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Short Communication

Analysis of Netrin 1 receptors during inner ear development

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ABSTRACT Netrin 1 plays key roles in axon guidance and neuronal migration during central nervous system (CNS) development. Outside the CNS, Netrin 1 has been shown to be involved in epithelial morphogenesis of various organs. We have shown that Netrin 1 is essential for inner ear semicircular duct formation, but the involvement of Netrin 1 receptors in this process has remained unknown. Netrin 1 receptors include members of the Deleted in colorectal cancer (Dcc), Unc5-homologue and integrin families. Here we have analysed the expression of these receptor genes during inner ear development and verified the inner ear phenotypes of several receptor mutant mice. Special interest was directed to receptors that could cooperate with Netrin 1 during semicircular duct formation. We show that *Neogenin* (*Neo1*), *Unc5c* as well as *integrin* $\beta 1$ (*Itgb1*) are expressed in periotic mesenchyme, while *Dcc, Unc5b, Unc5c, Itga3, Itga6* and *Itgb1* are expressed in different parts of the otic epithelium. In spite of the broad and strong expression of several receptors in ear region, none of the analysed receptor mutant embryos showed any defects in inner ear development.

KEY WORDS: *epithelial morphogenesis, semicircular duct, Dcc, Unc5, integrin*

Netrin1 in the inner ear

In mouse, semicircular ducts start to grow out from the dorsal otic vesicle as two-layered pouches of the dorsal epithelium around embryonic day (E) 10.5-11.0. These pouches then flatten and the opposing epithelial layers meet in the middle to form so-called fusion plates. The underlying basement membrane is disrupted, which allows the fusion plate cells to detach and form a single cell-layer that is subsequently cleared leaving behind the newly formed semicircular ducts at E12.5-13.0 (Martin and Swanson, 1993). The laminin-related secreted protein Netrin1 (Ntn1) is essential for the formation of the semicircular ducts. In *Ntn1*-/- embryos, the detachment of the fusion plate basement membrane does not occur properly. In addition, the proliferation of the adjacent mesenchyme is decreased and the two opposing fusion plate epithelia cannot approach each other normally (Salminen *et al.,* 2000). These defects result in the complete lack of posterior and lateral semicircular ducts. The superior duct forms but fails to grow to its normal size and shape (Salminen *et al.,* 2000).

Ntn1 expression in inner ear initiated at late E10.5 in the dorsolateral wall of the otic vesicle marking the future fusion plate area of the superior and posterior semicircular ducts (data not shown). At E11.5-12.5, *Ntn1* was strongly expressed in the areas destined to form the fusion plates but was excluded from the distal ends of the duct outgrowths. Expression extended also ventrally to the future saccule, utricle and cochlear duct. When the semicircular ducts had formed and the fusion plate cells cleared, *Ntn1* expression was maintained in the inner edges of the ducts (Fig. 1A,B; Salminen *et al.,* 2000). At E18.5, *Ntn1* continued to be expressed in the inner edges of the semicircular ducts and in the non-sensory epithelium of the utricle and saccule. No expression could be detected in any of the sensory epithelia or the vestibulocochlear ganglion (Salminen *et al.,* 2000). In postnatal cochlea, *Ntn1* expression was restricted to Reissner's membrane (Fig. 1T).

Ntn1 protein has been localised on the fusion plate forming cells and in the underlying basement membrane (Salminen *et al.,* 2000). Ntn1 is also thought to diffuse and form a concentration gradient at least in the developing chicken spinal cord (Kennedy *et al.,* 1994) and therefore, Ntn1 could interact with receptors expressed by the fusion plate epithelium itself and by the adjacent epithelial or mesenchymal cells.

Dcc family members in the inner ear

Ntn1 has been shown to bind to transmembrane proteins belonging to the Deleted in colorectal cancer (Dcc) and Unc-5 homologue (Unc5) families (Reviewed in Barallobre *et al.,* 2005).

Abbreviations used in this paper: Ntn1, Netrin1; Dcc, Deleted in colorectal cancer; Neo1, Neogenin, Unc5a-d, Unc5-homologue a-d; Itga, integrin alpha; Itgb, integrin beta; Pn1, postnatal day 1.

