

## RESEARCH ARTICLE

# Distinct functions for netrin 1 in chicken and murine semicircular canal morphogenesis

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## ABSTRACT

The vestibular system of the inner ear detects head position using three orthogonally oriented semicircular canals; even slight changes in their shape and orientation can cause debilitating behavioral defects. During development, the canals are sculpted from pouches that protrude from the otic vesicle, the embryonic anlage of the inner ear. In the center of each pouch, a fusion plate forms where cells lose their epithelial morphology and the basement membrane breaks down. Cells in the fusing epithelia intercalate and are removed, creating a canal. In mice, fusion depends on the secreted protein netrin 1 (Ntn1), which is necessary for basement membrane breakdown, although the underlying molecular mechanism is unknown. Using gain-of-function approaches, we found that overexpression of *Ntn1* in the chick otic vesicle prevented canal fusion by inhibiting apoptosis. In contrast, ectopic expression of the same chicken *Ntn1* in the mouse otic vesicle, where apoptosis is less prominent, resulted in canal truncation. These findings highlight the importance of apoptosis for tissue morphogenesis and suggest that *Ntn1* may play divergent cellular roles despite its conserved expression during canal morphogenesis in chicken and mouse.

**KEY WORDS:** Netrin 1, Inner ear, Canal morphogenesis, Vestibular system, Apoptosis, Fusion

## INTRODUCTION

Morphogenesis of a complex tissue is achieved by precise coordination of many cellular events. A stunning example is the formation of the inner ear, which develops from a simple sphere of epithelium known as the otic vesicle into an elaborate fluid-filled labyrinth featuring the cochlea of the auditory system, as well as the semicircular canals and macular chambers of the vestibular system. Semicircular canal formation requires particular accuracy and reliability, as the proper orthogonal orientation of the anterior, posterior and lateral canals is necessary for the perception of head position. Even slight changes in canal structure cause severe vestibular defects, including vertigo, dizziness and abnormal posture (Sando et al., 2001).

In amniotes, including chickens and mice, semicircular canals form from two epithelial outpocketings, or canal pouches, that

protrude from the dorsal region of the otic vesicle (Alsina and Whitfield, 2016). The vertical canal pouch gives rise to the anterior and posterior canals, while a separate lateral canal pouch generates the lateral canal. The pouches are transformed into hollow canals through controlled fusion of opposing epithelial walls at discrete sites called fusion plates. During this process, fusion plate cells lose their epithelial morphology, and the underlying basement membrane breaks down. The opposing epithelial layers then meet and fuse into a single layer that is cleared away, ultimately leaving a toroidal-shaped canal (Martin and Swanson, 1993). Although fusion is observed in all amniotes, it is unclear whether the same cellular mechanisms are employed across species. For example, apoptosis is required for fusion in chickens (Fekete et al., 1997), but does not appear to play a prominent role in mice or humans (Ceconi et al., 2004; León et al., 2004; Nishikori et al., 1999).

A key regulator of canal formation in mice is the laminin-related molecule netrin 1 (Ntn1), which plays diverse roles in axon guidance, survival, local cell adhesion and basement membrane integrity (reviewed by Cirulli and Yebra, 2007). During murine inner ear development, *Ntn1* is expressed at the fusion plate prior to and during fusion, and its expression is maintained in the canal rim after fusion (Abraira et al., 2008; Rakowiecki and Epstein, 2013; Salminen et al., 2000). In *Ntn1* mutants, the basement membrane remains intact at the fusion plate, and fusion fails to occur (Salminen et al., 2000). Complementarily, loss of the immunoglobulin superfamily protein *Lrig3* causes an expansion of the *Ntn1* expression domain in the lateral pouch, leading to early and ectopic basement membrane breakdown, expanded fusion and hence a truncated canal (Abraira et al., 2008). Removing one copy of *Ntn1* is sufficient to rescue the canal truncation in *Lrig3* mutants, suggesting that the phenotype is indeed caused by ectopic *Ntn1*. Moreover, changes in basement membrane breakdown and fusion correlate with changes in *Ntn1* expression in other mouse models (Hurd et al., 2012; Rakowiecki and Epstein, 2013), emphasizing the tight link between Ntn1 expression and canal morphogenesis.

As a highly multifunctional molecule, Ntn1 could regulate semicircular canal fusion in a number of ways. Netrin proteins are related to the laminins and localize to the basement membrane (Yurchenco and Wadsworth, 2004); in worms, *UNC-6*, the homolog of *Ntn1*, is required for loss of basement membrane integrity during anchor cell invasion in vulval development (Ziel et al., 2009). Ntn1 has also been shown to ensure adhesion between adjacent cell layers in the development of multiple organ systems (Srinivasan et al., 2003; Yebra et al., 2003). Finally, there is evidence suggesting that Ntn1 can serve as a trophic factor by blocking activation of dependence receptors such as deleted in colorectal cancer (Dcc) and Unc5 family members (Furne et al., 2008; Llambi et al., 2001; Mazelin et al., 2004; Newquist et al., 2013), although Ntn1 is not required for cell survival *in vivo* in mice (Bin et al., 2015; Williams et al., 2006; Yung et al., 2015). By acting

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on any of these processes, Ntn1 could directly influence the initiation of fusion, the act of fusion itself or the completion of fusion concomitant with cell clearance. However, despite its clear importance for canal formation in mice, the cellular mechanism by which Ntn1 regulates fusion and basement membrane integrity is still unknown.

To elucidate the primary function of Ntn1 during canal morphogenesis, we compared the effects of Ntn1 overexpression across species. Here, we confirm that expression of *Ntn1* in the fusion plate is conserved in chickens, and show that overexpression of chicken *Ntn1* (*cNtn1*) in chicks arrests fusion by interfering with apoptosis. This phenotype contrasts with the effects of ectopic expression of *cNtn1* in mice using a novel conditional expressor allele, which results in expanded fusion and thus canal truncation. Together, these findings provide new evidence supporting a role for Ntn1-sensitive apoptosis during tissue morphogenesis *in vivo* and highlight how the same molecule can influence divergent cellular processes during the development of conserved structures.

## RESULTS

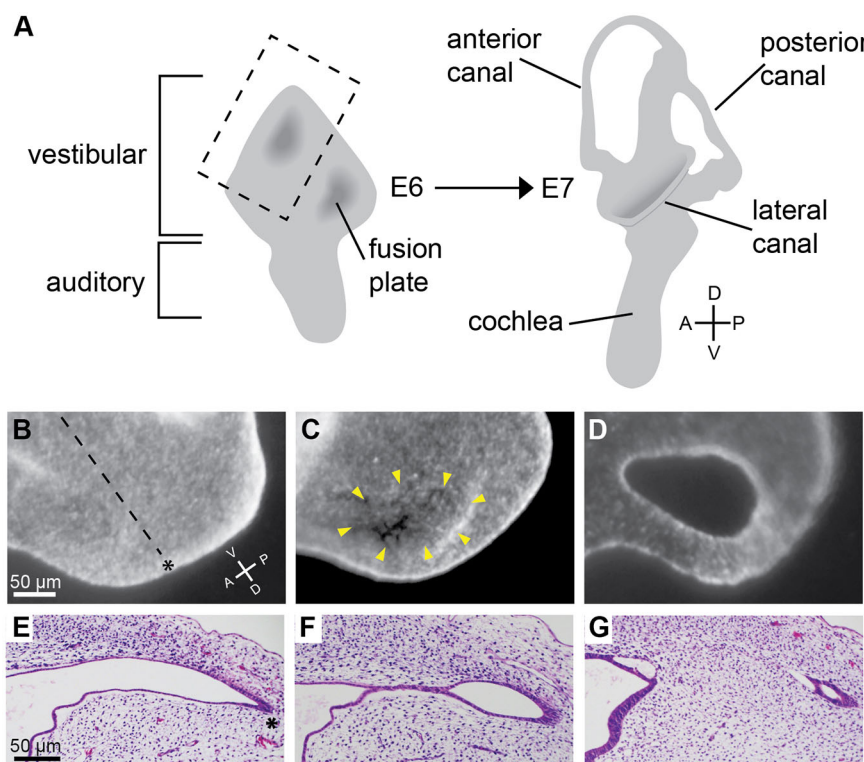
### Apoptosis precedes basement membrane breakdown in sites where netrin orthologs are expressed during canal morphogenesis in chicks

Although the cochlear duct varies widely in size and shape across species, semicircular canal morphology is strikingly conserved, with bony fish, amphibians, reptiles, birds and mammals all exhibiting three hollow canals grossly oriented in three dimensions of space (Baird, 1974). Embryological studies suggest that the overall sequence of canal development is the same across species, but significant differences in the underlying cellular mechanisms have also been observed (Bissonnette and Fekete, 1996; Haddon and Lewis, 1991; Martin and Swanson, 1993; Morsli et al., 1998; Streeter, 1906; Waterman and Bell, 1984). To better understand the cellular events that create canals in different species, we analyzed

canal development in chickens, which diverged from mice ~300 million years ago (Hedges, 2002).

Paint-filling and histological analysis of thin sections confirmed that the basic steps of semicircular canal formation in chicks closely mimic those of the mouse (Fig. 1), consistent with previous reports (Bissonnette and Fekete, 1996; Fekete et al., 1997; Martin and Swanson, 1993). The majority of canal morphogenesis occurs between embryonic days 6 and 7 (E6-7), which corresponds to Hamburger-Hamilton (HH) stage 27-30 (Bissonnette and Fekete, 1996; Hamburger and Hamilton, 1992) (Fig. 1A). By E6 (HH28-29), the vestibular components of the inner ear are discernable: the vertical and lateral canal pouches are present, and the opposing epithelial walls of the canal pouch are separated by a visible fluid-filled space (Fig. 1B,E). Next, a fusion plate forms in the center of the canal pouch where the opposing epithelial walls come together and fuse into a single layer (Fig. 1C,F). By E7 (HH30), the cells of the fusion plate have been removed such that cells at the edge of the fusion plate have re-formed an intact epithelial tube and mesenchymal cells occupy the location of the former fusion plate (Fig. 1D,G).

