diagram of a section through the developing rodent forebrain. Red arrows show radial migration. The lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) of the ventral forebrain generate the neurons of the basal ganglia and cortical interneurons; the latter follow tangential migratory routes to the cortex (gray arrows). (B) The dorsal forebrain gives rise to the cerebral cortex. In the dorsal forebrain (A; boxed area), neurons generated in the ventricular zone (VZ), migrated radially, guided by radial glia, through the intermediate zone (IZ) to split the preplate into the outer marginal zone (MZ) and inner subplate (SP) (c). CP, cortical plate. (adapted from (Nadarajah and Parnavelas, 2002))

migration is that of glutamatergic neurons which are generated in the VZ of the dorsal forebrain and then migrate radially along neuroepithelial/glial fibers to form the cortical plate (red arrows in Fig. 8A). During tangential migration, neurons migrate orthogonal to the direction of radial migration, using a variety of mechanisms. For example, GABAergic interneurons which originate in the lateral and caudal ganglionic eminences (LGE and CGE) in the basal forebrain migrate tangentially to the cortex (gray arrows in Fig. 8A) (Anderson et al., 1997).

Basic mechanisms of neuronal migration

Like other motile cells, neurons migrate by extending a leading process (a growth cone or a dendritic tip) that attaches to the substratum, such as radial glial cells or extracellular matrix. This is followed by a nuclear (or somal) translocation within this extended furrow, and then by retraction of the trailing process (Lambert de Rouvroit and Goffinet, 2001).

Leading edge extension and the actin treadmill

Leading edge extension is preceded by a highly dynamic instability that consists of forming and disassembling actin-based structures such as filopodia and lamellipodia that sense the attractive or repulsive molecules in the environment (Bradke and Dotti, 1999). The extension of the leading edge is regulated by the actin cytoskeleton localized at its tip. This actin network is under the influence of members of the Rho family of small GTPases, such as Cdc42 and Rac (Luo, 2000). In fibroblasts, Rac leads to assembly of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles (Hall, 1998), whereas Cdc42 induces actin-rich surface protrusions called filopodia.

It was proposed that chemoattractants or repellents act on the growth cone by activating locally Rac and/or Cdc42, or Rho to guide axonal growth and cell migration. For instance, activation of plexin-B1 by semaphorin 4D regulates the activity of the Rho guanine nucleotide exchange factors PDZ-RhoGEF and LARG, leading to RhoA activation and growth cone collapse in primary hippocampal neurons (Swiercz et al., 2002). Ephrin-A5 induces collapse of growth cones by activating RhoA and ROCK, and inhibiting Rac1 and Cdc42 (Shamah et al., 2001; Wahl et al., 2000). A novel RhoGEF, ephexin, has been identified downstream of the Ephrin receptor EphA and is required for Ephrin-A-induced growth cone retraction (Wahl et

al., 2000). The Netrin-1 receptor DCC ("deleted in colorectal cancer") promotes filopodia formation and cell spreading in embryonic rat spinal commissural neurons (Shekarabi and Kennedy, 2002) and induces neurite outgrowth in neuroblastoma cells (Li et al., 2002) by activating Cdc42 and Rac1. Robo activation by Slit activates the GAP: srGAP1 that inactivates Cdc42. This inhibits neurite growth and induces repulsion of migratory cells from the anterior subventricular zone of the forebrain (Wong et al., 2001).

The involvement of actin binding proteins in radial migration was highlighted by the study of human X-linked periventricular heterotopia. This disorder is characterized by failure of a subset of neurons to migrate externally, resulting in formation of nodules of ectopic neurons in the ventricular zone. Periventricular heterotopia can result from mutations in the X-linked gene *Filamin A (FLNA)*. *FLNA* encodes a protein that binds to actin and may be involved in the extension of filopodia during the movement of the cell along the glial fiber (Fox et al., 1998; Hatten, 2002; Parrini et al., 2006; Walsh and Goffinet, 2000).

Somal translocation, nucleokinesis and microtubules

Nucleokinesis uses control mechanisms different from leading edge extension and relies more on the microtubule cytoskeleton (Morris et al., 1998). Within the cell soma, a cage-like distribution of microtubules surrounds the nucleus. During the translocation of the nucleus of migrating cerebellar granules, "plus" ends of microtubules assemble at the growing tip of the leading edge; while the depolymerizing "minus" ends face the nucleus. Microtubule polymerization in the direction of the leading edge may force the extension of the leading process, whereas their depolymerization in front of the nucleus may assist nucleokinesis towards the front of migration. This model is consistent with the saltatory movement of migrating neurons, with a slow microtubule association phase reflecting the slow leading process extension, and the rapid nuclear translocation reflecting the rapid dissociation of tubulin subunits. The influx of cytosolic Ca^{++} seen during movement may reflect the need of calcium for tubulin polymerization (Rakic et al., 1996).

The role of microtubules in neuronal migration is illustrated by studies of migration disorders in human and mice. *LIS1*, the gene mutated in the Miller-Dieker syndrome (type I lissencephaly), a severe form of lissencephaly, encodes the non-catalytic β subunit of platelet-activating factor acetylhydrolase (PAFAH1 β 1), a G-

protein-like, trimeric brain-specific enzyme. LIS1 binds to retrograde dynein motor proteins and to microtubules and may stabilize the microtubule network during cell migration (Wynshaw-Boris and Gambello, 2001). Both in humans and mice, homozygous LIS1 mutations are lethal, and heterozygotes are characterized by a profuse migration disorder in the neocortex, hippocampus, olfactory bulb and cerebellum. Interestingly, Lis1 is homologous to the NUDF protein (Xiang et al., 1995). Lis1 also interact physically and biochemically with the mammalian orthologue of NUDC, and with mNudE and mNudeL during neuronal migration *in vivo* (Feng et al., 2000; Niethammer et al., 2000). Nud proteins are required for nuclear translocation in *Aspergillus nidulens* (Morris et al., 1998). This strong similarity suggests that the Lis1 gene product may have a function similar to that of NUDF and supports the hypothesis that nuclear migration (nucleokinesis) plays a key role in neuronal migration.

Female patients with *doublecortin* (*DCX*) mutations exhibit a 'double cortex' syndrome, in which a band of grey matter is embedded within the white matter beneath the normal cortex, whereas male *DCX* patients show lissencephalies rather similar to *LIS1*. The *Dcx* gene encodes Doublecortin which binds to tubulin and has a microtubule stabilizing activity *in vitro* (Francis et al., 1999; Olson and Walsh, 2002).

Cyclin-dependent kinase 5 (Cdk5) is a member of the cyclin dependent serine/threonine kinases. However, unlike other Cdks, it is not regulated by cyclins and does not regulate the cell cycle. In mammals, Cdk5 mRNA and protein are expressed in kidney, testes and ovary, but the highest level of expression is in postmitotic neurons (Ino et al., 1994; Tsai et al., 1993). Cdk5-associated kinase activity depends on the presence of regulatory subunits p35 and p39 (Qi et al., 1995). Mutant mice defective in Cdk5, or to a lesser extent its activator p35, have defects in radial migration, with inversion of cortical layering (Chae et al., 1997). P39 is another activator of Cdk5 and its loss causes less severe abnormalities. However a combined defect of p35 and p39 induces the same phenotype as Cdk5 inactivation (Ko et al., 2001) showing redundancy in the activation of Cdk5.

An ENU-induced mouse mutant with abnormalities in the laminar architecture of the hippocampus and cortex was shown to be due to a mutation in the guanosine triphosphate (GTP) binding pocket of alpha-1 tubulin (*Tuba1*). Two patients with *de novo* mutations in *TUBA3*, the human homolog of *Tuba1* and migration disorder were

then identified, further emphasizing the role of microtubules in neuron migration (Keays et al., 2007).

Recently, two proteins related to *Drosophila* Klarsicht, named Syne-1/nesprin-1 and Syne-2/Nesprin-2 were shown to collaborate with SUN1 and SUN2 in regulating neuronal migration in mouse (Zhang et al., 2009). Nesprins are huge proteins anchored to microtubules and to the nuclear envelope, where they interact with SUN. Presumably, these complexes assist in the microtubule-mediated progression of the nucleus during migration.

Retraction of the trailing process

In moving cells, tail detachment typically implicates myosin II and RhoA-mediated actomyosin contraction (Bellion et al., 2005). The role of microtubules in trail retraction is less clear as it varies according to cell type. Rapidly migrating cells, such as neutrophils (that do not have focal adhesions) and keratinocytes, are not affected by microtubule polymerization inhibitors such as nocodazole, contrary to fibroblasts and epithelial cells (Ridley, 2001). Microtubules target focal adhesions and induce their relaxation and dissolution in slow-moving cells, perhaps by activating Rac or Cdc42 (Kaverina et al., 1999), which may explain why microtubules are required for tail retraction in some cells (Ballestrem et al., 2000).

Guiding tangential and radial migration

Neurons that move tangentially extend a growth cone similar to axonal growth cones. Occasionally, this migration proceeds in apposition to other neurons or axons, and was formerly called "neuronophilic". Radially migrating neurons extend a dendritic tip that will become the dendritic pole and is closely apposed to and follows glial fibers; this mode of migration is often referred to as "gliophilic" (Rakic, 1990).

In line with the idea that tangentially migrating neurons extend an "axonal" growth cone, many molecules that influence tangential migration also regulate axonal guidance. Several attractive or repulsive guidance molecules have been identified and belong to four main families: Ephrins, Semaphorins, Slit, and Netrins (Fig. 9) (Flanagan and Vanderhaeghen, 1998; Jossin, 2004).

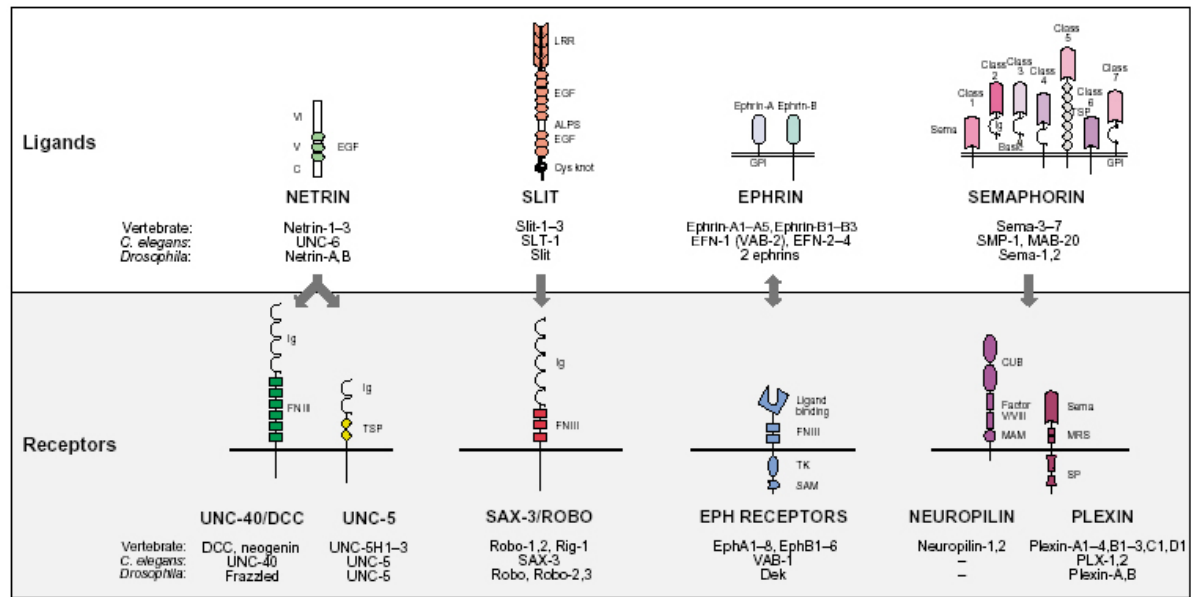


Fig. 9: Examples of families of guidance cues for axons and tangential migration and their receptors (Yu and Bargmann, 2001).

For instance, Slit, expressed in the VZ of the ganglionic eminences (GE), repels subventricular zone (SVZ) neurons that leave the GE to settle in the striatum or migrate to the cortex (Zhu et al., 1999). The striatum expresses a Semaphorin which repels neurons of the GE that express the neuropilin receptor, so that these neurons avoid the striatum and migrate to the cortex (Marin and Rubenstein, 2001).

Contrary to tangential migration, attractive or repulsive guidance cues have not been implicated in radial migration, which appears primarily guided by radial glia. Accordingly, the molecules involved in radial migration influence the interactions between neurons and glial cells, and include Astrotactin, Neuregulins, EGF receptors, Integrins. Thus far, however, very little is known of their molecular action on migration.

Integrin receptors consist of membrane-spanning heterodimers of α and β subunits, and more than 20 integrins receptors can be formed by combinations of 18 α and 8 β subunits. Mice deficient in most integrins subunits have been produced. Some of them, such as $\alpha 2$, $\alpha 5$ and $\beta 1$ die during early embryonic development, precluding analysis of cortical development (Schmid and Anton, 2003). Others have a normal cortex ($\alpha 1$, $\alpha 4$, $\alpha 7$, $\alpha 8$, $\alpha 9$, $\beta 2$, $\beta 3$, $\beta 5$, $\beta 6$ and $\beta 7$). However knockout mice for $\alpha 3$, $\alpha 6$, αv and a conditional mutant for $\beta 1$ exhibit cortical malformations, and some may be involved in neuron-radial glia interactions (Anton et al., 1999).

At the end of radial migration, immature neurons respond to a stop signal in the matrix, generated by Reelin. Secreted Reelin is cleaved and the central part of the protein is free to bind to two largely redundant receptors on the surface of migrating neurons, namely Vldlr and ApoER2. This triggers an intracellular signal that is still poorly understood but includes tyrosine phosphorylation of the adapter Dab1 by Src and Fyn kinases (Forster et al., 2006). Reelin signaling stops migrating neurons and instructs their postmigratory organization into architectonic structures at various places in the brain, mostly the cortex, hippocampus and cerebellum, but also other locations such as the facial nerve nucleus.

Hindbrain patterning and migration of facial branchiomotor neurons

Among the many neuronal migration events summarized above, that of facial branchiomotor neurons (FBM neurons) in the hindbrain is unique and intriguing. Inasmuch as the study of FBM neuron migration in PCP mutants is a main theme of my thesis, I will describe it in some detail, beginning with a brief introduction of hindbrain segmentation.

Outline of brain development and hindbrain segmentation

The central nervous system (CNS) originates from the ectoderm. At the end of gastrulation, the dorsal midline ectoderm that faces the notochord is induced to differentiate into the neural plate, a process named neurulation. The notochord secretes chordin, noggin as well as follistatin, which are all able to bind and neutralize the activity of Bmp4 and 7, thereby switching cell fate from the ectodermal to the neural lineage. The neural plate folds into the neural groove that closes to form the neural tube. Neural tube closure is defective in several PCP mutants, and thus controlled by PCP signaling. The rostral part of the neural tube develops into three primary vesicles: prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain), while the caudal part gives rise to the spinal cord. At later stages, five secondary vesicles are formed: the prosencephalon divides into telencephalon and diencephalon, and the rhombencephalon into metencephalon and myelencephalon. The mesencephalon becomes the midbrain, the metencephalon

differentiates into the pons and cerebellum, and the myelencephalon develops into the medulla.

The developing hindbrain is segmented along the anterior-posterior axis into eight segments called rhombomeres (r) r1-r8. Cells of a given rhombomere possess adhesive properties that do not allow them to locate in another rhombomere once they leave the cell cycle. Complementing this adhesive differential is an alternating periodicity of the expression domains of the Eph-like receptor tyrosine kinases and their ligands (Xu et al., 1995), and it is likely that rhombomere boundaries exert some repulsive action that complements the specific intrarhombomere adhesion.

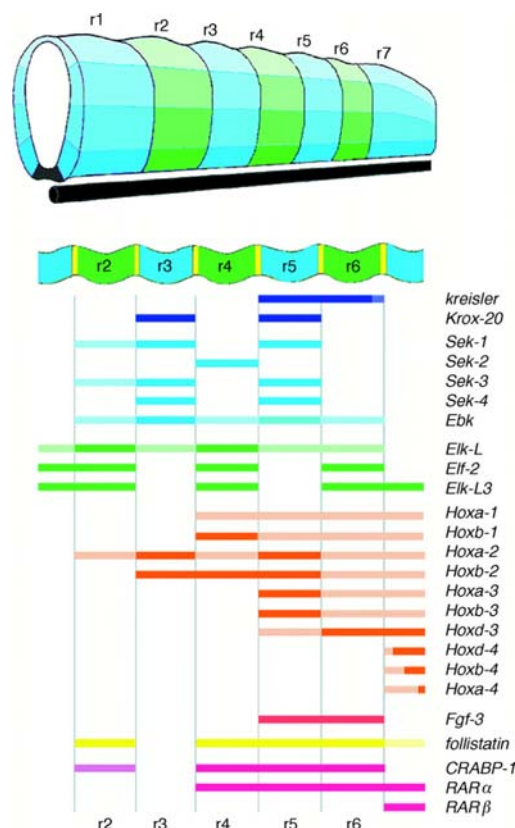


Fig. 10. Correlation of gene expression with rhombomere specification, compiled from analysis in mouse and chick embryos. (Top) Odd-numbered rhombomeres are indicated in blue and even-numbered rhombomeres in green, with each segment designated as r1 to r7. The rod-like notochord and overlying floorplate are indicated in black. The vertical yellow lines indicate the boundaries between rhombomeres. The patterns of gene expression are depicted in arbitrary colors with the darkest colors indicating the highest levels of expression. Related genes are indicated by the same color for convenience: *Hox* homeobox genes (orange), Eph family tyrosine kinase receptors (blue), Eph receptor ligands (green), retinoid- or signaling-related genes (magenta), and early expressed transcription factors (dark purple) (Lumsden and Krumlauf, 1996)

Another molecular correlate of this cellular patterning is provided by the segment-restricted expression domains of genes such as *Hox* genes, encoding transcription factors required for the formation and specification of segments (Fig. 10). Members of the *Hox* family have different expression patterns: except for *Hoxb1* that is specifically expressed in r4, each member is expressed with a sharp rostral border, from a certain rhombomere to the caudal hindbrain and spinal cord (Fig. 11B) (Lumsden and Krumlauf, 1996). Thus every rhombomere expresses a certain combination of different *Hox* genes that specifies its identity. *Hox* genes are not the only markers used in mapping rhombomeres. There are many markers, some more specific than others, such as *Hoxb1* in r4, *Egr2/Krox20* and *EphA4* in r3 and r5 (Gilardi-Hebenstreit et al., 1992; Guthrie, 2007; Murphy et al., 1989).

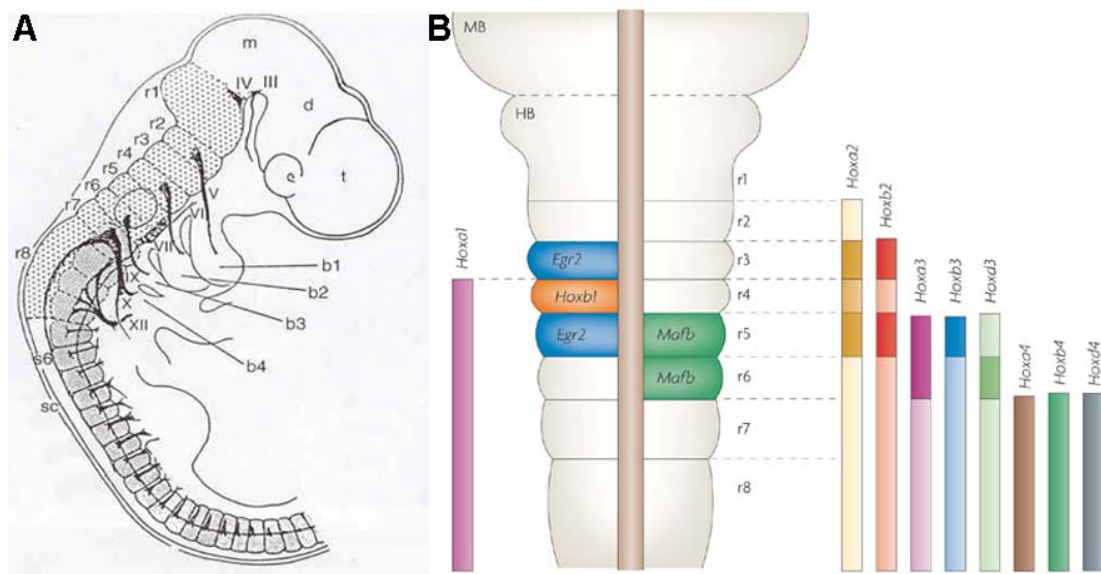


Fig. 11. Hindbrain segmentation and *Hox* gene expression domains. (A) Segmentation in the mouse or chick embryo. b1-b4, branchial arches; d, diencephalon; m, mesencephalon; r, rhombomeres; s, somites; sc, spinal cord; t, telencephalon; Roman numerals, cranial nerves. (B) The domains of mRNA expression of the *Hox* genes, *Egr2/Krox20* and *Mafb* in the hindbrain of the mouse embryo at embryonic day 11.5 (E11.5) - or chick embryo at E4. The bars labeled with different *Hox* genes show the corresponding expression domains, which extend from the caudal hindbrain and spinal cord up to specific rhombomere (r) boundaries. Darker shading indicates higher levels of expression. HB, hindbrain; MB, midbrain. (Guthrie, 2007)

FBM neuron migration

Cranial motor neurons are located in the brainstem (midbrain and hindbrain) and control eye, facial and head movement (Landmesser, 2001). There are three subpopulations of cranial motor neurons: branchiomotor (BM), somatic motor (SM), and visceral motor (VM) neurons, which have different origins and anatomical targets (Chandrasekhar, 2004; Song, 2007). Fig. 12A shows the organization of cranial motor neurons in the mouse hindbrain. Different sets of transcription factors are expressed by SM neurons and BM/VM neurons, such as *Isl1*, *Isl2*, *Hb9* and *Lhx3* in SM neurons, and *Isl1*, *Phox2a*, *Phox2b*, and *Tbx20* in BM/VM neurons (Song, 2007). FBM neurons are 7th cranial motor neurons that belong to the BM column.

FBM neurons innervate muscles responsible for facial expression that derive from the second pharyngeal arch (Chandrasekhar, 2004; Garel et al., 2000; Guthrie, 2007). They are generated near the midline in r4 at E10.5 and rapidly extend their axons to a lateral exit point and towards muscle targets. Then, at E11.5, their cell bodies initiate a complex caudal migration from r4 to r6. FBM neurons migrate in the subventricular region, and pass medial to the nucleus abducens (nVI) in r5 (Song et al., 2006), before moving laterally and dorsally in r6. Finally, they undergo a radial, gliophilic, Reelin- and Cdk5- dependent migration in r6 to reach their final, subpial location (Fig. 12B,C) (Chandrasekhar, 2004; Goffinet, 1984; Ohshima et al., 2002). Most FBM neurons finish their migration at E13.5. While migrating, FBM neurons leave their axon behind, and this results in formation of the ‘genu’ of the facial nerve, with facial motor axons looping around nVI and the medial longitudinal fasciculus (mlf). Caudal translocation of the soma of FBM neurons with looping of axons is conserved from fish to mammals, with important species-specific differences. For example it is blunted in chick (Gilland and Baker, 2005).

In zebrafish, the function of several PCP genes such as *van gogh-like 2* (*vangl2*), *frizzled3a* (*fzd3a*), *celsr2*, *prickle1a*, and *prickle1b* is necessary for normal caudal migration of FBM neurons (Bingham et al., 2002; Carreira-Barbosa et al., 2003; Jessen et al., 2002; Mapp et al., 2010; Rohrschneider et al., 2007; Wada et al., 2006). In mice, some Wnt/PCP genes (*Fzd3*; *Vangl2*, *Wnt5a*) and downstream signaling events have been implicated in regulating FBM neuron movement (Vivancos et al., 2009). These observations lead us to examine in detail FBM neuron migration in our *Celsr1-3* mutant mice.

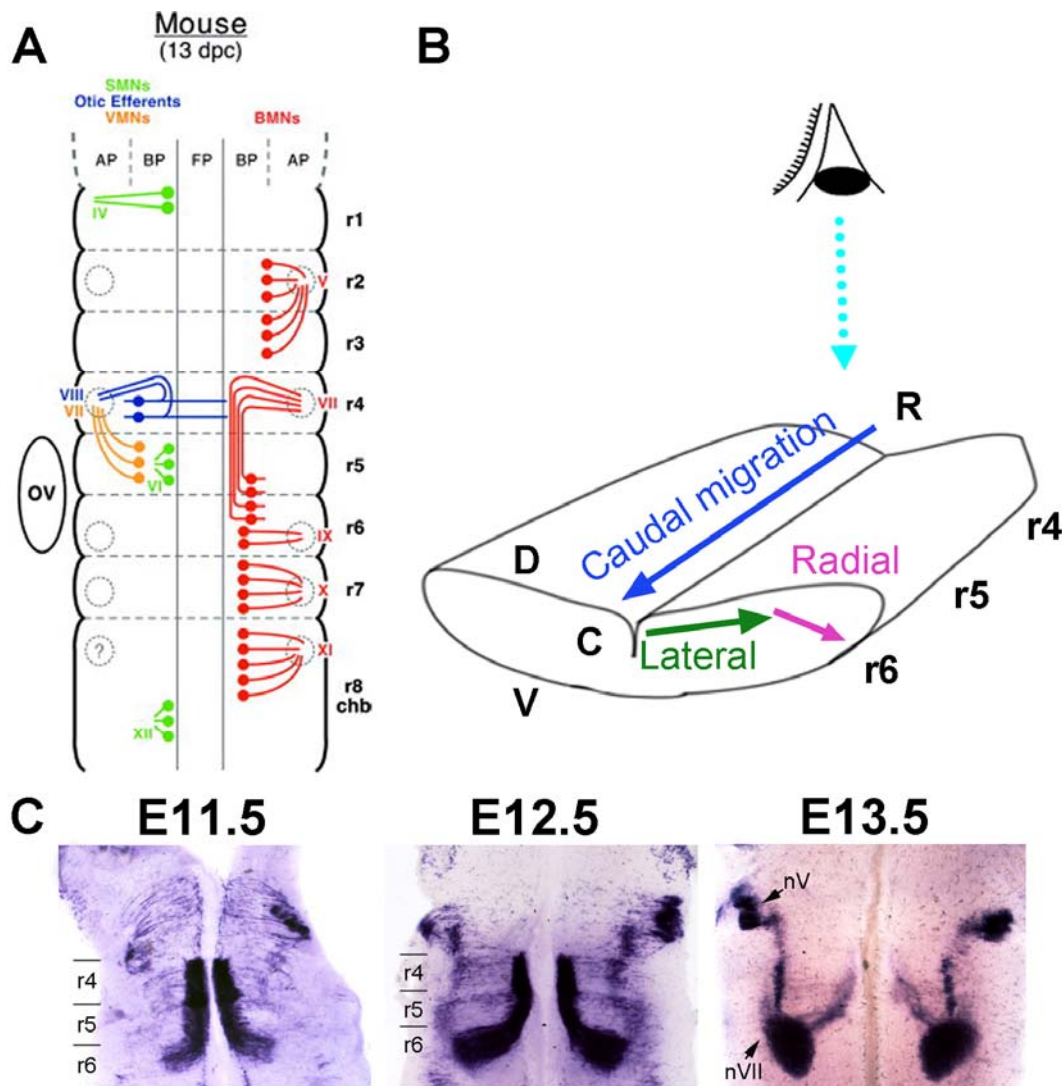


Fig. 12. Organization of cranial motor neurons in mouse hindbrain and FBM neuron migration. (A) Mouse embryo, 13 days post coitum (dpc). Hindbrains are depicted in open-book configuration (anterior to the top), with the dorsal most neural tube at the lateral margins of the diagrams. Bulges and broken grey lines reflect partition into rhombomeres from r1 to r8. Branchiomotor neurons (BMN) and their axons are shown in red, somatomotor neurons (SMN) in green, otic and lateral line efferent (VIII, OLe) neurons in blue, and visceromotor neurons (VMN) in yellow. For clarity, motor neuron populations are depicted on only one side of the neural tube. The nerve exit points of the BMN, VMN, and otic efferent axons are shown as grey circles and are located dorsally in the alar plate of the neural tubes. OV, otic vesicle (Chandrasekhar, 2004) (B) Three-dimensional summary diagram of flattened hindbrain showing directions of FBM neuron migration: caudal, lateral and radial. R: Rostral, C: Caudal, D: Dorsal, V: Ventral. (C) Flat-mount view of mouse hindbrain from E11.5 to E13.5 (as viewed from the eye position in panel B). FBM neurons were visualized by *in situ* hybridization with an Isl1 probe. FBM neurons initiate caudal migration from r4 to r6 at E11.5; most of them finish migrating and reach their final position in lateral r6 at E13.5.

1.4. Cilia and ciliogenesis

The second part of my work concerns the analysis of ciliogenesis in *Celsr2* and *Celsr3* mutant mice. To introduce this theme, I will provide a brief summary of cilia development.

Cilia are cell appendages that extend from surface in the extracellular space. A cilium consists of a specialized plasma membrane and a microtubule-based axoneme which emerges from a basal body embedded in the cell cortex (Beisson and Wright, 2003). Based on their structure and motility, cilia are classified into three types: motile, primary and nodal cilia (Davenport and Yoder, 2005).

