Planar cell polarity defects and defective Vangl2 trafficking in mutants for the COPII gene Sec24b

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SUMMARY
Among the cellular properties that are essential for the organization of tissues during animal development, the importance of cell polarity in the plane of epithelial sheets has become increasingly clear in the past decades. Planar cell polarity (PCP) signaling in vertebrates has indispensable roles in many aspects of their development, in particular, controlling alignment of various types of epithelial cells. Disrupted PCP has been linked to developmental defects in animals and to human pathology. Neural tube closure defects (NTD) and disorganization of the mechanosensory cells of the organ of Corti are commonly known consequences of disturbed PCP signaling in mammals. We report here a typical PCP phenotype in a mouse mutant for the Sec24b gene, including the severe NTD craniorachischisis, abnormal arrangement of outflow tract vessels and disturbed development of the cochlea. In addition, we observed genetic interaction between Sec24b and the known PCP gene, scribble. Sec24b is a component of the COPII coat protein complex that is part of the endoplasmic reticulum (ER)-derived transport vesicles. Sec24 isoforms are thought to be directly involved in cargo selection, and we present evidence that Sec24b deficiency specifically affects transport of the PCP core protein Vangl2, based on experiments in embryos and in cultured primary cells.

KEY WORDS: COPII-mediated protein trafficking, Planar cell polarity, Neural tube closure defect, Mouse genetics, Inner ear development, Cardiac defects

INTRODUCTION
Correct polarization and orientation of cells is of crucial importance for their function. In addition to apical-basal polarization, cells may be polarized in an orthogonal orientation, often but not exclusively in the plane of an epithelial sheet (Wu and Mlodzik, 2009). Loss of this planar cell polarization (PCP) has been correlated with developmental defects as well as with tumorigenesis (Humbert et al., 2003; Saburi et al., 2008; Zhan et al., 2008).

PCP signaling not only determines planar asymmetry of a cell, but also the ability of the cell to propagate this polarity to adjacent cells, thus establishing tissue polarity. It was originally discovered and studied in Drosophila, but in recent years the significance of PCP signaling for vertebrate development has become increasingly clear. Developmental processes involving PCP include the convergent extension movements that shape the embryo during gastrulation and neurulation, and the regular arrangement of structures as diverse as stereocilia in the organ of Corti and epidermal hairs (Wang and Nathans, 2007; Montcouquiol et al., 2003; Devenport and Fuchs, 2008). Mouse mutant embryos with a disrupted PCP pathway frequently suffer from the extreme neural tube closure defect known as craniorachischisis, which is rarely, if at all, encountered outside this context (Kiabar et al., 2001; Curtin et al., 2003) (reviewed by Copp et al., 2003). PCP pathways in insects and vertebrates share a core pathway that includes members of the dishevelled and frizzled gene families, but which is distinct from the b-catenin-dependent ‘canonical’ Wnt pathway (Lawrence et al., 2007).

In a random mutagenesis screen for genes essential for normal mouse development, we have identified a series of mutant lines on the basis of their abnormal appearance at day 10.5 of gestation. Of two mutants displaying craniorachischisis, one was shown to carry a novel mutant allele of the known PCP gene scribble (Scrib), whereas the second was found to be allelic with the Sec24b gene, scribble. Sec24b is a component of the COPII coat protein complex that is part of the endoplasmic reticulum (ER)-derived transport vesicles. Sec24 isoforms are thought to be directly involved in cargo selection, and we present evidence that Sec24b deficiency specifically affects transport of the PCP core protein Vangl2, based on experiments in embryos and in cultured primary cells.