Although epithelial fusion is a common feature of canal morphogenesis, there is evidence that different cellular mechanisms may be used to achieve the same outcomes in different species. For example, apoptosis is prominent at the fusion plate in chicks but not in mice (Fekete et al., 1997; Lang et al., 2000; Nishikori et al., 1999; Rakowiecki and Epstein, 2013), yet in both species, fusion is accompanied by a loss of basement membrane integrity (Kobayashi et al., 2008; Martin and Swanson, 1993). Curiously, when apoptosis was blocked by overexpression of *Bcl2* in the developing chicken inner ear, abnormally long fusion plates were observed where the opposing epithelial layers had come together, but had neither fused nor been cleared, leading to the suggestion that apoptosis may actually initiate basement membrane breakdown (Fekete et al., 1997). Conversely, cell detachment from the basement membrane may



**Fig. 1. Chicken semicircular canals form through fusion.** (A) A schematic of canal morphogenesis. By E6 (left), the vestibular and auditory regions of the inner ear are apparent, and the fusion plates are beginning to form (darker gray). Fusion is completed by E7 (right), creating the anterior, posterior and lateral semicircular canals of the mature vestibular system. (B-D) Higher magnification regions of the vertical canal pouch outlined in A in E6-E6.25 paint-filled ears ( $n=20$  ears). The dashed line indicates the plane of section in E-G, with the canal rim marked by an asterisk. (E-G) Hematoxylin and Eosin staining of sections through E6-E6.25 anterior canals ( $n=18$  animals). Canal formation starts with a canal pouch, with opposing epithelial walls of the pouch separated by a fluid-filled space (B,E). A fusion plate then forms in the center of the pouch (C, arrowheads), where the epithelial walls come together and form a single layer (F). Finally, the fusion plate is cleared (D), and mesenchymal cells occupy the area where the fusion plate was (G). The asterisk in E corresponds to the same region of the canal pouch marked by an asterisk in B. Scale bars: 50  $\mu\text{m}$ .

trigger apoptosis, an event called anoikis (Chiarugi and Giannoni, 2008), which raises the alternative possibility that apoptosis at the fusion plate in chicks occurs as a result of changes in basement membrane integrity.

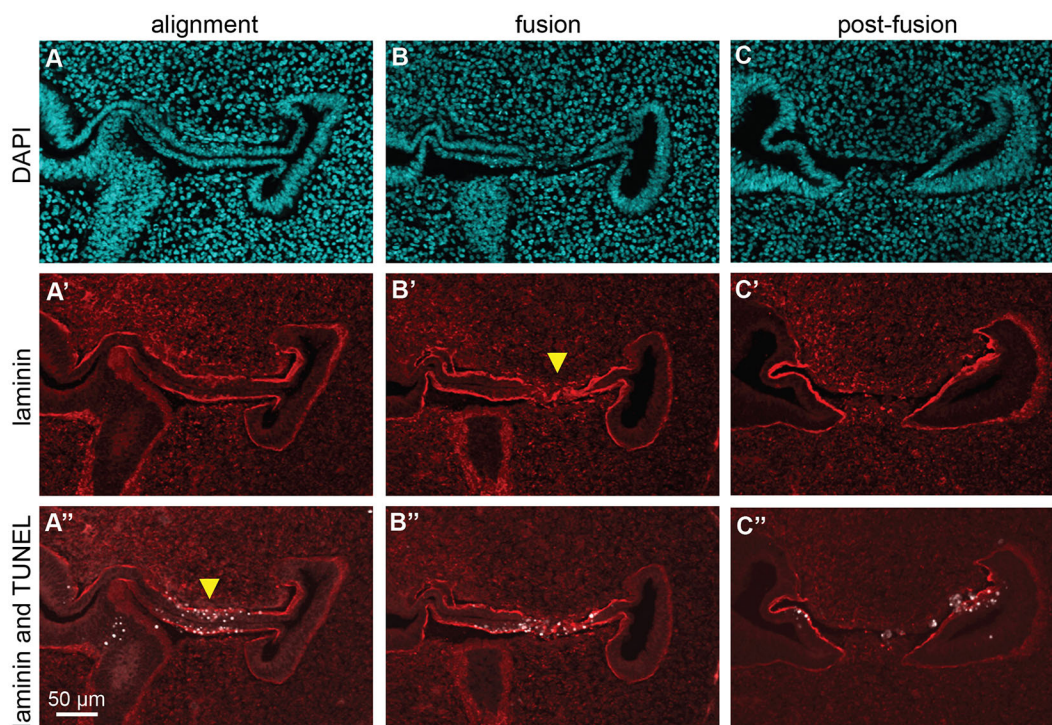
To gain insight into the relationship between basement membrane breakdown and apoptosis in the chick inner ear, we immunostained transverse sections of E6.25 (HH29–30) wild-type chick heads using the TUNEL assay and laminin antibodies ( $n=12$  animals) (Fig. 2). Owing to slight differences in the rate of overall development and the rapid progression of fusion during canal development, analysis at this stage allowed us to compare patterns of cell death and basement membrane breakdown before, during and after fusion at the same embryonic time point. We observed widespread cell death at the fusion plate and in the newly formed inner canal rim, as previously reported (Fekete et al., 1997; Lang et al., 2000). In addition, we found that cell death is present in the epithelium before fusion occurs and prior to the loss of basement membrane integrity (Fig. 2A–A''). Thus, apoptosis precedes breakdown of the basement membrane in chickens. This contrasts with mice, where apoptosis does not seem to be required for canal formation (Ceconi et al., 2004).

Given the apparent differences in how fusion occurs in chicken and mouse, we asked whether there might also be differences in the expression of known molecular regulators. In the mouse inner ear, localization of the extracellular protein Ntn1 is a major determinant of when and where basement membrane breakdown occurs during canal formation (Abraira et al., 2008; Salminen et al., 2000). In chickens, there are two netrin family members, *cNtn1* and *cNtn2*,

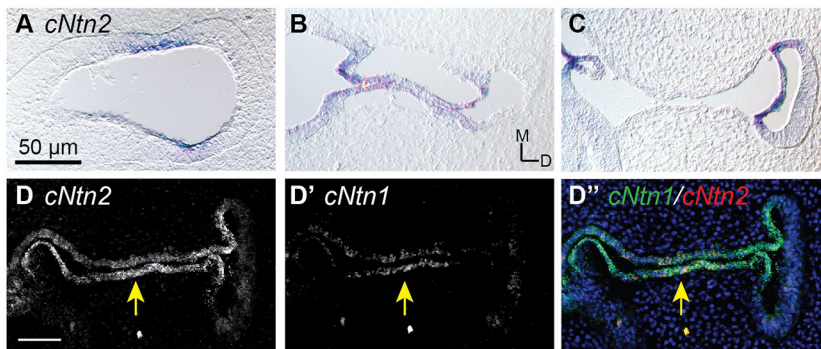
the combined expression of which equals that of *Ntn1* in mammals (Serafini et al., 1996). Previously, we showed that both *cNtn1* and *cNtn2* are expressed in the fusion plate (Abraira et al., 2010). To better define the spatiotemporal pattern of netrin expression, we used *in situ* hybridization to characterize *cNtn1* and *cNtn2* expression in transverse sections of embryonic chick heads. *cNtn2* was present in the presumptive fusion plate before it formed (Fig. 3A) ( $n=3$  ears), during fusion (Fig. 3B) ( $n=5$  ears) and then maintained in the inner canal rim after fusion was complete (Fig. 3C) ( $n=3$  ears). Double *in situ* hybridization shows that *cNtn2* overlaps with low levels of *cNtn1* in the fusion plate when the epithelia are aligned (Fig. 3D–D''). This time course is remarkably similar to the pattern of apoptosis during canal fusion (Fekete et al., 1997) (Fig. 2), with both *cNtn1* and *cNtn2* present where TUNEL signal is high prior to breakdown of the basement membrane. Thus, netrins are poised to influence the onset of basement membrane breakdown during canal formation in chicks, similar to mice (Abraira et al., 2008; Salminen et al., 2000).

### Overexpression of *cNtn1* in chick inner ears blocks fusion during canal formation

Given the similarities in netrin expression during canal formation in mouse and chicken, one simple model is that netrin plays the same core function in both species, despite the differing involvement of apoptosis. Because the two netrin genes are co-expressed in the canal pouches, we assayed netrin function through overexpression experiments, as complete loss of function would be hard to achieve in chickens, especially given the known potency of netrin, where



**Fig. 2. Apoptosis precedes basement membrane breakdown during canal fusion.** Immunostains of E6.25 sections ( $n=12$  animals) through the posterior canal before, during and after fusion. Because fusion occurs so quickly and individual eggs develop at slightly different rates, all stages of fusion can be captured from the same embryonic time point. DAPI (cyan) labels all cell nuclei (A–C). Laminin (red) labels the basement membrane (A'–C') whereas TUNEL (white) identifies apoptotic cells (A'–C''). Prior to fusion, when opposing epithelial walls are aligned but still distinct (A), apoptotic cells are already detectable in the epithelium that will form the fusion plate (A'', arrowhead), although the basement membrane is still intact (A'). During fusion, the opposing epithelial layers fuse into a single layer (B). This event is accompanied by basement membrane breakdown (B', arrowhead) and cell death (B''). Upon completion of fusion, the epithelial cells of the fusion plate are absent (C) and the basement membrane has re-formed around the inner rim of the new canal epithelium (C'). There is some cell death in the inner rims of the canal (C''). Scale bar: 50  $\mu\text{m}$ .