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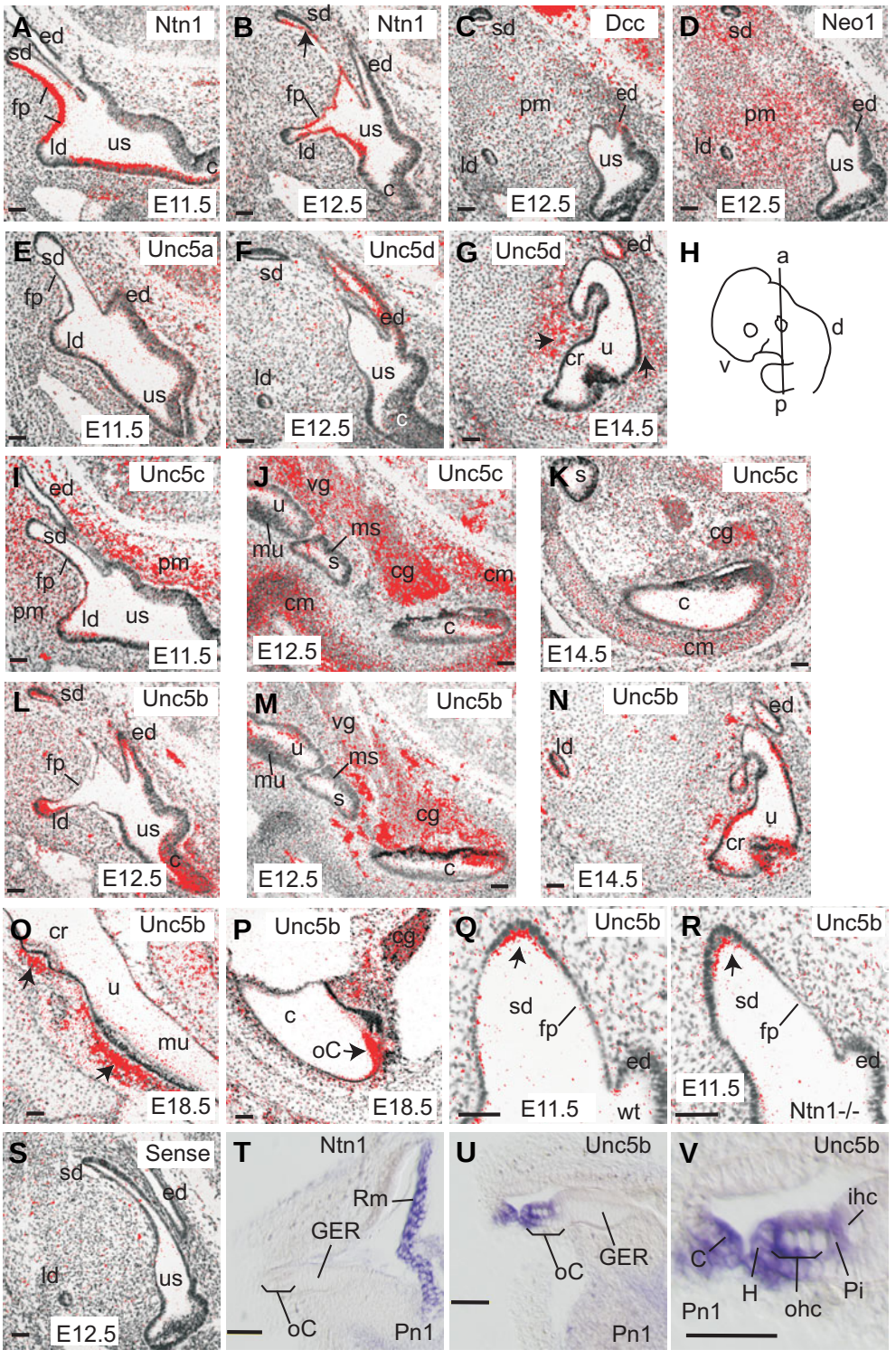

