The mouse Wnt/PCP protein Vangl2 is necessary for migration of facial branchiomotor neurons, and functions independently of Dishevelled

Derrick M. Glascoa,1, Vinoth Sittaramanea, Whitney Bryanta, Bernd Fritzschb, Anagha Sawanta, Anju Paudyalc,d, Michelle Stewartc, Philipp Andree, Gonçalo Cadete Vilhais-Netof, Yingzi Yangc,e, Mi-Ryoung Songg, Jennifer N. Murdochc,d, Anand Chandrasekhara, Anju Paudyalc,d, Michelle Stewartc, Philipp Andree, Gonçalo Cadete Vilhais-Netof, Yingzi Yangc,e, Mi-Ryoung Songg, Jennifer N. Murdochc,d, Anand Chandrasekhara

Introduction

Neuronal migration is an essential aspect of nervous system development, and contributes to the formation of functional neural networks and distinct neural layers in the mammalian brain. Radial migration, which accompanies the maturation of all neurons in the central nervous system, involves cell body translocation from the ventricular proliferative zone towards the outer (pial) surface of the brain, often along radial glial fibers. Several types of neurons also undergo tangential migration, which is glia-independent and orthogonal to the direction of radial migration (Hatten, 2002; Marin and Rubenstein, 2003; Nadarajah and Parnavelas, 2002).

In the embryonic hindbrain, facial branchiomotor (FBM) neurons undergo both radial and tangential migration, and thus make an excellent model system for studying neuronal migration mechanisms. In mice, FBM neurons are born in rhombomere 4 (r4) and by E10.5 begin to migrate caudally (tangentially) into r6, by multiple components of the Wnt/PCP pathway, but, importantly, may not require Dishevelled function. Since loss-of-function in some zebrafish Wnt/PCP genes does not affect caudal migration of FBM neurons, we tested whether this was also the case in mouse. Embryos null for Ptk7, a regulator of PCP signaling, had severe defects in caudal migration of FBM neurons. However, FBM neurons migrated normally in Dishevelled (Dvl) 1/2 double mutants, and in zebrafish embryos with disrupted Dvl signaling, suggesting that Dvl function is essentially dispensable for FBM neuron caudal migration. Consistent with this, loss of Dvl2 function in Vangl2Lp/+ embryos did not exacerbate the Vangl2Lp/+ neuronal migration phenotype. These data indicate that caudal migration of FBM neurons is regulated by multiple components of the Wnt/PCP pathway, but, importantly, may not require Dishevelled function. Interestingly, genetic-interaction experiments suggest that rostral FBM neuron migration, which is normally suppressed, depends upon Dvl function.

During development, facial branchiomotor (FBM) neurons, which innervate muscles in the vertebrate head, migrate caudally and radially within the brainstem to form a motor nucleus at the pial surface. Several components of the Wnt/planar cell polarity (PCP) pathway, including the transmembrane protein Vangl2, regulate caudal migration of FBM neurons in zebrafish, but their roles in neuronal migration in mouse have not been investigated in detail. Therefore, we analyzed FBM neuron migration in mouse looptail (Lp) mutants, in which Vangl2 is inactivated. In Vangl2Lp/+ and Vangl2LpLp embryos, FBM neurons failed to migrate caudally from rhombomere (r) 4 into r6. Although caudal migration was largely blocked, many FBM neurons underwent normal radial migration to the pial surface of the neural tube. In addition, hindbrain patterning and FBM progenitor specification were intact, and FBM neurons did not transmute into other non-migratory neuron types, indicating a specific effect on caudal migration.

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mouse mutants mammals. Therefore, we examined FBM neuron migration in whether these processes were also regulated independently in movements for nervous system development, we wondered dently in zebrafish. Due to the importance of these cellular convergence and extension movements are regulated indepen-
tently in zebrafish. These observations suggest that FBM neuron migration and a PCP process like PCP defects (Bingham et al., 2002; Jessen et al., 2002) (Fig. 8). Conversely, FBM neurons fail to migrate caudally in other Wnt/PCP mutants, defective for fzd3a, celsr2, pkib, and scrib, which exhibit no convergence and extension defects (Wada et al., 2005, 2006; Mapp et al., 2011) (Fig. 8). These observations suggest that FBM neuron migration and a PCP process like convergence and extension movements are regulated indepen-
dently in zebrafish. Due to the importance of these cellular movements for nervous system development, we wondered whether these processes were also regulated independently in mammals. Therefore, we examined FBM neuron migration in mouse mutants looptail and chuzhoi, which inactivate Van goghl-
like 2 (Vangl2) and Protein tyrosine kinase 7 (Ptk7), respectively, and exhibit strong defects in several PCP processes (Kibar et al., 2001; Lu et al., 2004; Montcouquiol et al., 2006; Murdoch et al., 2001; Paudyal et al., 2010).

Consistent with observations that FBM neuron migration and PCP processes may be regulated independently, it appears that Wnt/PCP signaling may itself play only a minor role in FBM neuron migration. First, although Wnt5a- and Wnt7a-coated beads can attract FBM neurons in hindbrain explants, these neurons still migrate caudally in Wnt5a and Wnt7a mutant embryos (Vivancos et al., 2009), suggesting a redundant or minor role for Wnts in this process, as shown previously with zebrafish wnt5b and wnt11 mutants (Jessen et al., 2002). Second, abrogation of PCP-specific Dishevelled (Dvl) function in zebrafish using a dominant negative approach had no effect on FBM neuron migration (Jessen et al., 2002), suggesting that Dvl function may be dispensable for caudal migration. We have tested this hypo-
thesis by extending the Dvl dominant-negative analysis in zebrafish, and by examining FBM neuron migration in Dvl knockout mice. Vivancos et al. (2009) found that FBM neurons failed to migrate caudally in Vangl2 mutant mice harboring the looptail (Lp) S464N mutation (Murdoch et al., 2001). We report here that in a different Lp mutant (D255E; Kibar et al., 2001), and also in Vangl2 knockout mice (Song et al., 2010), caudal migration of FBM neurons was abolished. FBM neurons also failed to migrate in Ptk7Lp-m1Jus mutant embryos. Importantly, FBM neurons migrated normally in Dvl1/2 double mutants, and in zebrafish embryos with disrupted dvl signaling. Our data demonstrate that FBM neuron migration and PCP processes are regulated independently in mouse, as in zebrafish, and that Dvl function is largely dispensable for caudal FBM neuron migration (Fig. 8). Interestingly, genetic-interaction experiments suggest that rostral FBM neuron migration, which is normally suppressed, depends upon Dvl function.