MATERIALS AND METHODS
Mouse strains and identification of mutants
Mutants were generated by random chemical mutagenesis and mapped using procedures essentially similar to those used by Kasarskis (Kasarskis et al., 1998) and to be published elsewhere in more detail (C.W., Van Gurp, L., H.F., C.K., Rieter, E., Verberne, M., Guryev, V., E.C. and F.M., unpublished). Briefly, C57BL/6 mice were injected three times at 1-week intervals with 60-80 mg/kg bodyweight of ethyl-N-nitrosourea (ENU) and after recovery, crossed to FVB/N mice. The resulting male founders were crossed with their daughters and embryos were visually inspected on embryonic day (E) 10.5. A SNP panel distinguishing C57BL/6 and FVB/N polymorphisms was used for positional cloning of the affected genes. Animal experiments were conducted under the approval of the Animal Care Committee of the KNAW.

Histology and immunostaining
Embryos and inner ears were dissected in PBS and fixed for 2 hours in 2% parafomaldehyde (PFA) for immunostaining and in 4% PFA overnight for Hematoxylin and Eosin stainings and in situ hybridizations. Cochleae were dissected and the organ of Corti was revealed by removing the stria vascularis. Stereocilia were visualized by staining with Rhodamin-
RESULTS AND DISCUSSION

Identification and mapping of two craniorachischisis mutants

Two mutant lines characterized by craniorachischisis, tentatively named 51206 and krabbel, were identified in a genetic screen in mouse (Fig. 1A-C). This phenotype is often associated with disruption of PCP signaling. Genetic mapping of the 51206 allele resulted in the identification of an interval on chromosome 15 between 71 and 83.2 Mbp, containing the scribble (Scrib) gene, which has previously been linked to craniorachischisis and gastroschisis (Rachel et al., 2002; Murdoch et al., 2001; Murdoch et al., 2003; Zarbalis et al., 2004). Sequencing of the 38 exons of Tspan2 revealed a C to T transition of nucleotide 4865 (ENSMUST000000012906) in exon 33, resulting in a nonsense mutation of arginine 1508 that truncates the protein by 158 amino acids (Fig. 1D). We conclude that this mutation is responsible for the affected phenotype. All conserved domains of Scrib, including the 16 leucine-rich repeats and four PDZ domains, are left intact by the mutation and a specific function of the carboxy-terminus has not been reported (Albertson et al., 2004), leaving the possibility that this is not a null allele, in spite of the strong phenotype. Quantitative PCR (qPCR) experiments (data not shown) revealed an approximately 65% decrease of Scrib mRNA in mutant embryos, presumably attributable to nonsense-mediated decay (NMD) (Stalder and Mühlemann, 2008). Therefore, the function of the protein encoded by the Scrib51206 allele might be affected both qualitatively and quantitatively.

Genetic mapping of the krabbel mutation identified a 9.24 Mb segment of chromosome 3 expected to contain the mutation. By sequencing the exons of the genes in this segment, we discovered a 1082 of the 1216 amino acids of the two Sec24b protein variants, the CAAX-YGFP construct was a gift of J. Bussmann (Hubrecht Institute); it encodes the Ras prenylation signal KLNNPDESQPGRSCSCKCVLSX fused to YGFP.

phenotype in well over 100 mutant embryos. According to the ENSEMBL database, the Sec24b gene (ENSMUSG000000098616) encodes two transcripts owing to alternative splicing, ENMUST000000012906 and ENMUST000000098616. The mutation changes a Ser codon to a stop codon, removing 1117 of the 1251 or 1082 of the 1216 amino acids of the two Sec24b protein variants, respectively. As the mutation is upstream from the alternatively spliced exon, the same residual 134-amino acid peptide remains, encoded by both splice variants. This allele must therefore represent a null (Fig. 1E). The expected NMD effect on Sec24b mRNA concentration was confirmed by qPCR, and estimated to be 72% (data not shown). While this manuscript was under review, Merte et al. (Merte et al., 2010) independently reported a different Sec24b mutant with a craniorachischisis phenotype. The existence of a second mutant allele definitely establishes the link between Sec24b and craniorachischisis.