Fig.1. Expression of *Dcc* and *Unc5* family members in the inner ear. *Radioactive RNA* in situ *hybridisation analysis of the expression of* Ntn1 **(A,B),** Dcc **(C)**, Neo1 **(D)**, Unc5a **(E)**, Unc5d **(F,G)**, Unc5c **(I-K)** *and* Unc5b **(L-P)** *performed on serial paraffin sections from E11.5 (A,E,I), E12.5 (B-D,F,J,L,M), E14.5 (G,K,N) and E18.5 (O,P) wild type mouse embryos. Arrow in (B) points to* Ntn1 *expression in the inner edge of a newly formed semicircular duct. Arrows in (G) point to* Unc5d *expression in the loose mesenchyme surrounding the utricle. Arrows in (O) point to* Unc5b *expression beneath the sensory organs. Arrow in (P) points to the organ of Corti area. The plane of sections is indicated in* **(H)**. *Analysis of* Unc5b *expression in sections from wild type* **(Q)** *and* Ntn1*-/- littermate embryos* **(R)** *at E11.5 showing that* Unc5b *remains restricted to the tip of the outgrowing semicircular duct (arrows). The expression domains are visualized as computer superimpositions of bright-field views showing the hematoxyline stained serial sections and dark-field views revealing the signal grains in red false-colour. No specific signal was detected with the* Ntn1 *sense probe at E12.5* **(S)**. *Radial vibratome sections through the cochleas of one-day old (Pn1) mice analysed with DIG-labelled* Ntn1 **(T)** *or* Unc5b *probes* **(U,V)**. **(V)** *A higher magnification of the organ of Corti area in (U). Scale bars in (A-G) and (I-S) represent 50 μm; in (T-V) 20 μm. Abbreviations: c, cochlea; C, Claudius cells; cg, cochlear ganglion; cm, condensing mesenchyme; cr, crista; ed, endolymphatic duct; fp, fusion plate epithelium; GER, greater epithelial ridge; H, Hensen's cells; ihc, inner hair cells; ld, lateral semicircular duct; ms, macula of the saccule; mu, macula of the utricle; oC, organ of Corti; ohc, outer hair cells; Pi, Pillar cells; pm, periotic mesenchyme; Rm, Reissner's membrane; s, saccule; sd, superior semicircular duct; u, utricle; us, utriculo-saccular space; vg, vestibular ganglion; wt, wild type; a, anterior; p, posterior; v, ventral; d, dorsal.*

During embryonic development, *Dcc* was strongly expressed in hindbrain but only a weak expression could be detected in the inner ear at E12.5, in the tip of the endolymphatic duct (Fig. 1C and data not shown). In addition, no defects were observed in the ears (n=4) of *Dcc-/-* embryos (data not shown) strongly suggesting that Ntn1/Dcc signalling does not play a major role in inner ear morphogenesis.

The close relative to *Dcc*, *Neogenin* (*Neo1),* was strongly expressed all over the dorsal periotic mesenchyme at all stages analysed (E11.5-18.5) while no expression in the epithelium could be detected (Fig. 1D and data not shown). The expression of *Neo1* in the mesenchyme adjacent to the fusion plate basement membrane suggested that it could participate in Ntn1 dependent signalling to promote proliferation and/or survival of the mesen-

chyme. However, no morphological defects were observed in *Neo1* deficient inner ears (n=2) at E17.5 (data not shown) suggesting again that the Dcc family receptors do not play important morphogenetic roles during inner ear development.

Unc5 family members in the inner ear

While no significant expression of *Unc5a* was detected at E11.5-18.5 (Fig. 1E and data not shown) the three other Unc5-family members were expressed in developing inner ear in variable levels and locations. *Unc5d* was transiently expressed in the endolymphatic duct at E11.5-14.5 (Fig. 1F,G). At E12.5-18.5 expression was detected also in a subset of vestibular and cochlear ganglion cells (data not shown). Especially strong expression at E14.5 was observed in the loose mesenchyme surrounding the vestibule (Fig. 1G). These mesenchymal cells will be removed during E14.5-18.5 to generate the perilymphatic space between the membranous labyrinth and the bony otic capsule.