Materials and methods

Animals

Mouse colonies were maintained and embryos collected according to the requirements of the Animals (Scientific Procedures) Act 1986 of the UK Government, and various institutional guidelines, including those of the University of Missouri Animal Care and Use Committee (ACUC). Zebrafish colonies were main-
tained and embryos collected following standard protocols approved by the University of Missouri ACUC.

Mouse lines and genotyping

The Vangl2Lp-m1Jus, Vangl2del, Celsr1Crsh, Dvl1tm1Awb, Dvl2tm1Awb, Ptk7Lp-m1Jus, and SE1::gfp mouse lines have been described previ-
ously (Curtin et al., 2003; Hamblet et al., 2002; Kibar et al., 2001; Lijam et al., 1997; Shirasaki et al., 2006; Song et al., 2010). The Dvl1 and Dvl2 lines were purchased from Jackson Labs. Genotyping of the Vangl2Lp-m1Jus allele (referred to as Vangl2Lp, from here on) was performed using primers 5'-GGTTCCAG-TTTGCCGTTCCTC-3' and 5'-CCCTCCCTCCCCAAAACCT-3', followed by sequencing of the PCR product to identify the T1228A muta-
tion. Genotyping of Celsr1Crsh was performed similarly using primers 5' AATTCAGGTAAGTGTTGGA-3' and 5' GTCACACT-
CAGTAGGTCC-3' to identify the A3119G mutation.

For timed matings, noon on the day of a copulation plug was defined as embryonic day (E)0.5. Embryos were staged using standard morphological criteria (Nagy, 2003) before fixation.

Zebrafish

Maintenance of zebrafish stocks, and collection and develop-
ment of embryos in embryo medium were carried out as described previously (Bingham et al., 2002; Westerfield, 1995). To facilitate analysis of branchiomotor neuron development, Tg(is1::gfp) fish (Higashijima et al., 2000) were used for all experiments.

RNA injection

The expression constructs encoding different Dishevelled (Dvl) and Daam1 proteins in pCS2 were obtained from indicated sources by Drs. Oni Mapp and Victoria Prince (University of Chicago) and kindly provided to us: Xenopus Dvl full-length, and Dvl deltaC (Tada and Smith, 2000); Dvl delta PDZ (Xdd1), Dvl delta N, and human N-Daam1 (Habas et al., 2001). RNAs were prepared and injected as described previously (Sittaramane et al., 2009). Embryos at the 1–4 cell stage were injected with RNA (200 pg/embryo) and examined at 24–48 hours post fertilization (hpf) for FBM neuron migration phenotypes. To monitor the amounts of protein produced from the various RNAs, ~50 embryos per treatment were collected at 20 hpf, when FBM neurons are migrating, solubilized in lysis buffer, and processed for Western blot analysis with anti-myc (all Dvl constructs) and anti-FLAG (N-Daam1) antibodies (anti-myc: Cell Signaling Tech-
nologies; anti-FLAG: Sigma–Aldrich) (Fig. S6).

In situ hybridization

Hindbrains were processed for in situ hybridization as described previously (Qu et al., 2010). For imaging, hindbrains were either mounted in open book preparations or embedded in OCT (Ted Pella) for cryostat sectioning. Riboprobes used in this study were Dvl3 (Tissier and Goffinet, 2006), Gata3 (Karvis et al., 2001), Hb9 (Qu et al., 2010), Mash1 and Math3 (Tiveron et al,
2003), Pk1 (Song et al., 2006), Tbx20 (Kraus et al., 2001), Vangl2 (Tissir and Goffinet, 2006), and Wnt5a (Song et al., 2006).

**Immunohistochemistry**

For NF160 neurofilament staining, mouse embryos were processed as previously described (Qu et al., 2010). Embryos were stored in 75% glycerol and imaged on an Olympus SZX12 stereomicroscope. Whole-mount immunohistochemistry of zebrafish embryos (zn5, GFP, myc antibodies) was performed and imaged as described previously (Sittaramane et al., 2009).

**Demarcation of rhombomere boundaries**

Rhombomere boundaries in the mouse wild-type hindbrain were defined using several criteria: In E12.5 embryos processed for Tbx20 in situ, the caudal limit of the trigeminal (nV) motor neurons was defined as the r2/3 boundary, and the sharp indentation in the lateral edge of the migrating column was defined as the r4/5 boundary (Fig. 2D). For E14.5 embryos, the approximate locations of r2, r5 and r6 were defined by the positions of the nV (r2) and FBM (r5, r6) neurons, respectively (Fig. 2C). For E12.5 embryos expressing GFP in cranial motor neurons, the r3/4 and r4/5 boundaries were defined as for Tbx20 in situ. These boundaries also coincided with the fan-shaped genu of GFABX axons extending laterally toward their exit points (Fig. 2A), and with the location of the abducens motor nucleus (nVI) in r6 (Fig. 2B).

Rhombomere boundaries in mouse mutant hindbrains were assumed to be coincident with those in wild-type embryos because the identities and sizes of rhombomeric compartments (r3–r5) were not affected in Vangl2Lp+/−, Vangl2Lp/Lp, Celsr1Crsh+/− and Celsr1Crsh/Crsh embryos (Qu et al., 2010; Thoby-Brisson et al., 2012). One exception was Dvl1/2 double mutant embryos where r3 appeared to be longer at the expense of r4 (Fig. 6D) with no appreciable change relative to wild-type in the overall length of the hindbrain region spanning r2–r6.

Rhombomere boundaries in zebrafish were established based on the characteristic patterns of zn5+/ve commissural axon fascicles (Bingham et al., 2002).

**Results**

**FBM neurons fail to migrate caudally in Vangl2 mutants**

In mouse, facial branchiomotor (FBM) neurons start differentiating in rhombomere (r) 4 at E10.5, as shown by expression of Tbx20, a marker for branchiomotor and visceromotor neurons (Coppola et al., 2005), (Fig. 1A; Song et al., 2006), and start migrating caudally shortly thereafter. By E12, there is a continuous stream of FBM neurons migrating caudally out of r4 into r5 and r6, where they undergo radial migration toward the pial surface (Fig. 1B; Garel et al., 2000). Previous studies in zebrafish and mice have demonstrated roles for several Wnt/PCP genes in rhombomere (r2–r5) were not affected in Vangl2Lp+/−, Vangl2Lp/Lp, Celsr1Crsh+/− and Celsr1Crsh/Crsh embryos (Qu et al., 2010; Thoby-Brisson et al., 2012). One exception was Dvl1/2 double mutant embryos where r3 appeared to be longer at the expense of r4 (Fig. 6D) with no appreciable change relative to wild-type in the overall length of the hindbrain region spanning r2–r6.