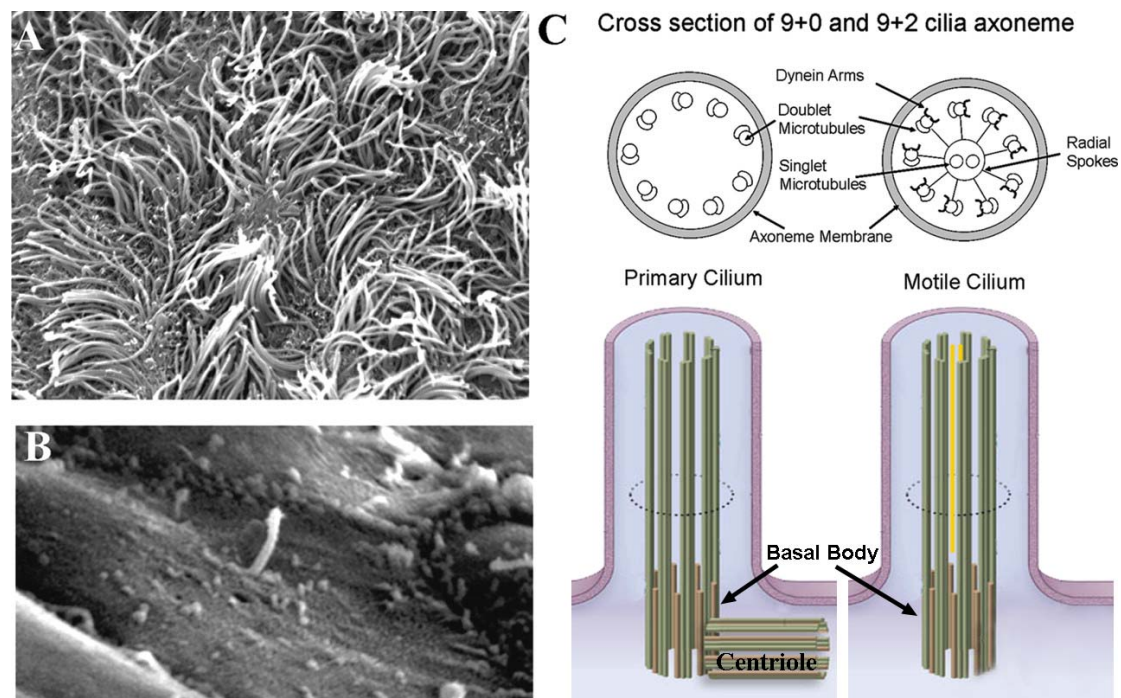


Fig. 13. Motile or immotile (primary) cilia are located on the surface of nearly every cell within the mammalian body. A: scanning electron microscopic (SEM) image of motile cilia present on mouse ependymal cells located along the lateral ventricles. B: SEM image displaying a solitary primary cilium projecting from the surface of an ectodermal cell in the developing limb bud of an E10.5 mouse embryo. C: structural differences determine the motility of a cilium. Motile cilia (right) consist of 9 doublet microtubules surrounding 2 inner singlet microtubules used to conduct force. Primary cilia (left) are lacking both singlet microtubules and dynein arms. Multiple, motile cilia are anchored on a basal body, similar to a centriole, whereas primary, monocilia are anchored to the centrosome (two centrioles). Adapted from (Ainsworth, 2007; Davenport and Yoder, 2005).

Primary cilia are found in most cells. They are monocilia with 9 surrounding doublet microtubules, and lack a central pair of microtubules which is necessary for generating motile force, hence their designation as 9+0 structure (Fig. 13 B,C) (Perkins et al., 1986). Primary cilia are anchored to a basal body, which is linked to an adjacent centriole by a striated connector (Fig. 14d). The basal body corresponds to the mother centriole. The basal body extends basal feet, which number between 1 and 5, and which probably anchor the cilium to the microtubule network (Fig. 14d-f) (Hagiwara et al., 2008).

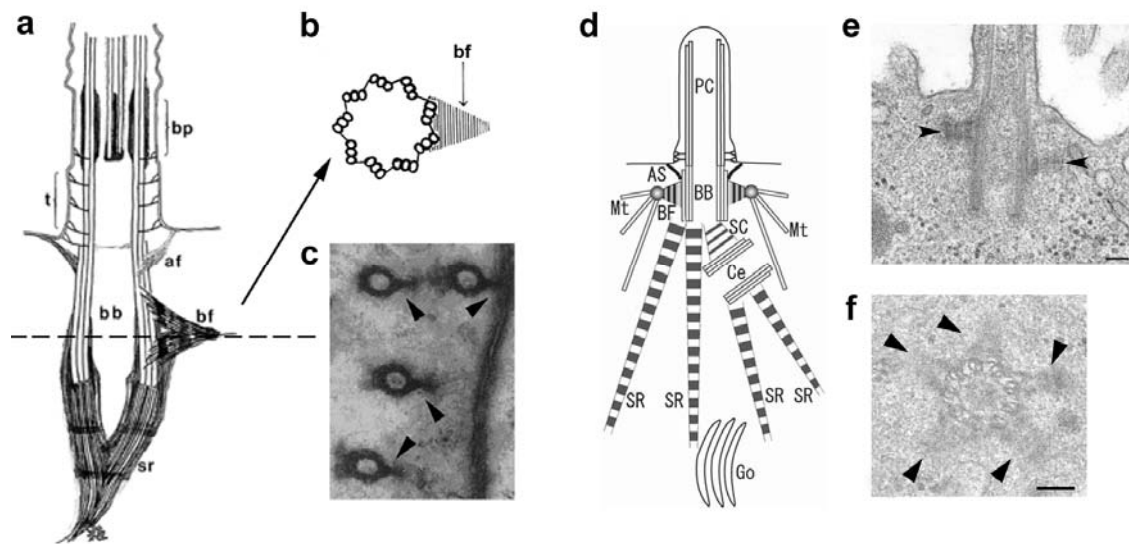


Fig. 14. Basal foot of motile and primary cilia. a-c, cilia structure and basal foot in motile cilia. (a) Schematic representation of ciliary and basal body (bb) regions of the mouse trachea. The basal body is connected to accessory structures: striated rootlet (sr), basal foot (bf), and anchoring fibers (af). The transition zone (t) of the cilium is between the basal body and basal plate (bp). (b) Transverse sections at the level of basal foot indicated in (a); Only one basal foot projects from the basal body in motile cilia and its orientation indicates the effective beating direction. (c) Transmission Electron Microscopy (TEM) picture showing basal feet (arrowheads). d-f, the situation in primary cilia. (d) A schema showing the primary cilium and its accessory structures. PC, primary cilium; BB, basal body; Ce, centriole; AS, alar sheet; BF, basal foot; SR, striated rootlet; SC, striated connector; Mt, microtubule; Go, Golgi apparatus. (e) The level of insertion of basal feet can vary (arrowheads). (f) Contrary to motile cilia, several basal feet project from the lateral side of the basal body (arrowheads) in primary cilia. Adapted from (Gibbons, 1961; Hagiwara et al., 2008; Kasuya and Miyoshi, 2001).

Several receptors such as the Shh receptor Patched-1 and Smoothened or PDGF receptors, were shown to be located on primary cilia (Rohatgi et al., 2007). It is generally believed that immotile primary cilia behave as “antennae” to receive extracellular signals. In the mammalian kidney, in retinal and olfactory receptors and in *C. elegans* neurons, primary cilia serve as chemo- or mechanosensors, and also act as coordinators of signaling pathways (Bae and Barr, 2008; Deltas and Papagregoriou, 2010; Satir et al., 2010).

Nodal cilia also have a 9+0 structure but, contrary to other monocilia, they move in a propeller-like fashion (Nonaka et al., 1998). Nodal cilia are essential for generating an asymmetric distribution of morphogenetic cues in the node, which is required for the left-right body axis specification (Bonnafe et al., 2004; Murcia et al., 2000; Watanabe et al., 2003). Interestingly, a role of PCP genes *Vangl1* and 2 in nodal cilia development and function was recently demonstrated (Song et al., 2010).

Motile cilia invest the apical surface of epithelial cells lining airways, reproductive tracts and cerebral ventricles. They have a central pair of microtubules surrounded by 9 doublet microtubules, an organization known as 9+2 (Fig. 13C) and are anchored to a basal body in the apical cortex. Contrary to primary cilia, this basal body is not flanked by a centriole and harbors only one basal foot, orientation of which indicates the effective direction of cilia beat (Fig. 14a-c). Motile cilia are usually concentrated to form apical tufts (Fig. 13A) and one of their functions is to generate directional flow at the surface of epithelial sheets. In the airways, motile cilia are crucial for clearing mucus and debris, and in genital tracts they may assist the transit of sperm and eggs (Salathe, 2007; Voronina et al., 2009). In the mouse brain at late embryonic stage and during the first postnatal week, neuroepithelial cells that line the cerebral ventricles leave the cell cycle and differentiate into a monolayer of ependymal cells. At birth, ependymal cells are not yet ciliated. Cilia tufts develop progressively from P5 and reach their mature shape at the end of maturation, when the apical surface bears dozens of cilia that beat in coordinated manner to facilitate the circulation of the cerebrospinal fluid (CSF), from sites of production in choroid plexuses to sites of absorption in subarachnoid spaces.

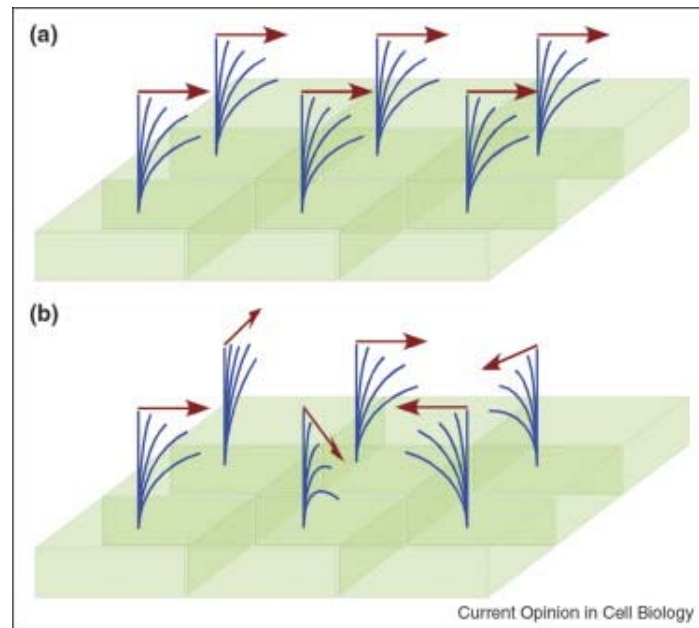


Fig. 15. Ciliary orientation must be aligned across multiple cells in a tissue in order to produce coherent overall flow of fluid. (a) When cilia are aligned, locally produced flows (red arrows) can sum to produce a coherent flow. (b) When cilia are randomly aligned, local flows run in different directions leading to overall chaotic motion of the fluid that resembles turbulence (Marshall and Kintner, 2008).

Ciliary basal feet are lateral expansions from the basal bodies and their direction can be seen easily in tangential electron microscopy sections. Basal feet point to the effective beating direction and can be used as indicator of basal body orientation (Fig. 14a-c) (Gibbons, 1961; Hagiwara et al., 2008; Wallingford, 2010). To generate an efficient directional flow, all cilia must beat coordinately in the same direction. If cilia in neighboring cells move in different directions, no coherent overall flow can occur (Fig. 15). Thus the basal bodies of cilia need to be aligned in the same orientation with respect to the tissue polarity. The orientation of basal bodies is imprecise during initial ciliogenesis. After cilia start to beat, the flow they produce generates a positive feedback that refines the alignment of cilia orientation (Fig. 16) (Marshall and Kintner, 2008; Mitchell et al., 2007). Thus far, little is known about how basal bodies become orientated in epithelial cells. Inasmuch as the arrangement of the basal body is a planar polarity event, PCP signaling pathways are possible candidates that may contribute to this process.

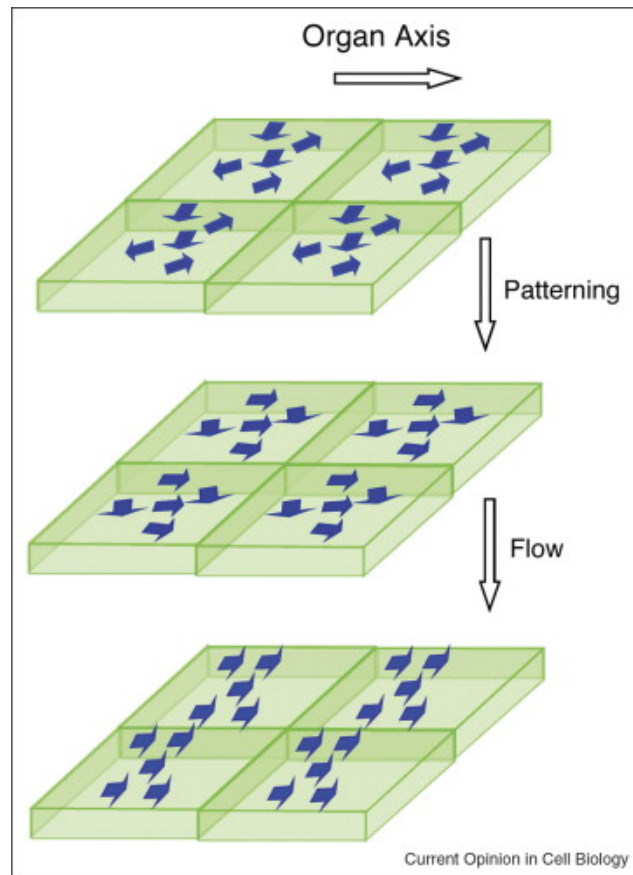


Fig. 16. Cilia orientation is acquired gradually via several processes. When cilia first form, the orientation of the basal body (arrows) is very imprecise (top diagram). Tissue patterning perhaps involving PCP signaling helps to align the orientation of cilia along a common tissue axis. Cilia-generated flow is then used as a self-organizing cue to refine orientation further, thus producing laminar, directed flow (Marshall and Kintner, 2008).

In the ciliated epidermis of larval *Xenopus*, morpholino downregulation experiments showed that *Disheveled1–3*, *Inturned* and *Fuzzy* are implicated in the control of the actin cytoskeleton and the apical docking of ciliary basal bodies, and that the planar orientation of multiciliate cells is disrupted when *Disheveled*, *Vangl2* and *Fuzzy* signaling are altered (Park et al., 2006; Park et al., 2008), indicating that PCP signaling and ciliogenesis are indeed linked

Several mutations in animals, particularly rodents, generate hydrocephalus attributed to defective ciliogenesis or cilia function. In mice, mutations in genes involved in the assembly or structure of ependymal cilia, such as *Mdnh5* (Ibanez-Tallon et al., 2004), *Ift88* (also known as *Tg737* or *Polaris*) (Banizs et al., 2005) and *Hy3* (Davy and Robinson, 2003; Lechtreck et al., 2008) affect ciliogenesis, alter CSF

dynamics and result in hydrocephalus. Several human diseases were recently shown to be due to defective cilia and are referred to as ciliopathies. They include polycystic kidney diseases, Bardet Biedel syndrome and Joubert syndrome (Lee and Gleeson, 2011). Hydrocephalus is present in some of them, but not all, for reasons that are not understood.

2. AIM OF THE PROJECT

As mentioned in the Introduction, the aim of the projects going on in our laboratory is to understand better the role of *Celsr1*, 2 and 3 and planar cell polarity signaling in mouse brain development. My contribution to that project was focused on two aspects:

- 1) Function of *Celsr1*, 2 and 3 in facial branchiomotor (FBM) neuron migration. This was addressed by examining FBM migration in *Celsr1*, 2 and 3 constitutive and conditional mutant mice.
- 2) Function of *Celsr2* and *Celsr3* in ependymal cilia development and hydrocephalus. *Celsr2* and 3 mutant mice develop severe hydrocephalus, and we aimed to try and understand the mechanism. This was performed by studying ependymal cilia structure using markers, SEM and TEM, together with functional assays.

3. RESULTS

3.1. Migration of facial branchiomotor neurons (FBM neurons)

The migration of FBM neurons is so far the only neuronal migration event known to be regulated by PCP. It was thus of interest to investigate this further in our PCP mutants. That study allowed us to define two mechanisms by which *Celsr1* on one hand, and *Celsr2-3* on the other hand, affect respectively the direction and the trajectory of FBM neuron migration.

3.1.1. Our studies on FBM neuron migration were carried out in collaboration with the laboratory of Dr Anand Chandrasekhar (U. Missouri). They have been published in *J. Neurosci.* (Qu et al., 2010).

I performed the experiments of Fig. 1 E-J, Fig. 2, Fig. 4, Fig. 5, Fig. 6 A-H and Fig. 8 as well as Supplementary Fig. S1, Fig. S2, Fig. S6 and Fig. S8. I participated in the design of the project and in writing the manuscript.

3.1.2. Unpublished results.

I collaborated to the production of novel *Celsr2* conditional and constitutive mutants and studied the FBM autonomy of *Celsr2* function using *Isl1-Cre* mice. I confirmed that *Celsr1* acts in FBM neuron precursors by studying *Celsr1|Nkx6.2* mutants.

Atypical Cadherins *Celsr1-3* Differentially Regulate Migration of Facial Branchiomotor Neurons in Mice

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During hindbrain development, facial branchiomotor neurons (FBM neurons) migrate from medial rhombomere (r) 4 to lateral r6. In zebrafish, mutations in planar cell polarity genes *celsr2* and *frizzled3a* block caudal migration of FBM neurons. Here, we investigated the role of cadherins *Celsr1-3*, and *Fzd3* in FBM neuron migration in mice. In *Celsr1* mutants (knock-out and *Crash* alleles), caudal migration was compromised and neurons often migrated rostrally into r2 and r3, as well as laterally. These phenotypes were not caused by defects in hindbrain patterning or neuronal specification. *Celsr1* is expressed in FBM neuron precursors and the floor plate, but not in FBM neurons. Consistent with this, conditional inactivation showed that the function of *Celsr1* in FBM neuron migration was non-cell autonomous. In *Celsr2* mutants, FBM neurons initiated caudal migration but moved prematurely into lateral r4 and r5. This phenotype was enhanced by inactivation of *Celsr3* in FBM neurons and mimicked by inactivation of *Fzd3*. Furthermore, *Celsr2* was epistatic to *Celsr1*. These data indicate that *Celsr1-3* differentially regulate FBM neuron migration. *Celsr1* helps to specify the direction of FBM neuron migration, whereas *Celsr2* and 3 control its ability to migrate.

Introduction

In the developing central nervous system, postmitotic neurons leave ventricular zones of precursor proliferation and migrate to their destinations. Several tightly regulated modes of migration have been described, including radial migration of excitatory neurons to the cortex, tangential migration of cortical interneurons, migration to the olfactory bulb in the rostral migratory stream, and migration of rhombic lip derivatives. A unique and intriguing example of neuronal migration is that of facial branchiomotor neurons (FBM neurons), which innervate muscles responsible for facial expression derived from the second pharyn-

geal arch (Garel et al., 2000; Chandrasekhar, 2004; Guthrie, 2007). FBM neurons are generated in rhombomere 4 (r4) and extend their axons from dorsal exit points toward muscle targets. Their cell bodies undergo a complex caudal, tangential migration from r4 to r6. They migrate in the subventricular region and pass medial to the nucleus abducens (nVI) in r5 (Song et al., 2006), before moving laterally and dorsally in r5–r6. Finally, they undergo a radial, gliophilic, Reelin- and Cdk5-dependent migration in r6 to reach their terminal, subpial location (Goffinet, 1984; Ohshima et al., 2002; Chandrasekhar, 2004). While migrating, FBM neurons leave their axons behind to form the “genu” of the facial nerve, with facial motor axons looping around nVI and the medial longitudinal fasciculus. Caudal translocation of the soma of FBM neurons with looping of axons is conserved from fish to mammals (except chicken), with some species-specific differences (Gilland and Baker, 2005).

In zebrafish, several planar cell polarity (PCP) genes such as *van gogh-like 2* (*vangl2*), *frizzled3a* (*fzd3a*), *celsr2*, *prickle1a*, and *prickle1b* are necessary for the caudal migration of FBM neurons (Bingham et al., 2002; Jessen et al., 2002; Carreira-Barbosa et al., 2003; Wada et al., 2006; Rohrschneider et al., 2007). In mice, some Wnt/PCP genes (*Fzd3*, *Vangl2*, *Wnt5a*) and downstream signaling events have recently been implicated in regulating FBM neuron movement (Vivancos et al., 2009). To understand better the role of PCP-related mechanisms in FBM neuron migration in mice, we studied the role of cadherins *Celsr1-3*. Mouse *Celsr1-3* are orthologs of *Drosophila flamingo/starry night* and are ex-

Received Jan. 8, 2010; revised April 29, 2010; accepted May 18, 2010.

This work was supported by grants from the following: Actions de Recherches Concertées (ARC-186), Fonds de la Recherche Fondamentale Collective (FRFC 2.4504.01), Fonds de la Recherche Scientifique Médicale (FRSM 3.4501.07), Interuniversity Poles of Attraction (SSTC, PAI p6/20), Région Wallonne, and the Fondation Médicale Reine Elisabeth, all from Belgium, (to F.T. and A.M.G.); University of Missouri Research Board (RB07-03) and National Institutes of Health (NS040449) (to A.C.). Y.Q. has a FRIA PhD fellowship and F.T. is a research associate at the Belgian National Fund for Scientific Research. We thank Jeremy Nathans for providing *Fzd3* mutant mice, Sonia Garel for providing *Hoxb1* and *EphA4* probes, Michele Studer for providing *Tbx20* probe, and Elaine Fuchs for anti-*Celsr1* antibody. D.M.G. and A.C. are indebted to Angelo Iulianella and Paul Trainor (Stowers Institute, Kansas City, MO) for training in several mouse embryological techniques and for providing *Hoxb1* and *Krox20* probes. D.G. thanks the Molecular Cytology Core facility (University of Missouri, Columbia, MO) for imaging assistance.

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DOI:10.1523/JNEUROSCI.0124-10.2010

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pressed in the developing nervous system in complementary patterns (Formstone and Little, 2001; Shima et al., 2002; Tissir et al., 2002). *Celsr* and *Fzd* proteins have established roles in PCP-related processes such as neural tube closure, organization of stereocilia of hair cells in the inner ear (Curtin et al., 2003; Montcouquiol and Kelley, 2003; Wang and Nathans, 2007), and skin hair patterning (Guo et al., 2004; Devenport and Fuchs, 2008; Ravni et al., 2009), as well as dendritic growth (Shima et al., 2004) and axon guidance (Wang et al., 2002; Zhou et al., 2008). Using *Celsr1^{Crsh}* (Curtin et al., 2003), *Celsr1^{KO}* (Ravni et al., 2009), *Celsr2^{KO}* (Tissir et al., 2010), *Celsr3^{KO}* (Tissir et al., 2005), and *Fzd3* (Wang et al., 2002) mutant mice, we demonstrate specific and different roles for *Celsr1* on one hand and *Celsr2,3* and *Fzd3* on the other hand in regulating the direction and the rostrocaudal extent of FBM neuron migration.

Materials and Methods

All animal procedures were carried out in accord with European and American guidelines and approved by the animal ethics committee of the University of Louvain and the animal care and use committee of the University of Missouri (Columbia, MO).

Mouse lines. The *Celsr1^{KO}*, *Celsr1^{Crsh}*, *Celsr3*, and *Fzd3* mutants and the *SEI::GFP* (where GFP is green fluorescent protein) transgenics were described previously (Wang et al., 2002; Curtin et al., 2003; Tissir et al., 2005; Shirasaki et al., 2006; Ravni et al., 2009). The *Celsr2* mutant is described by Tissir et al. (2010). For timed mating, noon on the day of plugging was defined as embryonic day 0.5 (E0.5). Embryos were dissected in ice-cold PBS and the appropriate embryonic stage was determined using morphological criteria before fixation.

In situ hybridization. Hindbrains were dissected and cut at the dorsal edge of the neural tube and fixed in 4% paraformaldehyde in PBS at 4°C overnight. For the *Isl1*, *Celsr1*, and *Hb9* genes, the following primer sets were used to amplify cDNA fragments, which were used as templates for synthesis of digoxigenin-labeled probe: *Isl1* cDNA [GenInfo Identifier (GI): 162287064], nucleotides 435–1383, primers 5'-TTCCCACTTTCTCCAACAGG-3' and 5'-ACGTGCTTTGTTAGGGATGG-3'; *Celsr1* cDNA (GI: 3800735), nucleotides 7812–8257, primers 5'-GACTGGCTGTGGCTTGGAC-3' and 5'-CGTTAGCAGAGTGGCCCCGAG-3'; *Hb9* cDNA (GI: 5733508), nucleotides 1176–1992, primers 5'-GAAGACGGAAGAGGAGCTGA-3' and 5'-GGGGGATGGGAAAGCTAAAT-3'. The other probes were as follows: *Fzd3* (Tissir and Goffinet, 2006); *EphA4*, *Hoxb1* (Garel et al., 2000); *Tbx20* (Coppola et al., 2005); *Krox20* (Marin and Charnay, 2000); *Gata3* (Karis et al., 2001). Hindbrains were permeabilized with proteinase K treatment, postfixed with 4% paraformaldehyde and 0.2% glutaraldehyde in PBS, and hybridized with the probe at 70°C overnight. Samples were washed in 50% formamide, 5× SSC, and 1% SDS at 70°C and then washed in 50% formamide, 2× SSC at 65°C and incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche) overnight at 4°C. The hybridization signal was revealed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride substrates. After bleaching, hindbrains were flat mounted on a slide glass for visualization and photographed; images were processed with Photoshop (Adobe).

β-Galactosidase staining. Embryos were fixed with 2% paraformaldehyde, 0.1% glutaraldehyde in PBS on ice for 20 min and stained with a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 2 mM MgCl₂ in PBS.

Immunohistochemistry. For neurofilament staining, samples were processed as described previously (Lee et al., 1995; Tiveron et al., 2003; Tissir et al., 2005) using anti-neurofilament-160 (NF-160; Sigma catalog #N5264), or 2H3 [Developmental Studies Hybridoma Bank (DSHB)] monoclonal antibodies. For immunofluorescence, the following primary antibodies were used: mouse anti-Isl1 (1/5000, DSHB), guinea pig anti-Celsr1 (1:200, a gift from Elaine Fuchs, Rockefeller University, New York NY), rabbit anti-Celsr1 C-terminal (1:400, this work), and rabbit anti-

cleaved caspase-3 (1:200, Cell Signaling). Secondary antibodies were goat anti-mouse IgG Alexa Fluor 594 (1:800, Invitrogen), goat anti-rabbit IgG Alexa Fluor 488 (1:800, Invitrogen), and goat anti-guinea pig IgG DyLight 488 (1:400, Jackson ImmunoResearch Laboratories). Preparations were examined with a Zeiss Axio Scope microscope and photographed with an Eclipse system (Nikon), and montages were edited with Photoshop (Adobe).

Celsr1 N-terminal (extracellular) and C-terminal (intracellular) antibodies. For the N-terminal antibody raised in rabbit, a 906 bp cDNA fragment encoding amino acids 1663–1964 of the *Celsr1* protein (GI: 22095546) was cloned into the His-tagged QE30 plasmid. The C-terminal antibody was raised in rabbit against the most membrane proximal region of the *Celsr1* cytoplasmic tail (Formstone et al., 2010).

Retrograde labeling of hindbrain neurons. To visualize cranial motor neurons, nylon filters coated with NeuroVue lipophilic dyes (Molecular Targeting Technologies) were applied to peripheral nerve projections as described previously (Fritzsche et al., 2005). NeuroVue Jade (emission maximum, 508 nm), Maroon (667 nm), and Red (588 nm) were used in this study. Confocal images were captured on a Zeiss LSM 510 Meta NLO system and processed using Zeiss software.

Results

Expression of *Celsr1-3* in the hindbrain

Celsr1 expression was studied by *in situ* hybridization (ISH) with a digoxigenin-labeled riboprobe and immunohistochemistry with anti-Celsr1 antibodies (Fig. 1). At E10.5, *Celsr1* mRNA was expressed in the ventricular zone and floor plate cells at all axial levels (6/6 embryos) (Fig. 1*A,B*). By E12.5, *Celsr1* expression in floor plate cells was downregulated in r4 and more anterior regions but maintained at high levels in and caudal to r5, adjacent to the migrating FBM neurons (4/6 embryo) (Fig. 1*C,D*). At the protein level, Celsr1 was strongly expressed in radial neuroepithelial cells located in the paramedian ventricular zone at E9.5–E10.5, with high signal in their pial end feet (Fig. 1*E*). Celsr1-positive cells were intermingled with emerging *Islet1*-positive FBM neurons in r4. Expression was also seen in radial cells in the alar plate. Using confocal imaging (Fig. 1*E*, inset), we found that Celsr1 protein may colocalize with *Isl1* in some FBM neurons at E9.5. From E10.5 to E11.5, Celsr1 expression was downregulated in neuroepithelial cell, but remained high in floor plate cells (Fig. 1*F–G*). At this stage, we did not find any Celsr1 and *Isl1* colocalization in FBM neurons. Expression of *Celsr2*, studied using the knocked-in β-galactosidase, was broader than that of *Celsr1*. The *Celsr2*-related signal was detected in the floor and alar plates in the neuroepithelium, as well as in newly generated FBM neurons (Fig. 1*H–J*). *Celsr3* expression, previously studied by ISH, is found in all postmitotic neurons but not in precursors, including in the hindbrain (Song et al., 2006; Tissir and Goffinet, 2006). Altogether, these results show that, in the developing hindbrain, *Celsr1* is expressed in neuroepithelial cells, *Celsr2* in both precursors and postmitotic neurons, and *Celsr3* in postmitotic neurons, a pattern similar to that reported in other regions of the nervous system (Formstone and Little, 2001; Shima et al., 2002; Tissir et al., 2002).