Unc5c showed a mainly mesenchymal expression in inner ear all through the development. It was already expressed in the mesenchyme surrounding the otic vesicle at E10.5 as well as in a small dorso-lateral domain of the vesicle epithelium (data not shown). At E11.5-14.5 strongest expression was detected in the periotic mesenchyme and the vestibular and cochlear ganglia (Fig. 1I-K). The mesenchymal expression became especially prominent during otic capsule condensation suggesting a role in this process (Fig. 1J,K). In otic epithelium, strongest expression was observed in the distal end of the lateral semicircular duct (Fig. 1I). Weak expression could also be detected in the distal parts of the superior and posterior ducts and the thin non-sensory lateral wall of the cochlear duct (Fig. 1I-K and data not shown). No expression could be observed anymore in the inner ear at E18.5 (data not shown). In spite of the strong mesenchymal expression, no defects in inner ear morphology could be observed in *Unc5c-/-* embryos (n=2) at E18.5 (data not shown).

From all Unc5 receptors, *Unc5b* showed the broadest expression pattern. *Unc5b* was detected at E10.5 in dorso-lateral and ventral parts of the otic vesicle as well as in the endolymphatic duct outgrowth (data not shown). At E11.5-12.5 *Unc5b* was strongly expressed in the distal parts of the outgrowing semicircular duct plates and remained there in the newly formed semicircular ducts until E14.5 (Fig. 1L,N and data not shown). Also in the endolymphatic duct, *Unc5b* expression was detected until E14.5 (Fig. 1N).

Unc5b was the only Ntn1 receptor expressed in the otic sensory epithelia. Strong expression was observed in developing semicircular duct cristae at E12.5-14.5 (Fig. 1N and data not shown) whereas no expression could be detected in the sensory areas of the saccule and the utricle (Fig. 1M,O). Instead, *Unc5b* was expressed in non-sensory parts of the utricle (Fig. 1M,N) as well as in mesenchymal cells underlying the different sensory epithelia of the vestibule (arrows in Fig. 1O). In the cochlea, *Unc5b* expression was confined to the medial pre-sensory epithelium and the adjacent mesenchyme at E12.5-14.5 (Fig. 1L,M) and at E18.5-Pn1 strong expression was detected in the hair and supporting cells of the organ of Corti as well as in the neighbouring Hensen's and Claudius cells (Fig. 1P,U,V). Strong expression of *Unc5b* was also observed in vestibular and cochlear ganglion cells (Fig. 1M,P).