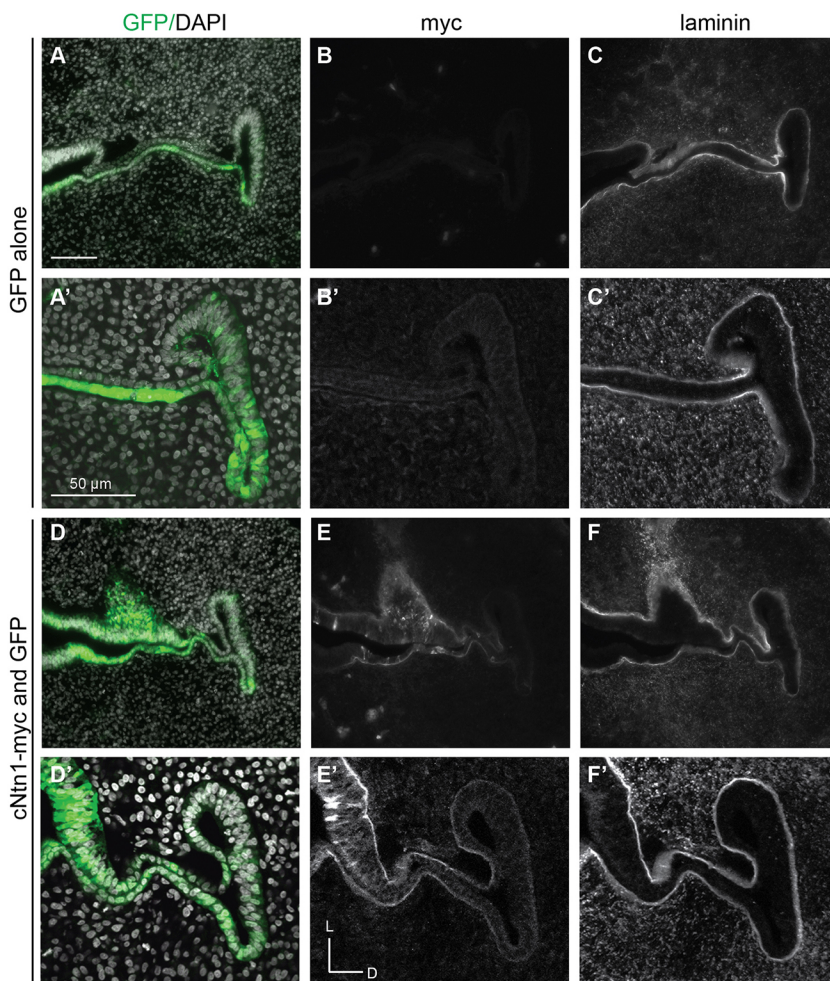


**Fig. 3. Netrins are expressed at the fusion plate in chicks.** (A-C) *In situ* hybridizations for chicken *cNtn2* on E5 (A), E6 (B) and E6.25 (C) sections through the emerging posterior canal. *cNtn2* is expressed in the presumptive fusion plate from an early age (A, E5 shown,  $n=3$  animals), and this expression pattern is maintained during (B,  $n=5$  animals) and after (C,  $n=3$  animals) fusion in the canal rim. (D,D') Double fluorescent *in situ* hybridization for *cNtn1* (D') and *cNtn2* (D) confirmed co-expression in the center of the pouch (arrows) before fusion has begun (E6-6.25,  $n=3$  animals). A merged image is shown in D''. *cNtn1* is present at lower levels than *cNtn2*. Scale bars: 50 μm.

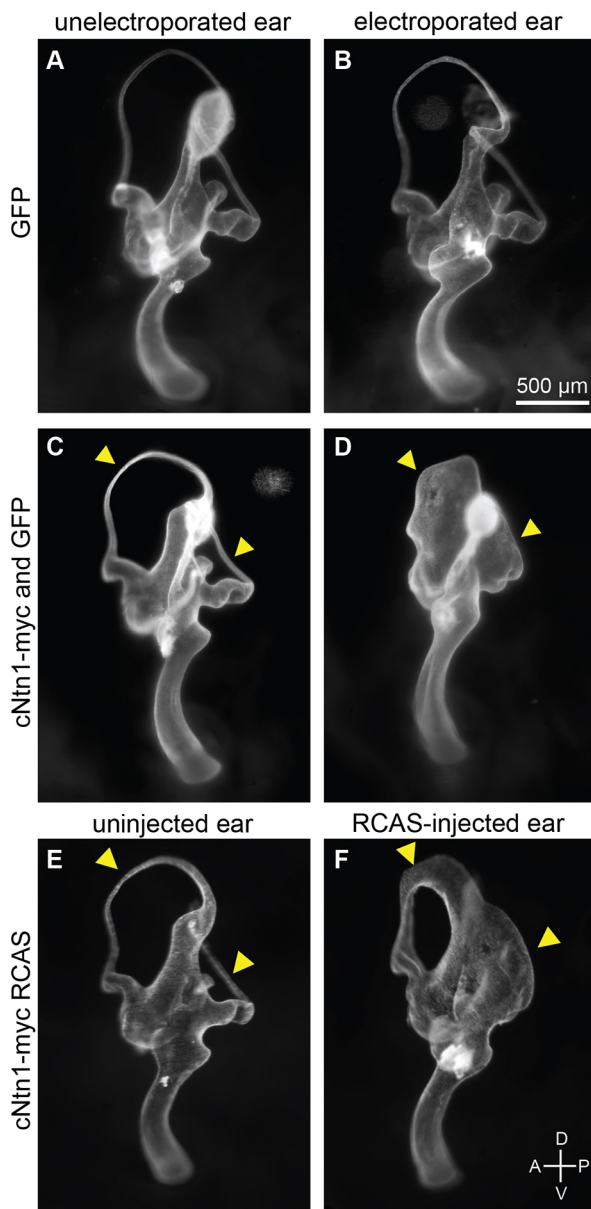
even a few molecules can elicit effects on guidance (Bin et al., 2015; Pinato et al., 2012; Yung et al., 2015). We electroporated either a GFP plasmid alone (control) or the GFP plasmid and a *cNtn1-Myc* construct *in ovo* at E2 (HH14-16) to drive overexpression in the dorsal otic epithelium of the right otic vesicle prior to canal fusion. This Myc-tagged Ntn1 protein has previously been shown to be biologically active in *in vitro* axon outgrowth assays (Serafini et al., 1994). Immunostaining transverse sections of chick inner ears at E6.25 (HH29-30) revealed that the majority of cells in the epithelia of all three canals had been electroporated, reflected by GFP expression (Fig. 4A,A',D,D') ( $n=7$  animals). Furthermore, whereas there was no detectable Myc signal in ears electroporated with GFP alone (Fig. 4B,B') ( $n=2$ ), the *cNtn1-Myc*-electroporated cells

successfully produced and secreted *cNtn1-Myc*, which accumulated together with laminin in the basement membrane (Fig. 4E-E') ( $n=5$ ).

To evaluate the effects of excess netrin on gross canal morphology, we electroporated the otic vesicle at E2 (HH14-16) and then paint-filled the inner ears at E7 (HH30), when canal fusion is normally complete. As expected, electroporation of GFP plasmid alone did not affect canal formation in any animal (Fig. 5A,B) ( $n=6$  animals). However, when *cNtn1-Myc* and GFP plasmids were both delivered, canal formation was disrupted every time (Fig. 5C,D) ( $n=8$  animals). Unexpectedly, the primary defect was an inhibition of fusion, ranging from a complete absence of fusion that resulted in the retention of canal pouches ( $n=16/24$  canals), to an arrest or delay



**Fig. 4. *In ovo* electroporation results in broad expression of cNtn1-Myc and GFP throughout the developing canal.** *In ovo* electroporation was performed in HH stage 14-16 embryos to drive expression of either GFP alone ( $n=2$  animals) or both GFP and *cNtn1-Myc* ( $n=5$  animals) in the dorsal otic vesicle. Immunostains of tissue sections through E6.25 posterior canals are shown in A-C and D-F, with higher power images in A'-C' and D'-F'. (A,A',D,D') DAPI (white) and GFP (green) identify cell nuclei and electroporated cells, respectively. (B,B',E,E') Immunostaining for Myc shows positive staining only in ears that have been co-electroporated with *cNtn1-Myc* (E,E'). (C,C',F,F') Laminin staining highlights the basement membrane, where *cNtn1-Myc* accumulates. Scale bars: 50 μm.



**Fig. 5. Overexpression of cNtn1-Myc inhibits canal fusion.** Paint-fills of E7 chick inner ears. (A,B) No differences in gross morphology were detected between the unelectroporated control left ear (A) and the GFP-electroporated right ear ( $n=6$  animals). (C,D) Compared with the unelectroporated control left ear (C), the *cNtn1-Myc*- and GFP-electroporated ears display a failure of fusion, resulting in either fatter canals (not shown) or the persistence of the canal pouches (C,D, arrowheads,  $n=8/8$  animals). (E,F) Overexpression of cNtn1-Myc using the RCAS virus system also arrested canal fusion ( $n=20/25$  animals). Arrowheads indicate canals. Scale bars: 500  $\mu\text{m}$ .

in fusion that resulted in fat canals ( $n=8/24$  canals). Similar results were obtained when a replication-competent avian retrovirus (RCAS) was used to deliver the same *cNtn1-Myc* construct to the developing ear at E3 (HH17) (Fig. 5E,F) ( $n=20/25$  animals). Thus, in contrast to what was predicted from mouse genetic studies, *cNtn1* overexpression inhibited fusion in the chick.