Inactivation of *Celsr1* perturbs the direction of FBM neuron migration

Given the intriguing pattern of *Celsr1* expression in the developing hindbrain, we tested its potential role in FBM neuron migration by analyzing *Celsr1* knock-out (Ravni et al., 2009) (Fig. 2) and *Celsr1^{Crsh}* mice that harbor an *N*-ethyl-*N*-nitrosurea-induced allele (Curtin et al., 2003) (Fig. 3).

We examined FBM neuron migration in E11.5–E13.5 embryos using ISH with an *Islet1* (*Isl1*) probe, an established marker for FBM neurons (Garel et al., 2000; Pattyn et al., 2003). In wild-

type (WT) embryos ($n = 16$) (Fig. 2*A, E, I, M*) at E11.5 (Fig. 2*A*), FBM neurons formed one longitudinal column at each side of the hindbrain in r4, where the facial nerve exits laterally. The rostral edge of the column was sharp, whereas FBM neurons migrated across the caudal border of r4 into r5 and r6. At E12.5 (Fig. 2*E*), FBM neurons were aligned in a migratory stream from r4 to r6, where they were engaged in lateral-dorsal and then radial migration, events that were essentially complete by E13.5, when a prominent facial motor nucleus (nVII) was clearly defined in lateral r6 (Fig. 2*I*).

In *Celsr1* knock-out (*Celsr1*^{KO/KO}) mice ($n = 12$) (Ravni et al., 2009) (Fig. 2*B, F, J, N*) at E11.5, some FBM neurons were confined to r4 and r5. Intriguingly, some also migrated rostrally into r3 and r2, a phenotype not seen previously in any vertebrate (Fritzsche, 1998; Chandrasekhar, 2004; Gilland and Baker, 2005). In addition, a cohort often migrated laterally in r4 (Fig. 2*B*, asterisk). While the rostral migration phenotype was fully penetrant, its expressivity varied among animals and even between the left and right sides of the body (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), perhaps reflecting stochastic variations as proposed for *Fzd6* mutant skin phenotypes (Wang et al., 2006). At E12.5 (Fig. 2*F*), while the abnormal lateral and rostral migratory streams persisted, caudally directed FBM neurons moved through r5, medially to the abducens (nVI) nucleus, before migrating laterally in r6 like their WT counterparts. Hence, a facial (nVII) nucleus formed in its normal location in lateral r6 by E13.5. In addition, rostrally migrating FBM neurons subsequently migrated laterally to form an ectopic nucleus adjacent to the trigeminal (nV) nucleus (Fig. 2*J*, arrowhead). The nVII nucleus was examined at P0 and in adults using neurofilament and Nissl staining (supplemental Figs. S2, S3, available at www.jneurosci.org as supplemental material). In WT mice at postnatal day 0 (P0), axons of all FBM neurons loop around the abducens (supplemental Fig. S2*A*, available at www.jneurosci.org as supplemental material). In *Celsr1*^{KO/KO}, only a part of the axons loop around the abducens (Fig. S2*B*). At P21, a facial nerve (nVII) nucleus was found in normal position in the pons (data not shown), and clusters of FBM-like neurons were found ectopically at the level of the trigeminal (nV) motor nucleus (supplemental Fig. S3*E*, available at www.jneurosci.org as supplemental material), indicating that the rostrally migrated FBM neurons may be integrated into local circuits.

FBM neurons also migrated aberrantly in *Celsr1*^{Crsh} mutants, which carry a missense mutation in the *Celsr1* coding sequence resulting in one amino acid substitution in the eighth cadherin repeat (Curtin et al., 2003). Importantly, the defects were very similar to those observed in *Celsr1*^{KO} embryos. In WT embryos, *Tbx20* (Coppola et al., 2005; Song et al., 2006) was expressed by FBM neurons throughout their migratory pathway from r4 to r6 from E10.5 to E12.5 (3/3 embryos) (Fig. 3*A*) (data not shown) as defined by *Isl1* expression (Fig. 2*A, E*). By E14.5, FBM neurons had completely migrated and formed the facial (nVII) nucleus in lateral-dorsal r6 (9/9 embryos) (Fig. 3*D*). In E12.5 *Celsr1*^{Crsh/+}

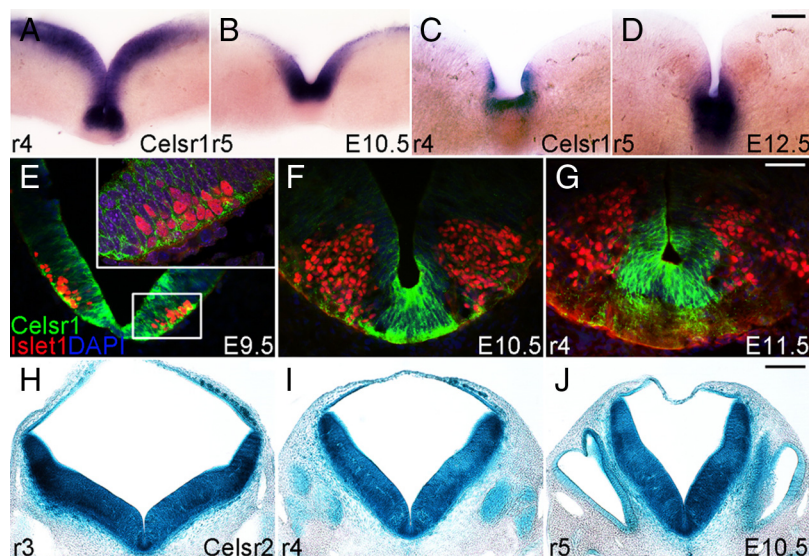
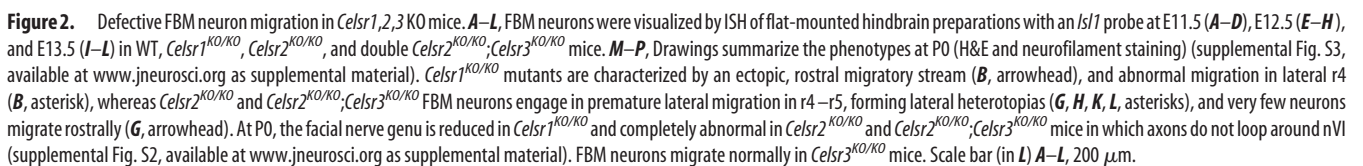


Figure 1. Expression of *Celsr1* and 2 in the developing hindbrain. *A–D*, ISH of *Celsr1* digoxigenin-labeled probe on hindbrain coronal sections. At E10.5 (*A, B*), *Celsr1* mRNA is expressed in the neuroepithelium and the floor plate. At E12.5 (*C, D*), *Celsr1* expression is downregulated in r4 but remains high in the floor plate of r5. *E–G*, Immunofluorescence using anti-*Celsr1* (green) and anti-*Isl1* (red) in r4. *Celsr1* protein expression is found in FBM neuron precursors in ventricular zones at E9.5 and restricted to floor plate from E10.5. Confocal analysis shows that *Celsr1* (green) may colocalize with *Isl1* (red) in FBM neurons at E9.5. *H–J*, Coronal sections in the mouse hindbrain processed for LacZ histochemistry to examine *Celsr2* expression in r4–r6. The *Celsr2*-related signal is detected in the basal and alar plates and in the neuroepithelium, as well as in all postmitotic neurons. Scale bars: (in *D*), *A–D*, 100 μ m; (in *G*), *E–G*, 50 μ m; (in *J*), *H–J*, 200 μ m.

heterozygotes, *Tbx20* ISH revealed abnormal, rostrally migrating columns of putative FBM neurons in r2 and r3, in addition to caudally migrating neurons in r5 and r6 (6/6 embryos) (Fig. 3*B*). By E14.5, the caudally migrating FBM neurons had formed the nVII nucleus in dorsal r6, and the rostrally migrating cells also formed a putative nVII nucleus in dorsolateral r2, adjacent to the trigeminal (nV) nucleus (6/8 embryos) (Fig. 3*E*). In *Celsr1*^{Crsh/Crsh} homozygotes, FBM neuron migration was greatly reduced compared to heterozygotes at E12.5 (2/2 embryos) (Fig. 3*C*) and E14.5 (5/5 embryos) (Fig. 3*F*). Nevertheless, by E14.5 an ectopic putative nVII nucleus was formed in r3 (2/5 embryos) (Fig. 3*F*) or a facial nucleus was seen in r5/r6 (3/5 embryos) (data not shown), indicating that FBM neurons in *Celsr1*^{Crsh/+/+} can migrate out of r4 in both directions in a similar fashion as *Celsr1*^{KO/+} and *Celsr1*^{KO/KO} mutants.

We used additional tools and reagents to characterize the FBM neuron migration defects in *Celsr1*^{Crsh} mutants. Neurofilament (NF-160) staining (Tiveron et al., 2003) revealed abnormal, rostrally migrating columns of putative FBM neurons in r2 and r3, in addition to caudally migrating neurons in r5 and r6 in *Celsr1*^{Crsh/+} and nonmigrated cells in r4 of *Celsr1*^{Crsh/Crsh} (supplemental Fig. S4*A–C*). Despite aberrant neuronal migration, motor innervation patterns in the periphery were unaffected (supplemental Fig. S4*D–F*). Retrograde labeling of FBM neurons from the second arch using NeuroVue dyes (Fritzsche et al., 2005) stained cell bodies in r2 and r3, confirming the aberrant rostral migration of FBM neurons in *Celsr1*^{Crsh/+} (4/6 embryos) (supplemental Fig. S4*H*, available at www.jneurosci.org as supplemental material). In some *Celsr1*^{Crsh/Crsh}, FBM neuron migration appeared completely blocked, with cells remaining confined to r4 (3/3 embryos) (supplemental Fig. S4*I*, available at www.jneurosci.org as supplemental material). To investigate further whether FBM neurons migrated in *Celsr1*^{Crsh/Crsh} homozygotes, we examined mutant phenotypes in the *SE1::GFP* background,



Given that the *Celsr1*^{KO} allele is null, the *Celsr1*^{Crsh/+} phenotype could result from haploinsufficiency or a dominant-negative effect. To further investigate this, we crossed the *Celsr1*^{Crsh} and *Celsr1*^{KO} strains to examine the FBM neuron migration phenotype of *Celsr1*^{Crsh/KO} transheterozygotes at E12.5 by using *Tbx20* ISH (Fig. 3J–M). Rostrally and caudally migrating cells were seen in *Celsr1*^{Crsh/KO} embryos (5/5), and the phenotype was very similar to that of *Celsr1*^{KO/KO} mutants (compare Figs. 2F, 3M). Thus, while the *Crsh* allele behaves like a null in *trans* with the KO allele, only *Celsr1*^{Crsh/+}, but not *Celsr1*^{KO/+}, embryos exhibit

migration defects, suggesting that the *Celsr1^{Crsh}* allele is dominant negative.

FBM neurons migrate prematurely into lateral r4–r5 in *Celsr2*, *Celsr2;Celsr3*, and *Fzd3* mutant mice

In zebrafish, knockdown of *celsr1a,b* has little effect on FBM neuron migration, whereas *celsr2* loss of function leads to a severe phenotype (Wada et al., 2006). Therefore, we tested the roles of mouse *Celsr2*, and *Celsr3* in FBM neuron migration. In *Celsr2^{KO/KO}* ($n = 27$) (Fig. 2C,G,K,O), the caudal migration stream was severely truncated. From E12.5, the majority of mutant FBM neurons migrated prematurely into lateral r4 and r5 (Fig. 2G). In contrast to WT neurons, *Celsr2^{KO/KO}* FBM neurons that migrated into r5 turned laterally rostral to the abducens nucleus and formed facial nuclei in lateral r5. Consequently, the neurons that migrated laterally within r4 and those that prematurely migrated laterally in r5 formed elongated facial nuclei spanning r4 and r5 (Fig. 2G,K). At postnatal stages, the facial nerve did not form a genu around nVI, since it looped laterally without turning around nVI (supplemental Fig. S2A,C).

In *Celsr3^{KO/KO}* mice ($n = 8$), FBM neurons migrated normally (data not shown). However, in *Celsr2^{KO/KO};Celsr3^{KO/KO}* ($n = 16$), in addition to abnormalities seen in *Celsr2^{KO/KO}* embryos, the facial nuclei were greatly reduced in size by E13.5 due to a marked reduction in the number of FBM neurons compared to their WT and *Celsr2^{KO/KO}* counterparts (Fig. 2, compare L to I and K). To test whether apoptosis was involved, we performed activated caspase-3 immunostaining at E12.5, 1 d before atrophy of the *Celsr2^{KO/KO};Celsr3^{KO/KO}* facial nucleus became evident. The number of apoptotic profiles in medial r4/r5 was considerably increased in *Celsr2^{KO/KO};Celsr3^{KO/KO}* embryos compared with WT and *Celsr2^{KO/KO}* embryos (Fig. 4A–D), and no apoptotic cells were detected among post-migrated FBM neurons in the facial nucleus (Fig. 4E, dotted area). This result suggests that the combined loss of *Celsr2* and *Celsr3* leads to cell death. To test whether neuronal death in double mutants could be due to axonal pathfinding defects, we carried out whole-mount staining of E10.5 embryos with anti-neurofilament antibodies (supplemental Fig. S8, available at www.jneurosci.org as supplemental material). Cranial axon outgrowth and cranial ganglia development in *Celsr2^{KO/KO};Celsr3^{KO/KO}* embryos were indistinguishable from those in WT and *Celsr2^{KO/KO}* mutant embryos, suggesting that cell death is not due to defective axonal outgrowth. Hematoxylin and eosin (H&E) and neurofilament staining at P0 confirmed that FBM neuron loss in *Celsr2^{KO/KO};Celsr3^{KO/KO}* mice resulted in a diminutive

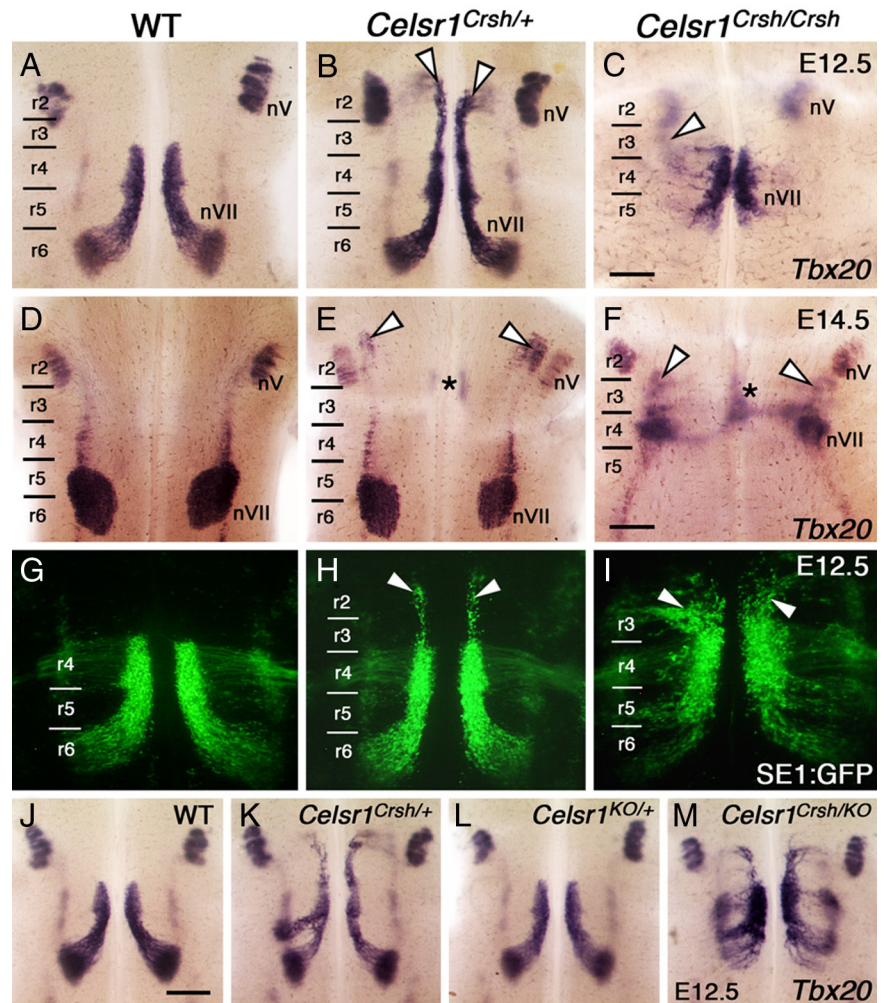


Figure 3. FBM neurons migrate rostrally in *Celsr1^{Crsh}* embryos. **A–M**, Ventricular side (**A–C**, **G–I**) and pial side (**D–F**) views of flat-mounted hindbrains processed for ISH using *Tbx20* (**A–F**, **J–M**) and imaging of neurons in *SE1::GFP* embryos (**G–I**). **A**, In a WT embryo at E12.5, FBM neurons are distributed throughout their migratory pathway spanning r4, r5, and r6. Within r6, the neurons migrate radially (laterally in this view) toward the pial surface to form the facial (nVII) nucleus. **B**, In a *Celsr1^{Crsh/+}* embryo, a large number of putative FBM neurons (arrowheads) migrate rostrally into r2 and r3. **C**, In a *Celsr1^{Crsh/Crsh}* embryo, putative FBM neurons mostly remain in r4 or migrate into r5. A few *Tbx20*-expressing cells are found in r3 (out of focus, arrowhead). The trigeminal nucleus (nV) develops normally in all embryos. **D**, In a WT embryo at E14.5, the facial (nVII) nucleus spans r5 and r6, and the trigeminal (nV) nucleus is found in r2. **E**, In a *Celsr1^{Crsh/+}* embryo, the nVII nucleus forms but is smaller than in *Celsr1^{+/+}* siblings. In addition, ectopic *Tbx20*-expressing cells are found in r3 (asterisk) and r2 (arrowheads) adjacent to the nV neurons. **F**, In a *Celsr1^{Crsh/Crsh}* embryo, a putative facial (nVII) nucleus forms in r4. Ectopic *Tbx20*-expressing cells are also found in medial (asterisk) and lateral (arrowheads) locations in the r2–r3 region. **G**, **H**, In *SE1::GFP* transgenic WT and *Celsr1^{Crsh/+}* embryos, the organization of FBM neurons is identical to that seen by *Tbx20* *in situ* (**A**, **B**), with rostral migration in heterozygotes (arrowheads). **I**, In a *Celsr1^{Crsh/Crsh}* embryo, the *SE1::GFP* reporter reveals extensive rostral FBM neuron migration into r3 and r2 (arrowheads), and few neurons migrate into r6. **J–M**, Siblings from a *Celsr1^{KO/+} × Celsr1^{Crsh/+}* cross. **J**, **L**, FBM neurons migrate normally in WT (**J**) and *Celsr1^{KO/+}* (**L**). **K**, Rostral neuron migration in *Celsr1^{Crsh/+}*. **M**, In a *Celsr1^{Crsh/KO}* transheterozygote, most FBM neurons remain in r4 and r5, with smaller numbers migrating into r2/r3 and r6. This phenotype is very similar to the migration pattern seen in *Celsr1^{Crsh/Crsh}* (**I**) and *Celsr1^{KO/KO}* (Fig. 2F). Scale bars: (in **C**, **A–C**, **G–I**, 300 μ m; (in **F**) **D–F**, 375 μ m; (in **J**), **J–M**, 375 μ m).

nVII nucleus that was displaced to a rostral position (supplemental Fig. S3C, available at www.jneurosci.org as supplemental material). Given the importance of *Fzd3* in PCP, we examined its expression using ISH. *Fzd3* was widely expressed in the hindbrain, both in neuroepithelial and postmitotic cells, including FBM neurons (Fig. 5A). In homozygous *Fzd3* mutant (*Fzd3^{KO/KO}*) mice, FBM neurons migrated prematurely in lateral r4 and r5 and formed a very diminutive nVII, as seen in *Celsr2^{KO/KO};Celsr3^{KO/KO}* mice (Fig. 5B–D) (Vivancos et al., 2009). The observation that *Celsr3^{KO}* alone generates no phenotype but enhances the *Celsr2^{KO}* phenotype, together with the observation that this

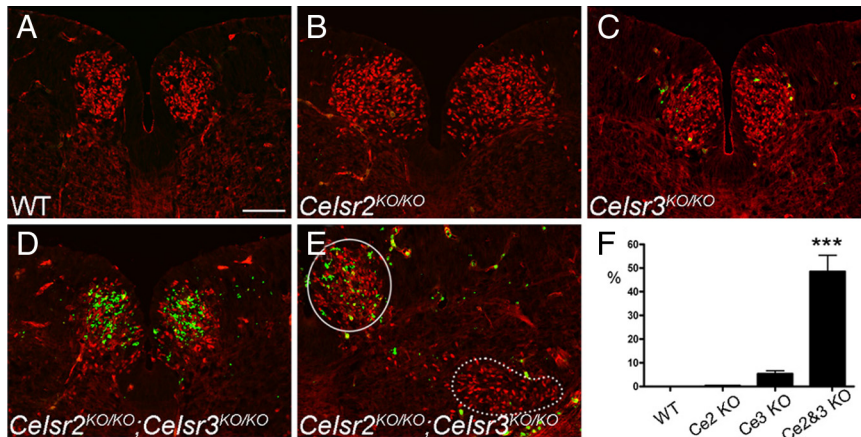


Figure 4. *Celsr2* and *Celsr3* are required for FBM neuron survival. **A–E**, FBM neurons shown with anti-Is1 (red) were stained for activated (cleaved) caspase-3 (green) (**A–E**) at E12.5. No apoptotic cells were found in WT (**A**) and *Celsr2*^{KO/KO} mice (**B**). In *Celsr3*^{KO/KO} (**C**), a few FBM neurons were positive for activated caspase-3. In *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} (**D–E**), the number of caspase-3 positive cells increased dramatically in FBM neurons located in the medial region (circle), but not when they finish lateral migration (dotted contour). **F**, Cell death, estimated as the ratio of green to red signals (ImageJ software), was 5% in *Celsr3*^{KO/KO} and 50% in *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} samples. ****p* < 0.001, Student's *t* test, Bonferroni correction. Error bars are SD. Scale bar (in **A–E**), 100 μm.

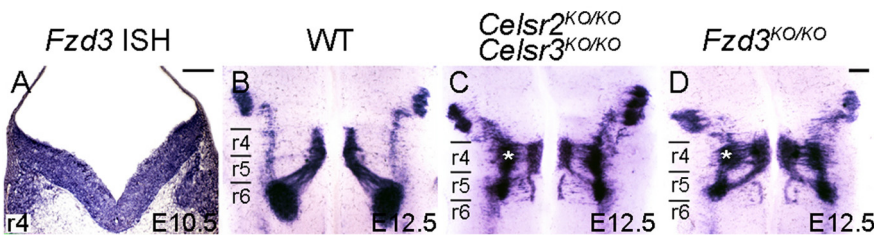


Figure 5. FBM neuron migration in *Fzd3* KO mice. **A**, Coronal section of E10.5 mouse hindbrain at the level of r4 processed for ISH with a *Fzd3* probe. *Fzd3* mRNA is found in both neuroepithelial cells and postmitotic cells, including FBM neurons. **B–D**, E12.5 hindbrains hybridized with an *Is1* riboprobe. Abnormalities of FBM neurons migration in *Fzd3*^{KO/KO} mutant mice (**D**) phenocopy those in double *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} mice (**C**). Note the premature lateral migration in r4 (asterisk). Scale bars, **A** (for **A**), **D** (for **B–D**), 200 μm.

phenotype is mimicked by *Fzd3*^{KO}, suggests strongly that *Celsr2* and *Celsr3* act redundantly during FBM neuron migration through mechanisms that probably involve *Fzd3*.

Mutations in *Celsr* genes do not affect rhombomere identity or neuronal specification

To test whether mutations in *Celsr* genes affect rhombomere specification and patterning, we performed ISH at E10.5 with *Hoxb1* (Fig. 6A–D) and *Eph4* (Fig. 6E–H), which label r4 and r3 plus r5, respectively (Murphy et al., 1989; Gilardi-Hebenstreit et al., 1992). The specification of r3–r6 and rhombomeric borders appeared normal in all mutants studied. Similarly, expression of *Krox20* in r3 and r5, and of *Hoxb1* in r4, was unaffected in *Celsr1*^{Crsh/+} mice (*Krox20*, *n* = 5; *Hoxb1*, *n* = 3) (Fig. 6I–L). Together, these results indicate that defective migration of FBM neurons in the *Celsr* mutants does not result from aberrant specification of rhombomere identity.

Another possibility is that many FBM neurons fail to migrate in *Celsr* mutants because they become misspecified as nonmigratory neurons. To test this, we examined the expression of *Gata3*, which marks inner ear efferent neurons that are specified in but do not migrate out of r4 (Karis et al., 2001), and *Hb9*, which identifies somatic motor neurons such as the abducens (nVI) neurons in r5 (Thaler et al., 1999). In r4 of E11.5 WT embryos,

there was a medial domain of high *Gata3*-expressing cells adjacent to the floor plate, a lateral domain of weaker expressing cells, and a narrow domain ventral to the FBM neurons connecting the two (Fig. 7A, A'). In all three backgrounds with defective FBM neuron migration (*Celsr1*^{Crsh/+}, *Celsr1*^{Crsh/KO}, *Celsr1*^{KO/KO} mutants), there was a marked increase in the number of *Gata3*-expressing cells in r4 (Fig. 7B–D). However, cross-sections revealed that the excess cells were located either in the lateral domain or the connecting domain and not within the FBM neuron domain (Fig. 7B'–D'), suggesting that non-migratory FBM neurons are not misspecified as *Gata3*-expressing cells. Similarly, *Hb9* was not expressed ectopically in r4 in nonmigratory FBM neurons in any of the mutant backgrounds (Fig. 7E–H), suggesting that the neuronal migration defects are not caused by transdifferentiation of FBM neurons into non-migratory cell types.

Celsr1 and *Celsr2* and 3 define a dual control of FBM neuron migration

The observation that *Celsr1* is expressed in neuroepithelial cells, including FBM neuron precursors, but not in postmitotic neurons, suggests that the function of *Celsr1* may be extrinsic to FBM neurons. To test this, we crossed conditional *Celsr1*^f mutants (Ravni et al., 2009) with *Isl1*-Cre mice (*Isl1*^{tm1(cre)Sev}) (Yang et al., 2006) that express the Cre recombinase very early and efficiently in postmitotic motor neurons to generate *Celsr1*;*Isl1* embryos in which *Celsr1* should be inactivated in FBM neurons as soon as they exit the cell cycle. None of the ectopic migration streams evident in constitutive *Celsr1*^{KO/KO} mutants were present in *Celsr1*;*Isl1* embryos in which FBM neurons migrated normally (Fig. 8A–C), suggesting that *Celsr1* regulates FBMN migration in a non-cell-autonomous manner.

Similarly, to determine the cell autonomy of the *Celsr3*^{KO} phenotype, we produced *Celsr2*^{KO/KO};*Celsr3*;*Isl1* mutant mice in which *Celsr2* is inactivated constitutively and *Celsr3* is inactivated only in FBM neurons upon *Isl1*-CRE expression. Comparative histological examination at E14.5 (Fig. 8D–F) and P0 (Fig. 8G–I) showed that the facial nucleus of *Celsr2*^{KO/KO};*Celsr3*;*Isl1* mutant mice was atrophic and morphologically indistinguishable from that in *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} and different from that of *Celsr2*^{KO/KO} mutants, which was dispersed along the rostrocaudal axis but not atrophic. This indicated that the action of *Celsr3* is cell autonomous. Both the phenotypic differences between *Celsr1*^{KO/KO} and *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} mice and the differences in cell autonomy suggest that *Celsr1* on one hand and *Celsr2* and *Celsr3* on the other hand regulate two different aspects of FBM neuron migration.

Do *Celsr1* and *Celsr2* act on FBM neurons in independent manner? To test for a putative interaction between the two proteins, we produced *Celsr1*^{KO/KO};*Celsr2*^{KO/KO} double-mutant embryos and studied the migration of FBM neurons and the

development of facial motor nucleus. FBM neurons underwent premature migration in lateral r4–r5, but almost no ectopic rostral migration was seen ($n = 8$) (Fig. 8L). This phenotype was indistinguishable from that in *Celsr2*^{KO/KO} embryos (Fig. 8K) but unlike that observed in *Celsr1*^{KO/KO} (Fig. 8J). These data show that the phenotype of *Celsr2* mutation masks that of *Celsr1* mutation, indicating that *Celsr2* is epistatic to *Celsr1*.