Interestingly, at all stages analysed, the expression of *Unc5b*

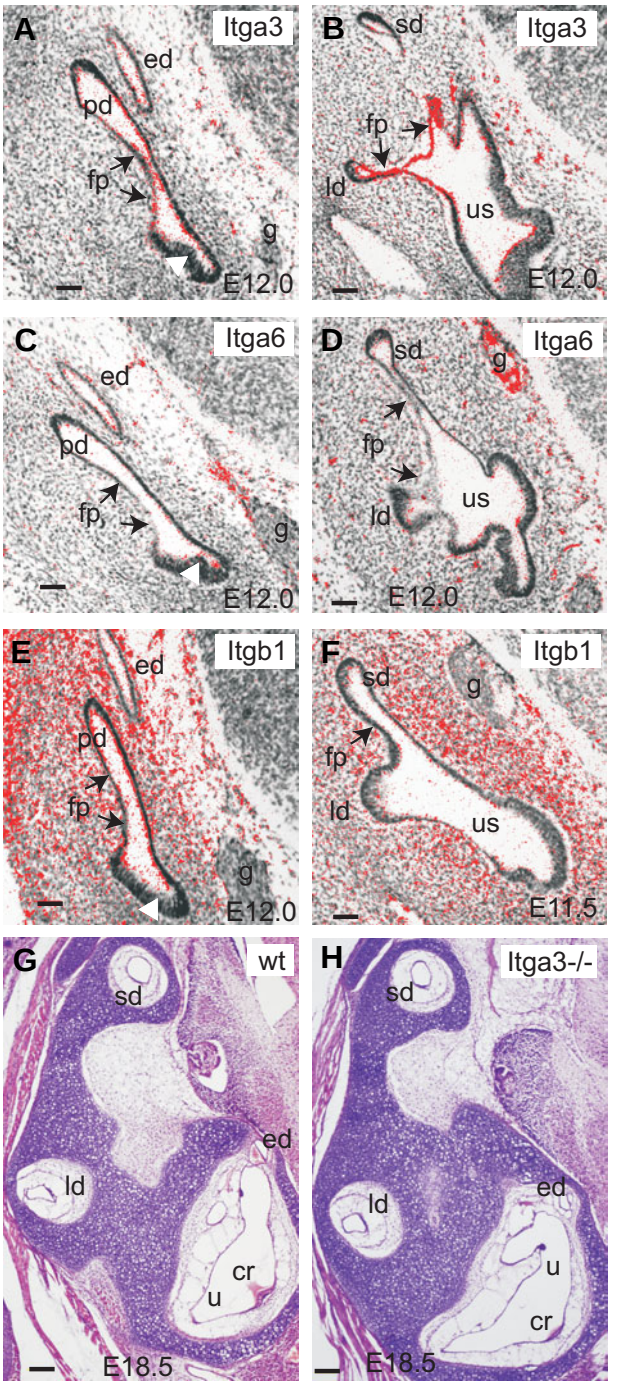

Fig. 2. Integrin subunits in the inner ear. *RNA* in situ *hybridisation analysis of* Itga3 **(A,B)**, Itga6 **(C,D)** *and* Itgb1 **(E,F)** *expression on sections from E12.0 (A-E) and E11.5 (F) mouse embryos. Arrows point to the fusion plate areas where* Itga3 *and* Itgb1 *signals could be detected. White arrowheads point to the thick presensory areas where* Itga6 *and* Itgb1 *were weakly expressed. Hematoxylene-eosin stained paraffin sections from E18.5 wild type* **(G)** *and* Itga3-/- **(H)** *littermates showing that no obvious differences were detectable in inner ear structures between the genotypes. Plane of sections is as in Fig. 1H and scale bar represents 50 μm. cr, crista ampullaris; ed, endolymphatic duct; fp, fusion plate; g, ganglion; ld, lateral semicircular duct; pd, posterior semicircular duct; sd, superior semicircular duct; u, utricle; us, utriculo-saccular space.*

was strikingly complementary to *Ntn1* and thus, the reduction of Ntn1 levels in *Ntn1-/-* embryos could cause an extension of *Unc5b* expression into neighbouring areas. We compared *Unc5b* expression in *Ntn1-/-* embryos (n=3) and wild type littermates at E11.5-12.5 and could not detect any alteration in expression level or distribution. In the outgrowing semicircular ducts, *Unc5b* was restricted to the tips whereas no expression could be detected in the fusion plate forming areas both in wild type and *Ntn1-/-* embryos (Fig. 1Q,R and data not shown). This showed that the *Ntn1-/-* ear phenotype is not derived from an extension of *Unc5b* expression into the fusion plate.

The *Unc5b* expression analysis suggested specific roles for this receptor in inner ear sensory development and especially in the developing auditory system. Since no defects have been observed in *Ntn1-/-* sensory development (Salminen *et al.,* 2000) Unc5b could play Ntn1 independent roles in these areas. Unfortunately, *Unc5b* deficient mouse embryos die too early to enable conclusive analysis of inner ear sensory development (Lu *et al.,* 2004).

Integrins in the inner ear

Since none of the Dcc or Unc5 receptors were co-expressed with *Ntn1* in the semicircular duct fusion plate epithelia, Ntn1 could function through other receptors or mechanisms to induce changes in epithelial morphology and to promote detachment of the fusion plate epithelium from the underlying basement membrane. Integrins are heterodimeric cell surface receptors consisting of α and β subunits that interact with various extracellular matrix molecules such as laminins, collagens and fibronectin (Reviewed in Kreidberg, 2000). Recently, integrins $\alpha6\beta4$ and $\alpha3\beta1$ have also been shown to bind Ntn1 (Yebra *et al.,* 2003) and all these subunits are produced in inner ear at some developmental stages (Davies and Holley, 2002).