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We first examined FBM neuron migration in the SE1::gfp background, in which all cranial motor neurons express GFP (Song et al., 2006). In WT embryos at E11.5 and E12.5, FBM neurons formed longitudinal streams of migrating cells spanning r4 to r6, then migrated dorso-laterally and radially within r6 (Fig. 2A and Fig. S1A; n=10 embryos). In contrast, FBM neurons largely failed to migrate caudally out of r4 in both Vangl2Lp+/− and Vangl2Lp/Lp embryos (Fig. 2B, C; Lp+/+ (n=17), Lp/Lp (8); Table S1), although some neurons migrated dorso-laterally, especially in the vicinity of the r4/r5 boundary (Fig. S1B, C). Interestingly, some GFP+/ve cells were also found laterally in r3 in several Vangl2Lp+/− and Vangl2Lp/Lp embryos, but never in WT embryos, suggestive of aberrant rostral migration (Fig. S1; Lp+/+ (39/47), Lp/Lp (9/26); Table S1). Tbx20 in situ revealed that by E12.5, WT FBM neurons migrated radially towards the pial surface within r6 to form the facial motor nucleus (nVII) (Fig. 2D and Fig. S2A–A′; n=7), which was fully formed by E14.5 (Fig. 2G; n=3). Interestingly, although FBM neurons in Vangl2Lp+/− remained mostly confined to r4, they still underwent their dorso-lateral and radial migrations to form elongated nuclei at the pial surface, spanning r3 to r5 (Fig. 2E, H and Fig. S2B–B′; E12.5 (n=8), E14.5 (4)). In Vangl2Lp/Lp mutants, FBM neurons remained within r4 and most failed to migrate radially to the pial surface of the neural tube (Fig. 2F, I and Fig. S2C–C′; E12.5 (n=3), E14.5 (6); see also Vivancos et al., 2009). Since craniorachischisis (open neural tube) occurs in
Vangl2Lp/+, but not Vangl2Lp/+ embryos (Murdoch et al., 2001; Torban et al., 2004b; Ybot-Gonzalez et al., 2007), the failure of FBM neurons to migrate caudally in looptail (ilp) mutants is not a secondary consequence of neural tube defects (Table S1).

The failure of FBM neuron migration in Vangl2Lp/+ embryos may reflect a dominant negative nature of the Vangl2Lp allele, which encodes a protein that fails to reach the plasma membrane (Iliescu et al., 2011; Torban et al., 2007). Therefore, we also examined FBM neuron migration in Vangl2 knocko ut mice (Vangl2del/+), where no Vangl2 protein is detected (Song et al., 2010). No FBM neurons migrated caudally in Vangl2del/+ embryos (Fig. 2C; n = 5). While a significant number of FBM neurons migrated caudally in Vangl2del/+ embryos and formed a facial motor nucleus in r6, a large number of neurons also failed to migrate out of r4 (Fig. 2K; data not shown; n = 11). In a few Vangl2del/+ embryos (3/11), Tbx20+ve cells were found in r3 and r2 (Fig. 2K) suggestive of aberrant rostral migration (Table S1). Together, the Vangl2del/+ and Vangl2del/+ phenotypes suggest that the caudal migration defects in these embryos result from haploinsufficiency.

Vangl2Lp/+ embryos (Fig. 2B) and Vangl2del/+ embryos (C), FBM neurons fail to migrate out of r4. GFP-expressing cells in r5 are abducens motor neurons (nVI; white arrowheads). (D)–(F), Ventricular views of hindbrains processed for Tbx20 ISH. In a WT embryo (D), FBM neurons migrate caudally from r4 into r6 (white arrowheads), then migrate radially to form the facial motor nucleus (nVII) in r6. In Vangl2Lp/+ embryos (E), most Tbx20-expressing cells remain in r4 in medial and lateral positions, reflecting a near-failure of caudal migration. In Vangl2del/+ embryos (F), caudal migration is completely abolished, with all FBM neurons located medially within r4. The apparent fusion of FBM populations across the midline may result from the defective floorplate and open neural tube in mutants. (G)–(I), Pial views. In a WT embryo (G), FBM neurons have completed migration into r5/r6 to form the facial motor nucleus (nVII). In Vangl2del/+ embryos (H), the facial motor nucleus (nVII) is elongated within r4/r5, and is confined entirely to r4 in Vangl2del/+ embryos (I), with some fused clusters (asterisk) at the midline. (J) and (K), Ventricular views of hindbrains processed for Tbx20 ISH. In a WT embryo (J), FBM neurons (white arrowhead) migrate in characteristic fashion. In a Vangl2del/+ embryo (K), FBM neurons span r3 (black arrowhead) to r5 (white arrowhead), and do not migrate into r6, with most of the neurons confined to r4. In a Vangl2del/+ embryo (L), FBM neurons do not migrate out of r4, with some cells migrating radially within r4 (white arrowheads).

FBM neurons are specified and differentiate normally in Vangl2Lp/+ embryos, but not Vangl2Lp/+ embryos (Murdoch et al., 2001; Torban et al., 2004b; Ybot-Gonzalez et al., 2007), the failure of FBM neurons to migrate caudally in looptail (ilp) mutants is not a secondary consequence of neural tube defects (Table S1).

We tested whether the FBM migration defects in Vangl2Lp/+ mutants were a consequence of improper progenitor specification. Math3 and Mash1 encode transcription factors essential for FBM progenitor specification and their subsequent migration, and
are expressed in longitudinal domains in the ventricular zone (Ohsawa et al., 2005; Tiveron et al., 2003). These expression domains were not affected in \( Vangl2^{+/+} \) and \( Vangl2^{Lp+/Lp} \) embryos (Fig. 3G–I; WT (4), Lp/ (n=3), Lp/Lp (2); Mash1: Lp/+ (2), Lp/Lp (2)), indicating that mutant FBM neurons are specified correctly. Furthermore, FBM neurons in both WT and \( Vangl2^{Lp} \) mutants expressed Phox2b, another branchiomotor and visceromotor differentiation marker (Pattyn et al., 2000), indicating that the non-migrating cells in the \( Vangl2^{Lp} \) mutants differentiate normally (Fig. 3B–F; Math3: Lp/+ (n=8), Lp/+ (7), Lp/Lp (3)). Interestingly, Ret7, a GDNF receptor that is normally expressed by FBM neurons in r6 (Garel et al., 2000), was expressed by non-migrated neurons in r4 of \( Vangl2^{Lp/+} \) and \( Vangl2^{Lp+/Lp} \) embryos (Fig. S3H, I; WT (n=3), Lp/+ (4), Lp/Lp (2)), suggesting that the entire program of FBM neuron differentiation proceeds normally in the absence of caudal migration.