Sec24b is a component of the COPII complex that has an essential role in generating secretory vesicles at the ER. These vesicles have a central function in the strictly regulated ER–Golgi secretory pathway (Salama and Schekman, 1995; Fromme and Schekman, 2008). Genetic work in yeast has uncovered a series of genes that encode the various constituents of the COPII coat (Kuehn and Schekman, 1997). Sec24, occurring as a component of Sec23-Sec24 dimer, has been linked specifically to a function in cargo selection. Sec24b is one of four paralogous genes, which are found in mouse, human and other vertebrate genomes. Knowledge of any distinct functional specificity of these four genes remains rather limited (see Wendeler et al., 2007). In the early embryo (at least E8.5-E10.5) all four paralogues, with the possible exception of Sec24d, are ubiquitously expressed (see Fig. S1 in the supplementary material).
**Sec24bkrabbel** mutants fail to undergo neural tube closure

At least 95% of **Sec24bkrabbel** mutant embryos displayed severe neural tube closure defects, whereas the remaining fraction had a weaker but similar phenotype. We observed gastroschisis in less than 5% of **Sec24bkrabbel** mutant foetuses, in contrast to the **Scrib**1206 mutant that displayed this failure of the ventral body wall to close in about half of the embryos (see also Murdoch et al., 2003).

**Aberrant aortic arch morphology in Sec24bkrabbel mutants**

Homozygous **Sec24bkrabbel** mutant foetuses die around day E17.5 of gestation and are externally recognized by blood-filled yolk sacs and local effusions (Fig. 1C, asterisk) indicative of disturbed blood circulation.

Several mouse mutants known to have deficient PCP signaling have been shown to have severe cardiac abnormalities (reviewed by Van den Hoff and Moorman, 2005; Henderson et al., 2006; Davis and Katsanis, 2007). Numerous aspects of cardiogenesis may be affected in PCP mutants, often including outflow tract abnormalities like double-outlet right ventricle, misplacement of the great arteries, aortic arch abnormalities and septation defects. Possible explanations for these aberrancies include defects in directed cell migration due to disrupted PCP signaling, deficient ‘myocardialization’ of some of the great vessels, and looping defects.

To see whether **Sec24bkrabbel** mutants display any of these abnormalities, we compared cardiac development of mutant and wild-type embryos. Fig. 2A compares PECAM-stained E16.5 hearts from a wild-type embryo and a **Sec24bkrabbel** mutant. Ventricles were abnormally small in mutants, whereas dissection of atria demonstrated approximately normal sizes in mutants (data not shown). In addition, as the appearance of the great outflow vessels gave some indication of their abnormal orientation in the mutant, we decided to investigate this further by analysis of histological sections. Transverse sections of E15.5 hearts revealed an abnormal arrangement of the vessels branching off from the aortic arch. Although in the wild-type heart (Fig. 2B,D) the subclavian artery can be seen to be located ventrally from the esophagus and trachea, in the mutant this artery branches off of the aortic arch at an abnormal position and is at a dorsal position from the esophagus and trachea (Fig. 2C,E), an anomaly known as retroesophageal subclavian artery (RSA). In addition, in sections at a more caudal level, we observed that the pulmonary artery was directed in an aberrant, rightward direction (see Fig. 2F,G).

RSA is among the abnormalities reported for embryos carrying mutations in PCP genes: Torban and colleagues (Torban et al., 2008) observed RSA in **Vangl2** mutants, where it is accompanied by severe cardiac septation defects. Interestingly, **Vangl2/Vangl1** double-heterozygous embryos had RSA as the predominant effect.

**Sec24bkrabbel** mutants have smaller cochleae and an abnormal arrangement of hair cells in the organ of Corti

A classic manifestation of planar polarity in mammals occurs in the development of the inner ear. PCP mutants may have shorter cochlear tubes and, in addition, misalignment of cochlear mechano-sensory receptors, i.e. the stereociliary bundles of the organ of Corti (Montcouquiol et al., 2003; Curtin et al., 2003).