#### Overexpression of cNtn1 in chick inner ears leads to a decrease in apoptosis

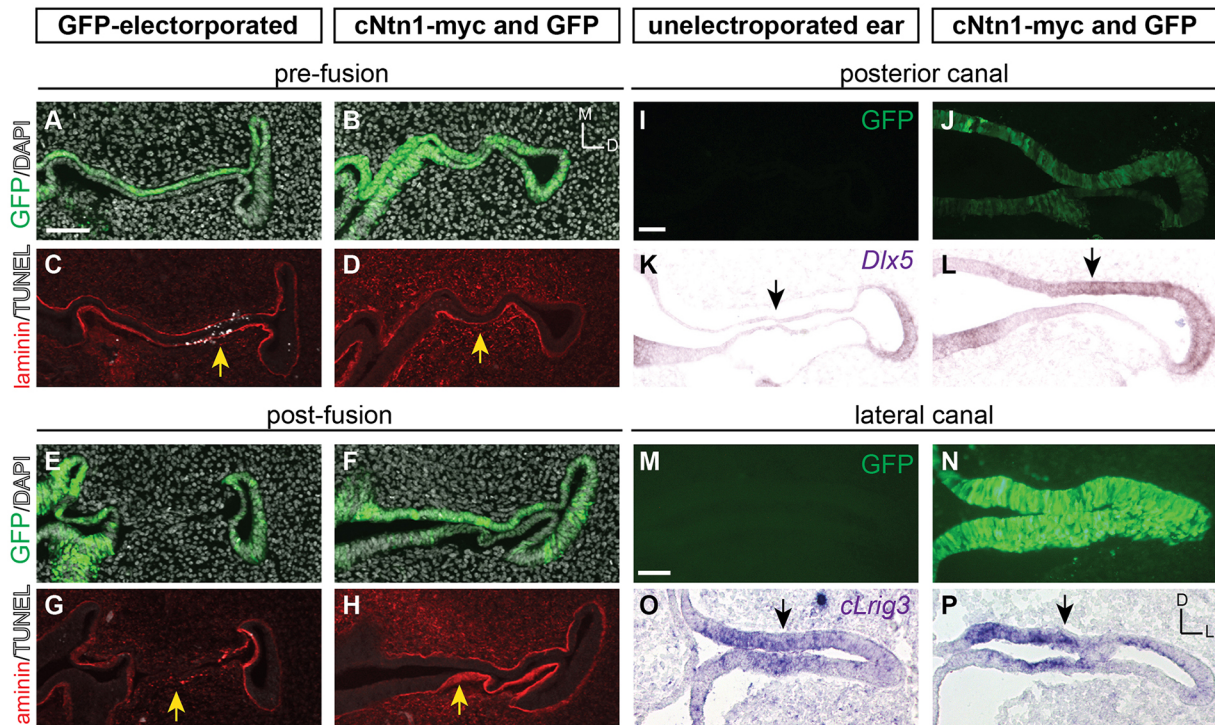
The effect of cNtn1-Myc overexpression is strikingly similar to what was observed when apoptosis was blocked by overexpression of

*Bcl2* in chicks (Fekete et al., 1997). As apoptosis during canal formation is more prominent in chickens than in mice, we wondered whether Ntn1 might have distinct roles in each species. We therefore investigated how overexpression of *cNtn1* interfered with cell death and basement membrane breakdown at E6.25 (HH29–30), the stage when fusion normally occurs. In control animals where the otic epithelium of the unelectroporated ear was aligned but not yet fused, the contralateral GFP-electroporated ear looked identical: the basement membrane remained intact and cell death was detectable using the TUNEL assay in the otic epithelium, where the fusion plate will form (Fig. 6A,C) ( $n=2$  animals). The GFP-electroporated ear also appeared normal after fusion (Fig. 6E,G) ( $n=2$  animals), consistent with the absence of a gross morphological defect in paint-fills of the inner ear (Fig. 5A,B).

In contrast, co-electroporation of cNtn1-Myc and GFP blocked both apoptosis and basement membrane breakdown in the fusion plate. *cNtn1-Myc*-electroporated ears exhibited an obvious reduction in the number of TUNEL-positive cells in the epithelium prior to fusion (Fig. 6B,D) ( $n=5/5$  animals) and also in animals where the unelectroporated ear had already fused (Fig. 6F, H) ( $n=5/5$  animals). In addition, the otic epithelium remained unfused, with an intact basement membrane (Fig. 6H). To quantify the effect on apoptosis, we compared the number of TUNEL-positive cells throughout the vertical pouch region on the electroporated versus unelectroporated sides. Apoptosis was obviously decreased in *cNtn1-Myc*-electroporated ears ( $n=7$  animals), with only  $22.6\pm 9.8\%$  (s.d.) of the number of TUNEL-positive cells found in unelectroporated ears ( $P=9.9\times 10^{-6}$  by *t*-test). In contrast, there was no noticeable effect on cell death in the control embryos ( $n=3$  animals), with as many ( $93.9\pm 3.2\%$ ) TUNEL-positive cells in the electroporated versus unelectroporated ears ( $P=0.43$  by *t*-test). Consistently, the average apoptotic ratios in *cNtn1-Myc*-electroporated animals were significantly lower than in GFP-electroporated controls ( $P=1.073\times 10^{-6}$  by *t*-test). Moreover, although the walls of the epithelium came together, fusion appeared arrested at an early stage, as evidenced by maintained expression of *Dlx5*, which is normally downregulated in the center of the pouch (Brown et al., 2005) ( $n=6$  animals) (Fig. 6I–L). We did not observe any premature downregulation of *cLrig3* expression in the center of the lateral canal pouch ( $n=3$  animals) (Fig. 6M–P), further hinting that *cNtn1* may act through a different pathway in chicks than in mice, where *Ntn1* and *Lrig3* mutually restrict each other's expression in this canal (Abraira et al., 2008). These differences do not appear to reflect functional differences between cNtn1 and cNtn2, as viral overexpression of cNtn2 had qualitatively similar effects on canal formation, resulting in a low incidence of fat canals ( $n=4/26$  ears) but no truncations (Fig. S1). This is consistent with the fact that cNtn1 and cNtn2 show similar effects on axon guidance *in vitro* (Serafini et al., 1994). Altogether, these results show that in chicks, excess netrin activity prevents fusion plate formation, accompanied by a severe decrease in apoptosis and subsequent basement membrane breakdown.

#### Overexpression of cNtn1 in mouse inner ears causes excessive canal fusion that phenocopies *Lrig3* mutants

Our results suggest that netrins play distinct roles during canal formation in chicken and mouse, despite conserved expression at the fusion plate. The apparent differences could be due either to intrinsic differences in the proteins or extrinsic differences in the responding tissue. For example, modest differences in the Ntn1 protein sequence might permit an anti-apoptotic function in chickens that has been lost in mice. Alternatively, the fusing



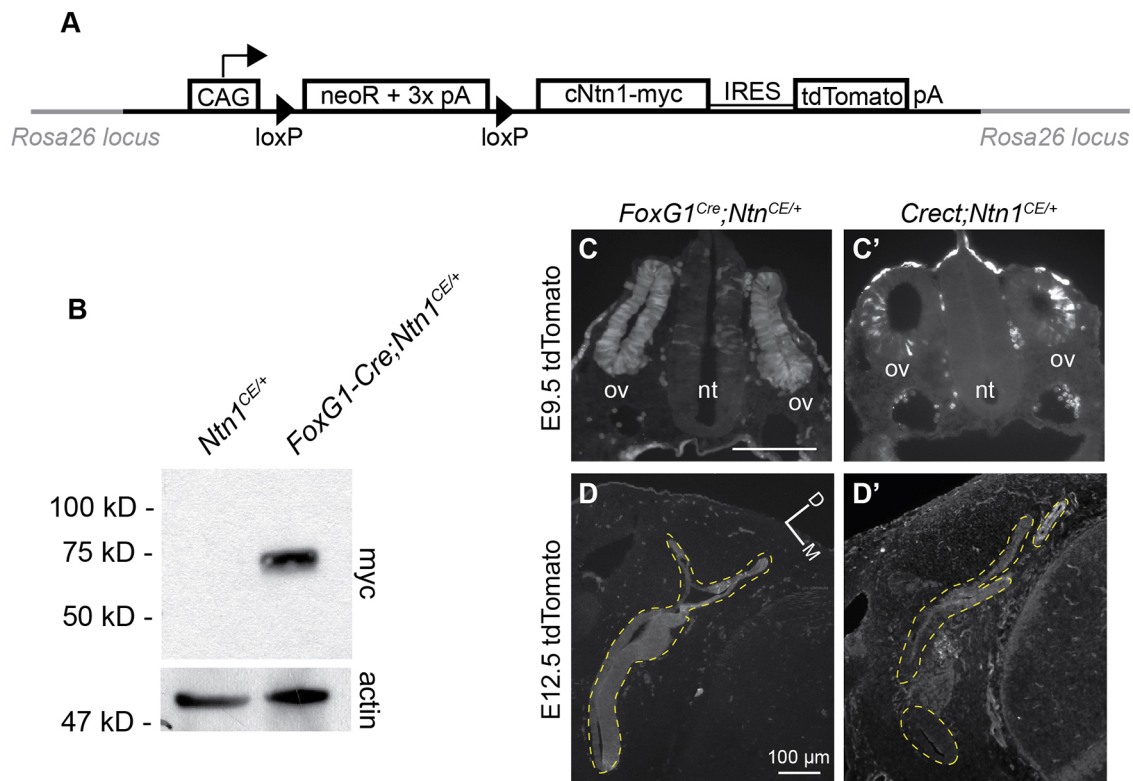
**Fig. 6. Overexpression of cNtn1-Myc arrests fusion.** Immunostaining and *in situ* hybridization of E6.25 tissue sections. (A,B,E,F) DAPI (white) labels cell nuclei and GFP (green) identifies electroporated cells. (C,D,G,H) Laminin (red) labels the basement membrane, whereas TUNEL (white) marks apoptotic cells. GFP-electroporated ears ( $n=2$  animals) display apoptosis in the presumptive fusion plate (C, arrow) and undergo fusion as expected (G, arrow). In contrast, TUNEL-positive cells were rarely observed in ears that were co-electroporated with *cNtn1-Myc* and GFP (D, arrow;  $n=5$  animals), although the opposing epithelial walls are still aligned. In the absence of cell death, the basement membrane remains intact (H, arrow), and fusion does not occur. The images in A-D depict adjacent sections from the same tissue shown in Fig. 4A-C. (I,J,M,N) GFP (green) confirms the presence of electroporated cells throughout the otic epithelium of the posterior (J) and lateral (N) canals, with no expression on the unelectroporated side of the same animal (I,M). In ears that were co-electroporated with *cNtn1-Myc* and GFP, *Dlx5* expression is maintained at the fusion plate, where it is normally downregulated in the unelectroporated ears (K,L, arrows). Meanwhile, *cLrig3* expression is maintained in similar patterns throughout the lateral canal (O,P, arrows), which has not yet begun to fuse at this stage of development. With the exception of (M-P), which show the lateral canal, all images depict sections through the posterior canal. Scale bars: 50  $\mu$ m.