Immunostaining of peripheral nerves with a neurofilament antibody (NF160) revealed similar projection patterns for the trigeminal (nV) and facial (nVII) nerves in E11.5 embryos of all genotypes (Fig. 3G–I; Vent (n=8), Lp/+ (7), Lp/Lp (3)), indicating that despite a failure of FBM neurons to migrate, their axons project and branch correctly, and innervate peripheral targets in vivo. Consistent with NF160 staining, FBM neurons were found in r4 in \( Vangl2^{Lp+/+} \) and \( Vangl2^{Lp+/Lp} \) mutants, although they were reduced in number in the latter (Fig. S3D–F; Lp/+ (n=3), Lp/ Lp (2)). This reduction may result from open neural tube defects in \( Vangl2^{Lp} \) embryos rather than due to failure of caudal migration, since the numbers of back-filled FBM neurons were qualitatively similar in wild-type and \( Vangl2^{Lp/+} \) embryos even though caudal migration was nearly absent in heterozygotes (Fig. S3D, E).

Finally, we investigated whether the \( Vangl2^{Lp} \) mutation affected the development of other neuronal types in the r4–r5 region. The rhombomere markers Hoxb1 (r4) and Krox20 (r3/r5) were expressed normally in \( Vangl2^{Lp/+} \) and \( Vangl2^{Lp+/Lp} \) embryos (see Fig. 1 in Thoby-Brisson et al., 2012), indicating that rhombomere patterning is not affected by the failure of the neural tube to close in \( Vangl2^{Lp/Lp} \) mutants, and that FBM neuron migration defects are not due to defective hindbrain patterning. Rhombomere 4 generates both FBM and inner ear efferent (IEE) neurons. Whereas, FBM neurons migrate caudally out of r4, IEE neurons, which express Gata3 (Karis et al., 2001), are confined to r4 (Fritzsch et al., 1993). In r5, the non-migratory abducens motor neurons express the somatomotor neuron marker Hb9 (Thaler et al., 1999). In WT embryos, Gata3 was found in a high-expressing medial domain adjacent to the floorplate and a weaker-expressing lateral domain, but not expressed in FBM neurons (Fig. S4A–A; n=6). This expression pattern was not

**Fig. 3.** Normal hindbrain development in looptail mutants. (A)–(F), ventricular views; (A’)–(C’), coronal sections. Math3 expression in r4 (white arrowhead) is predominantly in the motor neuron progenitor domain (pMN, dotted circle) in WT (A) and (A’) embryos, and is unaffected in \( Vangl2^{Lp} \) (B) and (B’) and \( Vangl2^{Lp/T} \) (C) and (C’) embryos. Mash1 expression in medial aspect of r4 is also limited to the pMN domain in the WT embryo (D), and is unaffected in \( Vangl2^{Lp} \) (E), and \( Vangl2^{Lp/T} \) (F) embryos. (G)–(I), Lateral views, rostral to the left, of NF160-stained embryos. In a WT embryo (G), the trigeminal (V) and facial (VII, white arrowhead) nerves project into the first and second branchial arches, respectively, and the vagus (X) nerve exits from the caudal hindbrain. These projections are essentially normal in \( Vangl2^{Lp} \) (H) or \( Vangl2^{Lp/T} \) (I) embryos.
affected in \textit{Vangl2} embryos (Fig. S4B–C; \textit{Lp}+/+ (n=8)), and was slightly reduced in the r4 medial domain in \textit{Vangl2} embryos (Fig. S4C–C; \textit{Lp}+/+ (n=1)), correlating with the absence of \textit{Tbx20}-expressing IEE neurons (Fig. S2C–C). Importantly, however, \textit{Gata3} was not expressed ectopically in FBM neurons (Fig. S4B and C). Similarly, \textit{Hh9} was not ectopically expressed in FBM neurons in r4 of \textit{Vangl2} and \textit{Vangl2} embryos (Fig. S4D–F; \textit{Lp}+/+ (n=10); \textit{Lp}+/ (5)). Together, these results indicate that the failure of FBM neurons to migrate caudally in \textit{Vangl2} mutants is not due to a misspecification as non-migratory neurons.

\textbf{Wnt/PCP genes are expressed normally in \textit{Vangl2} mutants}

In mouse \textit{Tbx20} mutants, FBM neurons fail to migrate caudally and poorly express several Wnt/PCP genes, including \textit{Vangl2}, \textit{Prickle1} (\textit{Pk1}) and \textit{Wnt11} (\textit{Song} et al., 2006). In zebrafish, \textit{PK1b} is expressed in FBM neurons, and is necessary for caudal migration (Rohrschneider et al., 2007). In addition, \textit{Wnt5a} is expressed in the vicinity of FBM neurons and may function as an attractive cue for caudal migration (Song et al., 2006; Vivancos et al., 2009). Therefore, we examined the expression of \textit{Wnt5a} and \textit{Pk1} in \textit{Vangl2} mutants. In WT hindbrains, \textit{Wnt5a} was expressed at the midline up to r3, then broadly in the neuroepithelium from r5 and caudally, with a sharp boundary at the r4/r5 border (Fig. 4A; \textit{n=4}; Vivancos et al., 2009). In \textit{Vangl2} embryos (Fig. 4C; \textit{n=4}), the boundary was less clear and overall expression levels appeared lower. However, \textit{Wnt5a} expression in \textit{Vangl2} embryos (Fig. 4B; \textit{n=4}), which have FBM migration defects, was indistinguishable from WT, indicating that the caudal migration defect in \textit{Vangl2} mutants is not due to altered \textit{Wnt5a} expression. \textit{Pk1} was expressed in FBM neurons in both WT and \textit{Vangl2} embryos (Fig. 4D–F; WT (n=2); \textit{Lp}+/+ (2); \textit{Lp}+/ (2)). Together, these data suggest that defective FBM neuron migration in \textit{Vangl2} mutants does not result from altered Wnt/PCP gene expression.