We verified the expression of *Itga3*, *Itga6*, *Itgb1* and *Itgb4* during semicircular duct formation at E11.5-12.5 to identify potential Ntn1 interacting subunits. *Itga3* expression could be detected all over the developing non-sensory vestibular epithelium at E11.5-12.5 (Fig. 2A,B and data not shown). An especially prominent expression was observed in the fusion plate forming epithelia (arrows in Fig. 2A,B). Instead, no expression was detected in the developing sensory areas (white arrowhead in Fig. 2A).

Itga6 was weakly expressed in the tips of the outgrowing semicircular ducts and in the thickened presensory epithelia of the utricle, saccule and cochlea as well as in the outermost cells of the vestibulocochlear ganglion (Fig. 2C,D and data not shown). No expression of *Itga6* in the fusion plate epithelium could be observed (arrows in Fig. 2C,D). *Itgb1* was expressed all over the otic area including the fusion plate epithelium and the periotic mesenchyme (Fig. 2E,F). In contrast, no expression of *Itgb4* could be detected (data not shown).

Our expression analysis showed that integrin $\alpha3\beta1$ was the only Ntn1 receptor expressed in the fusion plate epithelium and it could thus play a role in controlling the interactions between the fusion plate and the underlying basement membrane together with Ntn1. Integrin $\alpha3\beta1$ is highly expressed also in many other epithelial cells and it is known to bind mainly to laminin-5 and thought to be involved in organising and stabilising the basement membrane. Inactivation of *Itga3* causes a loss of $\alpha3\beta1$, which leads to a thinner and disrupted basement membrane in several organs such as kidney, lung, skin and submandibular gland (Reviewed in Kreidberg, 2000). Ntn1 interaction with integrin $\alpha3\beta1$ could in fact cause a destabilisation of the integrin-laminin interactions and in this way lead to the detachment of the epithelium from the basement membrane.

To verify whether the inactivation of *Itga3* caused developmental defects in semicircular duct formation we prepared histological sections of E18.5 *Itga3-/-* (n=3) and control littermate embryos. In all analysed embryos, the semicircular duct formation and growth had occurred normally (Fig. 2G,H) suggesting that Ntn1 can function in fusion plate detachment without integrin $\alpha3\beta1$.

Adhesion defects have been observed when Ntn1 receptors are missing (*Neo1-/-* mammary glands) or functionally blocked (integrins in pancreatic cell cultures) (Srinivasan *et al.,* 2003; Yebra *et al.,* 2003). No loss of adhesion has however been observed during *Ntn1-/-* inner ear development and the adhesion mediating Ntn1 receptors, Neo1 (Srinivasan *et al.,* 2003) and integrin $\alpha6\beta4$ (Yebra *et al.,* 2003) are not present on inner ear epithelium. Instead, we have suggested that high amounts of Ntn1 protein could be required locally for the detachment of the basement membrane underlying the semicircular duct fusion plate epithelium (Salminen *et al.,* 2000). Whether there is a yet unknown «detachment mediating receptor» for Ntn1 in inner ear remains to be identified. Alternatively, Ntn1 may function in a receptor-independent manner and interfere directly with the laminin network (Hedgecock and Norris, 1997; Salminen *et al.,* 2000). Since the other epithelial organs where Ntn1 plays a morphogenetic role do not undergo any fusion events, Ntn1 may play a specific role in inner ear. Interestingly, also other axon guidance cues such as EphB2 and ephrin-B2 have a very specific role in inner ear vestibule, where they control endolymph fluid production (Cowan *et al*., 2000; Dravis *et al.,* 2007). Thus, both Ntn1 and Eph/ephrin systems critically participate in fine-tuning inner ear morphogenesis. How these factors integrate into distinct regulatory networks controlling inner ear morphogenesis (Reviewed in Fritzsch *et al.,* 2006) is however currently unknown.