epithelium may respond differently to netrin in each species. In fact, the effects of Ntn1 on fusion in mice were inferred from analysis of the *Lrig3* mutant mouse, where *Ntn1* is ectopically expressed outside the fusion plate (Abraira et al., 2008). Moreover, in these animals, defects were limited to the lateral canal, raising the possibility that the pro-fusogenic functions of netrin are only uncovered in certain contexts. For example, other genes downstream of *Lrig3* could influence the effects of Ntn1.

To better define the origin of the divergent activities of netrin in chickens and mice, we asked whether chicken Ntn1 is able to promote fusion in mice, where apoptosis is not a prominent feature of canal morphogenesis. We generated a Cre-dependent *cNtn1* conditional expressor strain (*Ntn1<sup>CE/+</sup>*) by inserting the coding sequences for cNtn1-Myc and IRES-tdTomato downstream of a *CAG* promoter and a floxed stop cassette in the *Rosa26* locus (Fig. 7A). *Ntn1<sup>CE/+</sup>* mice were crossed to a *Foxg1<sup>Cre</sup>* mouse line, which mediates recombination in the otic epithelium beginning around  $\sim$ E8.75 (Hébert and McConnell, 2000). As *Foxg1<sup>Cre</sup>* is also active in the developing forebrain, we tested for expression of Myc-tagged netrin protein by performing western blots of E12.5 forebrain lysate, which confirmed that the allele produces full-length protein in a Cre-dependent manner, as designed (Fig. 7B) ( $n=2$  animals). Additionally, in transverse sections of E9.5 and E12.5 *Foxg1<sup>Cre/+</sup>; Ntn1<sup>CE/+</sup>* heads ( $n=2$  per age), the entire otic epithelium was tdTomato-positive (Fig. 7C-D), indicating successful ectopic expression of *cNtn1* throughout the inner ear.

Cre-negative animals were not positive for tdTomato at either age, as expected (data not shown;  $n=4$  animals/age).

To assess the effects of ectopic chicken *Ntn1* on mouse semicircular canal morphogenesis, we paint-filled ears from *Foxg1<sup>Cre/+</sup>; Ntn1<sup>CE/+</sup>* and control embryos at E14.5, when canal fusion is normally complete. No canal defects were observed in controls (Fig. 8A; Fig. S1A) ( $n=15$  *Ntn1<sup>CE/+</sup>* and 11 *Ntn1<sup>CE/CE</sup>* ears from 15 animals). In contrast, although a large fraction of *Foxg1<sup>Cre/+</sup>; Ntn1<sup>CE/+</sup>* ears were unaffected, several showed defects in canal formation ( $n=11/49$  ears from 28 animals; 9/28 mice showed a defect in at least one ear), ranging from a thinning to a complete truncation of one of the canals (Fig. 8B,C,F). Defects were most common in the lateral canal ( $n=7/11$  affected ears), but were also observed in the anterior ( $n=2/11$ ) and posterior ( $n=2/11$ ) canals. These phenotypes are indicative of excessive canal fusion. Consistent with the structural defects, two out of three *Foxg1<sup>Cre/+</sup>; Ntn1<sup>CE/+</sup>* mice that survived to adulthood exhibited circling and head bobbing, behaviors that are characteristic of impaired vestibular function (Movie 1). Moreover, similar results were obtained upon crossing *Ntn1<sup>CE/+</sup>* to an alternative Cre driver, *Crect* (Forni et al., 2011), which in our hands drives recombination in the otic epithelium beginning from E9.5, albeit more sparsely than *Foxg1<sup>Cre</sup>* (Fig. 7C', D') ( $n=2$  E9.5 embryos,  $n=3$  E12.5 embryos). Paint-fills of E14.5 *Crect/+; Ntn1<sup>CE/+</sup>* ears revealed lateral canal defects in 56% of all ears (Fig. 8D-F) ( $n=19/34$  ears from 20 animals; 14/20 animals affected), with nine ears showing truncations and 10 ears showing



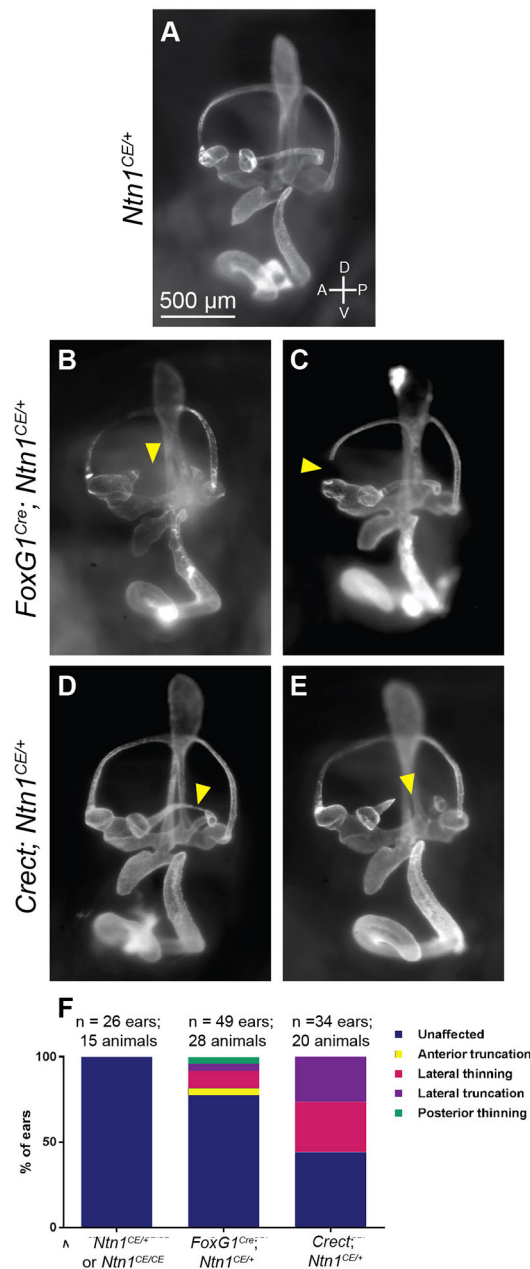
**Fig. 7. Generation and validation of *Ntn1*<sup>CE</sup> allele.** (A) Schematic of the *Ntn1*<sup>CE</sup> allele, which contains a Cre-dependent CAG promoter driving a *cNtn1-Myc-IRES-tdTomato-pA* cassette inserted into the *Rosa26* locus. (B) Western blots of E12.5 forebrain lysate from *Ntn1*<sup>CE/+</sup> and *Foxg1*<sup>Cre</sup>; *Ntn1*<sup>CE/+</sup> embryos. Blotting for Myc reveals production of cNtn1-Myc only in the presence of Cre. Actin is shown as a loading control. (C-D') Transverse sections of otic vesicles (ov in C,C'; outlined in yellow in D,D') at E9.5 (C,C') and E12.5 (D,D') immunostained for tdTomato. At E9.5, *Foxg1*<sup>Cre</sup> drives recombination of the *Ntn1*<sup>CE</sup> allele throughout the otic vesicle (C; *n*=2 animals), whereas *Cre<sup>ct</sup>* results in a sparser pattern of recombination (C'; *n*=2 animals). These expression patterns are maintained at E12.5 (D,D'; *n*=2 for *Foxg1*<sup>Cre</sup>, *n*=3 for *Cre<sup>ct</sup>*). nt, neural tube. Scale bar: 100 μm.

obvious thinning. Thus, *Cre<sup>ct</sup>* led to a more-penetrant phenotype despite the salt-and-pepper pattern of ectopic *Ntn1* expression. Unlike what was observed using *Foxg1*<sup>Cre/+</sup>, neither the anterior nor posterior canals were affected, perhaps due to differences in the spatiotemporal pattern of Cre activity in these two drivers. For both *Cre* lines, some animals exhibited defects in only one ear, suggesting that the incomplete penetrance is not due to the mixed genetic background. We also did not observe new canal defects in *Foxg1*<sup>Cre</sup> embryos harboring two copies of the *Ntn1*<sup>CE</sup> allele (*n*=2/10 ears showed lateral canal truncations, in 1/6 animals) (Fig. S2A, B). Overall, these experiments demonstrate that ectopic expression of *cNtn1* in mouse promotes fusion, as predicted by the *Lrig3* mutant analysis (Abraira et al., 2008). This indicates that the activity of ectopic netrin in chickens is not due to differences at the protein level. Instead, there must be differences in how tissues respond.