Discussion

Our results show that *Celsr1*, *Celsr2*, *Celsr3*, and *Fzd3* play critical roles in FBM neuron migration in mice. In zebrafish, *celsr2*, *celsr1a*, and *celsr1b* regulate FBM neuron migration out of r4 such that combined loss of function results in a block of caudal FBM neurons migration, with all neurons confined to r4 (Wada et al., 2006). In contrast, we show that in the mouse *Celsr1* on one hand and *Celsr2,3* and *Fzd3* on the other regulate FBM neuron migration in two different ways: *Celsr1* helps specify the caudal direction of FBM neuron migration in a non-cell-autonomous manner, whereas *Celsr2,3* and *Fzd3* regulate the trajectory that FBM neurons follow from r4 to r6, as well as

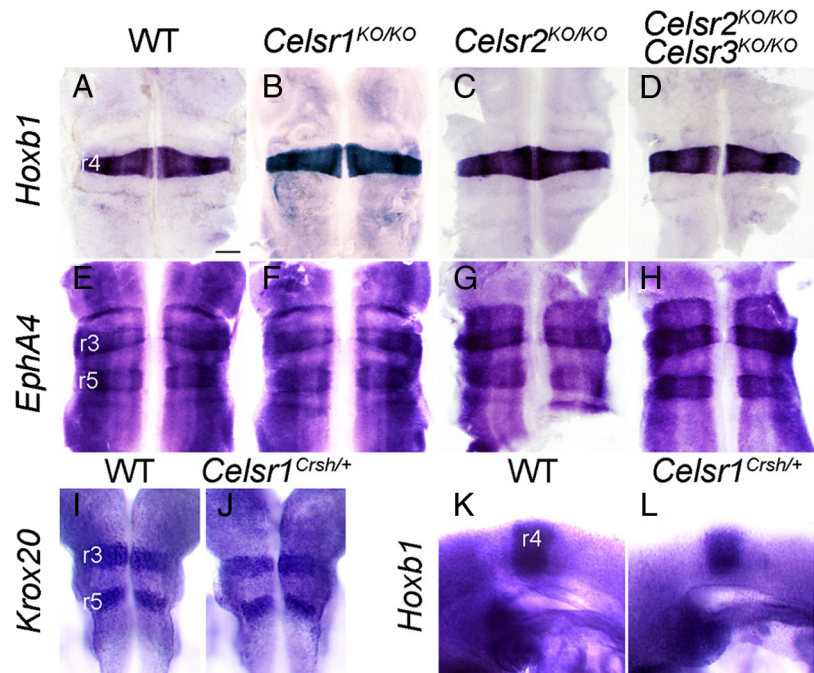


Figure 6. Normal hindbrain patterning in *Celsr* mutant mice. **A–H**, Hindbrains at E10.5 were processed by ISH with *Hoxb1* (**A–D**) to label r4 and *EphA4* (**E–H**) to label r3 and r5. As compared with the WT (**A**, **E**), no difference was seen in *Celsr1*^{KO/KO} (**B**, **F**), *Celsr2*^{KO/KO} (**C**, **G**), or *Celsr2*^{KO/KO}; *Celsr3*^{KO/KO} (**D**, **H**) mice. **I–J**, Dorsal views of E9.5 hindbrains from WT (**I**) and *Celsr1*^{Crsh/+} (**J**) hybridized with *Krox20* probe. **K**, **L**, Lateral view of whole-mount embryos WT (**K**) and *Celsr1*^{Crsh/+} (**L**) hybridized with *Hoxb1* probe. As in *Celsr1*^{KO/KO}, *Celsr2*^{KO/KO}, and *Celsr2*^{KO/KO}; *Celsr3*^{KO/KO}, the patterning of r3–r5 is preserved in *Celsr1*^{Crsh/+}. Scale bar (in **A**–**L**), 200 μ m.

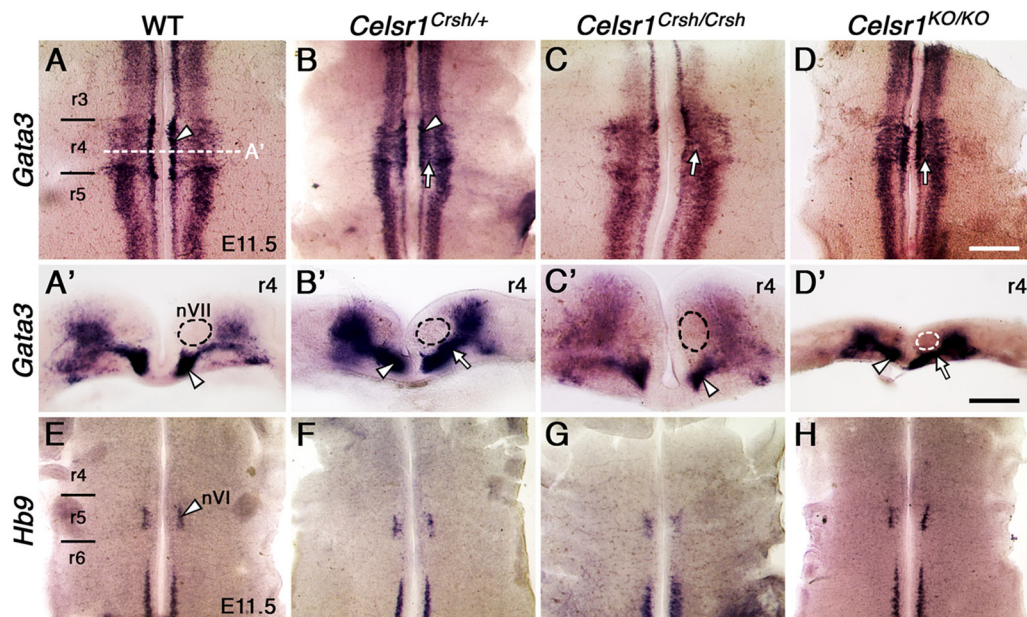


Figure 7. Normal neuronal specification in *Celsr1*^{Crsh} and *Celsr1*^{KO} mutants. Ventricular side views (**A–H**) and cross-sections (**A'–D'**) of flat-mounted E11.5 hindbrains processed for ISH of *Gata3* (**A–D**, **A'–D'**) and *Hb9* (**E–H**) expression. **A'–D'** show cross-sections in r4 of the respective embryos (**A–D**) at the approximate level indicated in **A**. Thickness of the hindbrain tissue varied considerably between embryos (compare **C'** and **D'**) due to genotype and slight stage differences. **A**, In a WT embryo two domains of *Gata3* expression are evident, a high expression medial domain (arrowhead) and a weaker-expressing lateral domain. The cross-section at the r4 level (**A'**) shows the inner ear efferent (IEE) neurons (arrowhead) with the position of FBM (nVII) neurons outlined in black, adjacent to the *Gata3*-expressing cells. **B**, In a *Celsr1*^{Crsh/+} embryo, many *Gata3*-expressing cells (arrow) bridge the gap between the two expression domains in r4. Cross-section in r4 (**B'**) reveals a continuous column (arrow) connecting the IEE domain (arrowhead) to the lateral domain, but these cells are outside the region of FBM neurons (black outline). **C**, **D**, In *Celsr1*^{Crsh/Crsh} and *Celsr1*^{KO/KO} embryos, *Gata3*-expressing cells (arrows) found in between the two expression domains are located outside the region of the FBM neurons (outlines in **C'** and **D'**). **E–H**, There is a bilateral cluster of *Hb9*-expressing cells (arrowhead) in r5, corresponding to the abducens motor (nVI) nucleus in WT (**E**) and *Celsr1*-deficient embryos (**F–H**). Importantly, there are no ectopic *Hb9*-expressing cells in r4 in the mutants (**G**, **H**), even though there are a large number of nonmigrated FBM neurons in these embryos. Scale bars: (in **D**) **A–H**, 400 μ m; (in **D'**), **A'–D'**, 200 μ m.

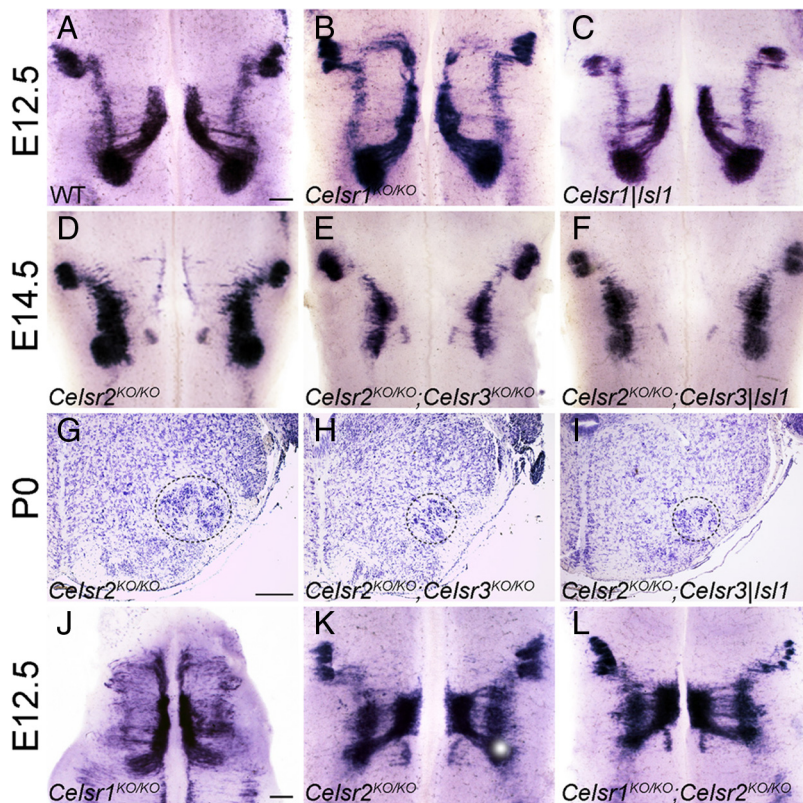


Figure 8. Cell autonomy and epitaxis in the *Celsr* gene family. **A–C**, In *Celsr1/Is11* conditional KO mice (**C**), no rostral migration of FBM neurons was seen, a situation similar to that of WT (**A**) but unlike *Celsr1*^{KO/KO} constitutive mutants (**B**) (E12.5, *Isl1* ISH). Scale bar (in **A–F**), 200 μ m. **D–I**, Compared to *Celsr2*^{KO/KO} (**D**) hindbrain, the number of FBM neurons is similarly reduced in double *Celsr2*^{KO/KO}; *Celsr3*^{KO/KO} (**E**) and double *Celsr2*^{KO/KO}; *Celsr3/Is11* (**F**) (E14.5, *Isl1* ISH). This is confirmed in Nissl-stained coronal sections at P0. The number of FBM neurons (dotted contour) is diminished in double *Celsr2*^{KO/KO}; *Celsr3*^{KO/KO} (**H**), and double *Celsr2*^{KO/KO}; *Celsr3/Is11* (**I**) as compared with single *Celsr2*^{KO/KO} (**G**). Scale bar: (in **G–I**), 200 μ m. **J–L**, Unlike in *Celsr1*^{KO/KO} (**J**), no rostral migration occurs in *Celsr1*^{KO/KO}; *Celsr2*^{KO/KO} double-mutant mice (**L**). Instead, premature FBM neuron lateral migration is seen as in *Celsr2*^{KO/KO} (**K**) (E12.5, *Isl1* ISH). Scale bar: (in **J–L**), 200 μ m.

their survival. *Celsr3* acts in a cell-autonomous manner and *Celsr2* is epistatic to *Celsr1*.

Celsr1 regulates directionality of FBM neuron migration

When *Celsr1* function is compromised, FBM neurons can still migrate caudally out of r4, but many move rostrally. *Isl1*- and *Tbx20*-expressing cells display a continuous distribution in medial r2–r4. The size of the facial nucleus in r6 is diminished when the number of these cells increases, whereas that of the trigeminal motor nucleus is comparable to its wild-type counterpart. Altogether, these data suggest that *Isl1*- or *Tbx20*-positive cells located in medial r2–r4 are not translocated trigeminal motor neurons. All mouse and zebrafish mutants studied thus far exhibit complete or partial block of FBM neuron caudal migration, but never direction reversal (Chandrasekhar, 2004; Cooper et al., 2005; Wada et al., 2005; Nambiar et al., 2007; Rohrschneider et al., 2007; Song, 2007). Interestingly, only a subset of FBM neurons migrates rostrally in *Celsr1* mutants. One possibility is that *Celsr1* functions redundantly with other molecules to specify directionality. Alternatively, the *Celsr1* mutant phenotype may reflect heterogeneity within the FBM neuron population such that only a subset is depends on *Celsr1* for specification of caudal direction of migration. Intriguingly, in zebrafish only the earliest migrating FBM neurons express Tag1 protein (Sittaramane et al., 2009), suggesting that there may be molecular heterogeneity among FBM neurons. Whether such heterogeneity could underlie differential responses to

Celsr1 remains to be explored. Nevertheless, the role for *Celsr1* in regulating the caudal direction of migration is particularly significant, given that no other gene has been shown to perform a similar function.

Our observations suggesting that the *Celsr1*^{Crsh} allele encodes a protein with a dominant-negative activity is supported by analysis of the skin and cultured keratinocytes in *Celsr1*^{Crsh} mutant mice (Devenport and Fuchs, 2008). In the skin, homophilic interactions between *Celsr1* molecules appear to drive PCP signaling. The mutant protein encoded by *Celsr1*^{Crsh} is produced but no longer polarized along the anterior posterior axis, which perturbs the subcellular distribution of the PCP proteins *Stbm/Vangl2* and *Fzd6*. Furthermore, *Celsr1* and *Vangl2* interact physically (Devenport and Fuchs, 2008), and this interaction is abolished by the *Looptail* mutation in *Vangl2*, which disrupts PCP (Kibar et al., 2001; Murdoch et al., 2001; Montcouquiol et al., 2003), and blocks FBM neuron migration (Vivancos et al., 2009) (D. M. Glasco, B. Fritzsch, J. N. Murdoch, and A. Chandrasekhar, manuscript in preparation). Altogether, our observations in *Celsr1* mutants suggest that PCP genes of the *Celsr*, *Fzd*, and *Vangl* families may regulate interactions between cells and thereby specify directionality in cell migration along the rostrocaudal axis (Goodrich, 2008). It would be instructive to examine the subcellular distribution of PCP proteins in FBM neuron precursors in WT and *Celsr1* mutant mice, as well as the role of other PCP genes such as *Fzd6* in FBM neuron migration.

Celsr1 regulates migration of FBM neurons in a non-cell-autonomous manner

Celsr1 is expressed broadly in ventricular zone precursors of the developing hindbrain (Tissir et al., 2002), and our data demonstrate a particularly high expression in the ventricular zone where FBM neurons are generated. To probe the cell autonomy of *Celsr1* function in FBM neuron migration, we used the *Isl1-Cre* line to specifically inactivate *Celsr1* in these cells. In contrast to *Celsr1*^{KO/KO} mice, FBM neurons migrated normally in *Celsr1/Is11* mice. Although an FBM neuron-autonomous effect of residual *Celsr1* protein inherited from precursors cannot be formally excluded, the fact that no *Celsr1* protein was detected in early FBM neurons clearly indicates non-cell-autonomous action. Thus, *Celsr1* specifies caudal movement of FBM neurons indirectly by acting in neuroepithelial precursors or in radial cells, both of which have their cell bodies in ventricular zones. In line with this, in zebrafish PCP proteins *Frizzled3a* and *Celsr2* also regulate migration of FBM neurons non-cell autonomously, probably through their function in the neuroepithelium (Wada et al., 2006). Although the role of radial neuroepithelial cells as a substrate to guide radially migrating neurons is well known, it is more difficult to figure out how they could regulate tangential migration. The non-FBM neuron-autonomous action of *Celsr1*

implies that *Celsr1*, expressed in radial cells but not in FBM neurons, would mediate heterophilic interactions, which runs counter to evidence implicating *Celsr* and Flamingo cadherins in homophilic interactions (Takeichi, 2007). We deem it more likely that *Celsr1* acts at the level of FBM neuron precursors, even though the mechanism whereby an action in precursors can impact the migration of daughter cells is unclear. Crosses to inactivate *Celsr1* specifically in FBM neurons precursors (*Nkx6.2-Cre*) or in r4, where FBM neurons are generated (*Hoxb1-Cre*), might be useful to test this hypothesis. In the zebrafish neural tube, rostrocaudal PCP is lost during and re-established after division of precursors, and this transient loss of planar polarity is considered important for neural tube closure (Ciruna et al., 2006). FBM neurons are generated almost synchronously at E9.5–E10.5 (Taber-Pierce, 1973; Goffinet, 1984). One may hypothesize that rostrocaudal PCP, acting during the last division, may impact the migration of daughter cells. For example, the orientation of the mitotic spindle could influence the intracellular location of the centrosome or the Golgi in daughter cells, which would in turn specify the direction of migration. Consistent with this view, the asymmetric distribution of Leu-Gly-Asn and Numa, two proteins involved in spindle orientation, is lost in neuron progenitors that are defective in the PCP protein Vangl2 (Lake and Sokol, 2009). It would be interesting to compare the planar orientation of mitotic spindles in r4 and/or the localization of the Golgi or centrosome in early FBM neurons at E9.5–E10.5 in WT and *Celsr1* mutant mice.

Roles of *Celsr2*, *Celsr3* and *Fzd3* in FBM neuron migration

Inactivation of *Celsr2* reduces severely the caudal movement of FBM neurons, and added inactivation of *Celsr3* impacts further on their migration and survival, indicating redundancy between the two proteins. The phenotype of the double *Celsr2,3* KO mice closely resembles that of *Fzd3*^{KO}, as shown also by others (Vivancos et al., 2009), and of *Mash1,Math3* double KO mice (Ohsawa et al., 2005). The redundant role of *Celsr2* and *Celsr3* in FBM neuron migration and the probable association with *Fzd3* fits in with similar roles proposed in zebrafish (Wada et al., 2006). It is also consistent with their synergistic role in axon guidance (Wang et al., 2002; Tissir et al., 2005, 2010). The *Celsr2,3-Fzd3* system regulates FBM neuron migration by promoting their locomotion along the midline until they pass the abducens nucleus, after which they initiate their lateral displacement. Importantly, mutant neurons that fail to reach r5–r6 remain able to move laterally and differentiate locally into neurons with similar features as FBM neurons. The mediolateral displacement may be a default behavior of differentiated FBM neurons in response to as yet unidentified attractive cues expressed laterally, such as Wnt or Bmp proteins, and/or repulsive signals from the midline region, such as Shh or Slit factors (Guthrie, 2007).

As in *Celsr2* mutant mice, almost no caudal FBM neuron migration occurs in chick and some other nonmammalian vertebrates where the facial (nVII) nucleus forms normally but facial axons do not loop around nVI nucleus (Studer, 2001; Gilland and Baker, 2005). Intriguingly, the chicken genome contains *Celsr1* and *Celsr3* but lacks *Celsr2*, indicating that loss of *Celsr2* might be the origin of the peculiar anatomy of nVII and its axons in birds.

Whereas *Celsr2* and *Celsr3* act redundantly, combined mutations of *Celsr1* and *Celsr2* phenocopy the *Celsr2*^{KO/KO} phenotype. The fact that *Celsr2* is epistatic to *Celsr1* provides additional support to the view that two genes regulate FBM neurons migration in specific, sequential manners. The *Celsr1* system may regulate planar orientation of precursor divisions, which could provide

a directionality cue to the FBM neurons. The second system, involving *Celsr2*, *Celsr3*, and *Fzd3* would be required for the acquisition of a rostrocaudal migratory phenotype and for maintenance of FBM neurons. For example, activation of this pathway may allow FBM neurons to respond to Wnt proteins (Vivancos et al., 2009). Studies of FBM neuron migration in other PCP mutants and the use of *in vitro* systems that faithfully recapitulate migration events (Vivancos et al., 2009) may provide ways to test these models further.

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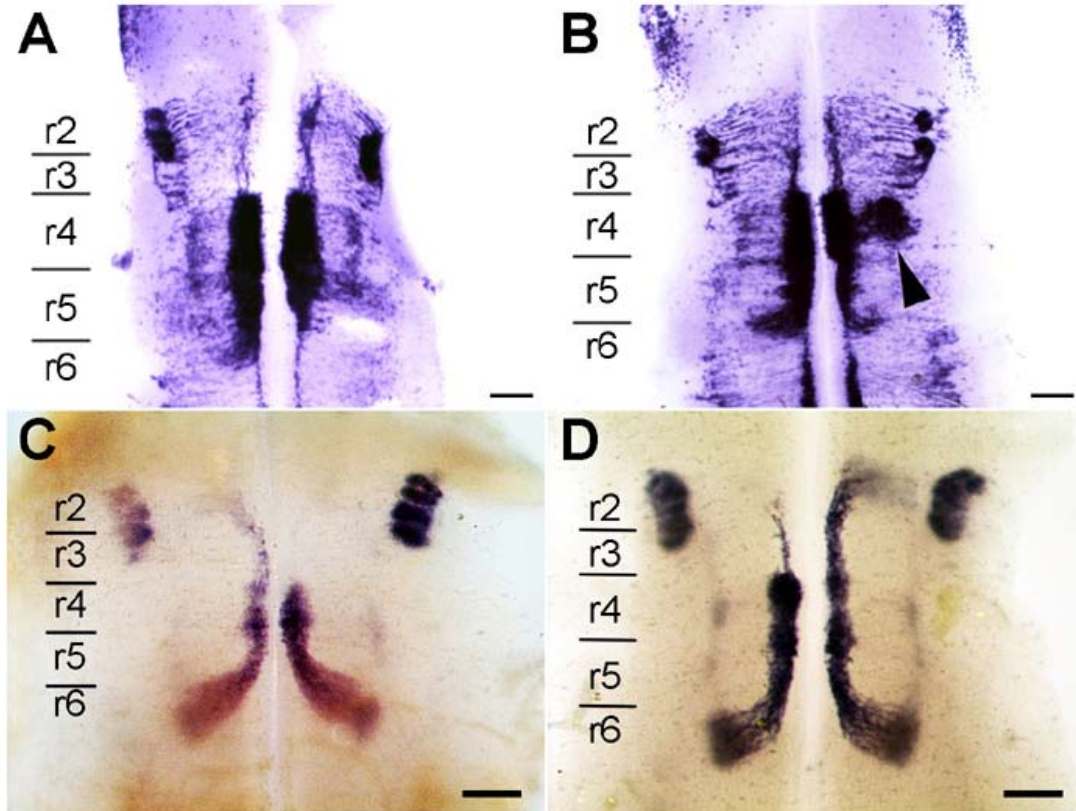
Supplemental Material

Figure S1. Phenotypes in *Celsr1* mutant mice vary from animal to animal and are often asymmetric.

In situ hybridization with an *Isl1* probe in *Celsr1*^{KO/KO} embryos at E11.5 (A-B) and with *Tbx20* in *Celsr1*^{Crsh/+} embryos at E12.5 (C-D). Asymmetries in the rostral abnormal stream are evident in A-D. Some asymmetric ectopic lateral migration is illustrated in (B) (arrowheads). Bars: 200 μ m (A-B) and 250 μ m in (C-D).

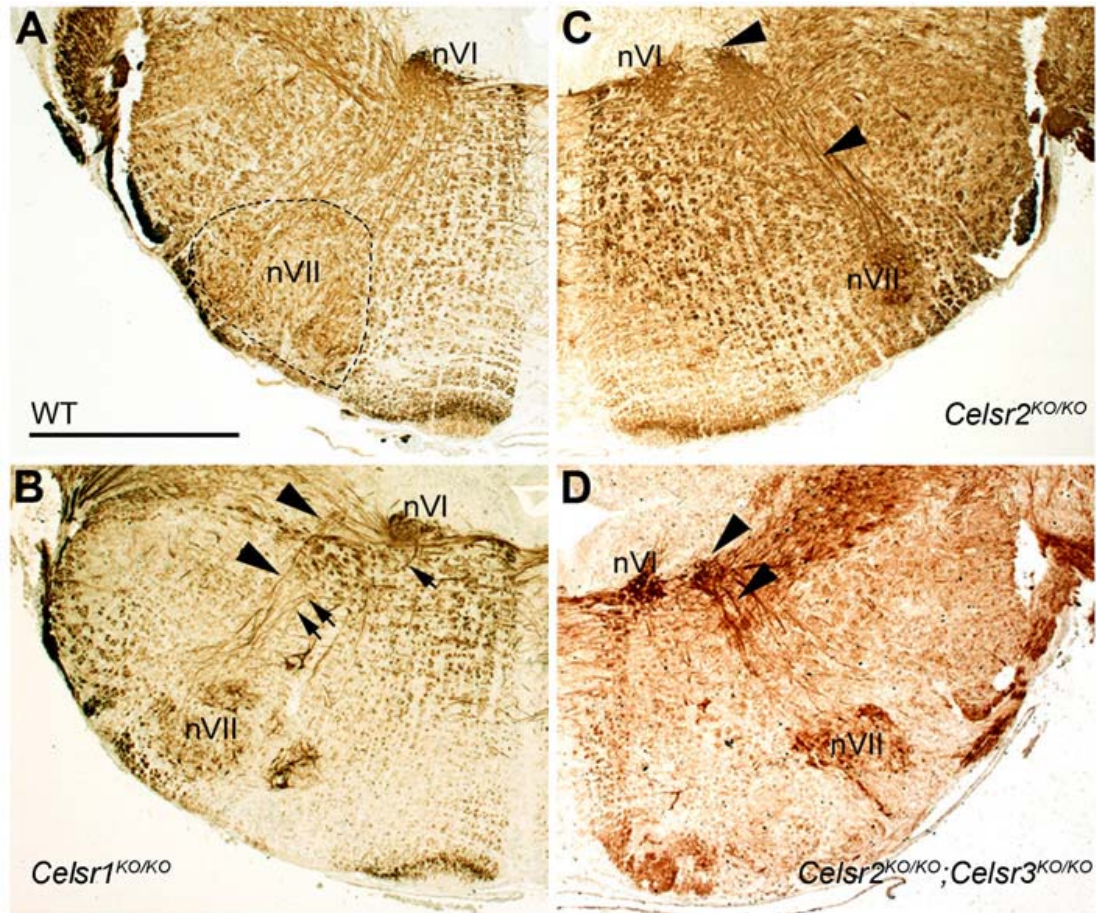


Figure S2. Abnormal trajectories of FBM axons in *Celsr* mutants. Neurofilament immunohistochemistry of coronal sections in hindbrain at P0. In wildtype (A), axons from the facial nucleus (nVII) loop around the abducens nucleus (nVI), forming the genu, before turning laterally. In *Celsr1*^{KO/KO} mice (B), some axons form a partial genu (arrows), whereas others turn laterally to nVI (arrowheads). In *Celsr2*^{KO/KO} (C) and *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} (D) there is no genu *stricto sensu*; instead, FBM axons turn in a position lateral to nVI (arrowheads). Bar = 500 μ m.

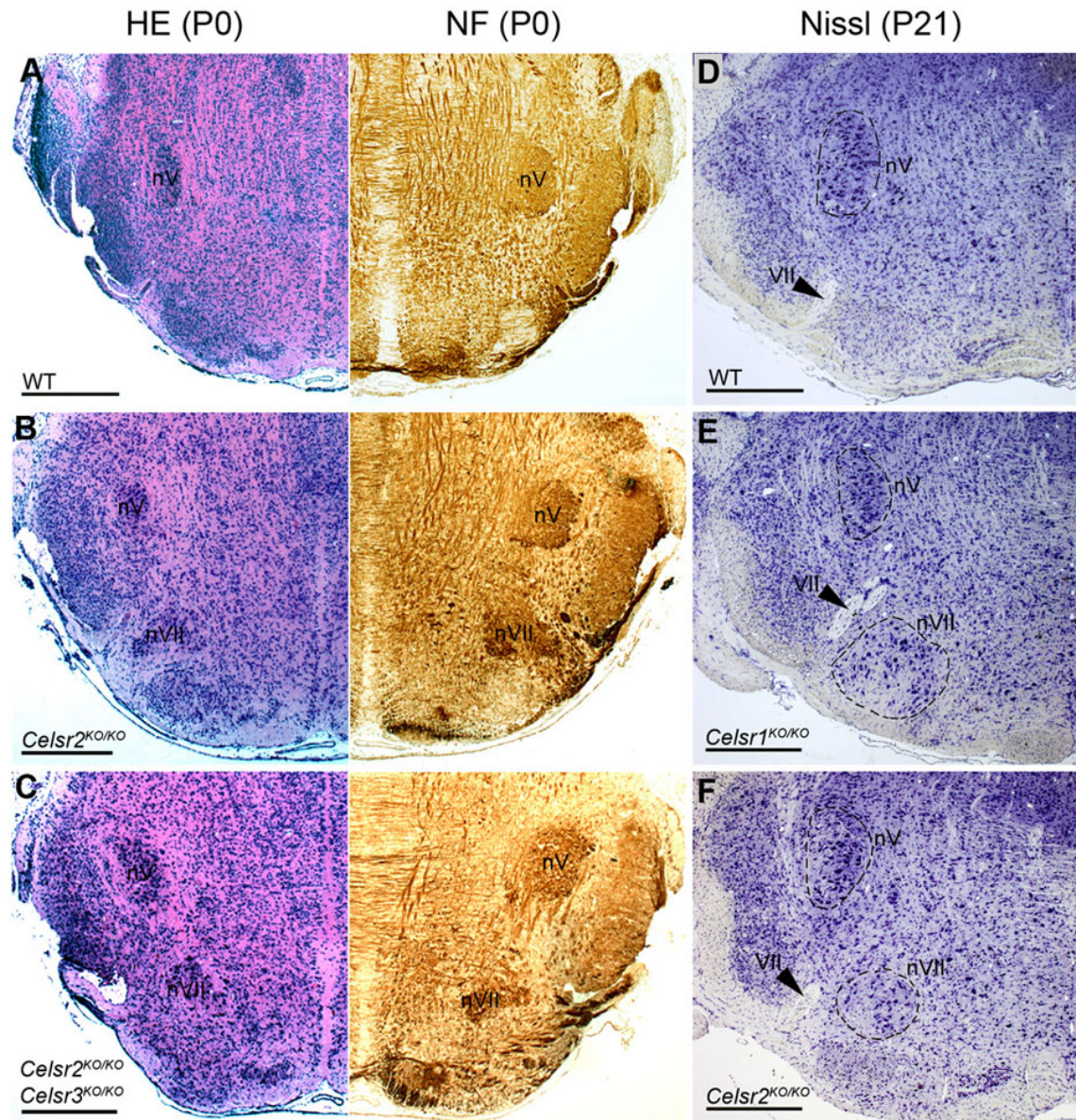


Figure S3. Postnatal FBM neurons are scattered along the rostrocaudal axis in *Celsr* mutants (A-C)

Coronal sections at P0 at the level of the trigeminal motor nucleus (nV) stained with hematoxylin-eosin (HE, left) and anti-neurofilament antibodies (NF, right). In *Celsr2*^{KO/KO} (B) and *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} (C), FBM neurons (nVII) are found in ectopic position at the level of nV. (D-F) Nissl-stained coronal sections of adult hindbrains showing a rostral shift of the facial nucleus (nVII) in *Celsr1*^{KO/KO} (E), and *Celsr2*^{KO/KO} mice (F). Ectopic nVII is found at the same level as the exit point of the facial nerve (VII, arrowheads) and the trigeminal nucleus (nV). Bar = 500 μm.