Experimental procedures

Mouse breeding and genotyping

The breeding of *Ntn1+/-* and *Itga3-/-* mice and their genotype determination from genomic DNA were performed as before (Kreidberg *et al.,* 1996; Salminen *et al.,* 2000). The embryos were taken from timed matings between two *Ntn1+/-* or *Itga3+/-* mice and the day on which a vaginal plug was detected was assigned as 0.5. All animal experiments were approved by the Committee of experimental animal research of the University of Helsinki.

RNA in situ *hybridization*

For radioactive RNA *in situ* hybridization on paraffin sections, mouse embryos from time-pregnant females were treated and sectioned as described before (Salminen *et al.,* 2000; Lilleväli *et al.,* 2004). The preparation of ^{35}S labelled riboprobes and the RNA *in situ* hybridization were carried out as described previously (Salminen *et al.,* 2000). Images where the red colour represents mRNA expression were produced as before (Lilleväli *et al.,* 2004). Non-radioactive RNA *in situ* hybridisations on dissected whole cochleas from postnatal mice were performed using digoxigenin-UTP (Roche) labelled RNA probes (Wilkinson, 1993) and the stained cochleas were cut radially with vibratome.

The cDNA fragments used to generate *Ntn1*, *Dcc*, *Neo1*, *Unc5a*, *Unc5b* and *Unc5c* probes have been described before (Keino-Masu *et al.,*

1996; Serafini *et al.,* 1996; Ackerman *et al.,* 1997; Leonardo *et al.,* 1997). Complementary DNA fragments for five additional probes were generated with reverse transcription polymerase chain reaction (RT-PCT) from E12.5 mouse embryonic total RNA and cloned into pGEM-TEasy vector (Promega). The following oligonucleotide primers were used for RT-PCR:

Unc5d
forward: 5'-GAACAGCATCAATAGGAATTTATCCG-3' and
reverse: 5'-CATATAATAAGTGCTGGTGTCATGTAC-3'.

Integrinα3
forward 5'-CTGGAATCTCCTTCAAGACCTTG-3' and
reverse 5'-CTACCATCAACATGGAGAACAAG-3',

Integrinα6
forward 5'-GAGCAAGCTATGAAATCTGAAGAT G-3' and
reverse 5-'CTCTGGCTGCAGGAGAACATCAG-3',

Integrinβ1
forward 5'-GTGCACAGGAGTGCTCCCACTTC-3' and
reverse 5'-GCACACTGTGACCTCAGCAGAC-3',

Integrinβ4
forward 5'-GGTCCAGGAAGATCCATTTCAACT G-3' and
reverse 5'-CATTGGCTGCAGTCATTCTGTGC-3'.

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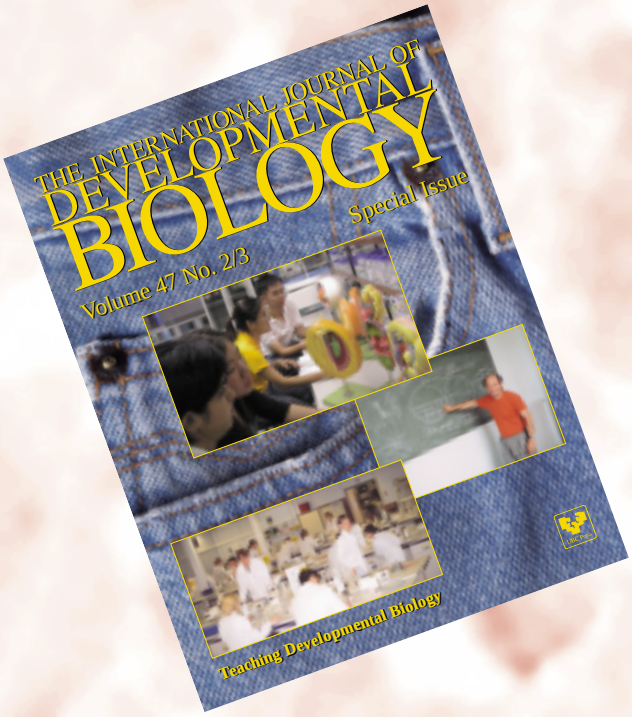

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