Dissection at E16.5 of inner ears of **Sec24bkrabbel** mutant foetuses revealed that mutant cochleae were markedly smaller than those from wild-type and heterozygous embryos. Although an extensive analysis of the vestibular system is beyond the scope of the present study, we tentatively conclude that it appears relatively normal (Fig. 3A,B). To obtain an objective quantitative measure for reduction of cochlear duct length, we measured the length of the organ of Corti by means of stainings for the hair cell marker Myo7A (Fig. 3C). This confirmed that the lengths of organ of Corti in **Sec24b** and **Scrib** mutants were reduced by a statistically significant degree (Fig. 3D). Comparable observations have previously been reported for PCP mutants, including **Vangl2** (Torban et al., 2008) and double mutants involving **Dvl1-3** genes (Wang et al., 2005; Etheridge et al., 2008).

This prompted us to investigate in these cochleae the stereociliary
bundle orientation, as at this stage PCP is normally manifest from an asymmetrical localization of the kinocilium establishing a precisely oriented chevron arrangement of stereociliary bundles towards the abneural side of the sensory epithelium (Rida and Chen, 2009).

Phalloidin staining of the dissected organ of Corti at E17.5 at basal (Fig. 3E-H) and middle (Fig. 3I-K) regions (as defined by white lines in Fig. 3C) of the cochlear duct of wild types confirmed this highly organized pattern (Fig. 3E,I), but in the cochleae of mutants, a significant degree of disorganization was observed. Locally, a fourth row (Fig. 3G,J; square brackets) of outer hair cells appeared, or only two rows were seen (data not shown). In the single row of inner hair cells, cells were frequently positioned out of line (Fig. 3F,J; arrows). In addition, in the three rows of outer hair cells, stereociliary bundle orientation appeared to be rather frequently misaligned (Fig. 3F; asterisks). The severity of these aspects of the Sec24bkrabbel phenotype were mild compared with those reported for the PCP mutants mentioned above (Wang et al., 2005; Etheridge et al., 2008) and with Scrib51206 (Fig. 3H). In conclusion, a rather severe external abnormality of the cochlea is accompanied by a relatively mild phenotype in the organ of Corti.

Genetic interaction between Sec24bkrabbel and Scrib51206

Evidence for involvement of two genes in a common pathway can be obtained by asking whether their phenotypes increase synergistically in double mutants. We crossed Sec24b mutants with the Scrib51206 mice, as well as with Vangl2looptail mice (Kibar et al., 2001), but no neural tube defects were seen in either type of double-heterozygous embryos. Also, in other aspects of the phenotype, Sec24b−/−; Scrib+/− embryos were indistinguishable from wild-type embryos. However, Merte and colleagues (Merte et al. 2010), using their Sec24b mutant line, reported significant incidence of open neural tube in Sec24b−/−; Vangl2−/− double-heterozygous embryos, confirming a link with PCP signaling. As both Sec24b mutants are almost certainly nulls, differences in the genetic backgrounds of the mice involved are the most probable explanation for this unexpected disparity.