Initially defined as axon guidance cues, netrins also influence cell adhesion, polarity and survival, depending in part on which receptors are expressed (Lai Wing Sun et al., 2011). For example, Ntn1 stabilizes cell-cell interactions via neogenin in the mammary epithelium (Srinivasan et al., 2003) and controls cell adhesion via integrin receptors in the developing pancreas (Yebra et al., 2003). Several netrin receptors, including Dcc and Unc5 family members, also function as dependence receptors, triggering cell death in the absence of a ligand (Mehlen and Mazelin, 2003). Thus, differences in the pattern of receptor expression could lead to distinct outcomes in each species. However, we found that neogenin, which is the only Dcc family member in chickens (Friocourt et al., 2017; Phan et al., 2011),

shows the same general pattern of expression as in mice, with high levels in the periotic mesenchyme and low levels throughout the otic epithelium (Fig. S3A,A') (Matilainen et al., 2007). Expression of all four *Unc5* family dependence receptors was also similar in chickens and mice. None was enriched in the fusion plate, though both *Unc5b* and *Unc5d* were transcribed in the statoacoustic ganglion, and *Unc5c* was present at low levels in the periotic mesenchyme (Fig. S3B-D'). *Unc5a* was not detected at all.

To further explore possible differences at the receptor level, we revisited these in mice. Although several known Ntn1 receptors are expressed in the otic epithelium and/or surrounding mesenchyme, canals develop normally in mice lacking *Dcc*, neogenin (*Neo1*), *Unc5b*, *Unc5c*, integrin- $\alpha 3$  (*Itga3*) or integrin- $\alpha 6$  (*Itga6*) (Matilainen et al., 2007; Abraira et al., 2008). We considered the possibility that *Dcc* and *Neo1* might function redundantly to mediate the pro-fusogenic functions of Ntn1. However, all three canals were present in inner ears of *Dcc*<sup>-/-</sup>; *Neo1*<sup>Gt/Gt</sup> double mutant mice (Fig. S2C) (*n*=4 ears from two animals), further supporting the previous proposal that Ntn1 sculpts the canals through a novel receptor or perhaps in a receptor-independent manner (Abraira et al., 2008; Matilainen et al., 2007). Finally, we asked whether residual Ntn1 in hypomorphic gene trap mutants might mask a trophic role by examining *Ntn1* null mutants (Yung et al., 2015). However, canal fusion was arrested in *Ntn1* null inner ears (*n*=8 ears from four animals) just as in hypomorphic mouse mutants (Abraira et al., 2008) (Fig. S2D). In summary,



**Fig. 8. Overexpression of cNtn1-Myc in mice leads to excess fusion and canal truncations.** Paint-fills of E14.5 mouse inner ears. Whereas control *Ntn1<sup>CEI/+</sup>* (A,  $n=15$  ears) and *Ntn1<sup>CEI/CEI</sup>* (not shown;  $n=11$  ears) mice develop with normal canal morphology, some *FoxG1<sup>Cre/+</sup>; Ntn1<sup>CEI/+</sup>* ears display thinning (not shown;  $n=5/49$  ears) or a complete truncation of the lateral canal (B, arrowhead;  $n=2/49$  ears). Rare anterior (C, arrowhead;  $n=2/49$  ears) and posterior (not shown;  $n=2/49$  ears) defects were also observed. Overall, nine out of 28 animals (32%) showed a defect in at least one ear. *Cre<sup>+</sup>; Ntn1<sup>CEI/+</sup>* ears also show defects in canal morphogenesis, but only in the lateral canal (D,E). Both thinning (D, arrowhead;  $n=10/34$  ears) and complete truncations (E, arrowhead;  $n=9/34$  ears) were observed. The overall distribution of defects is illustrated in F. In this case, 14 out of 20 animals (70%) showed a defect in at least one ear. Scale bar: 500  $\mu$ m.

despite clear similarities in gross features of canal morphogenesis and a conserved pattern of netrin expression, fusion can be modulated via different Ntn1-sensitive cellular mechanisms in chicks and mice, underscoring the versatility of this potent molecule throughout evolution.

## DISCUSSION

In contrast to the cochlea, the organization of the vestibular apparatus has changed minimally during evolution, with three orthogonal canals present long before the divergence of birds and mammals (Baird, 1974). Additionally, many of the same molecules and developmental mechanisms are used to pattern and form the canals across vertebrate species (Alsina and Whitfield, 2016). Given how similar the sequence of canal development is between chickens and mice, it was not surprising to find expression of netrins in the fusion plate in both species. However, these basic similarities seem to be accompanied by salient differences that affect how netrin functions. Here, we show that the conserved molecule Ntn1 can have divergent effects on semicircular canal formation in chicken and mouse. In addition to providing new evidence for the ability of Ntn1 to influence survival, our experiments shed light on the complexity of Ntn1 function *in vivo* and highlight how intricate biological structures can be sculpted through distinct cellular processes.

Although canal formation depends crucially on regulated fusion of opposing epithelia across species, our results suggest that there may be a variety of ways to reach this same basic outcome. In mice, the onset of fusion is characterized by restricted basement membrane breakdown and loss of epithelial morphology at the fusion plate (Martin and Swanson, 1993). Apoptotic cells have rarely been detected in the mouse fusion plate, and fusion can still occur in the absence of apoptosis (Ceconi et al., 2004; Nishikori et al., 1999; Rakowiecki and Epstein, 2013). In contrast, we find in chickens that TUNEL-positive cells are abundant at the fusion plate before the walls meet and before basement membrane breakdown, consistent with the suggestion that localized cell death may initiate the fusion process (Fekete et al., 1997). A similar phenomenon occurs in the developing mammalian palate, where apoptosis promotes basement membrane breakdown and hence the fusion of epithelial ‘shelves’ at the midline of the oropharyngeal cavity, resulting in a continuous palate (Cuervo and Covarrubias, 2004; Cuervo et al., 2002; Mori et al., 1994). Although the role for apoptosis in these and other fusing epithelia remains unresolved (Ray and Niswander, 2012), our findings suggest that cell death-triggered basement membrane degradation, which has been dubbed ‘cataptosis’ (Cuervo and Covarrubias, 2004), could be a common developmental mechanism. Further potential mechanisms underlying fusion may remain to be discovered: notably, though canal development in zebrafish also relies on fusion, neither cell death nor mesenchymal proliferation accompanies the event (Alsina and Whitfield, 2016; Bever and Fekete, 1999; Haddon and Lewis, 1991; Waterman and Bell, 1984).

The differences in apoptosis during canal formation in chicken and mouse are accompanied by unexpected differences in the activity of netrins, which are highly conserved at the protein level and localized to the fusion plate in both species. In mice, *Ntn1* levels correlate strongly with the timing and extent of basement membrane breakdown (Abraira et al., 2008; Salminen et al., 2000). As predicted from their homology to laminins, netrin proteins consistently localize to the basement membrane (Yurchenco and Wadsworth, 2004), raising the intriguing possibility of direct effects on its integrity. However, although netrins likewise localize to the basement membrane in the chick inner ear, we find that overexpression of *cNtn1* in chicks does not enhance basement membrane breakdown, but instead prevents apoptosis and leads to arrested rather than accelerated fusion. Strikingly, overexpression of *cNtn1* in mice was indeed able to promote excessive fusion, causing canal truncations. Thus, the same Ntn1 protein can exert opposite effects in different contexts.



The fact that the same netrin protein can enhance cell survival in some contexts but not others may help explain conflicting findings regarding the possible functions of Ntn as a trophic factor. In *Drosophila*, *NetAB* double mutants display increased neuronal cell death that can be prevented by broad expression of NetB but not NetA, suggesting that NetB can act as a trophic factor (Newquist et al., 2013). However, although *Unc5A* serves as a dependence receptor in the mouse spinal cord (Williams et al., 2006), cell survival is unchanged in *Ntn1* mutant mice (Bin et al., 2015; Williams et al., 2006; Yung et al., 2015), raising questions about the importance of Ntn1 as a trophic factor in developing mammals. Our results provide new evidence that ectopic Ntn1 can influence survival *in vivo*, at least in some environments. One possibility is that our overexpression studies uncovered a trophic function normally mediated by a different family member, such as the Ntn2-3 family member Ntn3, which is distantly related to cNtn2 (Friocourt et al., 2017) and also binds to Dcc, albeit with lower affinity than Ntn1 (Wang et al., 1999). If Ntn3 does serve a trophic function in some contexts, however, it is unlikely to be a major player in canal morphogenesis in mice, where apoptosis does not seem to be required (Cecconi et al., 2004).

Regardless of how Ntn proteins normally contribute to cell survival, our findings have important medical implications, as increased Ntn1 expression is associated with breast, lung and pancreatic cancers (Delloye-Bourgeois et al., 2009a,b; Fitamant et al., 2008; Link et al., 2007; Mehlen et al., 2011). Conversely, several Ntn1 receptors exhibit loss of heterozygosity and downregulation in multiple cancers (Bernet et al., 2007; Fearon et al., 1990; Thiebault et al., 2003). Thus, even if Ntn1 does not normally regulate apoptosis, the presence of extra Ntn1 may enable tumor cells to overcome these dependence receptors and survive. Together with the newly available floxed null alleles of *Ntn1* (Bin et al., 2015; Brunet et al., 2014; Yung et al., 2015), the generation of a conditional *cNtn1* expressor allele offers a useful tool for testing test which other tissues are sensitive to ectopic Ntn1 during development and tumorigenesis.