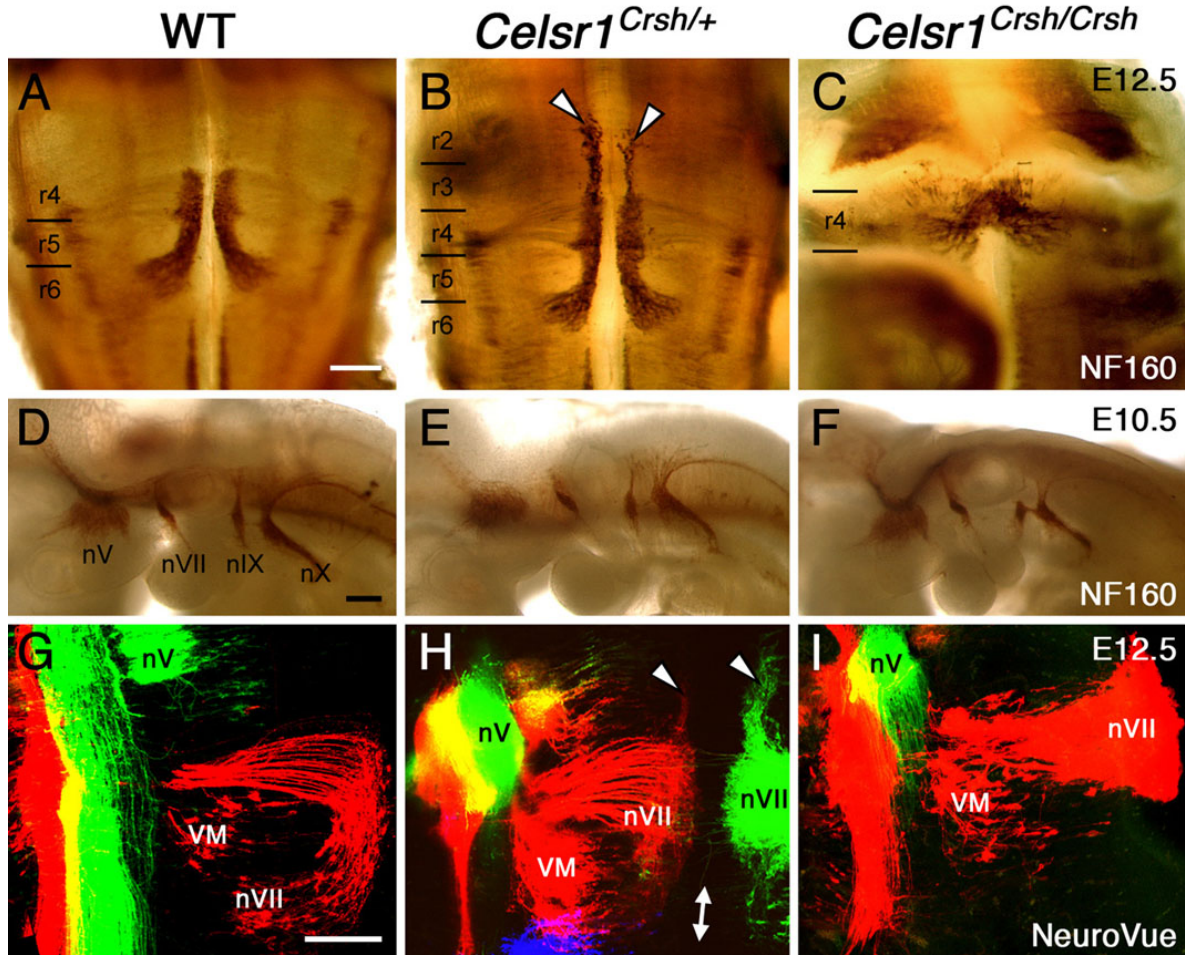


Figure S4. FBM neurons migrate rostrally in *Celsr1^{Crsh}* embryos. Ventricular side views of flat-mounted E12.5 hindbrains processed for neurofilament NF160 immunostaining (A-C) and retrograde labeling with NeuroVue dyes (G-I). (A) In a WT embryo, neurofilament staining labels FBM neurons in r4-r6, and their axons exiting the hindbrain from r4. (B) In a *Celsr1^{Crsh/+}* embryo, putative FBM neurons (arrowheads) migrate rostrally into r2 and r3, while a large number of neurons also migrate correctly into r5 and r6. (C) In a *Celsr1^{Crsh/Crsh}* embryo, putative FBM neurons remain in the r4 region. (D-F) Lateral views, with rostral to left, of NF160-stained E10.5 embryos. The peripheral projections of cranial nerves V, VII, IX, and X are very similar between wild-type (D) and *Celsr1*-deficient embryos (E, F). (G-I) Retrograde labeling of motor neurons using NeuroVue lipophilic dyes applied to nerve exit points. Trigeminal motor neurons (nV, green) were labeled from the r2 exit point. FBM neurons (nVII, red) and visceral motor neurons (VM, red) were labeled from the r4 exit point. The positions of nV and VM mark the locations of r2 and r5, respectively. (G) In a WT embryo, FBM neurons migrate through r5 to form nVII in lateral r6.

(H) In a *Celsr1*^{Crsh/+} embryo, many FBM neurons (arrowheads) are located in r2 and r3. FBM neurons on the right side (nVII, green) were labeled with dye applied at the r4 exit point on the right side. Two-sided arrow indicates midline. (I) In a *Celsr1*^{Crsh/Crsh} embryo, the FBM neurons (nVII) mostly remain in r4. Bars = 250 μ m

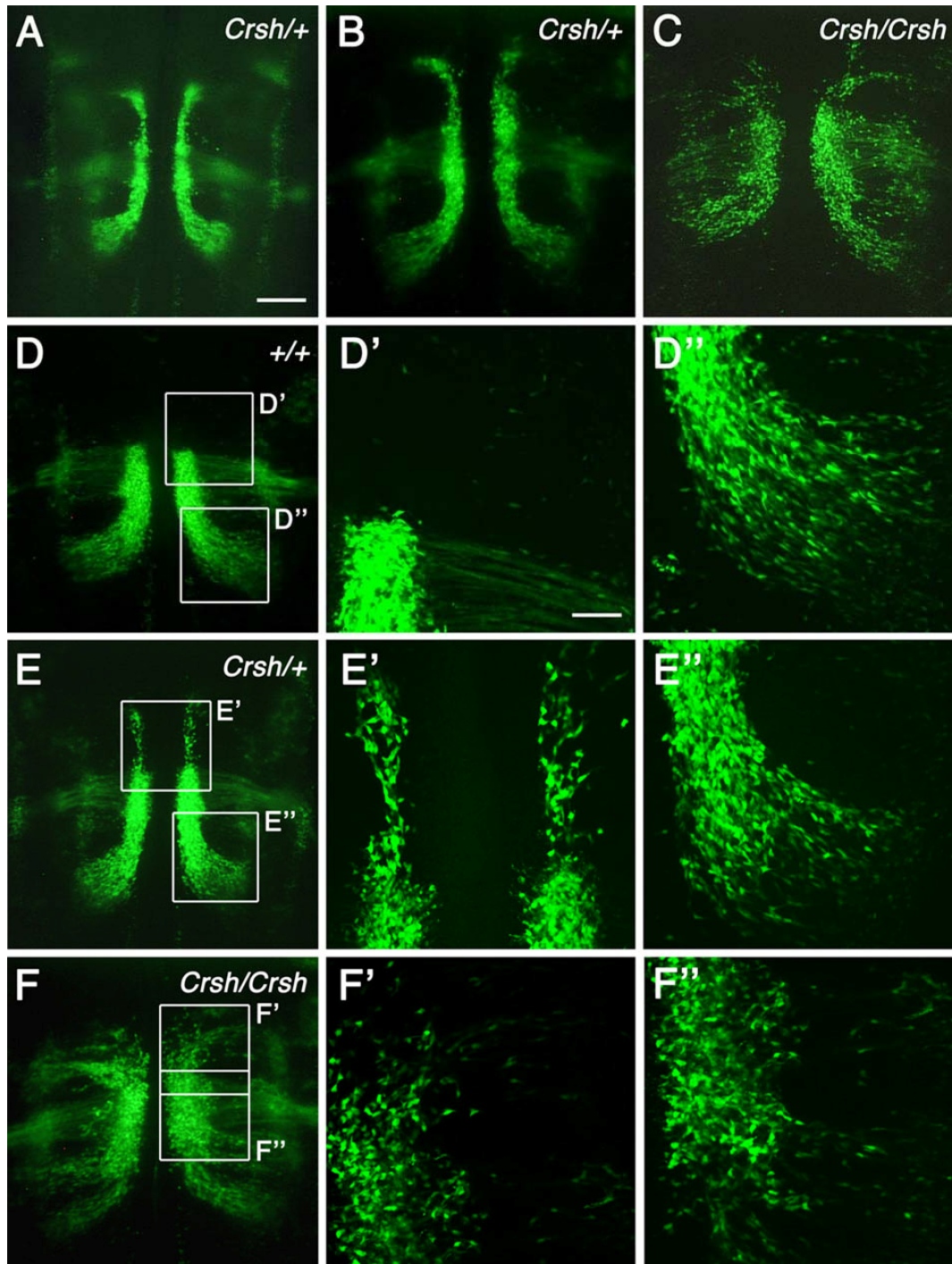


Figure S5. FBM neurons migrate rostrally in *Celsr1^{Crsh}*; *SE1::GFP* embryos. Ventricular side views of live, flat-mounted E12.5 hindbrains from *SE1::GFP* embryos (A, B) Additional examples of *Celsr1^{Crsh}* showing rostral FBM neuron migration into r2 and r3, while a large number of neurons also migrate

correctly into r5 and r6. (C) Additional example of *Celsr1*^{Crsh/Crsh}, with FBM neurons migrating rostrally and caudally out of r4. (D-F) Embryos shown in Fig. 3G-I. For each panel, the right-side panels show higher magnification views of the regions indicated by the boxes. (D''-F'') In +/+ and *Celsr1*^{Crsh/+} embryos, FBM neurons migrate caudally into r6 in orderly streams, but are more disorganized in the *Celsr1*^{Crsh/Crsh}. (D'-F') Whereas FBM neurons migrate rostrally out of r4 only in *Celsr1*^{Crsh/+} and *Celsr1*^{Crsh/Crsh} embryos, their movement appears to be disorganized in the latter. Bars: in A, 300 µm for A-F; in D', 100 µm for D'-F', D''-F''.

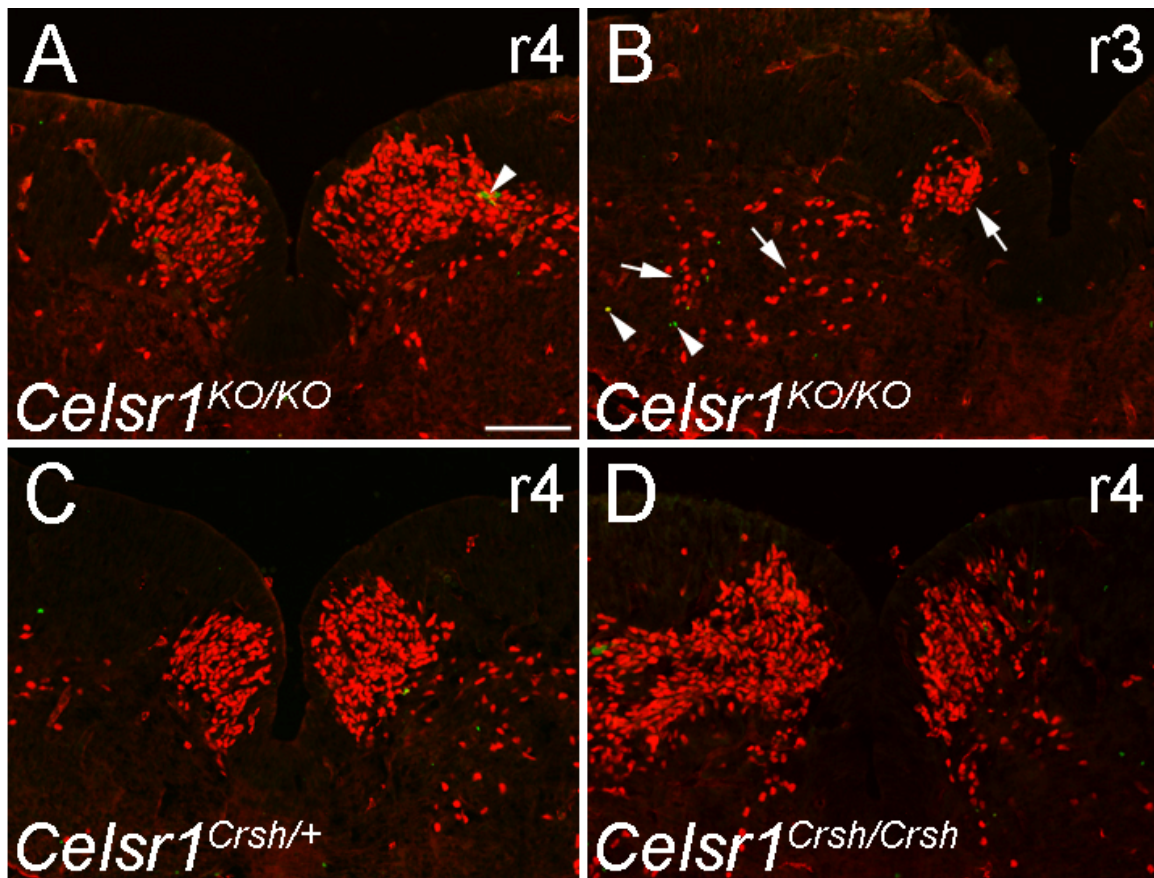


Figure S6. Apoptosis in FBM neurons is not increased in *Celsr1* mutants. E12.5 hindbrain sections stained with anti-Isl1 to label FBM neurons (red) and with anti-cleaved caspase-3 to detect apoptosis (green). In *Celsr1*^{KO/KO}, rare apoptotic profiles (arrowheads) were detected in regions where FBM neurons were located, either in normal position in r4 (A) or in ectopic position (arrows) in r3 (B). Similarly, very little apoptosis was detected in *Celsr1*^{Crsh/+} (C) or *Celsr1*^{Crsh/Crsh} (D) embryos. Bar = 100 μm.

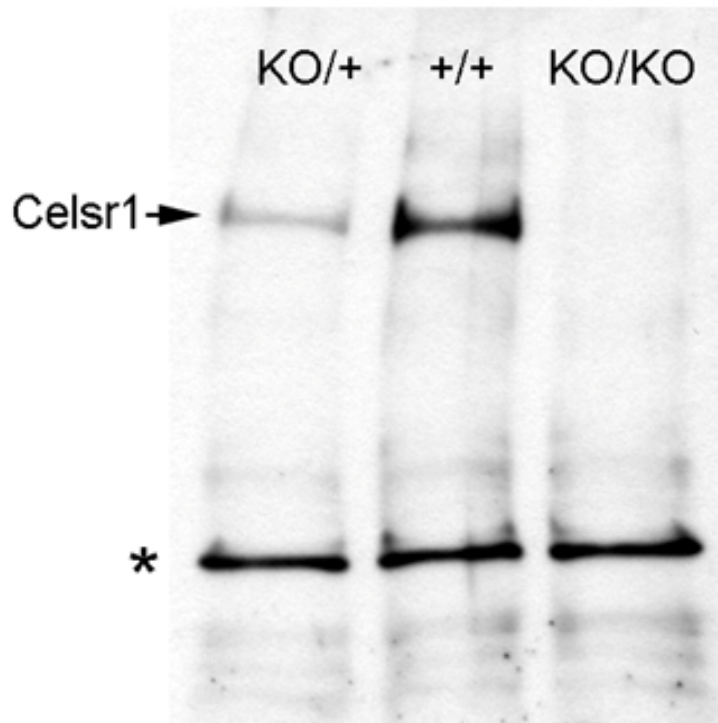


Figure S7. *Celsr1*^{KO} is a null allele. Western blot analysis of brain extracts from WT (+/+), *Celsr1*^{KO/+}, and *Celsr1*^{KO/KO} using an N-terminal antibody detected a band of the predicted size in the WT (middle) and heterozygous (left) but not in homozygous mice (right). Note that the intensity of the lower (asterisk), non specific band is unchanged in the three genotypes.



Figure S8. The early outgrowth of cranial nerves is normal in *Celsr2*^{KO/KO} and *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} embryos. (A-C) lateral views of Neurofilament-stained E10.5 embryos. The peripheral projections of cranial nerves VII and VIII are similar in WT (A), *Celsr2*^{KO/KO} (B), and *Celsr2*^{KO/KO};*Celsr3*^{KO/KO} embryos (C).

Unpublished data

To study the cell autonomy of *Celsr2* function in FBM neuron migration, we first produced *Celsr2* conditional mutant mice. We inserted LoxP sites into introns that flank exons 16-28. Cre expression is predicted to delete the GPS domain and the seven transmembrane segments and to induce a reading frame shift (Fig. 17 A, B). However, the conditional allele containing the neomycin (neo) cassette (Ce2 F_n) yielded a phenotype similar to the Ce2 Δ/Δ mutant (Fig. 18 B, C). Fortunately, the neo cassette is flanked with Frt sites, and could be removed in crosses with Rosa-flp mice. Following removal of the neo cassette, mice homozygous for the conditional *Celsr2* allele (Ce2 F/F) were entirely normal (Fig. 18 E). We then crossed the Ce2 F mice with P_{gk}-Cre mice to obtain a new constitutive knockout allele (Ce2 -). Homozygous (Ce2^{-/-}) mice harbored FBM neuron migration defect similar to those in Ce2 Δ/Δ homozygous mice (Fig. 18 B, D). To determine the cell autonomy of *Celsr2*, we produced Ce2|*Isl1* mutant mice, in which *Celsr2* is inactivated specifically in FBM neurons upon *Isl1*-Cre expression. The FBM neuron caudal migration in Ce2|*Isl1* mutants was blunted, and nVI was unmasked, similarly in Ce2^{-/-} and in Ce2 Δ/Δ mutants (Fig. 18 B, D, F, arrows shows nVI). Thus, *Celsr2* regulates FBM neuron migration in a FBM neuron autonomous manner.

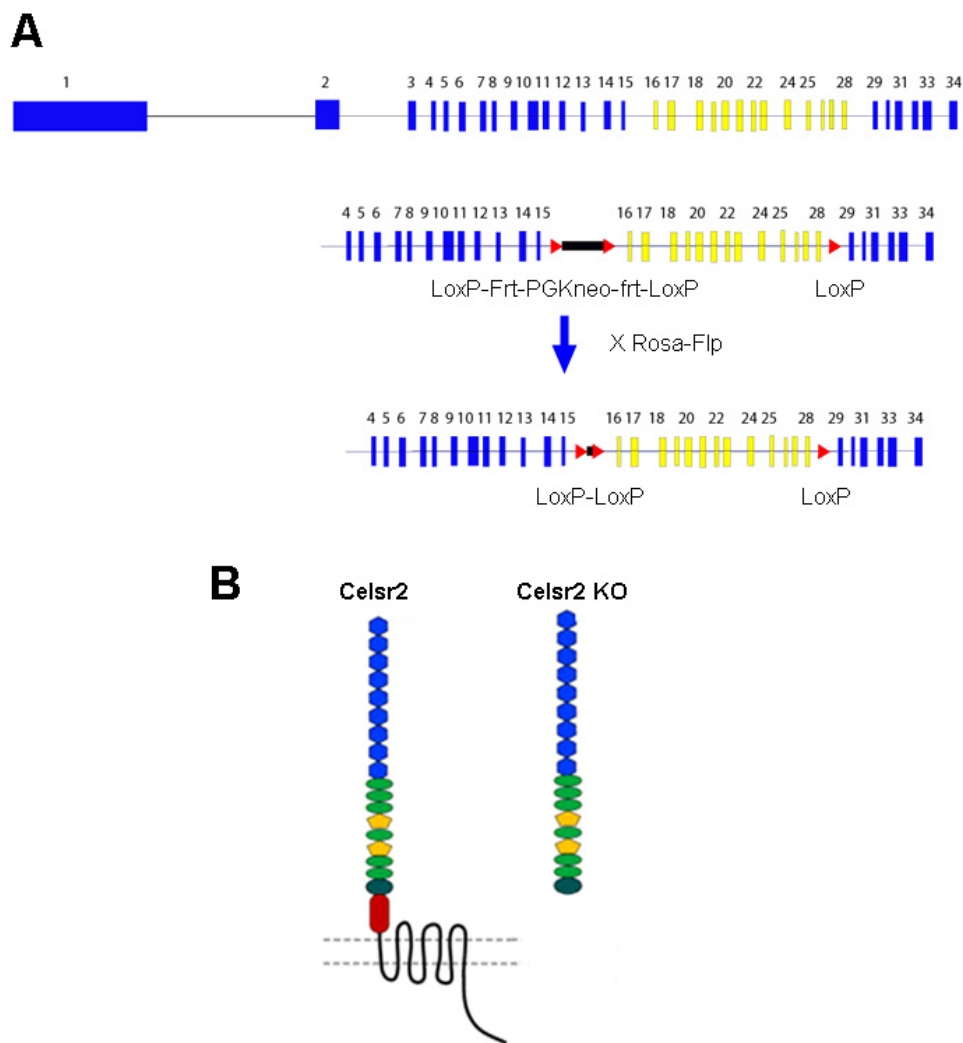


Fig. 17. Celsr2 conditional allele. (A) Exons 16-28, which encode the GPS domain and the seven transmembrane segments, were flanked by LoxP sites to allow their deletion by Cre recombinase. Crossing with Rosa-Flp mice removed the neo cassette in Ce2 Fn allele to generate Ce2 F allele. (B) Deletion of exons 16-28 not only removes GPS and transmembrane domains, but also induces a reading frame shift in the C-terminal sequences.

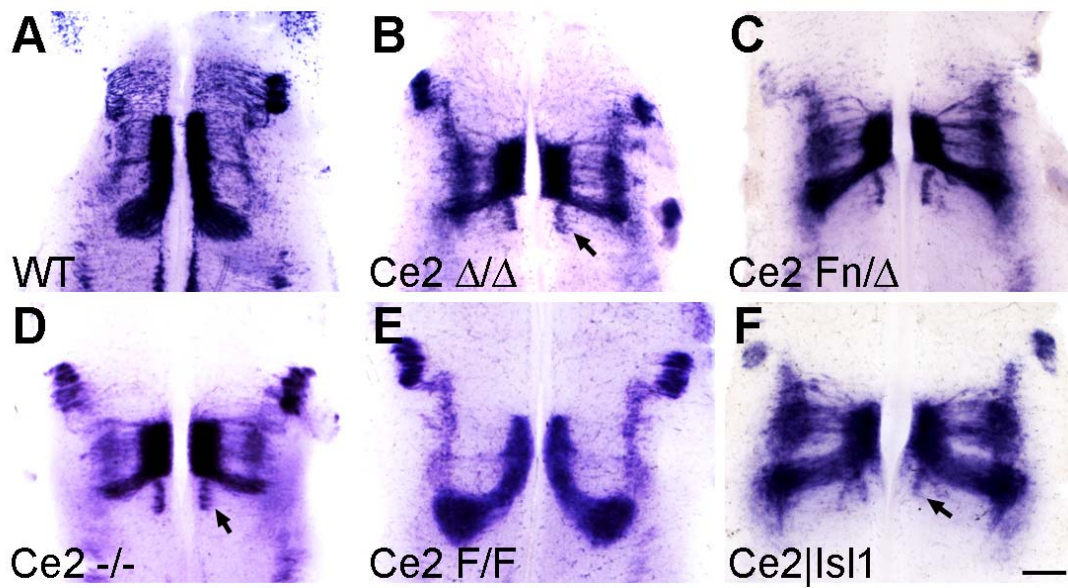


Fig. 18. FBM neuron migration in mutant mice with different *Celsr2* alleles. E12.5 hindbrains hybridized with an *Is11* riboprobe. (A-C) Similar abnormalities of FBM neurons migration are observed in *Ce2Fn/Δ* and *Ce2Δ/Δ* mice. (D) *Ce2-/-* mutants harbor FBM neuron migration defects similar to those in *Ce2Δ/Δ*. (E) FBM neuron migration is normal in *Ce2F/F* mice. (F) *Ce2|Is11* has similar FBM neuron migration defects as in *Ce2Δ/Δ* or *Ce2-/-*. Arrows show unmasked nVI in r5. Scale bar, 200μm.

By producing *Ce1|Isl1* mutants, we proved that *Celsr1* function in FBM neuron migration is non-FBM neuron-autonomous. To verify that the *Celsr1* mutant defect was due to *Celsr1* function in FBM neuron precursors, we generated *Ce1|Nkx6.2* mutants to delete *Celsr1* in FBM neuron precursors. Abnormal rostral FBM neuron migration was found in *Ce1|Nkx6.2* mutants (Fig. 19, fifteen out of sixteen mutants showed abnormal rostral migration). This confirmed the hypothesis that *Celsr1* acts in FBM neuron precursors.

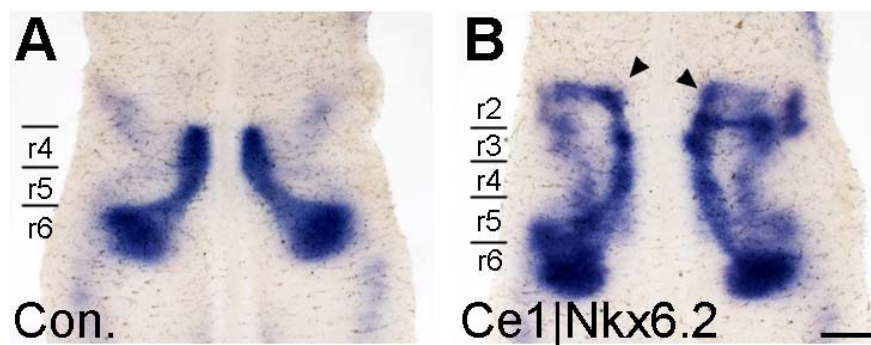


Fig. 19. FBM neuron migration in *Ce1|Nkx6.2* mutants. E12.5 hindbrains hybridized with an *Isl1* probe. Compared to control embryos, abnormal rostral migration (arrowheads) is seen in *Ce1|Nkx6.2* mutants. Scale bar, 200 μ m.

3.2. Role of *Celsr2* and *Celsr3* in the development of ependyma cilia and hydrocephalus

It was previously observed in our laboratory that *Celsr2* mutant mice develop variable degree of hydrocephalus that increases with age. While generating double mutants *Celsr2* and *Celsr3*|*Emx1* to study the development of the internal capsule, I realized that those animals have dramatic hydrocephalus, leading to early lethality. About at the same time, papers by the Wallingford and Kintner groups hinted at a role of PCP proteins in ciliogenesis in frog epidermal cells. We thus decided to investigate ependymal ciliogenesis in normal, *Celsr2* and double *Celsr2-3* mutants.

This work has been published in Nat. Neurosci. (Tissir et al., 2010).

I generated and typed the mice, and performed the experiments shown in Fig. 2 C, Fig. 4, Fig. 5, and Fig. 6 as well as Supplementary Fig. 5. I also participated to the design of the other experiments.

Lack of cadherins *Celsr2* and *Celsr3* impairs ependymal ciliogenesis, leading to fatal hydrocephalus

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Ependymal cells form the epithelial lining of cerebral ventricles. Their apical surface is covered by cilia that beat in a coordinated fashion to facilitate circulation of the cerebrospinal fluid (CSF). The genetic factors that govern the development and function of ependymal cilia remain poorly understood. We found that the planar cell polarity cadherins *Celsr2* and *Celsr3* control these processes. In *Celsr2*-deficient mice, the development and planar organization of ependymal cilia are compromised, leading to defective CSF dynamics and hydrocephalus. In *Celsr2* and *Celsr3* double mutant ependyma, ciliogenesis is markedly impaired, resulting in lethal hydrocephalus. The membrane distribution of Vangl2 and Fzd3, two key planar cell polarity proteins, was disturbed in *Celsr2* mutants, and even more so in *Celsr2* and *Celsr3* double mutants. Our findings suggest that planar cell polarity signaling is involved in ependymal cilia development and in the pathophysiology of hydrocephalus, with possible implications in other ciliopathies.