\textbf{Ptk7, which interacts genetically with \textit{Vangl2}, is necessary for FBM neuron migration}

\textit{Ptk7} is a PCP gene encoding a transmembrane, tyrosine kinase-like protein (Mossie et al., 1995) that genetically interacts with \textit{Vangl2} during PCP events like neural tube closure and stereociliary bundle orientation in the inner ear (Lu et al., 2004; Paudyal et al., 2010). Therefore, we examined FBM neuron migration in the mouse \textit{Ptk7} mutants (\textit{Ptk7} 

\textbf{Dishevelled function is dispensable for caudal migration of FBM neurons}

In mice, \textit{Wnt5a} and \textit{Wnt7a} can act as chemoattractants for FBM neurons in an explant assay; however, their in vivo roles are less clear since FBM neurons migrate caudally out of r4 in \textit{Wnt5a} and \textit{Wnt7a} mutants, with a slight defect in dorso-lateral migration in r5/r6 in \textit{Wnt5a} mutants (Vivancos et al., 2009). In zebrafish, several Wnt/PCP genes (\textit{vangl2}, \textit{pk1a}, \textit{pk1b}, \textit{fad3a}, \textit{cebr1-3}, \textit{scrib}) are necessary for FBM neuron migration (Bingham et al., 2002; Carreira-Barbosa et al., 2003; Jessen et al., 2002; Rohrschneider et al., 2007; Wada et al., 2005, 2006), but \textit{wnt5b} and \textit{wnt11} appear to be dispensable (Jessen et al., 2002). Importantly, over-expression of \textit{Xdd1} (Sokol, 1996), a dominant negative form of \textit{Dishevelled} (\textit{Dvl}), the central downstream
signaling mediator of the Wnt/PCP pathway, has no effect on caudal migration of FBM neurons (Jessen et al., 2002). To test directly a role for Dvl function in mice, we examined FBM neuron migration in Dvl knockout mice (Etheridge et al., 2008; Hamblet et al., 2002; Wang et al., 2005).

E12.5 and E14.5 embryos from Dvl1\(^{+/−}\); Dvl2\(^{+/−}\)/C0; Dvl1\(^{+/−}\); Dvl2\(^{−/−}\)/C0; Dvl1\(^{−/−}\); Dvl2\(^{+/−}\)/C0; Dvl1\(^{−/−}\); Dvl2\(^{−/−}\)/C0 embryos (n=6), some of which

Fig. 5. FBM neurons fail to migrate caudally in chuzhoi mutants. Ventricular (A)–(C) and pial (D)–(F) views of embryos processed for Tbx20 ISH. In E12.5 WT (A) and Ptk7\(^{chz/+}\) (B) embryos, FBM neurons migrate caudally from r4 into r6 (arrowheads) to form the facial motor nucleus (nVII). In Ptk7\(^{chz/chz}\) mutants (C), FBM neurons completely or largely fail to migrate out of r4, with a few neurons migrating caudally into r5 (white arrowhead). By E14.5, FBM neurons in WT (D) and Ptk7\(^{chz/+}\) (E) embryos have completed their caudal and radial migrations to form facial motor nuclei (nVII) in r6. In Ptk7\(^{chz/chz}\) mutants (F), the facial motor nuclei are rostrally displaced and located in r4 (8/8 embryos). The location of the trigeminal motor neurons (nV) in r2 is not affected in mutants.

Fig. 6. Caudal migration of FBM neurons is not affected by loss of Dishevelled function. Ventricular (A)–(D) and pial (E)–(H) views of embryos processed for Tbx20 ISH. In E12.5 control Dvl1\(^{+/−}\); Dvl2\(^{+/−}\) embryos (A), FBM neurons migrate caudally from r4 into r6 (arrowhead) to form the facial motor nucleus (nVII). This caudal migration occurs normally in Dvl1\(^{+/−}\); Dvl2\(^{−/−}\) (B), Dvl1\(^{−/−}\); Dvl2\(^{+/−}\) (C), and Dvl1\(^{−/−}\); Dvl2\(^{−/−}\) mutants (D), despite of neural tube closure defects in many of these embryos. By E14.5, FBM neurons in control embryos (E) have completed their caudal and radial migrations to form facial motor nuclei (nVII) in r6. Likewise, these nuclei are formed in r6 in Dvl-deficient embryos including Dvl1\(^{+/−}\); Dvl2\(^{−/−}\) (F, 2/2 embryos), Dvl1\(^{−/−}\); Dvl2\(^{+/−}\) (G, 6/6 embryos), and Dvl1\(^{−/−}\); Dvl2\(^{−/−}\) mutants (H, 4/4 embryos). Neural tube defects were seen in Dvl1\(^{−/−}\); Dvl2\(^{−/−}\) mutants. I–L, Dorsal views of 48 hpf Tg(is1:glp) zebrafish embryos processed for anti-GFP and zn5 immunohistochemistry. In control embryos (I), GFP-expressing FBM neurons migrate caudally from r4 into r6 (arrowhead) and r7 to form the facial motor nucleus (nVII). zn5 staining labels rhombomere boundaries. The trigeminal (nV) and vagal (nX) motor neurons are located in r2 and r3, and the caudal hindbrain, respectively. Inset shows intact embryo with normal extension of the body axis. In embryos injected with Dvl-delPDZ (J), Dvl-delC (K), and N-Daam1 (L) RNA (200 pg/embryo), FBM neurons are able to migrate caudally (arrowheads) out of r4 despite convergence and extension defects resulting in a shortened body axis (insets). Positions of the nV and nX neurons are not affected by these treatments.
exhibit hindbrain neural tube defects (3/6). Importantly, FBM neurons also migrated caudally into r6 in Dvl1/2 double mutants (Fig. 6D and H; n=4 for each age), which exhibit profound PCP defects including a fully open neural tube and misorientation of stereociliary bundles (Etheridge et al., 2008; Hamblet et al., 2002; Wang et al., 2005; Table S1). Similar to Vangl2lox/lox; Dvl2−/−, FBM neurons in r4 of double mutants were initially fused at the midline (Fig. 6D). By E14.5, however, no Tbx20-expressing cells were found at the midline, and a well-defined facial motor nucleus was formed in r6 (Fig. 6H) indicating that FBM neurons undergo essentially normal caudal and radial (dorso-lateral) migrations in Dvl1; Dvl2 double knockout embryos. Since three Dvl genes (1–3) are expressed in partially overlapping patterns in the mouse embryo (Tissir and Goffinet, 2006), Dvl3 could potentially compensate for loss of Dvl1/2 function in FBM neurons in double mutants. However, Dvl3 expression is not affected in double mutants, and is restricted to the ventricular zone adjacent to the migrating FBM neurons at E12.5 (Fig. S5B; n=2) and post-migrated neurons at E14.5 (Fig. S5D; n=1). These results suggest strongly that caudal migration does not require Dvl function in FBM neurons.