As our ectopic expression studies suggest a role in survival, it is surprising that *cNtn1* and *cNtn2* are normally expressed at the canal fusion plate, exactly where apoptosis occurs. One possibility is that the presence of netrins puts the apoptotic cells into a suspended state, so that they can initiate basement membrane breakdown and migrate towards each other before ultimately dying during the resorption phase of canal development. This could occur either directly, for example by binding to dependence receptors, or indirectly through effects on expression of *Dlx5*. In fact, it has previously been noted that *Dlx5* expression, like *Ntn1*, presages the pattern of apoptosis in the chicken inner ear (Brown et al., 2005). It is also possible that netrins do in fact control basement membrane integrity in chickens as in mice, but that differences in the level or duration of expression lead to distinct phenotypes in the two species. For example, in chickens, excess levels of netrin protein might activate an alternative pathway that prevents apoptosis and hence masks subsequent netrin-mediated basement membrane breakdown.

Arguing against this possibility, we consistently observed arrested fusion in chickens and accelerated fusion in mice, despite differences in how and when cNtn1-Myc was provided. For example, we did not observe examples of ectopic fusion in inner ears sparsely infected with either RCAS-cNtn1 or RCAS-cNtn2, suggesting that neither the identity nor level of netrin protein determines the nature of the phenotype. Similarly, no new phenotypes were revealed in the mouse inner ear when we drove

expression of *cNtn1* using either the broadly active *Foxg1<sup>Cre</sup>* driver or the sparser *Crect* driver, nor did we observe new phenotypes in *Foxg1<sup>Cre</sup>* embryos harboring two copies of the *Ntn1<sup>CE</sup>* allele. Inner ear morphology was also qualitatively similar in *Ntn1* null animals and in hypomorphic mouse mutants (Abraira et al., 2008). The resilience of the mouse inner ear to ectopic Ntn1 was unexpected, but suggests that additional post-translational mechanisms may operate, for example limiting the accumulation of Ntn1 protein in the basement membrane. Because is not possible to measure Ntn1 protein levels reliably in a structure as small as a canal pouch, we cannot compare the degree of overexpression across experiments. However, the fact that canal fusion was arrested in a variety of gain-of-function situations in chicks suggests that different levels of Ntn1 do not exert different cellular effects per se, consistent with the absence of dose-dependent differences in loss-of-function mouse strains, as well as the consistent signs of ectopic fusion in multiple gain-of-function mouse lines.

Variation in Ntn1 activity may also be modulated by the availability of receptors. Unfortunately, the identity of receptors relevant for canal fusion remains unknown in both chicken and mouse. Both Dcc and Unc5 family receptors are expressed in the otic epithelium and/or mesenchyme in the murine inner ear (Abraira et al., 2008; Matilainen et al., 2007). However, fusion proceeds normally in all mouse mutants that have been examined so far (Abraira et al., 2008; Matilainen et al., 2007), including *Dcc*; *Neol* double mutants (Fig. S2), which carry a null allele of *Dcc* and a severely hypomorphic allele of *Neol* (Fazeli et al., 1997; Kam et al., 2016). Chickens do not seem to have a *Dcc* gene (Friocourt et al., 2017) and instead rely solely on *Neol*, at least for axon guidance (Phan et al., 2011). It is tantalizing to consider whether this fundamental difference in the repertoire of netrin receptors is responsible for the distinct effects of *cNtn1* upon overexpression in chicks and mice. However, we did not find any obvious differences in the patterns of expression for *Neol* or any of the *Unc5* family genes in chickens; as in mice, none showed enriched expression in the fusion plate (Fig. S3). No inner ear loss-of-function studies have been reported for any of these receptors in chicken, likely due to the challenges of creating null mutants in this model system. Once appropriate tools are available, it will be crucial to examine whether Ntn1 and its receptors are required for normal patterns of apoptosis in the chick inner ear. In the meantime, our results emphasize the flexibility of development, where similar structures can be created even when evolutionarily conserved proteins are confronted with varying cellular contexts.

## MATERIALS AND METHODS

### Animals

The coding sequence of chicken netrin 1 (*cNtn1*) with a C-terminal Myc tag (Serafini et al., 1994) was cloned into the *AscI* site of a *Rosa26* targeting vector (Ctd), which contains a floxed stop and an IRES-tdTomato under the control of a CAG promoter. The targeting construct was linearized using *SgfI*, electroporated in J1 ES cells (derived from the 129S4/SvJae strain) and selected under G418 for 1 week. Recombinant clones were identified by PCR using primers to amplify the 5' and 3' homology regions of the *ROSA26* locus. To verify Cre-mediated induction of cNtn1 expression, ES clones were transfected with Cre-GFP, GFP or no DNA using Lipofectamine 2000 (Life Technologies), and cell lysates were used for western blot analysis. Genotyping was performed using PCR primers that amplify *tdTomato*. Mice were maintained on a mixed background.

For mice, noon on the day of the vaginal plug was considered to be embryonic day (E) 0.5. For chicks, the time at which specific pathogen-free fertilized eggs (Charles River) were placed into a humidified incubator at 37°C was designated as time zero. All animal work was conducted in compliance

with protocols approved by the Institutional Animal Use Care Committee at Harvard Medical School. The number of animals used for each experiment was based on previous experience studying similar phenotypes.

### Paint-filling

Embryonic heads were fixed overnight in Bodian fix at 4°C and washed and dehydrated in 100% ethanol at room temperature. Samples were then rinsed briefly in methyl salicylate and cleared overnight in methyl salicylate at room temperature. Heads were hemisectioned along the midline, and the cochleae were injected from the medial side with White-Out (Bic) diluted to 0.025% with methyl salicylate using a pulled glass pipette and a Hamilton syringe. Samples were imaged using a Nikon SMZ800 dissection scope.

Canal defects in chicks were scored blind to treatment by comparing the injected ear to the control ear of the same animal. Chick samples were excluded from paint-fill analysis if the control ear had not yet completed fusion or if both the control and experimental ear were not clearly paint-filled. For analysis of canal morphology in paint-filled mouse ears, each ear was scored individually blind to genotype. In most instances, both ears from a single embryo were clearly paint-filled and included in the analysis.

### In situ hybridization and imaging

Standard *in situ* hybridization for *cNtn2* and *cLrig3* (Abraira et al., 2010), and *Dlx5* (Sienknecht and Fekete, 2009) was performed as previously described and imaged on an Olympus BX-63 upright microscope. Fluorescent double *in situ* hybridization was performed using RNAscope (Advanced Cell Diagnostics, ACD) (Wang et al., 2012). All chicken Ntn and Ntn receptor probes were custom designed (ACD). Embryonic tissue was fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and placed through a cryoprotective sucrose gradient before embedding and freezing in Neg-50 (Thermo-Scientific). Sections (14 µm) were rinsed with PBS to remove any Neg-50 and then treated with protease III (ACD). Subsequent steps followed the manufacturer's protocol for the RNAscope Fluorescent Multiplex Kit. Slides were imaged on a Leica Sp8 confocal microscope.

### In ovo electroporations, immunohistochemistry and imaging

Full-length *cNtn1* with a C-terminal Myc tag (Serafini et al., 1996) was cloned into an *EcoRI* site in a modified pCAGGS vector (Niwa et al., 1991). GFP in the same vector was used as a control. *In ovo* electroporation was performed using HH stage 14–16 chick embryos (Hamburger and Hamilton, 1992). Eggs were windowed and plasmid solution (1 µg/µl) containing 0.1% Fast Green for visualization was injected into the cavity of the otic vesicle with a fine glass micropipette. Positive and negative electrodes were positioned to achieve electroporation of the dorsal region of the otic vesicle, and five 25 ms pulses of 5 V were applied using a CUY21 electroporator (NEPA Gene).

Embryonic tissue was fixed overnight at 4°C in 4% PFA in PBS and placed through a cryoprotective sucrose gradient before embedding and freezing in Neg-50 (Thermo-Scientific). Sections (14 µm) were incubated in blocking solution (3% bovine serum albumen in PBS) for 1 h at room temperature and incubated overnight at 4°C with permeabilization solution (1% bovine albumin serum and 0.1% Triton-X in PBS) and primary antibodies directed against: laminin (1:750, Sigma, L9393), Myc (1:200, Santa Cruz, 56633) and phosphohistone 3 (PH3) (1:500, Santa Cruz, SC-8656-R). All of these antibodies are widely used and have been well-characterized. Sections were counterstained with DAPI (1:10,000) and mounted using Fluoromount-G mounting media (Southern Biotech).

For labeling with TUNEL and anti-laminin or anti-GFP, slides were post-fixed in 1% PFA/PBS for 15 min and permeabilized in 0.5% Triton-X-100, processed with the ApopTag-Red fluorescent TUNEL kit (Millipore) and then immunolabeled. Images were collected on a Nikon Eclipse E800 fluorescent microscope equipped with a digital camera. TUNEL-positive foci of varying sizes were counted from every third section (each 15 µm thick) throughout the non-sensory parts of the vertical (anterior and posterior) pouches/fusion plates/canals and the common crus. Apoptotic profiles within cristae anlagen were excluded. Because the unelectroporated ears varied in staging from early fusion through completed fusion, and full fusion plates display the largest apoptotic domains, control-side counts

varied widely between embryos. Thus, within-animal normalization was used by calculating the left/right ratio of TUNEL-positive foci. GFP expression in the same sections confirmed widespread plasmid transfections of the right-side otic epithelium in all samples.