Cilia are organelles that protrude from the apical surface of most eukaryotic cells. They are classified into three groups according to their structure and motility¹. Primary monocilia, which are present in most cells, lack a central pair of microtubules (9+0 structure), and have several roles in mechanosensation and cell signaling. Nodal cilia have a 9+0 structure but, unlike primary cilia, they move and generate an asymmetric distribution of morphogenetic cues in the node, thereby contributing to laterality². The third group comprises motile 9+2 cilia that cover the epithelial cells that line airways, reproductive tracts and cerebral ventricles. Motile cilia are crucial for clearing mucus and debris in the airways and might assist the transit of sperm and eggs in genital tracts^{3,4}. In the early postnatal mammalian brain, neuroepithelial cells that line the cerebral ventricles leave the cell cycle and differentiate into a monolayer of ependymal cells. At the end of maturation, the apical surface of ependymal cells bears dozens of cilia that beat in coordinated manner to facilitate the circulation of the CSF, from sites of production in choroid plexuses to sites of absorption in subarachnoid spaces. In mice, mutations in genes involved in the assembly or structure of ependymal cilia, such as *Mdnah5* (ref. 5), *Ift88* (also known as *Tg737* or *Polaris*)⁶ and *Hy3* (refs. 7,8) affect ciliogenesis, alter CSF dynamics and result in hydrocephalus. However, little is known about the genetic factors that govern the polarization of ependymal cilia and the relationship between polarity and the development and function of these organelles.

Planar cell polarity (PCP), also known as tissue polarity, controls the polarization of epithelial cells in a plane perpendicular to their apicobasal axis. It was initially described in *Drosophila*, where it affects

the stereotypic arrangement of cuticular hairs, sensory bristles and ommatidia through the control of so-called 'core PCP genes' that include *Van Gogh*, *Frizzled*, *Flamingo* (also known as *Starry Night*), *Disheveled*, *Diego* and *Prickle*^{9,10}. In mammals, PCP designates processes that control the development of polarized cellular structures and involve at least one core PCP gene ortholog¹¹. PCP-dependent events include neural tube closure^{12,13}, hair bundle orientation in inner ear cells^{13,14} and hair follicle patterning in the skin^{15–17}. PCP-like mechanisms have also been implicated in dendrite morphogenesis¹⁸ and axon guidance^{19,20}. In the ciliated epidermis of larval *Xenopus*, morpholino downregulation experiments showed that *Disheveled1–3*, *Inturned* and *Fuzzy* are implicated in the control of the actin cytoskeleton and apical docking of ciliary basal bodies and that the planar orientation of multiciliated cells is disrupted when *Disheveled*, *Vangl2* and *Fuzzy* signaling are altered^{21,22}, indicating that PCP signaling and ciliogenesis are linked.

In this work, we studied mice in which *Celsr2* and *Celsr3*, the murine orthologs of the *Drosophila* core PCP gene *Flamingo*, had been inactivated and show that deficiency of these two seven-pass transmembrane cadherins markedly disrupts the genesis of ependymal cilia, as well as the cellular partition of the PCP proteins *Vangl2* and *Fzd3*, leading to lethal hydrocephalus.

RESULTS

Celsr2 mutant mice develop progressive hydrocephalus

We have previously described conditional and constitutive *Celsr3* mutant mice²³. We generated the mutant *Celsr2* allele by inserting a

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Received 7 January; accepted 1 April; published online 16 May 2010; doi:10.1038/nn.2555

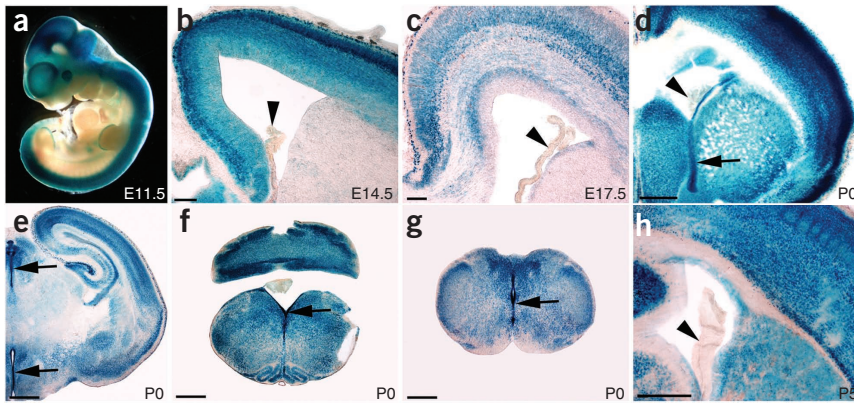


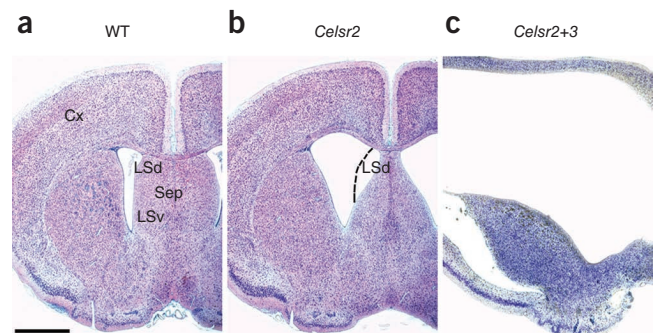
Figure 1 *Celsr2* is expressed in differentiating ependymal cells but not in choroid plexus. (a–h) Expression of *Celsr2* depicted by beta-galactosidase histochemistry in E11.5 embryo (a), and in brain coronal sections at E14.5 (b), E17.5 (c), P0 (d–g) and P5 (h). Widespread staining was observed in the central nervous system at E11.5, and in all brain regions, with highest levels in the layer lining cerebral ventricles at P0 (arrows in d–g). Note that no staining was found in choroid plexus (arrowheads in b–d and h). Scale bars represent 100 μ m (b,c) and 500 μ m (d–h).

targeting vector containing an internal ribosomal entry site (IRES) and the *LacZ* gene into exon 23 of the *Celsr2* gene, preserving the endogenous splice acceptor (Supplementary Fig. 1). RT-PCR and sequencing showed that the *Celsr2* transcript (Genbank 114050894) was interrupted at nucleotide 7,107 and extended by the *IRES-LacZ-neo* sequence (Supplementary Table 1; see also the partial sequence of the recombinant cDNA in Supplementary Data). If any truncated protein is produced, it should lack all transmembrane segments encoded by nucleotides 7,123–7,851, as well as the cytoplasmic tail (nucleotides 7,852–8,763). We analyzed neonatal brain extracts from wild-type, mutant and heterozygous mice by western blot using an anti-*Celsr2* antibody²⁴ and an antibody against beta-galactosidase. The anti-*Celsr2* antibody detected a high molecular weight band in wild-type tissue. This band was less intense in extracts from heterozygous mice, and absent in those from homozygous mice, whereas the beta-galactosidase protein was present in extracts from heterozygous and homozygous mutant mice, but not wild-type mice, confirming that *LacZ* was expressed instead of *Celsr2* (Supplementary Fig. 1). Using the knocked-in beta-galactosidase reporter, we monitored the expression of *Celsr2* in heterozygous mice. Consistent with published data^{24–26}, *Celsr2* expression was detected in all brain areas, from E11.5 to P5 (Fig. 1a–h).

Celsr2 mutant mice develop progressive hydrocephalus

Celsr2 mutant mice were viable and fertile, except for some females that had vaginal atresia. At birth, their brains showed no flagrant morphological abnormalities, suggesting that *Celsr2* is not essential for cerebral embryonic development. However, progressive ventricular dilation appeared between P5 and P10, with variable severity, and became obvious in all mutant mice at P21 (Fig. 2). The lateral ventricles were enlarged, and the septum had an abnormal triangular shape, due to reduction of the dorsal part of the lateral septum.

Figure 2 *Celsr2* and *Celsr2+3* mutant mice display hydrocephalus. (a–c) Nissl-stained coronal sections from wild-type (WT, left), *Celsr2* (middle) and *Celsr2+3* (right) mice at P21. Combined inactivation of *Celsr2* and *Celsr3* in ependymal cells results in severe hydrocephalus and extreme thinning of the cerebral cortex and basal forebrain (c). Cx, cortex; LSd, dorsal part of lateral septum; LSv, ventral part of lateral septum; Sep, septum. Scale bar represents 1 mm.



We did not observe any stenosis or constriction at the level of the foramen of Monro or of the aqueduct. The subcommissural organ (SCO), which is thought to be involved in non-communicating hydrocephalus, was normal and synthesized the SCO-Spondin, a component of Reissner's fibers (Supplementary Fig. 2). Abnormalities in the choroid plexus can lead to hydrocephalus⁶. To check whether *Celsr2* could be involved in the development of the choroid plexus, we analyzed its expression by beta-galactosidase staining at different time points and did not detect any signal in the choroid plexus (Fig. 1b–d,h). We examined the choroid plexus by transmission electron microscopy (TEM) and immunofluorescence with acetylated tubulin antibodies and found no difference between *Celsr2* mutants and control littermates (Supplementary Fig. 3).

In contrast, expression of *Celsr2* was very high in cells lining all cerebral ventricles and the central canal of the spinal cord (Fig. 1d–g), which differentiate into ependymal cells. To assess whether loss of *Celsr2* function affects ependymal differentiation, we used immunofluorescence to examine the ependymal markers S100, CD24 and vimentin^{27–29} (Fig. 3 and Supplementary Fig. 4). At P0, none of the three markers was expressed in cells in the ventricular zone that were destined to form the prospective ependymal layer. At P5, we detected S100, CD24 and vimentin immunoreactivity in ependymal cells lining the septal ventricular surface in control (Supplementary Fig. 4) and *Celsr2*-deficient samples (Supplementary Fig. 4). At P10 and P21, we found S100 and CD24 in ependymal cells all around the lateral ventricles (septal, striatal and cortical surfaces), and saw no difference between controls and *Celsr2*^{−/−} mutants (Fig. 3a,b,d,e and Supplementary Fig. 4). Similarly, vimentin was expressed in ependymal cells and, even though the signal was higher in *Celsr2* mutant mice than in controls, we found no difference in the number of vimentin-positive cells (Fig. 3g,h and Supplementary Fig. 4). These results rule out the possibility that hydrocephalus could be due to defective differentiation of ependymal cells in *Celsr2*-deficient mice.

The migration of young neurons, generated in the subependymal zone (SEZ) of the mature brain, depends on cerebrospinal fluid flow and is altered in hydrocephalic Tg737 mice (ref. 30). To study the relationship between hydrocephalus and neurogenesis in the SEZ, we performed immunofluorescence using doublecortin (Dcx, a marker of neuroblasts (A cells)), and GFAP (a marker of B1 cells)^{27–29}. Labeling for Dcx and GFAP was slightly higher in *Celsr2* mutant mice than in controls (Supplementary Fig. 4).

Figure 3 Ependymal cilia abnormalities in *Celsr2* and *Celsr2+3* mutant mice. (a–i) P21 brain sections were stained with DAPI (left), and by immunofluorescence for ependymal markers (second column) S100 (a–c), CD24 (d–f) and vimentin (g–i) and for acetylated alpha tubulin (third column). In *Celsr2* and *Celsr2+3* samples, ependymal cells show many ciliary defects, including shorter cilia and reduced number of cilia per cell (arrowheads), as well as absence of cilia (arrows). Scale bar represents 20 μ m.

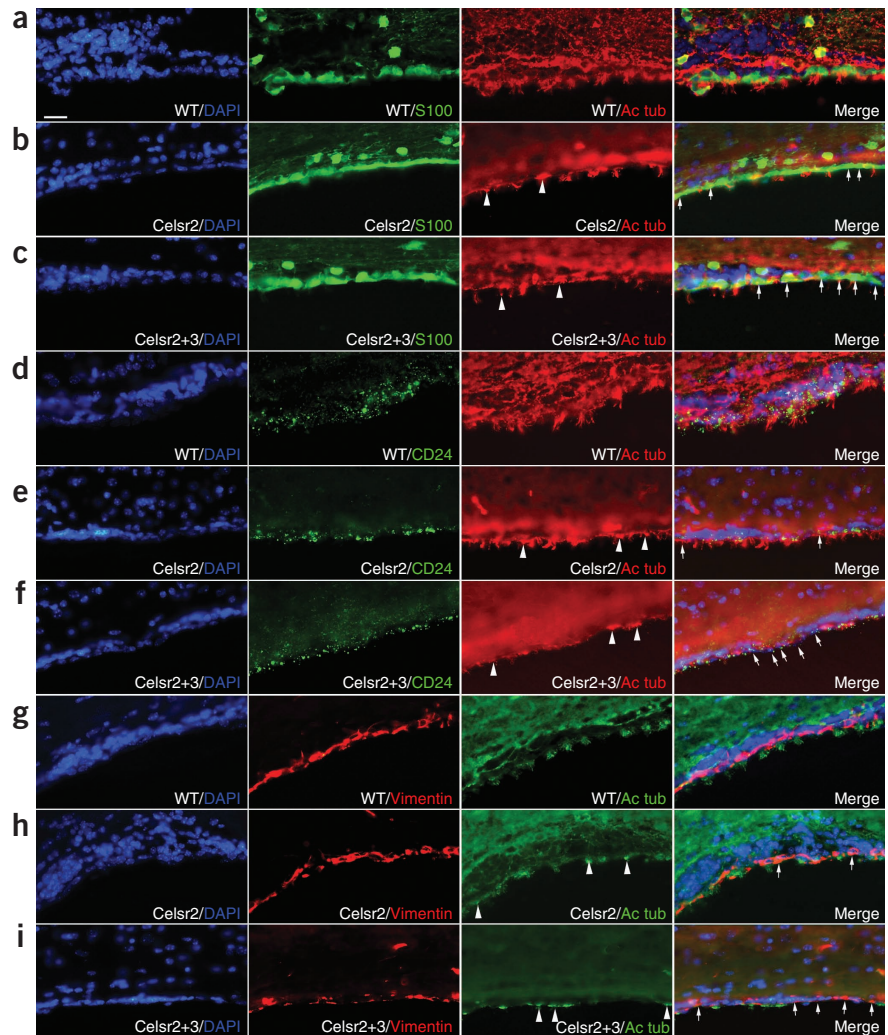
Ciliary defects in *Celsr2* mutant mice

As *Celsr2* was highly expressed in the ependymal layer (Fig. 1d–g), and impaired cilia function is a prevalent cause of hydrocephalus^{6,7}, we investigated ependymal cilia at P21 in the lateral ventricles by double staining with the cilia marker acetylated alpha tubulin and the ependymal markers S100, CD24 and vimentin (Fig. 3). Compared to wild-type mice, the number of cilia and ciliary tufts was clearly reduced in *Celsr2*^{−/−} mice, in which many ependymal cells labeled by S100, CD24 or vimentin had shorter and/or fewer cilia, and sometimes no cilia at all (Fig. 3).

These results indicate that some cilia fail to form, or form normally and then degenerate in *Celsr2*-deficient mice. To distinguish between these possibilities, we studied cilia development at different stages with scanning electron microscopy (SEM; Fig. 4). In newborn (P0) wild-type animals, we found only one primary cilium (Fig. 4a). Tufts of cilia appeared around P5, and increased gradually in number, in a caudal-to-rostral gradient, during the first postnatal week (Fig. 4b,c), to reach their mature density at P10 (Fig. 4d)³¹. At this stage, there were fewer cilia tufts in *Celsr2*^{−/−} mice (Fig. 4e) than in wild-type mice. Some mutant cells had stunted cilia (Fig. 4e) and others had a reduced number of cilia (arrowheads). At the end of maturation (P21), cilia abnormalities were arresting. Whereas the mean number of cilia tufts in wild-type mice was 15.8 per mm², and all pointed approximately in the same direction (Fig. 4j), the density of cilia tufts dropped to 6.7 per mm² in *Celsr2*-deficient mice, and their orientation was less uniform, with tufts from adjacent cells occasionally pointing in opposite directions (Fig. 4k). To quantify variations in the orientation of cilia tufts, we used SEM montages and plotted mean tuft orientation in cells with clearly identifiable tufts located in comparable areas of lateral ventricles in wild-type (439 cells) and *Celsr2* mutant mice (464 cells). The mean orientation was arbitrarily set to zero. Using circular statistics (Oriana program) we found that mutant ependymal cells had a wider distribution of tuft orientations than the wild-type cells ($P < 0.001$, Watson U² test; Fig. 4l,m). Together, these results show that cilia fail to form normally in *Celsr2* mutant mice, and that residual cilia tufts display abnormal organization at the end of maturation.

Celsr2 mutation affects cilia at the single-cell level

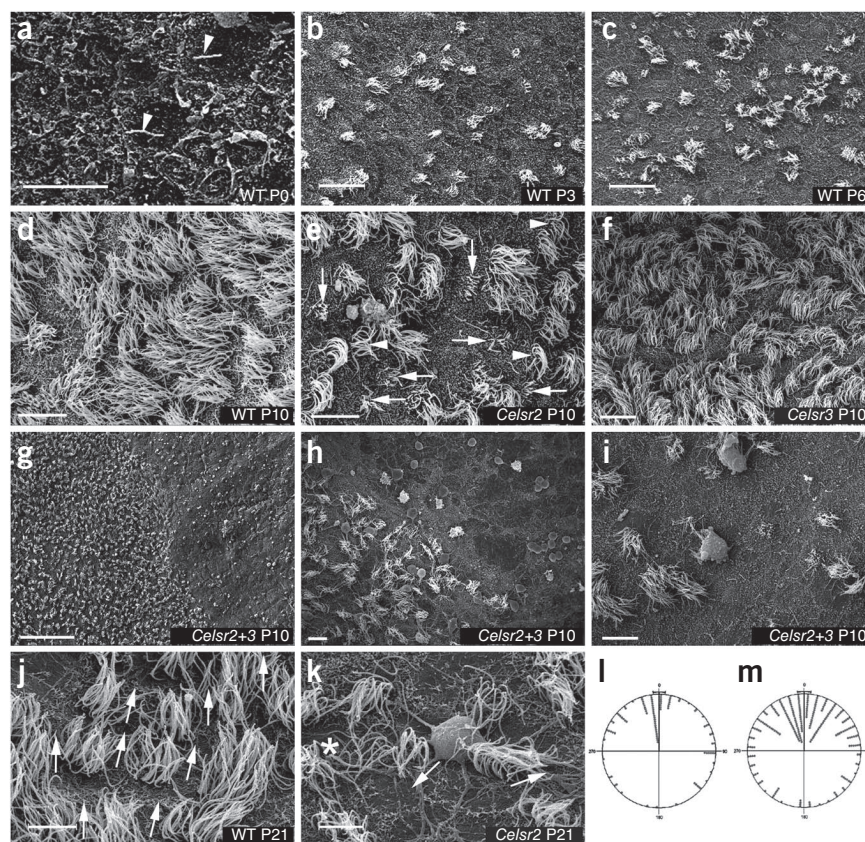
To study the effects of *Celsr2* inactivation on cilia development at the individual cell level, we used TEM in sections cut tangentially to the surface of the lateral ventricle, and examined the orientation of basal



feet (Fig. 5). These structures extend laterally to basal bodies, point to the effective stroke of cilia beats^{32,33} and are considered a landmark of cilia polarity³⁴. In wild-type cells (Fig. 5a), only 8.5% of basal feet showed divergent orientation with respect to their neighbors (82 basal feet from 15 cells were examined; in 10 cells, all basal feet had uniform orientation; in the 5 others, 1 or 2 basal feet per cell showed divergent orientation). In *Celsr2* mutant cells (Fig. 5b), 31.8% of the basal feet deviated from the common orientation (47 out of 148 basal feet examined in 14 cells; each cell had at least one basal foot with divergent orientation). Inasmuch as some mutant cilia degenerated prematurely or did not develop at all (Fig. 5e) and were thus not considered, the disorganization of basal feet in *Celsr2* mutants is probably underestimated.

During assembly of motile cilia, basal bodies form deep in the cytoplasm and are transported to the apical surface by actin-dependent mechanisms^{33,35,36}. To study how *Celsr2* inactivation influences ependymal ciliogenesis, we took sections perpendicular to the ventricular surface to examine by TEM the position of basal bodies relative to the apical surface of ependymal cells at P10. In wild-type samples, basal bodies formed a single row just beneath the apical surface (Fig. 5d). By contrast, procentrioles and basal bodies, together with densely osmiophilic profiles that probably correspond to deuterosomes, were found at deeper, more basal levels in *Celsr2* mutant cells (Fig. 5e). To quantify these differences,

Figure 4 Ciliogenesis is altered in *Celsr2* and *Celsr2+3* deficient mice. (a–k) SEM micrographs of ependymal cells lining the roof of lateral ventricles at P0 (a, arrowheads show monocilia), P3 (b), P6 (c), P10 (d–i) and P21 (j,k). Cilia develop progressively after birth and reach maturity by P10. At this stage, the number of cilia tufts is reduced and cilia have many morphological abnormalities in *Celsr2* (e) and *Celsr2+3* (g–i) mutants as compared to wild-type (d) and *Celsr3* mutants (f). Arrows show shorter cilia, and arrowheads cilia tufts with reduced number of cilia. In the mature brain, individual cilia converge to a common orientation in the wild type (arrows in j), but cilia tufts of mutant mice (k) are less regularly oriented and, in some cases, individual cilia in a given cell do not point in the same direction (asterisks). (l,m) Dispersion of cilia tuft orientation around the mean in wild-type (l) and *Celsr2* mutant mice (m). Scale bars represent 5 μ m (a,j,k), 10 μ m (d–f, h–i), 20 μ m (b,c) and 100 μ m (g).



we measured the distance between basal bodies and the apical surface and calculated the proportion of embedded basal bodies (defined as basal bodies located below the first row, more than 500 nm from the surface). In *Celsr2*^{-/-} mice, 54% of basal bodies were embedded versus 5% in wild type mice (Fig. 5g; χ^2 test, $P < 0.001$). Most embedded basal bodies were not associated with ciliary shafts. However, fully formed cilia were occasionally seen deep in the cytoplasm (Fig. 5h). Presumably, ectopic cilia and embedded basal bodies are fated to premature degeneration, which might account for the reduced density of cilia in adult *Celsr2* mutant ependymal cells.

Celsr2 mutation impairs cilia function

In rodents, mutations that affect ciliary motility lead to hydrocephalus^{5–8,37}. The ultrastructure of residual *Celsr2* mutant cilia were unremarkable and displayed the typical 9+2 structure of motile cilia (Supplementary Fig. 5). This indicated that *Celsr2* mutation has no direct effect on the axoneme structure and raised the question of the motility of cilia in *Celsr2* mutants. To investigate this, we prepared brain slices from 12-day-old animals and recorded cilia movement with a high-speed camera. We found two classes of movement in wild-type mice. One movement is more dynamic than the other, and reminiscent of seaweed drifting on restless waves. The other is a smaller motion that resembles a

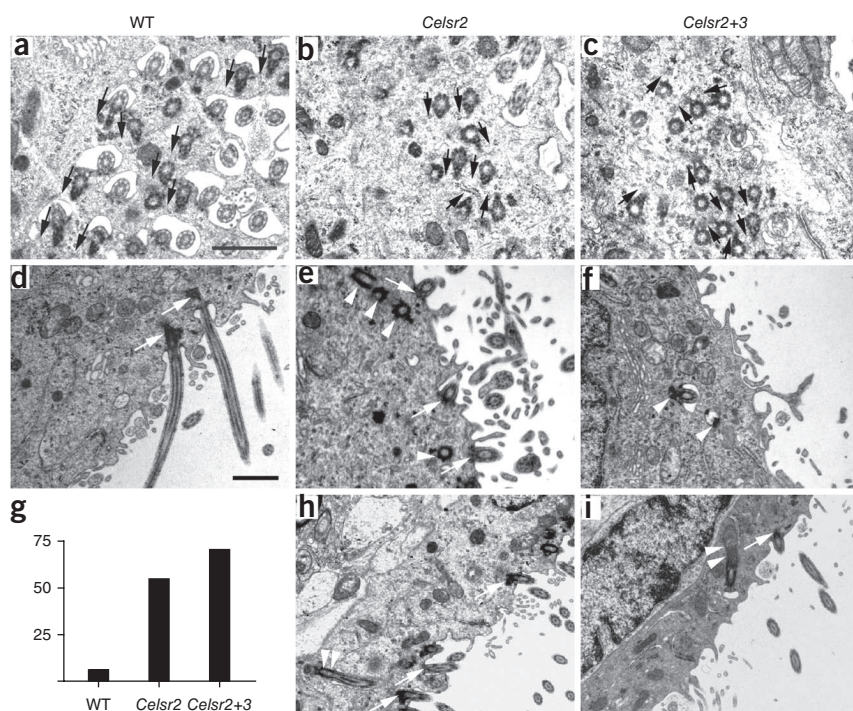
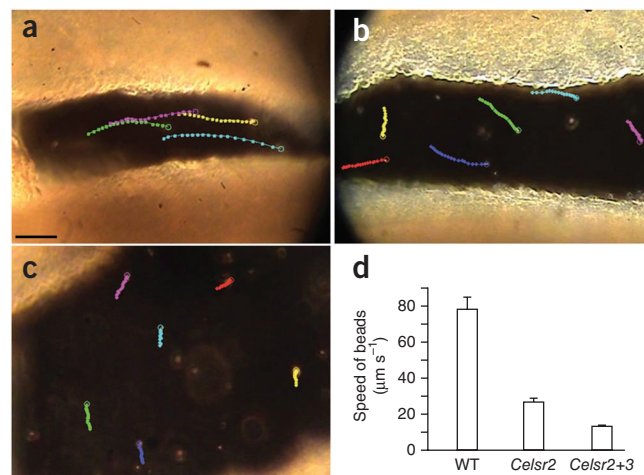


Figure 5 Inactivation of *Celsr2* and 3 affects polarization and apical positioning of cilia. (a–c) TEM analysis of tangential sections of ependymal cells from wild-type (a), *Celsr2* (b) and *Celsr2+3* animals (c). Arrows indicate the orientation of basal feet which show dispersed orientations in mutants. (d–f,h,i) Ependymal cells in radial sections, from wild-type (d), *Celsr2* (e,h) and *Celsr2+3* (f,i) mice. Arrows point to normally positioned basal bodies, arrowheads to embedded basal bodies and double arrowheads to ectopic cilia. (g) Graph showing the proportion of basal bodies with abnormal apicobasal position. Scale bars represent 500 nm (a, applies to b–c) and 1 μ m (d, applies to e,f,h,i).

Figure 6 *Celsr2* and *Celsr2+3* deficiency affects fluid flow. Latex beads were added to brain slices from P8 mice and their movement in the lateral ventricle was recorded with a video camera. Movies were fragmented and sequential images were analyzed using Retrac 2 software. Each bead was tracked for 720 ms and the time between two dots is 40 ms. Circles indicate track end-points. (a–d) In the wild type (a), bead motion was uniform and trajectories formed almost parallel lines. In *Celsr2* (b) and *Celsr2+3* (c) mutants, tracks were shorter than in the controls and beads moved in divergent directions (Watson U² test, $P < 0.05$; **Supplementary Table 2**). The speed was calculated for each genotype and is shown as mean $\pm$ s.e.m. in d; $P < 0.0001$. Scale bar represents 20 μ m.

‘wiggling pigeon’s neck’. Both classes were found in *Celsr2* mutant cells that harbored intact cilia, indicating that cilia movements were not grossly affected by *Celsr2* deficiency (**Supplementary Movies 1–4**).

Could the reduced number of cilia and cilia tufts result in inefficient CSF dynamics despite the preserved cilia beat at the single cell level? To address this question, we studied the flow generated by cilia beats in lateral ventricles in vibratome slices, taken from mice aged between P0 and P21. Ependymal cilia were beating from P5 onwards, but they generated a significant flow only from P8 (**Fig. 6** and **Supplementary Movie 5**). When latex beads were added to the medium of wild-type slices at P7–10 (**Fig. 6a**; $n = 13$), their movement was consistently directed from the caudal to the rostral pole of the lateral ventricles (see Online Methods), with a mean bead velocity of 78.3 ± 35.6 (SD) μ m s⁻¹. In *Celsr2*-deficient slices (**Fig. 6b**; $n = 19$), the overall movement of beads was slower and less clearly directed toward the rostral pole than in the wild type, and the mean bead velocity was 26.6 ± 9.7 μ m s⁻¹. Frequently, beads showed a circling movement without any clear resultant displacement along the rostrocaudal axis (**Supplementary Movie 5**). Both directions and velocities, compared respectively using Watson U² (**Supplementary Table 2**) and Student’s *t*-test (**Fig. 6d**), were significantly different between the two genotypes. Although the size of the ventricle could affect the speed of bead movement, our data indicate that inactivation of *Celsr2* impairs the efficiency of cilia beats, which could compromise CSF dynamics and thereby contribute to hydrocephalus.