Our previous studies with a dominant negative Dishevelled construct (Xdd1) in zebrafish embryos also suggested that FBM neuron migration is Dvl-independent (Jessen et al., 2002). To further validate these findings, we interfered with Dvl signaling using additional constructs, and examined their effects on caudal migration of FBM neurons using isl1:gfp zebrafish embryos, which express GFP in branchiomotor neurons (Higashijima et al., 2000). Western blot analysis confirmed the presence of mutant proteins in 20 hpf embryos during the period of neuronal migration (Fig. S6B). For embryos injected with Dvl-delPDZ RNA, we examined whether over-expression of the dominant negative construct (Fig. 6D), giving the appearance of a stronger migration defect (Fig. 6J), Jessen et al., 2002). Importantly, over-expression of additional dominant negative constructs, Dvl-delC (lacking the DEP domain and C-terminal; Tada and Smith, 2000) and N-Daam1 (lacking Formin-homology domains to bind Dvl and activate downstream signaling; Habas et al., 2001), generated CE defects, but failed to disrupt caudal FBM neuron migration (Fig. 6K and L; summarized in Fig. S6A). Since the dominant negative constructs would interfere with Dvl-mediated signaling notwithstanding the expression of multiple Dvl genes, these data suggest strongly that caudal migration of FBM neurons does not require Dishevelled function.

Since FBM neuron migration is Vangl2-dependent (Fig. 2), and Vangl2 and Dvl2 physically interact (Torban et al., 2004b), we further tested a potential role for Dvl function in migration by examining whether Dvl2 and Vangl2 interact genetically for this process. It was demonstrated previously that Dvl2 and Vangl2 interact genetically during neurulation, with Vangl2lox/lox; Dvl2−/− embryos showing craniorachischisis while Vangl2lox/lox; Dvl2+/− embryos have closed neural tubes (Wang et al., 2006). Therefore, we tested whether a reduction in Dvl signaling by lowering Dvl2 copy number exacerbated the FBM migration defect of Vangl2lox/lox embryos. Removal of one or both copies of Dvl2 in a Vangl2+/− background had no effect on migration of FBM neurons out of r4 (Fig. 7B and C; Dvl2+/− (n=14), Dvl2−/− (n=14)) (Fig. 7D), suggesting that Vangl2 and Dvl2 do not genetically interact to regulate caudal FBM neuron migration. Since Vangl2lox/lox; Dvl2−/− embryos had fully open neural tubes (Wang et al., 2006; data not shown), the clusters of non-migrated FBM neurons in r4 were fused across the midline (Fig. 7F), as in Vangl2lox/lox (Fig. 7G) and Dvl1−/−; Dvl2−/− embryos (Fig. 6D), giving the appearance of a stronger migration defect than in Vangl2lox/lox; Dvl2+/− embryos (Fig. 7E). Interestingly, putative

Fig. 7. Genetic interactions between Vangl2lox/lox, Dvl2, and Celsr1lox/lox for FBM neuron migration. The caudal migratory streams of FBM neurons are labeled with white arrowheads, while rostrally-migrating neurons in Celsr1-deficient embryos are labeled with black arrowheads. The location of the trigeminal (nV) motor nucleus (demarcating r2) is noted with an asterisk in every preparation except G. (A)–(C), Removing Dvl2 genes from a wild-type background (A) did not affect FBM neuron migration (B) and (C). (D)–(F), Removing Dvl2 genes from a Vangl2lox/lox background (D), did not exacerbate the migration defect of Vangl2lox/lox embryos, with thick streams of FBM neurons migrating caudally into r5 (E) and (F), compared to Vangl2lox/lox embryos (G). Fusion of FBM clusters across the midline may be a consequence of defects in neural tube and floorplate development in these embryos. (H)–(J), While removing one copy of Dvl2 had no effect on the abnormal rostral migration of FBM neurons (black arrowhead, I), as seen in Celsr1lox/lox embryos (H), removing the second copy of Dvl2 completely blocked rostral migration (J), but not caudal migration. Rostral migration was also blocked in Vangl2lox/lox; Celsr1lox/lox embryos (K).
migration of FBM neurons into r3, noted in some Vangl2<sup>li</sup> embryos (Fig. 7D; 3/4), was never seen in Vangl2<sup>li</sup>; Dvl2<sup>−/−</sup> embryos, suggesting that the ectopic migration may be Dvl-dependent.

To test this possibility, we crossed Dvl2 mutants with Crash mice, which carry a mutation in an extracellular cadherin repeat of the atypical cadherin Celsr1 (Torban et al., 2007) that results in a subset of FBM neurons migrating rostrally into r2 and r3 (Qu et al., 2010). In Celsr1<sup>Crsh</sup> embryos, a significant number of FBM neurons migrated rostrally into r2/r3, although most migrated caudally into r6 (Fig. 7H; n = 13; Table S1; Qu et al., 2010). Importantly, while many FBM neurons migrated rostrally in Celsr1<sup>Crsh</sup>; Dvl2<sup>−/−</sup> embryos (Fig. 7I; 12/14), this defect was completely suppressed in Celsr1<sup>Crsh</sup>; Dvl2<sup>−/−</sup> embryos (Fig. 7J; n = 5). These data suggest that while caudal FBM neuron migration is likely independent of Dvl activity, the aberrant rostral migration seen in Celsr1<sup>Crsh</sup> embryos requires Dvl function. Finally, the rostral migration defect was also suppressed in Vangl2<sup>li</sup>; Celsr1<sup>Crsh</sup> transheterozygotes (Fig. 7K; n = 3; Table S1), indicating further that aberrant rostral migration is a response to Wnt/PCP signaling since it is sensitive to reduction in both Dvl and Vangl2 function.