We then analyzed embryos that were homozygous for Sec24bkrabbel and heterozygous for Scrib51206. The neural tube defect of the Sec24b mutant, already extreme in single mutants, did not further deteriorate. Analyses of the cochleae from these compound mutants, however, showed significant differences, although these
were not evident from measurements of the Myo7A domains (Fig. 3C,D). We compared phenotypes in basal and middle areas of cochleae from these Sec24bkrabbel+/–, Scrib51260+/– embryos (Fig. 3G,K) with those of wild-type (Fig. 3E,I) and Scrib51260–/– (data not shown) embryos, as well as with Sec24bkrabbel–/– embryos (Fig. 3F,J). Although Phalloidin stainings of hair cell patterns were indistinguishable, Phalloidin staining patterns seen in Sec24bkrabbel–/–, Scrib51260+/– organs of Corti revealed stronger disruption of hair cell alignment than in those of Sec24bkrabbel–/–, as was particularly evident in the inner ear cell row. Quantification of this effect was performed by counting the occurrence of abnormal inner hair cells in subsets of 70–130 cells from basal and middle regions of five cochlear ducts of both genotypes. The results, summarized in Fig. 3L, indicate higher percentages of abnormally positioned cells in the compound mutants compared with homozygous Sec24bkrabbel mutants. We quantified the effect by counting, for each individual row of cells separately, the percentage of cells that were misoriented by 30° either left or right. This revealed, as shown in Fig. S2 in the supplementary material, significantly higher percentages of misoriented cells in the single row of inner hair cells. In the three outer hair cell rows, differences were not statistically significant, although only by a narrow margin for row 3. To avoid ambiguity in cell row identification, we did not include cells of regions where an abnormal number of inner hair cells occurred; this might have had some impact on the numbers.

Therefore, removing one Scrib allele from a wild-type background is not enough to bring on an affected phenotype, but removing it from a Sec24bkrabbel–/– background aggravates the phenotype. Involvement of Scrib in PCP signaling, suggested by its PCP-like phenotype, has been established by demonstrating genetic and physical interaction between Scrib and the PCP-core gene Vangl2 (Montcouquiol et al., 2003; Montcouquiol et al., 2006). Their Drosophila orthologs Scrib and Stbm/Vang were recently shown to interact genetically and physically as well (Courbard et al., 2009). Genetic interaction with Scrib therefore implicates Sec24b in the PCP signaling pathway.

**How does Sec24b link protein trafficking to planar polarity?**

Having concluded that inactivation of Sec24b leads to disruption of the PCP pathway we asked the question of how deficiency of a COPII coat protein could lead to a defect in cell polarity. It would seem probable that this must somehow reflect a disruption of regulated protein trafficking. As Sec24 proteins are known to be involved in cargo selection (Mancias and Goldberg, 2008), an obvious hypothesis would be that transport of PCP-related membrane proteins depends relatively strongly on the Sec24b subunit. To test this possibility directly, we investigated the subcellular location of the PCP proteins Vangl2 and Ptk7 in two tissues that are affected by the Sec24b mutation, the presumptive neural tube around the 3–8 somite stage, at the onset of neurulation, and the organ of Corti at E17.5.

Wholemount immunolocalization demonstrated that Vangl2 is expressed throughout the presumptive neural tube of wild-type and heterozygous Sec24bkrabbel E8.25 embryos and localizes to the plasma membrane in wild types (Fig. 4A–C; see also Fig. S5A–I; Fig. S3A–C in the supplementary material). By contrast, in neural folds of E8.25 homozygous mutant Sec24bkrabbel embryos, we detected abnormal Vangl2 expression or localization (Fig. 4D–F; see also Fig. S5J–R; Fig. S3D–F in the supplementary material). As these neural cells have large nuclei surrounded by a relatively thin shell of cytoplasm, it is not trivial to distinguish location to the plasma membrane from cytoplasmic location. We used an antibody against E-cadherin to demarcate the plasma membrane. At higher magnifications, a distinctly different
location is seen of E-cadherin (red signal in Fig. 4A-F) and Vangl2 (green). Arrows in Fig. 4F indicate examples of Vangl2 staining not located at the plasma membrane, which is not seen in wild-type embryos (Fig. 4C). In other experiments, the level of detected Vangl2 appeared to be lower in mutant neural folds (see Fig. S3 in the supplemental material). Staining of the PCP-related protein Ptk7 was identical in mutant and wild-type neural folds (Fig. 4G,H).