Celsr2 and 3 have redundant functions in ciliogenesis

Celsr3 expression is not detected in ventricular zones during embryonic development^{24,25} (**Fig. 7a,b**), but is initiated postnatally in cells that line the lateral ventricles, when they become postmitotic and start differentiating into ependymal cells²⁵ (**Fig. 7c,d**). We have evidence that *Celsr2* and *Celsr3* have redundant functions in neuronal migration and axon guidance, indicating that they might similarly cooperate in ependymal ciliogenesis. To test this possibility, we studied the ependymal cells of lateral ventricles in double *Celsr2/Celsr3* mutant mice. Constitutive *Celsr3* mutant mice die at P0 (ref. 19). Therefore, we produced *Celsr3|Emx1* mice, in which Cre is expressed from the *Emx1* promoter and deletes loxP-flanked *Celsr3* in the *Emx1*-expressing dorsal telencephalon, including the ependymal layer in the roof of lateral ventricles²³. In these mice, referred to as ‘*Celsr3*’, cilia were indistinguishable from those in wild-type cells (**Fig. 4d,f**). The density of cilia tufts was 15.6 per mm², compared with 15.8 per mm² for the wild type, confirming that mutation of *Celsr3* alone did not perturb ependymal cilia development. However, when regional *Celsr3* inactivation was combined with deficiency of *Celsr2* in *Celsr2*^{-/-}/*Celsr3|Emx1* mice, referred to as ‘*Celsr2+3*’, a severe hydrocephalus appeared from P5. The hydrocephalus was much more pronounced than in *Celsr2* mutant animals (**Fig. 2b,c**). Even though *Celsr3* is ablated only in *Emx1*-expressing ependyma, this led to extreme thinning of the cerebral cortex, with formation of a discontinuity in the dorsal hemisphere, and death of animals around weaning time (**Fig. 7e,f**). The number of Dcx- and GFAP-positive cells was higher in *Celsr2+3* mutants than in controls (**Supplementary Fig. 4**). Immunofluorescence analysis of S100, CD24 and vimentin indicated that ependymal cells differentiated normally (**Supplementary Fig. 4**) and persisted in the mature mutant brain (**Fig. 3c,f,i**), although their shape was stretched and some cells might have degenerated because of increased pressure, as reflected by vimentin immunoreactivity (**Fig. 3i**). Large areas of the

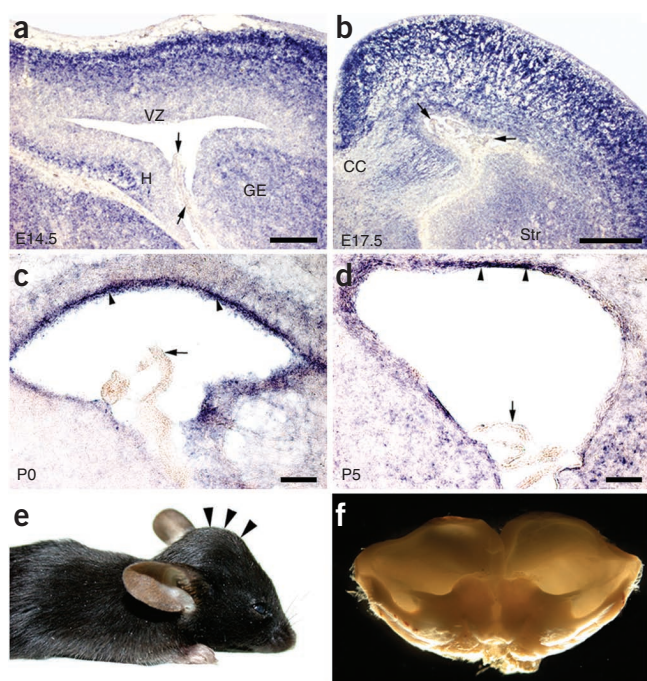
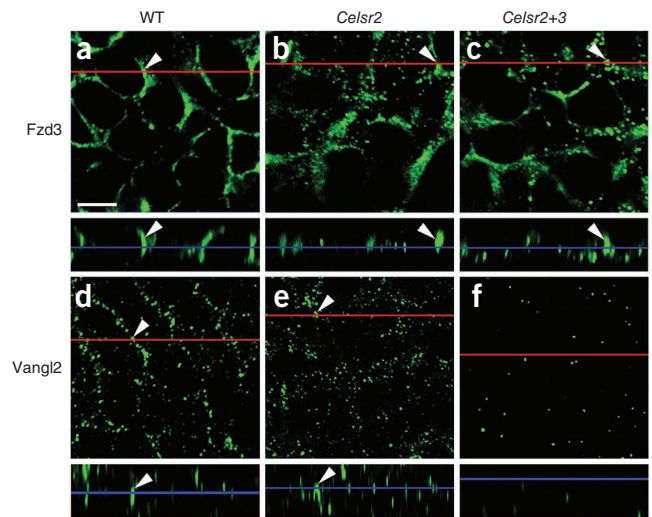


Figure 7 Combined mutation of *Celsr2* and *Celsr3* causes lethal hydrocephalus. (a–d) Coronal sections hybridized with *Celsr3* digoxigenin-labeled probe. *Celsr3* is not expressed in the ventricular zones at E14.5 (a), but is turned on postnatally in cells of the prospective ependymal layer (c,d, arrowheads). Note the absence of *Celsr3* transcript in the choroid plexus (arrows in a–d). (e) *Celsr2+3* mutant mice have a dome-like appearance of the head caused by hydrocephalus, and their brains (f) show extreme thinning of cerebral cortex and holes in telencephalic walls. CC: corpus callosum; GE: ganglionic eminence; H: hippocampus; Str: striatum; VZ: ventricular zone. Scale bar represents 200 μ m.

Figure 8 The cellular distribution of Fzd3 and Vangl2 is perturbed in *Celsr2* and *Celsr2+3* mice. (a–f) Confocal immunofluorescence imaging showing the cellular distribution of Fzd3 (a–c, horizontal sections in upper panels, and vertical sections in lower panels) and Vangl2 (d–f), in the ventricular zone at P5. The polygonal pattern that reflects localization of Fzd3 (a) and Vangl2 (d) at the lateral plasma membrane in normal mice (arrowheads) is modified in *Celsr2* (b,e) and *Celsr2+3* (c,f) mutants. The Z sections (rows 2 and 4) are at the level of red lines in horizontal sections (rows 1 and 3), which were recorded at the position of blue lines in vertical sections. Scale bar represents 5 μ m.



dorsal lateral ventricles were completely devoid of cilia (Fig. 4g–i), preventing any accurate estimation of cilia tuft density. These cilia-free zones persisted until death, indicating defective rather than delayed ciliogenesis. In cells with residual cilia, the percentage of basal feet with abnormal orientation reached 43.16% (60 of 139 scored basal feet; Fig. 5c). These abnormalities were comparable to those reported in mice with defective cilia motility, such as *Hydin*-deficient mice⁷. We found that 70% of the basal bodies were embedded in the basal cytoplasm (Fig. 5f,g). Ectopic cilia had a tilted configuration and their tips were not oriented apically, contrasting with the upright alignment of apical cilia in wild-type and ectopic cilia in *Celsr2* mutant cells (Fig. 5i). When latex beads were added to freshly prepared brain slices from *Celsr2+3* mice (Fig. 6c; $n = 2$), they drifted very slowly along different directions in the enlarged ventricle, with an estimated velocity of $13.0 \pm 4.2 \mu\text{m s}^{-1}$ versus $78.3 \pm 35.6 \mu\text{m s}^{-1}$ for the wild type. This difference was statistically significant using the Watson U² test (Supplementary Table 2) and Student's *t*-test (Fig. 6d).

Emx1-Cre is expressed not only in ependyma but also in cortical glutamatergic neurons and glial cells. To confirm that the effects observed were due to *Celsr3* deficiency in the ependyma, we produced double mutant *Celsr2*^{−/−}/*Celsr3*^{−/−}*Nex* mice, in which *Celsr3* is inactivated in cortical neurons but not in ependyma³⁸. The brains of these mice were indistinguishable from those in *Celsr2*^{−/−} animals ($n = 5$), showing that the contribution of *Celsr3* to the double mutant phenotype is probably due to its expression in ependymal cells.

Fzd3 and Vangl2 distribution is altered in *Celsr* mutants

Studies in the skin showed that PCP signaling is essential for hair follicle polarization and orientation^{15–17}. *Celsr1* and Vangl2 form physical complexes *in vitro*, and *Celsr1* is required for the correct targeting of Vangl2 and Fzd6 to the plasma membrane¹⁷. To gain insight into the mechanisms of action of *Celsr2* and *Celsr3*, and to assess whether their function could be mediated by PCP, we studied the distribution of Fzd3 and Vangl2, two key components of the PCP pathway that are expressed in ependymal cell precursors^{14,39,40} (Fig. 8). In wild type brains at P5, Fzd3 and Vangl2 signals displayed polygonal patterns, restricted to the lateral membrane, with a continuous (Fzd3) or punctate distribution (Vangl2; Fig. 8a,d). In *Celsr2*^{−/−} tissue, Fzd3 and especially Vangl2 were only partially targeted to the cell membrane. Rather, more signal was associated with intracellular vesicles (Fig. 8b,e). In *Celsr2+3* double mutants, the impaired distribution of Fzd3 was even more evident, whereas Vangl2 was downregulated (Fig. 8c,f). The restricted polygonal pattern upon labeling of the junctional protein ZO-1 was fully preserved in *Celsr2* and *Celsr2+3* mutants, showing that the abnormal distribution of Vangl2 and Fzd3 was not secondary to defective junction organization (Supplementary Fig. 6).

DISCUSSION

Using *in vivo* approaches in mice, we found that *Celsr2* and *Celsr3*, two murine orthologs of the core PCP gene *Flamingo* in *Drosophila*,

are key regulators of ciliogenesis in the ependyma, and that their inactivation leads to lethal hydrocephalus. In *Celsr2* and *Celsr2+3* mutant ependyma, cilia never develop in normal numbers and show abnormalities in their morphology, position and planar organization. Ciliary basal feet are disoriented, and basal bodies and cilia assemble ectopically deep in the cytoplasm. These defects do not primarily affect cilia motility; however, they alter ependymal function, leading to ventricular dilation. The lateral plasma membrane localization of Vangl2 and Frizzled3, two established PCP proteins, is disrupted in ependymal cell precursors in *Celsr2* and *Celsr2+3* mutant mice, providing a strong indication that *Celsr2* and *Celsr3* regulate ciliogenesis through PCP signaling.

Previous studies in frog larval epidermal cells provided the first evidence of a link between PCP signaling and cilia development. In these cells, morpholino knockdown of *Inturned*, *Fuzzy* and *Disheveled1–3* affect cilia polarity and ciliogenesis by preventing normal docking of basal bodies to the apical cytoplasm^{21,22}. Although these studies indicate a relationship between ciliogenesis and PCP signaling, *Inturned* and *Fuzzy* are not considered bona fide core PCP genes, and, besides PCP, *Disheveled1–3* also regulate canonical and non-canonical Wnt signaling. Moreover, being near tetraploid, *Xenopus laevis* cells might express PCP gene orthologs other than those that are identified in *Xenopus tropicalis* and whose sequences are used for morpholino design. In mice, mutations in genes that encode proteins implicated in cilia structure or development, such as *Bbs1*, *Bbs4* and *Ift88*, affect the 'V shaped' orientation of actin stereocilia bundles at the surface of inner ear cells, an organization that is governed by the directed growth of a monocilium during development^{39,41}. This phenotypic trait is typically found in mice with mutations in the PCP genes *Celsr1*, *Frizzled3*, *Frizzled6* and *Vangl2* (refs. 11,13,14). Furthermore, conditional inactivation of the ciliary gene *Kif3a* in neural progenitors and radial glia affects the orientation of ciliary basal feet and planar polarity of ependymal cells⁴². These data suggest that the link between PCP and ciliogenesis, described in amphibian epidermal cells, is conserved in mammals. Our observations that the core PCP genes *Celsr2* and *Celsr3* are essential for the correct positioning of cilia at the apical surface of ependymal cells, for cilia function as well as for CSF dynamics, extend and clarify this view.

Presumably, PCP signals act on the cytoskeletal machinery that is required to assemble cilia at the cell surface, a mechanism highly reminiscent of that involved in the growth of the actin shafts of hairs in *Drosophila* wing cells⁴³. That PCP signaling acts upstream from

cilium assembly is also supported by observations that PCP proteins are normally distributed in cochlear cells in mice with mutation of the 'ciliary gene' *Ift888* (ref. 39). Factors that regulate apical basal body docking remain poorly understood. Our observations that cilia can form deep in the cytoplasm in *Celsr2* and *Celsr2+3* mutant ependymal cells and that ectopic basal bodies can still serve as seeds for cilia formation indicate that apical docking is not absolutely required for cilia assembly *per se*. Studies of ciliogenesis in the quail oviduct showed that localization of basal bodies to the apical cell domain depends on actin dynamics³⁵. In line with this, ciliogenesis is defective in *Foxj1* mutant mice, owing to abnormal basal body anchoring to the actin-based apical cytoskeleton and to failure of RhoA-mediated apical actin enrichment^{44–47}. The hypothesis that PCP signaling regulates the apical actin network formation that is required for docking of basal bodies and normal ciliogenesis predicts that mutations in other genes that control PCP in mice should similarly lead to cilia dysfunction and hydrocephalus. Unfortunately, unlike *Celsr2* mutants, mice with mutations in other PCP genes, such as *Frizzled3* and *6* (ref. 40) and *Vangl2* (ref. 12) all die perinatally, before ependymal cilia develop. The generation of conditional mutant mice for PCP gene orthologs would provide additional tools to address these issues. In addition to defective docking, we also find impairment of the planar organization of cilia tufts in mature *Celsr* mutant ependymal layers. This phenotype could reflect a direct effect of *Celsr* mutations, but it might also be a consequence of disturbed CSF flow³⁴ in these hydrocephalic mice.

Finally, our findings underscore the role of PCP signaling in ependymal function and hydrocephalus. In man, CSF flow is commonly ascribed to a pressure gradient, generated by blood pulsations, between the sites of production and absorption. In contrast to rodents, cilia beats are usually not considered a key determinant of human CSF dynamics, nor a relevant pathophysiological factor of hydrocephalus⁴⁸. Our results add to a growing body of evidence that the impact of cilia on CSF dynamics is extremely important in rodents and probably not negligible in man, and invite additional scrutiny of ependyma and cilia function in patients with communicating hydrocephalus. Abnormalities of cilia are also associated with cystic pathology in the kidney, liver and pancreas^{1,49}. PCP genes such as *Celsr1* and *2*, *Dishevelled1* and *2*, *Frizzled3* and *Vangl1* and *2* are expressed in these organs^{24,50}, indicating that PCP signaling might be involved in the polarity and function of their cilia, in both normal and pathological conditions.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank K. Jones for *Emx1-Cre* mice, G. Meyer for the antibody to SCO-spondin, and K.-A. Nave for NEX-Cre mice. We also thank V. Bonte, I. Lambermont and E. Paitre for technical assistance and M.F. Boulanger, Y. Houbion, M.-J. Vertez and R. Vigneron for help with electron microscopy. This work was supported by grants from the Actions de Recherches Concertées (ARC-186), FRFC 2.4504.01, FRSM 3.4501.07, Interuniversity Poles of Attraction (SSTC, PAI p6/20), the Fondation médicale Reine Elisabeth, the Wallonne and Bruxelloise régions from Belgium and from the Japan Science and Technology Corporation. F.T. is a research associate at the Belgian Fonds National de la Recherche Scientifique.

AUTHOR CONTRIBUTIONS

F.T. characterized the *Celsr2* mutant allele, studied *Celsr2* and *Celsr3* expression and carried out histological and immunofluorescence studies; Y.Q. performed SEM and TEM with the help of Y.P.; L.Z. and J.L. studied cilia function in brain slices;

K.K. monitored cilia beats and analyzed data with D.S. and T.F.; M.M. provided the *Vangl2* antibody and commented on the manuscript; D.S. and P.C. helped with the confocal microscopy and commented on the manuscript; T.U. analyzed the ciliary beat data, provided the *Celsr2* antibody and commented on the manuscript; F.T. and A.M.G. designed research, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mutant mice. All animal procedures were carried out in accordance with European guidelines and approved by the animal ethics committee of the Université catholique de Louvain. The *Celsr2* mutant allele (*Celsr2^{tm1Dgen}*) was generated by Deltagen and *Celsr2* heterozygous mutant mice were obtained from the Jackson Laboratory. *Celsr3* and *Celsr1* mutants have been described²³.

RT-PCR. Total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel) and converted to single-stranded cDNA using the ImProm-II reverse transcriptase (Promega) with random primer ($0.5 \mu\text{g ml}^{-1}$) in $20 \mu\text{l}$ of $1\times$ ImProm-II buffer (6 mM MgCl_2 , 0.5 mM each dNTP, and 2 U recombinant RNasin RNase inhibitor). For subsequent PCR amplification, cDNA samples were incubated with 500 nM of each of the sense and antisense primers and 1 U of recombinant Go Taq polymerase (Promega) in $20 \mu\text{l}$ of $1\times$ PCR buffer.

Beta-galactosidase staining. Animals were perfused with 2% paraformaldehyde, 0.1% glutaraldehyde in phosphate-buffered saline. Dissected brains were post-fixed for 1 h at room temperature, sliced using a vibratome and stained with a solution containing 1 mg ml^{-1} X-gal, 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ and 2 mM MgCl_2 in phosphate-buffered saline.

Western blot, histology and immunohistochemistry. For western blots, we used mouse antibody to beta-galactosidase (1:200, Hybridoma Bank) and mouse antibody to *Celsr2* (supernatant)²⁴. For histological examination, 8- μm -thick paraffin sections were stained with cresyl violet or hematoxylin and eosin (H&E). For immunohistochemistry, we used 10- μm -thick sections or 100- μm -thick vibratome slices with the following primary antibodies: rabbit antibody to SCO-spondin (1:1,000, gift from G. Meyer, Universidad de la Laguna, and E.M. Rodriguez, Universidad Austral de Chile), rabbit antibody to S100 (1:500, DakoCytomation), mouse antibody to acetylated alpha tubulin (1:2,500, Sigma), mouse antibody to vimentin (1:1,000, Sigma), rat antibody to CD24 (1:500, BD Pharmingen), rabbit antibody to GFAP (1:500, Chemicon), rabbit antibody to doublecortin (1:500, Cell signaling), goat antibody to Fzd3 (1:25, R&D), rabbit antibody to Vangl2 (1:500, M.M.) and rabbit antibody to ZO-1 (1:100, Zymed). Secondary antibodies were goat antibody to mouse-AlexaFluor488 and AlexaFluor568,

goat antibody to rabbit-AlexaFluor488 and AlexaFluor568, donkey antibody to goat AlexaFluor488 and chicken antibody to rat AlexaFluor488 (1:400, all from Invitrogen). Immunofluorescence was performed using standard procedures; preparations were examined with a LSM510 confocal microscope (Zeiss).

Recording and analysis of cilia beats. We observed cilia in freshly prepared, 300- μm -thick vibratome sections at P7, P8 and P10. Sections were maintained in DMEM containing 20 mM HEPES in air as described⁷, or in artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 3 mM KCl, 26 mM NaHCO_3 , 2 mM CaCl_2 , 1 mM MgSO_4 , 1.25 mM KH_2PO_4 , 25 mM glucose and 10 mM HEPES, pH 7.4) under water-saturated 95% oxygen and 5% CO_2 . A maximum of 1 h elapsed between death of animals and the end of observations, and both methods yielded similar results. We added 1 μm latex beads (Sigma L2778) to the medium before video recording. Movies were fragmented into individual images corresponding to 40 ms, and beads were tracked in selected frame series using the Retrac 2 program to estimate speed and direction. Directions (30–40 tracks for each genotype) were compared using circular statistics, Watson U^2 test (Oriana program).

For high-speed recording of individual cilia beats, we used a 100 $\times$ objective and Nomarski optics. Movies were acquired at 200 frames per second using a digital high-speed camera (HAS-220; Ditect) and analyzed using Move-MN software (Library Inc).

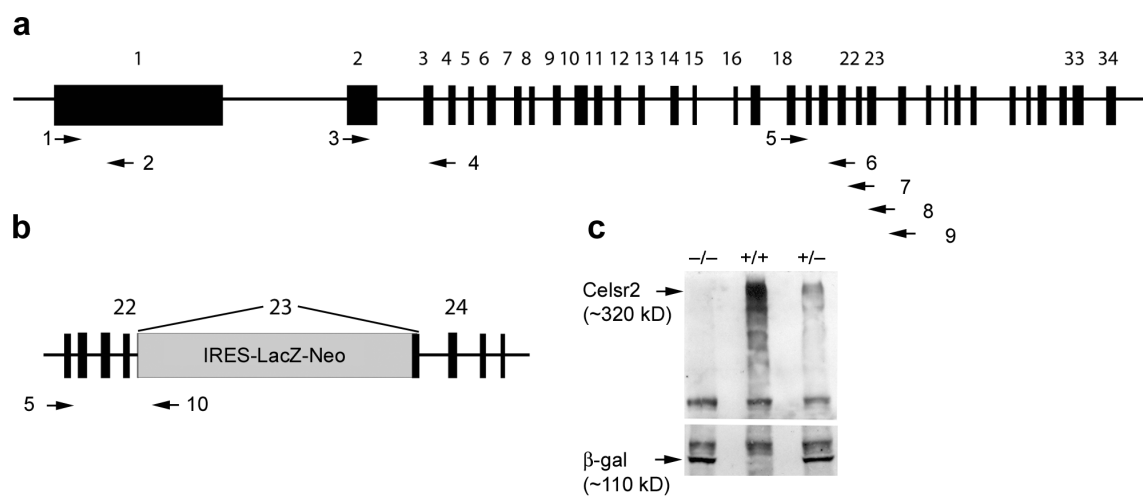
Electron microscopy. Brains were fixed by perfusion of the animal under deep anesthesia, with 2.5% glutaraldehyde in phosphate or cacodylate buffer (0.1 M, pH 7.5), and postfixed for 1 h in the same fixative. For SEM, 300–500- μm -thick horizontal slices containing the roof of the lateral ventricles were cut with a vibratome, dehydrated and prepared for observation using a Jeol 7500F scanning electron microscope. Blocs for TEM were rinsed, postfixed in osmium and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate, and observed with a Philips Tecnai 10 transmission electron microscope.

Statistics. Circular statistics were carried out with the Oriana program, using the Watson U^2 test to compare dispersion of orientations. Bead velocities in brain slices experiments were compared using Student's t test.

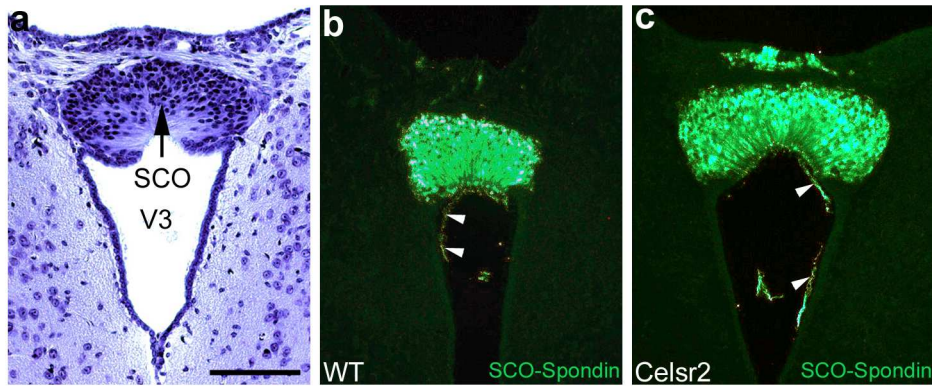
Lack of cadherins *Celsr2* and *Celsr3* impairs ependymal ciliogenesis, leading to fatal hydrocephalus

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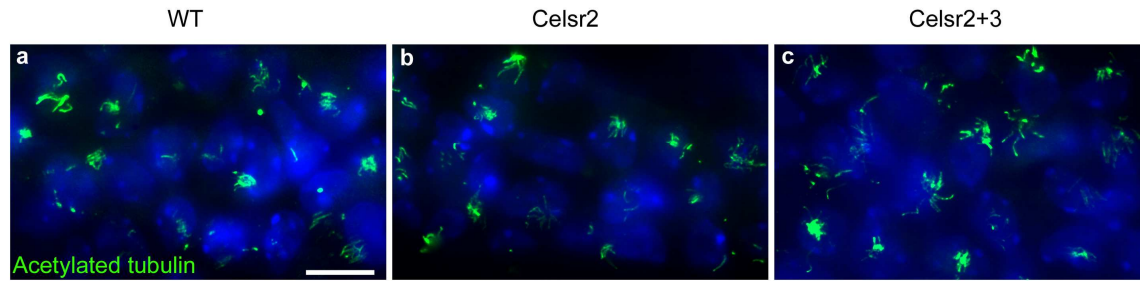
Supplementary figures:



Supplementary Fig. 1: Targeted insertion of LacZ into *Celsr2* locus. (a) *Celsr2* has 34 exons (rectangles); arrows 1-9 indicate positions of primers used in RT-PCR (see **Supplementary Table S1**). (b) The IRES-LacZ-Neo cassette was inserted in exon 23 downstream from nucleotide 7102 (Genbank 114050894). (c) Western blot analysis of brain extracts from wild type, heterozygous, and homozygous brain. *Celsr2* (top) was present in wild type and heterozygotes, and replaced by beta galactosidase (bottom) in mutant mice.

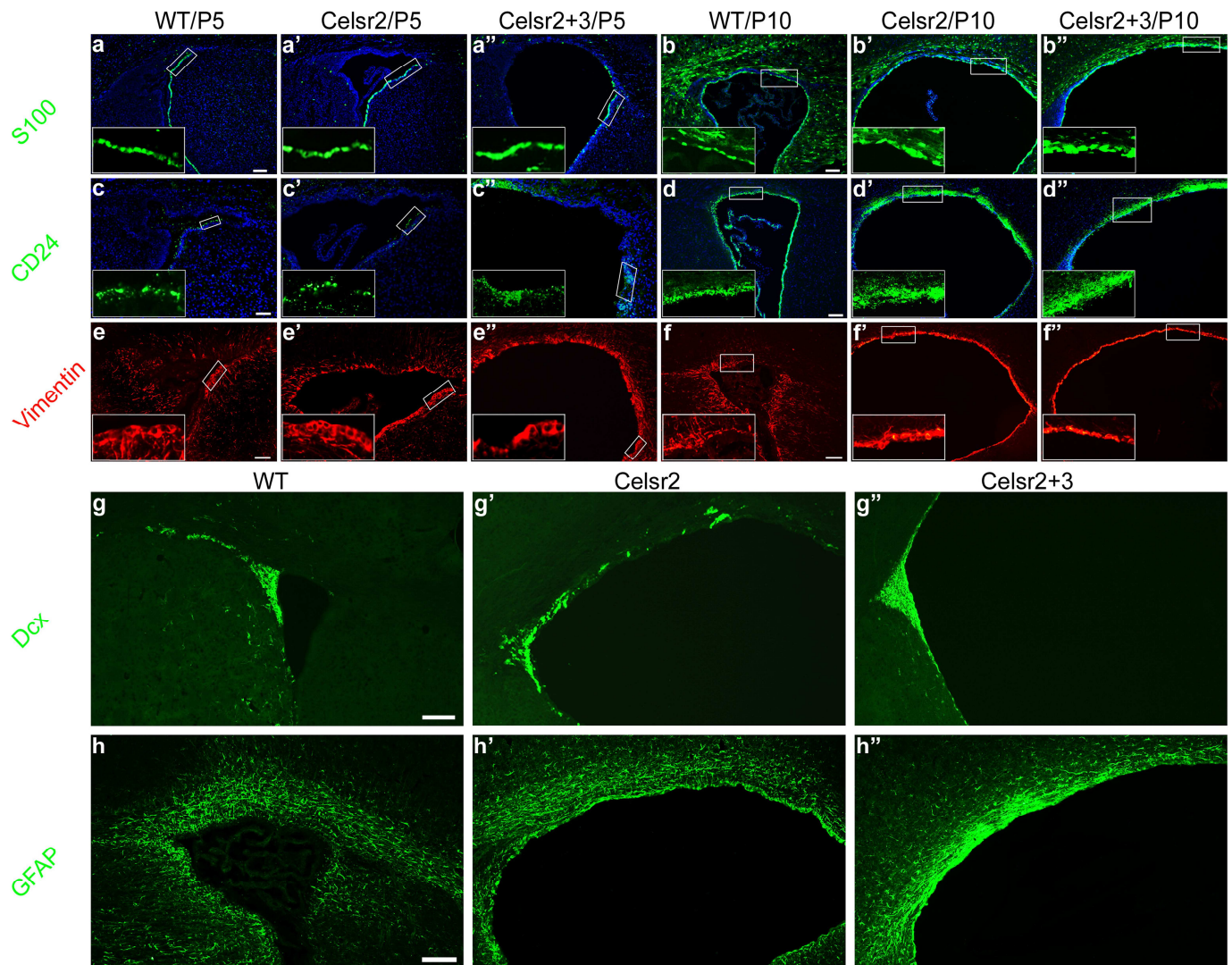


Supplementary Fig. 2: The subcommissural organ (SCO) develops normally in *Celsr2* mutant mice. (a) Nissl stained coronal section at the level of the SCO at P10. (b,c) Sco-spondin immunofluorescence shows that the SCO synthesizes the glycoprotein SCO-spondin, which aggregates and forms the Reissner's fiber (arrowheads). V3: third ventricle. Scale bar : 100 μ m.



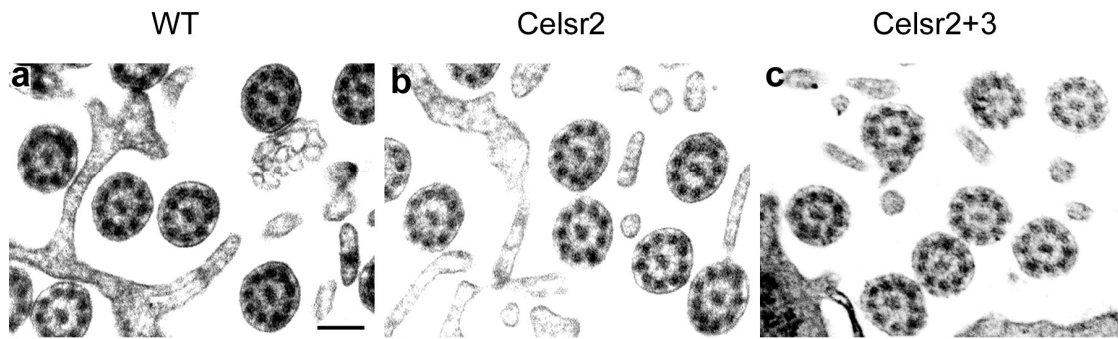
Supplementary Fig. 3: Choroid plexus displays normal cilia in *Celsr2* and *Celsr2+3* mutant mice.

Immunofluorescence for acetylated alpha tubulin reveals mono and grouped cilia on epithelial cells in choroid plexus. No difference was observed between WT (**a**), *Celsr2* (**b**), and *Celsr2+3* (**c**). Nuclei are counterstained with DAPI. Scale bar: 10 μ m.