**Discussion**

Our detailed characterization of FBM neuron migration defects in looptail mutants has highlighted the specificity of the phenotype within the hindbrain. We have shown that the failure of motor neuron migration is not a result of defective specification or differentiation of the neurons. Moreover, expression of some Wnt/PCP genes is not affected in mutants. Importantly, we find that caudal migration of FBM neurons is insensitive to severe reduction of Dishevelled function, suggesting that this mode of migration may be independent of Wnt/PCP signaling (Fig. 8).

**Effects of Vangl2 mutations on FBM neuron migration and PCP events**

In both Vangl2<sup>del</sup> and Vangl2<sup>li</sup> embryos, most FBM neurons fail to migrate caudally, a fully penetrant phenotype for both alleles. Since no Vangl2 protein is detected in Vangl2<sup>del</sup> mice (Song et al., 2010), the Vangl2<sup>del</sup> phenotype is likely due to haploinsufficiency, with a high level of Vangl2 activity required for caudal FBM neuron migration. The Vangl2<sup>li</sup> allele encodes either a non-functional or a dominant negative protein. Some studies suggest that the Vangl2<sup>li</sup> allele is null because the mutant protein fails to reach the plasma membrane (Iliescu et al., 2011; Merte et al., 2010; Torban et al., 2007) and is unlikely to compete with WT Vangl2 for intracellular binding partners, especially since mutant Vangl2 does not bind Dvl in vitro (Torban et al., 2004b). Other studies suggest that the Vangl2<sup>li</sup> allele is dominant negative since the mutant protein decreases wild-type Vangl2 phosphorylation, which is essential for its function (Gao et al., 2011; Song et al., 2010). Nonetheless, regardless of the nature of the Vangl2<sup>li</sup> allele, FBM neurons largely fail to migrate caudally in Vangl2<sup>li</sup> embryos suggesting that FBM neuron migration is quite sensitive to the level of Vangl2 function. Similar migration defects were noted in Vangl2<sup>li</sup> embryos in a previous study (Vivancos et al., 2009) using a different lp allele (S464N; Murdoch et al., 2001), consistent with a dose-dependent role for Vangl2 in this process. Interestingly, no defects in caudal migration were seen in zebrafish embryos heterozygous for either of two mutations in vangl2 (trilobite alleles: tri<sup>ls20f</sup> and tri<sup>ls240b</sup>; Bingham et al., 2002) including one that results in no detectable transcript (tri<sup>ls16f</sup>; Jessen et al., 2002). However, since biochemical or genetic compensatory mechanisms cannot be ruled out to explain the zebrafish data, we favor the idea that high levels of Vangl2 activity are required for caudal migration of FBM neurons.

Although some PCP events like stereocilia orientation are affected in Vangl2<sup>li</sup> and Vangl2<sup>del</sup> embryos (Montcouquiol et al., 2006; Song et al., 2010), other PCP processes are not affected since both Vangl2<sup>del</sup> and Vangl2<sup>li</sup> embryos have normal, closed neural tubes. Importantly, however, both exhibit strong and comparable caudal migration defects (Table S1). If one assumes that Vangl2 regulates FBM neuron migration and PCP processes through the same molecular pathways, these data suggest that FBM neuron migration is more sensitive to levels of Vangl2 than neural tube closure. Alternatively, Vangl2 may perform unique and separable functions, acting through independent pathways, for neuronal migration and neural tube closure, since these cellular processes have been uncoupled in Vangl2 heterozygotes.

**Site of Vangl2 function for FBM neuron migration**

In zebrafish embryos, vangl2 is expressed ubiquitously in the neural tube and non-neural tissues at the onset and early stages of FBM neuron migration (Jessen and Solnica-Krezel, 2004; Park and Moon, 2002). Genetic mosaic analysis with zebrafish trilobite mutants indicates that vangl2 functions primarily in a non-cell autonomous manner to regulate FBM neuron migration (Jessen et al., 2002; Walsh et al., 2011). In contrast to zebrafish, mouse Vangl2 expression in the hindbrain is restricted to the ventricular zone and to differentiating FBM neurons (Song et al., 2006; Fig. 1).
Therefore, it is possible that Vangl2 functions within FBM neurons in mice. Consistent with this idea, conditional inactivation of Tbx20 in facial motor neurons leads to the failure of caudal migration, concomitant with the loss of Vangl2 expression in these neurons (Song et al., 2006).

Alternatively, Vangl2 may function non-cell autonomously in mice, as in zebrafish, to regulate FBM neuron migration. Cell transplantation experiments in zebrafish indicate a role for vangl2 in floorplate cells for neuronal migration, and FBM neurons fail to migrate out of r4 in a mouse mutant lacking floorplate cells (Sittaramane, V., Glasco, D. et al., in preparation). Since Vangl2 is transiently expressed in mouse floorplate cells between E9.5-10.5, prior to FBM neuron migration, we cannot rule out a floorplate-associated role for Vangl2 in this process. The role of Vangl2 in specific hindbrain cell types can be addressed by generating tissue-specific knockouts using the Vangl2flox allele (Song et al., 2010).

In zebrafish, FBM neurons use the laminin-expressing basement membrane as a substrate for their caudal migration from r4 to r6/r7 (Grant and Moens, 2010). In contrast, mouse FBM neurons migrate caudally in close proximity to the ventricular zone from r4 into r6, where they migrate radially to the pial surface. Interestingly, lamininα5 is expressed along the ventricular zone in the mouse hindbrain (Coles et al., 2006), whereas in zebrafish, laminins are restricted to the basement membrane (Grant and Moens, 2010). The hindbrain expression domains of mouse Lamininα5, Vangl2 and Celsr1 overlap, and in zebrafish, lamininα1 genetically interacts with vangl2 to regulate FBM neuron migration (Sittaramane et al., 2009). Therefore, it is possible that Vangl2 and Celsr1 interact with Laminins and other molecules at the ventricular surface of the mouse hindbrain to guide the caudal migration of FBM neurons.