Hypothetically, the affected phenotype of the mutant could have consequences leading indirectly to abnormal behaviour of the Vangl2 protein. To confirm that abnormal Vangl2 location is not caused by such a ‘downstream’ effect, we show normal Ptk7 (see Fig. S4E-H,K,L in the supplemental material) and Vangl2 (see Fig. S4A-D,I,J in the supplemental material) protein location in cells in the neural folds of E8.5 Scrib+/− embryos and E17.5 Scrib+/− cochlea.

In the organ of Corti, where, as shown above, the phenotype is relatively mild, we observed normal Ptk7 staining patterns in wild-type embryos and Sec24b mutants (Fig. 3O,P). The Vangl2 protein was detected mostly in supporting cells and no convincing differences between wild types and mutants were seen, neither in subcellular location nor in overall levels (Fig. 3M,N). Accumulated Vangl2 is at this stage relatively easy to detect in supporting cells, whereas it is more difficult to perceive hair cells where it is more diffusely located. More appropriate stages to analyze this aspect of the phenotype are unfortunately beyond the lethal stage (around E17.5) of this mutant. Patterning of the organ of Corti at E17.5 is immature and membrane-bound Vangl2 is difficult to detect.

**Transport of Sec24b is impeded in mutant primary fibroblasts**

To corroborate these results with a methodologically independent approach, we transfected primary embryonic fibroblasts (MEFs) prepared from wild-type and Sec24bΔkrebbe1 mutant embryos with a Vangl2-eGFP fusion construct (Montcouquiol et al., 2006). An advantage of these cells is the larger volume of the cytoplasm, making the distinction between cell membrane and cytoplasm easier to detect. In approximately 75% of Vangl2-eGFP-transfected wild-type cells (as judged from the presence of any detectable fluorescence at all), the fluorescent signal clearly demarcated the plasma membrane, often in addition to its diffuse presence throughout the cytoplasm (Fig. 4I).

By contrast, in mutant MEFs this signal at the plasma membrane was never seen in three experiments performed in duplicate (Fig. 4J,K). The cytoplasmic fluorescence in these mutant cells was in some cases more strongly concentrated in areas surrounding the nucleus. Transfection with two additional constructs encoding fusion proteins known to localize to the plasma membrane, Tspan2-eGFP and CAAX-YFP resulted, as expected, in clear fluorescent signal at the plasma membrane (Fig. 4L-O) of MEFs of both genotypes. These data therefore confirm a defect in transport of the Vangl2 protein to the cell membrane.

**Conclusions**

Our observations show that loss-of-function of Sec24b leads to developmental defects affecting the spinal cord, the heart and the inner ear, resulting in a phenotype characteristically seen in mutants deficient for components of the PCP signaling pathway. We demonstrate that in mutant neural tube cells, as well as in mutant MEFs, the PCP core protein Vangl2 fails to locate to the cell membrane, presumably because of defective export from the ER. Absence of Vangl2 from the cell membrane is responsible for, or at least contributes to, the development of craniorachischisis. This observation indicates a surprising level of specificity in the mechanism of selective cargo exit from the ER. Even if, as we cannot exclude, transport of additional PCP proteins like Vangl1 is also compromised, the absence of other obvious defects in Sec24b mutants makes this a remarkably restricted phenotype. Interestingly, strong evidence for physical interaction between Vangl2 and Scrib (Montcouquiol et al., 2003; Montcouquiol et al., 2006) and their Drosophila orthologs (Courbard et al., 2009) indicates that aberrant Vangl2 localization might also have an effect on the function of Scrib, even though this non-membrane protein is not expected itself to depend on ER-to-Golgi trafficking.

Clearly, functional Vangl2 deficiency is not complete, as is evident from the weaker phenotype in the organ of Corti and heart of Sec24b mutants compared with Vangl2 mutants, as well as from our protein localization studies. The milder heart and ear phenotypes also suggest that in those tissues other factors, possibly other Sec24 isoforms, contribute to the specificity of cargo selection or that other PCP components like Vangl1 reach higher levels and are therefore better capable to compensate the phenotype.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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