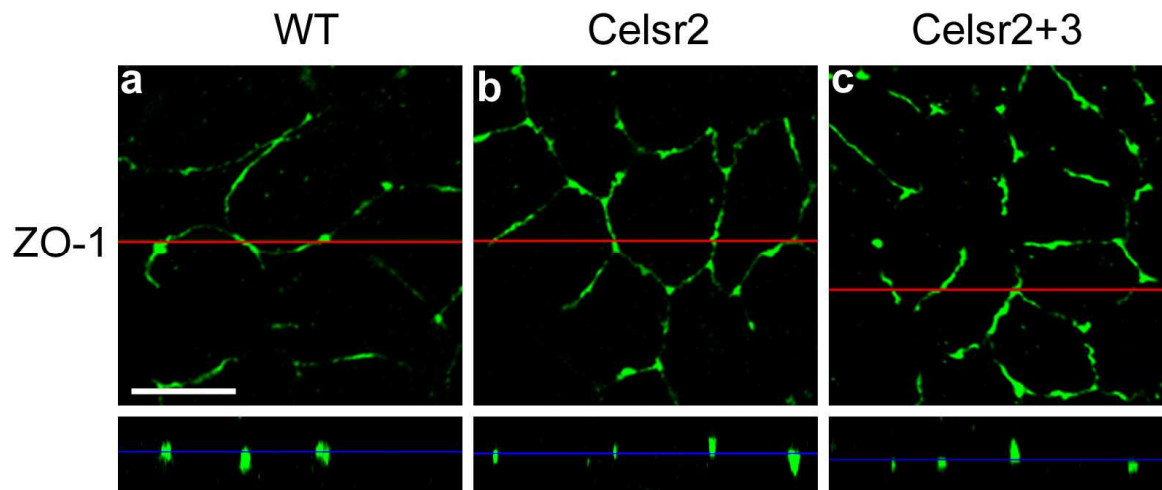


Supplementary Fig. 4: Ependymal cells differentiate normally in *Celsr2* and *Celsr2+3* mutant mice. (a–f'')

Immunofluorescence for S100 (a–b''), CD24 (c–d'') and vimentin (e–f'') show that ependymal cell lining lateral ventricles differentiate in similar ways in WT (a,b,c,d,e,f), *Celsr2* (a',b',c',d',e',f'), and *Celsr2+3* (a'',b'',c'',d'',e'',f'') mutant mice. (g–h''): Coronal sections of mature brain stained for Doublecortin (g–g'') and GFAP (h–h''). The number of Doublecortin and GFAP positive cells is increased in the subependymal layer of *Celsr2* (g',h') and *Celsr2+3* (g'',h'') as compared to WT (g, h) mice. (i–k''): Immature neurons, stained for doublecortin (DCX). Compared to WT (i,j,k), the number of DCX positive cells is increased in the subependymal niche of *Celsr2* (i',j',k') and *Celsr2+3* (i'',j'',k'') mutant mice. Scale bar: 50 μm in (c–c'', e–e'', and i–i''), 100 μm for other panels



Supplementary Fig. 5: Ependymal cilia have normal ultrastructure. TEM micrographs of cross sections of cilia from the WT (a), *Celsr2*^{-/-} (b) and *Celsr2*⁺³ (c). Mutant cilia have the typical (9+2) structure of motile cilia. Scale bar: 100 nm



Supplementary Fig. 6: Normal distribution of ZO-1. (a–c) immunofluorescence showing that the subcellular localisation of ZO-1 is preserved in *Celsr2* (b) and *Celsr2+3* (c) mutant ependyma. The Z sections (rows 2) are at the level of red lines in horizontal sections (rows 1). Scale bar: 5 μ m

Supplementary Movies 1–4: Cilia beat exhibits two types of movements in the WT tissue, namely “seaweed” (**movie 1**) and “pigeon neck” (**movie 2**), and both are preserved in *Celsr2* deficient tissue (“seaweed”, movie 3; “pigeon neck”, movie 4). Images were acquired at 200 frames/s. The playback rate of each the movie is 50 frames/sec, and each movie is made of 150 frames.

Supplementary Movie 5: Movement of latex beads in lateral ventricle of WT and *Celsr2* mutant tissue. In contrast to WT where beads move from the caudal to rostral pole, there is no significant displacement of beads in the mutant. Images were acquired using a standard video camera, at 25

Supplementary Table 1: RT-PCR analysis of the *Celsr2* recombinant mRNA

Primers	Targeted region	Amplification of the mutant cDNA
1: 5'-CGAACCTCTGGCTCTACACC-3' 2: 5'-GTTCTCCCTGAGGCTCTCCT-3'	188-903	+
3: 5'-CCAGAACGCTTTCTCTCACC-3' 4: 5'-CCACAAAGTCGTGCTTCTCA-3'	3412-4252	+
5: 5'-TCATCTCTGTAGTGCGCCTG-3' 6: 5'-ACCATGGAGGGCATCTCTCT-3'	6503-6647	+
5: 5'-TCATCTCTGTAGTGCGCCTG-3' 7: 5'-AGACACAGATGGGCTTGGTC-3'	6503-6961	+
5: 5'-TCATCTCTGTAGTGCGCCTG-3' 8: 5'-GCTCTCGTTACGGAAGACGAC-3'	6503-7041	+
5: 5'-TCATCTCTGTAGTGCGCCTG-3' 9: 5'-AAGGGAGGTCAGCCTGGTTG-3'	6503-7309	–
5: 5'-TCATCTCTGTAGTGCGCCTG-3' 10: 5'-CCTTATTCCAAGCGGCTTCG-3'	Junction <i>Celsr2</i> -IRES-LacZ–Neo.	+

cDNA from *Celsr2*^{-/-} was used as template to amplify fragments at different positions of *Celsr2* transcript. Whereas all primer pairs located downstream of nucleotide 7071 yielded positive bands, primers flanking, or upstream of nucleotide 7071 failed to amplify any sequence. The reverse primer “10” from the IRES sequence was used together with the forward primer “6” to clone and characterize the insertion site. The sequence of the junction between *Celsr2* and the cassette IRES-LacZ-Neo is provided below.

Supplementary Table 2: Comparison of bead track orientation using circular statistics, Watson's U^2 test

Genotypes	P value
WT versus <i>Celsr2</i>	< 0.001
WT versus <i>Celsr2</i> +3	< 0.001
<i>Celsr2</i> versus <i>Celsr2</i> +3	< 0.02

Supplementary data: Partial sequence of mutant *Celsr2-LacZ-Neo* cDNA:

GTCAGTGGCACTGGCGGCTGGTCCGCCCCGAGGCTGCGAAGTCGTCTTCCGTAACGAGAGCCACGTCAGC
TGCCAGTGCAACCATATGACAAGCTTTGCTGTGCTTATGGATATGTCACGACGAG**AGCTCGCGGTTGAG**
GACAAACTCAAGCTTCCCGGGCTGCAGCCAAGCTATCGAATTCCGCCCCCCCCCTCTCCCTCCCCCCCC
CCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGG

Underlined: exon 22

Bold: splice acceptor of exon 23

Italic: start of the IRES-LacZ-Neo sequence

4. DISCUSSION

Our results show that *Celsr1* on one hand, *Celsr2*, *3* and *Fzd3* on the other hand regulate FBM neuron migration in two different ways: *Celsr1* specifies the caudal direction of FBM neuron migration, whereas *Celsr2*, *3* and *Fzd3* regulate the trajectory of FBM neuron migration from r4 to r6. *Celsr1* functions in a non-FBM neuron autonomous manner; in contrast, *Celsr2*, *3* act in a FBM neuron autonomous way. These data, together with the observation that *Celsr2* is epistatic to *Celsr1*, indicate that the *Celsr* family acts as a ‘dual system’, most probably with other partners that include *Fzd3* and possibly *Fzd6*, as well as probably *Vangl1-2*, *Dvl1-3* and *Prickle-like 1-2* (Wang and Nathans, 2007). Analysis of ependymal ciliogenesis confirms that view: *Celsr2* and *Celsr3* act redundantly and affect cell differentiation.

4.1. PCP and neuron migration

Loss of *Celsr2* function results in severely reduced FBM neuron caudal migration: none of the FBM neurons in *Celsr2* mutants can pass beyond the abducens nucleus in r5 and reach r6. However, their ability to migrate laterally and radially is not affected, indicating that the mechanisms which regulate caudal and lateral/radial migration are different, as proposed before (Chandrasekhar, 2004). *Celsr3* mutants alone have no defects of FBM neuron migration, but when this mutation is added to *Celsr2*, it enhances the *Celsr2* phenotype in that the number of remaining FBM neurons is much reduced due to massive apoptosis. Thus *Celsr2* and *Celsr3* have redundant roles and functions in neuronal migration and survival. The phenotype of double *Celsr2* & *3* mutant mimics *Fzd3* mutants, suggesting that a PCP pathway is involved, which is in line with findings in zebrafish (Wada et al., 2006). Specific deletion of *Celsr2* and *3* in FBM neurons using conditional mutant alleles and *Isl1-Cre* proves that both *Celsr2* and *Celsr3* act in a FBM neuron autonomous manner. A function of PCP proteins in FBM neuron migration could involve noncanonical Wnt signaling (Vivancos et al., 2009). Studies of FBM neuron migration in other PCP mutants and the use of in vitro systems that faithfully recapitulate migration (Vivancos et al., 2009) may provide ways to understand those mechanisms further. Some growth factors and signaling cascades, such as VEGF and transcription factors *Hoxb1*, *Krox20* and *Egr2* (Chandrasekhar, 2004; Garel et al., 2000; Schwarz et al., 2004), regulate FBM neuron

migration directly or indirectly, for example by altering rhombomere identity. We believe that they are likely to function in parallel to, rather than as an integral component of PCP signaling. A recent work investigated the action of Prickle1b during FBM neuron migration in zebrafish. The action of Prickle1b is farnesylation-dependent and requires import to the nucleus, which is independent from its interaction with other PCP proteins at the membrane (Mapp et al., 2011).

4.2. Hypothesis for future work: PCP and oriented cell division

Our results show that *Celsr1* specifies the direction of FBM neuron migration. Loss of *Celsr1* function leads to near randomization of migration direction: FBM neurons migrate not only in caudal but also in rostral and lateral directions, a phenotype not seen before in any other vertebrates. Using immunohistochemistry with Celsr1 antibody we detected Celsr1 expression in precursors, but not in FBM neurons themselves, indicating that *Celsr1* functions in a non-FBM neuron-autonomous manner. Conditional *Celsr1* deletion experiments support this idea. In *Celsr1|Isl1* mice, *Celsr1* is specifically deleted in FBM neurons and no rostral migration stream can be observed. In contrast, when *Celsr1* is deleted in FBM neuron precursors by *Nkx6.2-Cre*, abnormal rostral migration is seen in *Celsr1|Nkx6.2* mutants. Thus, *Celsr1* indeed regulates FBM neuron migration direction in a non-FBM neuron-autonomous manner. This raises the question how Celsr1 can regulate migration direction when it is not expressed in the migrating neuron itself. One possible explanation is that *Celsr1* acts through oriented cell division (OCD) in FBM neuron precursors, thereby imprinting some directional cues to daughter cells. The orientation of cell division depends on cell intrinsic and extrinsic polarity cues. PCP pathways were suggested to be involved in linking cell division orientation (Segalen and Bellaiche, 2009; Segalen et al., 2010) with extrinsic polarity cues in both vertebrates and invertebrates. Inasmuch as cell intrinsic polarity cues are known to be regulated by polarity complexes (Siller and Doe, 2009), PCP signaling might act in concert with them. To discuss OCD further, I will review briefly the polarity complexes.

Cell polarity determinants

Cell polarity is morphologically evident in epithelial, including neuroepithelial cells, and in neurons. Indeed, a polar, vectorial organization is present in almost all metazoan cells. Epithelial cells are polarized into an apical and a basolateral domain separated by the junction belt, and this organization is crucial to their physiology. Similarly, polarization of neurons with somatic, dendritic and axonal domains is basic to their function. In epithelial and neuroepithelial stem cells, polarity is intimately linked to proliferation, particularly asymmetric divisions. Some major molecular systems that underlie vectorial polarization of individual cells in different contexts are evolutionarily conserved from nematodes and flies to mammals.

The ‘Par’ and ‘Crumbs’ complexes are implicated in determination of the apical and junctional domains, whereas ‘Scrib’ is involved in basolateral determination. These three systems interact together as well as with others, particularly Notch/Numb and mTor. Inasmuch as these complexes have been reviewed in detail elsewhere (Assemat et al., 2008), we will give a brief introduction, focusing on their relation with OCD. Due to their intimate link to asymmetric divisions, especially in the neuroepithelium, we also present an overview of ‘Pins’, a complex implicated in asymmetric divisions.

The Par apical complex (Par3/Par6/aPKC/Cdc42)

The ‘Par’ system (for ‘partitioning defective’), was identified in *C. elegans* where it regulates asymmetric divisions of the early zygote (Kemphues et al., 1988). Among six Par genes, Par3 and Par6 belong to the apical Par complex localized to tight junctions, that also includes also atypical PKC (iota/lambda and zeta), as well as Cdc42. A mechanism for localization of the Par complex is as follows. Cadherin-mediated interactions in the cell adhesion region activate Cdc42, which results in phosphorylation of aPKC. Activated aPKC then phosphorylates LGL (a component of the basolateral Scrib complex), which is released from PAR6/aPKC and degraded by the proteasome. This results into restricted localization of PAR6/PAR3/aPKC apically, and LGL basolaterally.

The Crumbs apical complex (Crb/Pals1/PatJ)

Crumbs (Crb) is a *Drosophila* gene discovered for its role in cuticle formation (Nusslein-Volhard et al., 1985), and identified as a key controller of epithelial polarity

(Tepass et al., 1990). Stardust (Pals1 in mammals) is activated by Crb, and cells lacking crumbs or stardust fail to form a continuous zonular adherens and to maintain cell polarity. The mammalian ortholog, Pals1 (“Protein associated with Lin7”, “Nagie oko” in zebrafish) was recently shown to play a role in asymmetric cell division (Srinivasan et al., 2008), as well as in neuroepithelial stem cells (Kim et al., 2010).

The Pins complex and OCD

Cell polarization is linked to OCD, often leading to asymmetric division: when division occurs with a spindle parallel to the polarity vector, daughter cells inherit different cortical determinants and follow different fates. Symmetric divisions would occur when spindles are orthogonal to polarity vectors, or when cells are not or weakly polarized. Pins (Partner of Inscuteable) complexes seem to dictate the orientation of spindles and thereby couple spindle orientation to polarity cues. In *Drosophila*, the three components of that complex are: i) Mud; ii) Pins; iii) Gαi. In mammals, the ortholog components are: i) Numa1; ii) Gpsm1, 2 & 3(LGN-AGS3); iii) Gα. In flies, Inscuteable (Insc) acts like a bridge between the Par and the Pins complexes. It binds to Pins and thereby modulates heteromeric G protein signaling (Schaefer et al., 2000). Mud is recruited to the apical cortex and localized at spindle poles (similarly to NUMA) (Fig. 20). In Mud mutant flies, cortical polarity is normal but spindles fail to align with the polarity axis, disturbing asymmetric division (Siller et al., 2006). Mammalian Pins orthologs link the microtubule interacting protein NUMA to heterotrimeric G proteins, and this regulates mitotic spindle organization (Du and Macara, 2004; Du et al., 2001).

The Scribble (Scrib) basolateral complex (Scrib/Dlg/Lgl)

Scribble (Scrib) was identified in *Drosophila* as an epithelial polarity gene localized in the basolateral domain, and is required for apicobasal determination (Bilder and Perrimon, 2000). Partners of Scrib are Lgl (lethal giant larvae) and Dlg (lethal disk large). Dlg is involved in the Pins-Dlg-Khc73 pathway that regulates spindle orientation in *Drosophila* neuroblasts besides the Gα-Pins-Mud pathway (Fig. 20) (Siegrist and Doe, 2005). Inactivation of Lgl1 in mice disturbs asymmetric neural precursor divisions, generates defects in cell cycle exit and increases cell proliferation (Klezovitch et al., 2004).

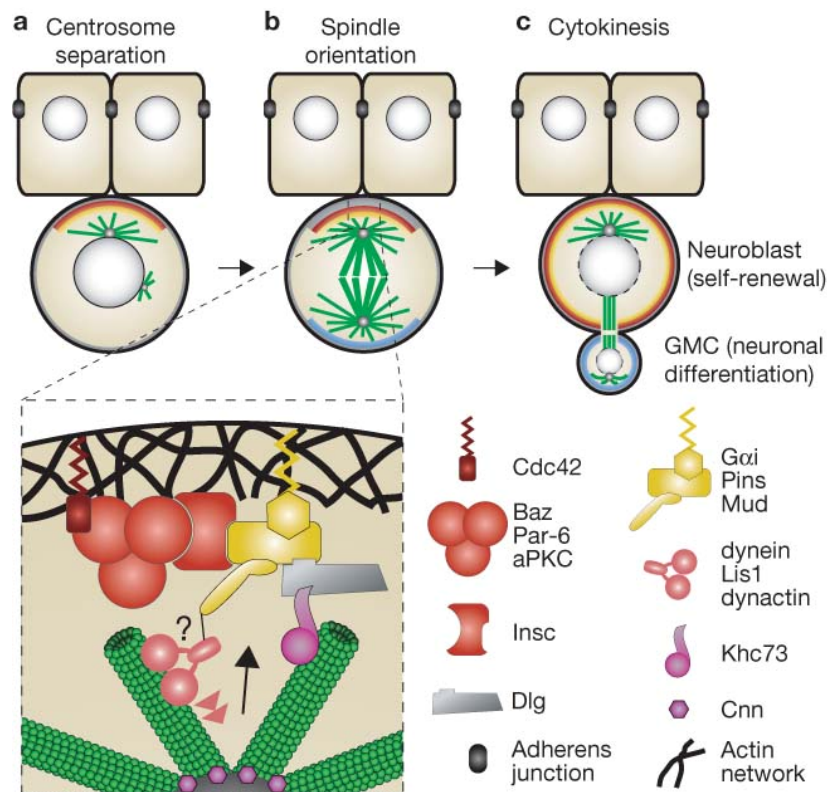


Fig. 20. Spindle orientation and positioning in the *Drosophila* neuroblast. (a) Late interphase/prophase. Par-proteins (Baz, Par-6 and aPKC) and Cdc42 (associated through Par-6) are enriched at the apical cortex. One centrosome is anchored at the apical cortex by Gαi–Pins–Mud (possibly through dynein–dynactin, although this remains to be tested) and by Pins–Dlg–Khc73. The second centrosome nucleates few microtubules and migrates basally. (b) Prometaphase/metaphase. Tight coupling of the spindle to the apical/basal polarity axis requires proteins Khc73, a kinesin-related + ended motor, and dynein, a – ended motor; Khc73 binds Dlg and may facilitate cortical microtubule anchoring, whereas spindle positioning forces on microtubules are probably due to dynein complex activity. The Insc protein directly binds Baz/Par3 and Pins, thereby coupling Par polarity with Mud- and Dlg–Khc73-dependent spindle positioning pathways. (c) Anaphase. The mitotic spindle becomes asymmetric leading to unequal sized daughter cells. Light red arrowheads indicate direction of dynein motion; bold black arrows indicate the direction of the net spindle positioning force. (Siller and Doe, 2009)

Oriented cell division and FBM neuron migration direction

It seems reasonable to propose that polarity complexes and PCP pathways integrate intrinsic and extrinsic cues to orient cell division. One well studied model is the sensory organ precursor (pI) in *Drosophila*. The pI precursor divides along the anterior-posterior axis to generate a posterior pIIa cell and an anterior pIIb cell (Gho and Schweisguth, 1998). The core PCP protein Fz and its effector Dsh are localized in the apical posterior side of the pI cell, with Vang and Pk at the anterior side. This asymmetry restricts the posterior localization of the Par complex, and anterior localization of Pins, both of which are required for the anterior localization of daughter cell fate determinants Numb, Neuralized (Neur) and Partner of Numb (Pon) (Bellaiche et al., 2001a; Bellaiche et al., 2001b; Le Borgne and Schweisguth, 2003; Wirtz-Peitz et al., 2008). Recent studies show that Dsh interacts directly with Mud and that a Fz-Dsh-Mud-Dynein pathway is used in the pI cell to align the spindle along the anterior-posterior axis (Segalen et al., 2010). A similar mechanism is used to regulate spindle orientation in zebrafish (Segalen et al., 2010). In mammals, the asymmetric distribution of LGN and Numa, two proteins involved in spindle orientation, is lost in neuron progenitors that are defective in the PCP protein Vangl2 (Lake and Sokol, 2009). Taken together, these observations suggest a possible mechanism that would orient cell division in precursors and influence the direction of migration of FBM neurons. By regulating mitotic spindle orientation, PCP proteins could lead to asymmetric segregation of directional cues in FBM neurons. This is likely to be reflected in the intracellular location of the centrosome or the Golgi, both of which are usually found in the leading edge of migrating neurons (Fig. 21). To test that hypothesis, it would be interesting to study comparatively in *Celsr1* mutant and WT embryos the localization of Par complexes and NuMA, the planar orientation of mitotic spindles in precursors, as well as the localization of the Golgi and centrosome in early FBM neurons in rhombomere 4. Those studies are made difficult, however, by the necessity to define rhombomere 4 (e.g. HoxB1 antibody) in all experiments, by the small size and high density of cells at that stage, and by the technical challenge associated with in vivo electroporation of small (E9.5) embryos.

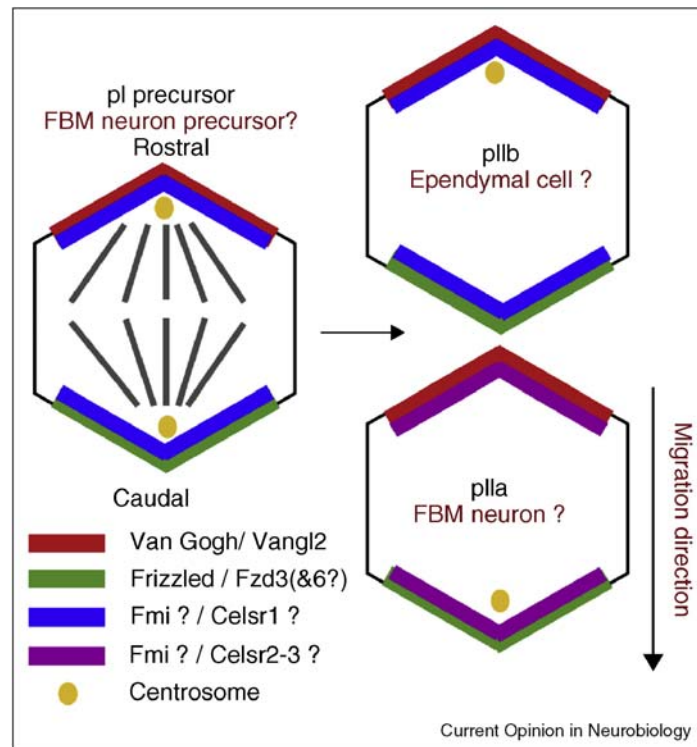


Fig. 21. Tentative model for regulation of asymmetric divisions and neurogenesis. In *Drosophila*, the sensory organ precursor pI divides asymmetrically into pIIb and pIIa. The division occurs along the rostrocaudal axis, and a PCP signal aligns the spindle along that axis, via Van Gogh and Flamingo (rostral distribution), and Frizzled/Flamingo (caudal distribution). A similar mechanism could apply to facial branchiomotor (FBM) neuron migration. During facial FBM neuron generation, a precursor would divide asymmetrically and give rise to an ependymal (or another) cell, and a FBM neuron. Precursor division would align along a PCP axis, under control of Celsr1 together with Fzd3 and Vangl2. This would specify the direction taken by migrating neurons. The ability to migrate is conferred by another PCP signal generated by Celsr2,3 with Fzd3 and Vangl2 (Tissir and Goffinet, 2010)

4.3. PCP and ciliogenesis: putative mechanisms.

Our *in vivo* study of *Celsr2* and *3* mutant mice shows that both proteins have critical functions in ependyma ciliogenesis, and that loss of function results in lethal hydrocephalus. In *Celsr2* and *Celsr2+3* mutant mice, ependymal cells differentiate normally, at least initially, but cilia never develop in normal numbers and display abnormalities in their morphology, position and planar organization. Ciliary basal bodies fail to dock normally to the subapical cortex in *Celsr* mutant cells. In some cases, cilia extend from deeply located basal bodies can be found embedded in ependymal cells, showing that apical docking is not absolutely required for cilia assembly. This ciliogenesis defect is similar to that induced in frog larval epidermal cells, where morpholino knockdown of *Inturned*, *Fuzzy* and *Disheveled1-3* prevent normal basal body docking to the apical cytoplasm (Park et al., 2006; Park et al., 2008). That study provided the first evidence linking ciliogenesis and PCP signaling. It is reasonable to assume that PCP pathways may affect basal body apical docking through their effects on the cytoskeletal machinery. During ciliogenesis in multiciliated cells, duplicated centrioles migrate towards the apical surface and finally transform into basal bodies at the subapical cytoplasm (Dawe et al., 2007). Previous studies indicate that the actin-myosin network is involved in centriole migration in these cells. Agents that inhibit actin polymerization or myosin function can prevent centriole migration in ciliogenic oviducts (Boisvieux-Ulrich et al., 1987, 1990). Similarly, in airway epithelial cells, formation of an apical web-like structure of actin is required for basal body docking, and this process is dependent on Rho-mediated actin assembly (Huang et al., 2003; Pan et al., 2007). In line with these aspects, the function of *Inturned* and *Dvl* in basal body docking was shown to be related to actin assembly and Rho activity (Park et al., 2008). Together with the involvement of Rho GTPase and the actin regulator formin protein Daam1 in PCP-mediated CE (Habas et al., 2003; Habas et al., 2001; Marlow et al., 2002), *Celsr2* and *3* may affect ciliogenesis through actin network assembly. Rho GTPase and formins are good candidates to study the mechanisms of *Celsr2* and *3* function in basal body apical docking.

Ciliary basal feet point to the effective beating direction and can be used as indicators of basal body orientation (Gibbons, 1961; Wallingford, 2010). Recently the

parallel alignment of all basal bodies in a multi-ciliated cell was named rotational planar polarity (Mirzadeh et al., 2010). *Celsr2* and *Celsr2+3* mutant mice also show defects in ‘rotational’ planar polarity: basal feet in the same cell point in different directions. This phenotype could be caused by lack of *Celsr2* and 3 signaling, which regulates basal feet position. It could also be secondary to inefficient CSF flow, since some studies show that basal body orientation can be influenced by fluid flow (Guirao et al., 2010; Mitchell et al., 2007). Study of orientations of basal feet during ependymal cilia development from the beginning of basal feet appearance may answer this question. High speed camera recordings demonstrate that individual cilia beatings are not affected in *Celsr* mutants. Nevertheless, functional assay of cilia beating in brain slice shows defective general flow, which can be caused by reduced cilia number and disoriented basal feet. The incapacity of cilia to generate effective CFS circulation in *Celsr2* and *Celsr2+3* mutants results into severe hydrocephalus, leading to death in *Celsr2+3* mutants.

In *Celsr* mutants, the cellular distribution of *Fzd3* and *Vangl2* is perturbed, indicating that other core PCP proteins are involved in ciliogenesis and basal feet orientation. Due to the perinatal death of *Fzd3* and *Vangl2* mutant mice, studies of ependymal cilia development cannot be performed in these constitutive mutants. The generation of conditional alleles of other core PCP genes would provide ideal tools to address this issue further. PCP genes such as *Vangl1*, *Vangl2* and *Dvl* are also demonstrated to have functions in nodal cilia in frog, zebrafish and mice (Antic et al., 2010; Borovina et al., 2010; Hashimoto et al., 2010; Song et al., 2010). The left/right patterning is disturbed in *Dvl* and *Vangl* mutants due to abnormal cilia positioning (Hashimoto et al., 2010; Song et al., 2010). Thus PCP signaling is probably a common machinery used in ciliogenesis, cilia positioning and basal feet orientation in different types of cells bearing different types of cilia. It would be interesting to study *Celsr* function, not only in motile cilia, but also in nodal cilia or primary cilia.

Other ongoing studies of *Celsr1* function in ependymal cilia indicate that, like during FBM neuron migration, *Celsr1* regulates ependymal cilia development in a different manner than *Celsr2*, 3. *Celsr2* and 3 have important roles in cilia development during ependymal cell maturation, while *Celsr1* probably plays a role in ependymal precursor cells. The hypothesis that *Celsr* family function in a dual system, with *Celsr1* in precursors, and *Celsr2* and 3 mostly in postmitotic cells, is under investigation.

5. ANNEXES

In addition to the two papers mentioned above, I also collaborated to the following projects:

1. Zhou L, Gall D, **Qu Y**, Prigogine C, Cheron G, Tissir F, Schiffmann SN, Goffinet AM. (2010) Maturation of "neocortex isole" in vivo in mice. J Neurosci. 30(23):7928-39. (Zhou et al., 2010)
2. Ravni A, **Qu Y**, Goffinet AM, Tissir F. (2009) Planar cell polarity cadherin Celsr1 regulates skin hair patterning in the mouse. J Invest Dermatol. 129(10):2507-9. (Ravni et al., 2009)
3. Zhou L, **Qu Y**, Tissir F, Goffinet AM (2009) Role of the atypical cadherin Celsr3 during development of the internal capsule. Cereb Cortex. 19 Suppl 1:i114-9. (Zhou et al., 2009)

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