Caudal migration of FBM neurons may be independent of Wnt/PCP and Dishevelled signaling

FBM neuron migration defects have been described in multiple Wnt/PCP mutants in both mice and zebrafish (Bingham et al., 2002; Carreira-Barbosa et al., 2003; Mapp et al., 2010; Qu et al., 2010; Vivancos et al., 2009; Wada et al., 2005, 2006). However, it is unclear whether FBM neuron migration is a PCP-dependent process. Our finding that caudal FBM neuron migration is blocked in Ptk7 mutants is consistent with a role for Wnt/PCP signaling since Ptk7 genetically interacts with Vangl2, and is a regulator of PCP signaling (Lu et al., 2004; Paudyal et al., 2010). Furthermore, Wnt5a is expressed in a graded fashion from r5 and caudally, suggesting that it may act as an attractive cue to migrating FBM neurons (Vivancos et al., 2009). In hindbrain explant cultures, some FBM neurons migrate towards Wnt-coated beads. However, Wnt5a mutant embryos show only very minor defects in caudal FBM neuron migration and Wnt7a mutants show no migration defects. These observations suggest that Wnts (and PCP signaling) may play a minor or redundant role in regulating FBM neuron migration (Vivancos et al., 2009). Interestingly, mosaic analyses of vangl2, scrb, pk1b, and Nancy-Horan syndrome-like 1b (nhs1b) in particular host environments have uncovered PCP-dependent and PCP-independent mechanisms underlying FBM neuron migration (Walsh et al., 2011).

To directly test the role of PCP signaling in FBM neuron migration, we examined Dishevelled-deficient embryos. FBM neurons migrate normally in Dvl1/2 double mutants despite a fully open neural tube, a hallmark PCP defect (Table S1). This suggests either that FBM neuron migration and PCP events such as neural tube closure are regulated independently, or that caudal neuronal migration requires relatively low levels of Dvl/PCP signaling. Although mice express three Dvl genes in partially overlapping patterns (Tissir and Goffinet, 2006), Dvl3 expression is not affected in Dvl1/2 double mutants, and is excluded from FBM neurons (Fig. S6), suggesting strongly that caudal migration does not require Dvl function within FBM neurons. However, we cannot rule out that Dvl3 compensates for loss of Dvl1-2 function in Dvl1/2 double mutants by acting non-autonomously during neuronal migration. Since Dvl2/3 double mutants die by E9.5, prior to FBM neuron migration (Etheridge et al., 2008), testing a putative role for low-level, non-autonomous Dvl signaling in neuronal migration would necessitate generating triple mutants using conditional alleles. Importantly, however, disruption of Dvl signaling in zebrafish embryos with three different dominant-negative reagents generated only PCP defects (shortened body axis) and had no effect on FBM neuron migration. Since these constructs have the potential to interfere with the functions of multiple Dvl genes not only in motor neurons but also in surrounding tissues, our results suggest strongly that FBM neuron migration is independent of Dvl function (Fig. 8).

Whereas our Dvl data suggest that FBM neuron migration requires lower (or zero) levels of Dvl/PCP signaling (relative to that needed for neural tube closure), our Vangl2flox–/+ data indicate that higher levels of PCP signaling are required (Table S1). Since these putative signaling requirements are mutually exclusive, we instead favor the model that FBM neuron migration and PCP processes are regulated independently of each other, with neuronal migration being independent of Dvl function. In this scenario, Vangl2 may possess a unique and separate function, not required for PCP, which acts in an alternate Dvl-independent pathway to regulate FBM neuron migration. Consistent with this idea, vangl2 genetically interacts with non-PCP genes such as tagl, lamininα1, and hdac1 to regulate FBM neuron migration in zebrafish (Nambari et al., 2007; Sittaramane et al., 2009). Intriguingly, Vangl2 genetically and physically interacts with Pk1b (unpublished data cited in Mapp et al. (2010)), which is expressed in migrating FBM neurons (Rohrschneider et al., 2007), and regulates migration primarily through a novel PCP-independent pathway at the nucleus (Mapp et al., 2011).

Migration of FBM neurons at r3/r4 boundary is Dvl-dependent

Whereas the ability of FBM neurons to migrate caudally appears to be Dvl-independent, our data suggest that Dvl signaling must be reduced to prevent them from migrating rostrally into r3. We showed previously that in Celsr1Csh+/− mutants, a subset of FBM neurons migrates rostrally into r2/r3, though most FBM neurons migrate caudally into r6 (Qu et al., 2010). Since Wnt5a is also expressed along the midline in r2/r3 in addition to the caudal hindbrain (Fig. 4; Vivancos et al., 2009), we propose that mouse Celsr1 normally functions to prevent FBM neurons at the r3/r4 boundary from migrating rostrally (in a Dvl-dependent manner) towards the chemoattractive Wnt5a source in r2/r3. Consistent with this, we found that loss of Dvl2 function rescued the rostral migration defect in Celsr1Csh+/− embryos (Fig. 7). Similarly, loss of Vangl2 function in Celsr1Csh+/− embryos (Vangl2flox–/+; Celsr1Csh+/−; transheterozygotes) also rescued the rostral migration defect (Fig. 7). Importantly, anterograde NeuroVue labeling experiments indicate that the FBM neurons migrating rostrally in Celsr1Csh+/− mutants originate exclusively from the r4 territory immediately adjacent to the r3/r4 boundary rather than from random or more caudal locations in r4 (D. Glaso and A. Chandrasekhar, unpublished observations). These results suggest that migration of FBM neurons at the r3/r4 boundary is especially sensitive to Dvl/Vangl2-dependent signaling, and that Celsr1 function is necessary to block inappropriate rostral migration towards the Wnt5a source in r3. In contrast, migration of FBM neurons arising in the rest of r4 is either only weakly sensitive to or independent of Dvl signaling. This
model may also explain why FBM neurons are attracted to Wnt-coated beads placed laterally near the r3/r4 boundary (Vivancos et al., 2009), since FBM neurons at this boundary would be most sensitive to Wnts, and free to migrate laterally toward the beads through a Celsr1-negative domain. Consistent with this model, Wnt5a-coated beads fail to attract FBM neurons in Dvl2 mutants (W. Bryant, L. Reustle, and A. Chandrasekhar, unpublished observations). Thus, it appears that subsets of mouse FBM neurons may exhibit differential sensitivity to Dvl signaling depending upon their position within r4.

In conclusion, our work has defined a role for Vangl2 in the caudal migration of FBM neurons that appears to be independent of PCP signaling mediated by Dishevelled. Our data also suggest that Wnt/PCP signaling can induce some FBM neurons to migrate rostrally but that this response is normally suppressed. It will be of interest to understand the mechanisms underlying the suppression of the rostral migration response, as well as the nature of the PCP-independent pathways regulating caudal migration.

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Appendix A. Supporting information

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References