### RCAS virus production and injections

Full-length *cNtn1* and *cNtn2* with a C-terminal Myc tag (Serafini et al., 1994) were each cloned into *Clal* sites of a replication-competent avian sarcoma retrovirus (RCAS) vector. As a control, the netrin 1 signal sequence was fused to GFP (ss-GFP) and cloned into the same vector. RCAS virus was produced using chicken fibroblast cells from well-characterized lab stocks, as previously described (Hollenbeck and Fekete, 2003). Viruses with titers of at least  $2.1 \times 10^8$  infectious units were used.

On the morning of the third day of egg incubation, approximately 5 ml of albumin was removed using a syringe with an 18-gauge needle, and the embryo was allowed to settle away from the top of the eggshell. A small window was then cut in the top of the eggshell to expose the embryo for manipulation. Injections were performed later that day at HH stage 18–19. To aid in visualization of the embryo, 0.2 ml of India ink (Winsor & Newton) diluted 1:10 in Ringers solution plus penicillin-streptomycin was injected into the yolk underlying the embryo using a syringe and a 32-gauge needle. A Picospritzer (Parker) attached to a pulled glass capillary was used to inject RCAS virus into the lumen of the otic vesicle. Fast Green (0.25%) was added to the virus to allow for visualization of the injection.

### Western blots

E11.5 forebrains and virally transfected DF-1 cells were treated with 200 µl of lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1× Pefabloc SC PLUS protease inhibitor (Roche)]. Standard western blotting protocols were followed using primary antibodies for Ntn1 (1:500, R&D, MAB1109) and Myc (1:500, Santa Cruz, SC-56633). To control for loading, blots were stripped (Thermoscientific Product 2105) and re-probed for actin (1:10,000, Abcam). The Ntn1 antibody detects a band that disappears in null mutant animals (Yung et al., 2015); the other antibodies are routinely used and have been extensively characterized in the literature.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: A.M.N., V.E.A., D.M.F., L.V.G.; Methodology: A.M.N., S.O., A.R.Y., M.I.G., V.E.A., D.M.F.; Validation: A.M.N.; Formal analysis: A.M.N., A.R.Y., V.E.A., D.M.F., L.V.G.; Investigation: A.M.N., S.O., A.R.Y., T.d.R., V.E.A., D.M.F.; Resources: T.d.R., E.C.A.; Writing - original draft: A.M.N., A.R.Y., L.V.G.; Writing - review & editing: A.M.N., A.R.Y., D.M.F., L.V.G.; Visualization: A.R.Y., L.V.G.; Supervision: G.C.S., D.M.F., L.V.G.; Project administration: L.V.G.; Funding acquisition: G.C.S., D.M.F., L.V.G.

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### Supplementary information

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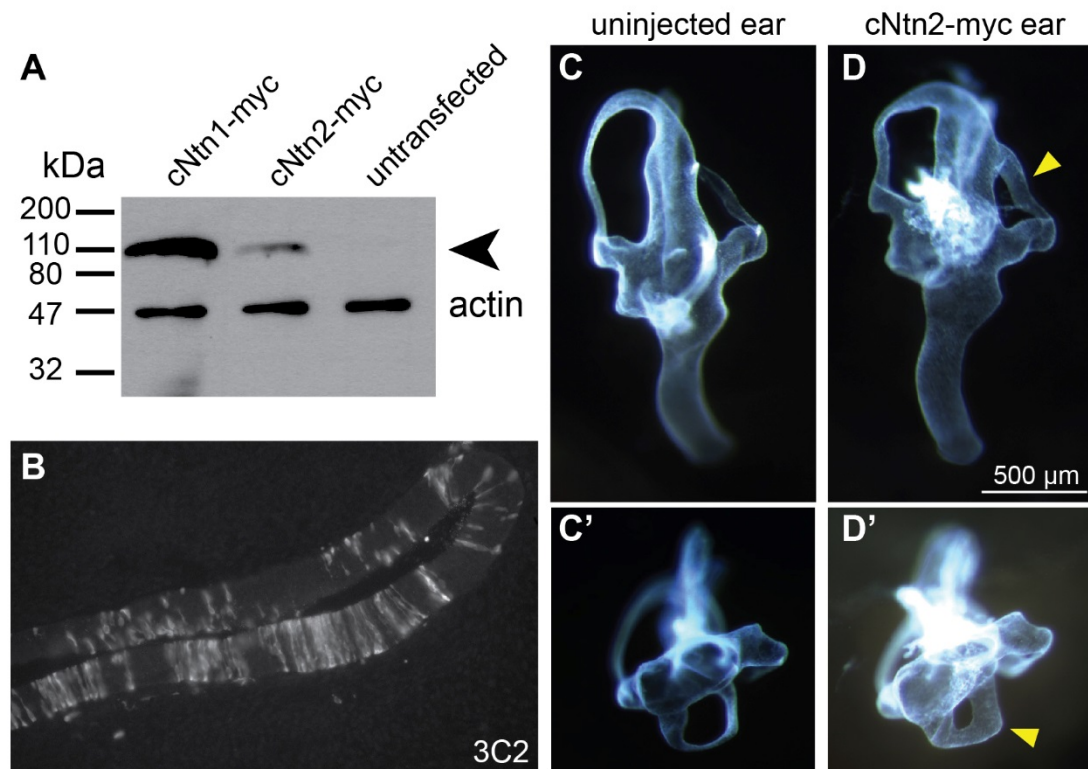
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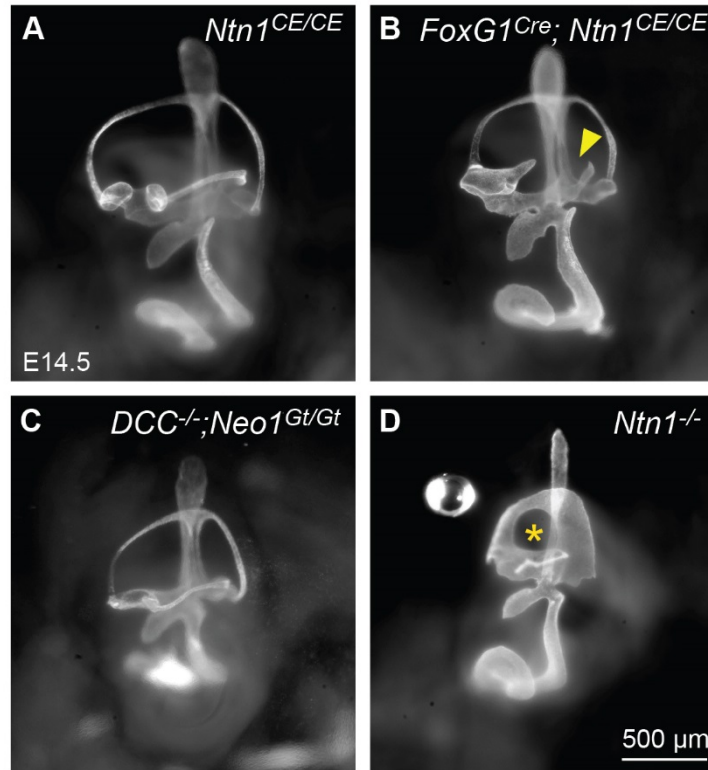
## Supplementary Information



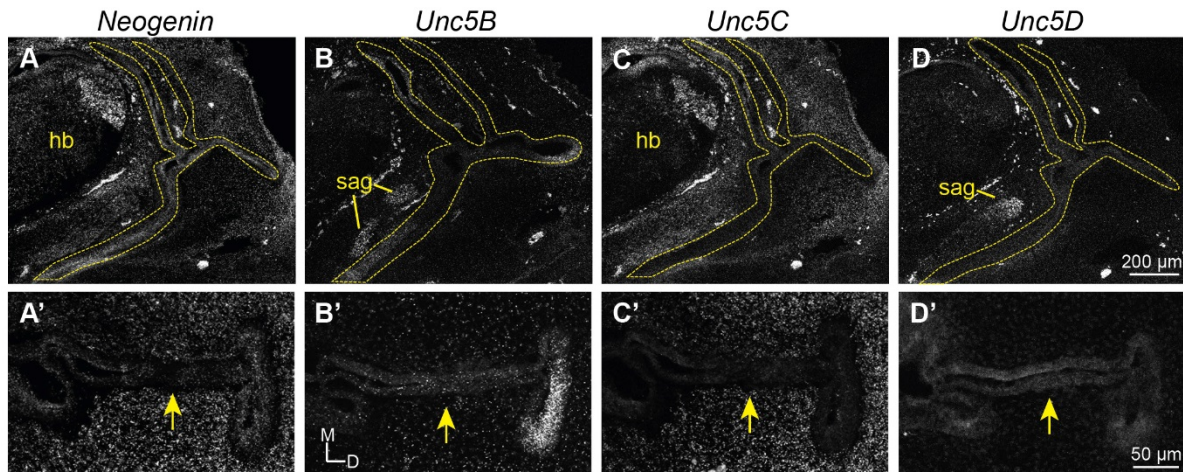
**Movie S1. Overexpression of cNtn1-myc causes circling behavior in adult mice. A *FoxG1<sup>Cre</sup>;Ntn1<sup>CE/+</sup>* mouse circles around the cage and around its littermate.**



**Figure S1: Overexpression of cNtn2-myc can inhibit fusion.** (A) A Western blot using anti-myc and anti-actin antibodies to probe DF1 cell lysates infected with cNtn1-myc or cNtn2-myc RCAS, along with untransfected cells. Both cNtn1-myc and cNtn2-myc were present at the expected size (arrowhead). c-Ntn2-myc levels were noticeably lower. (B) Transverse section through an ear injected with cNtn2-myc RCAS at E3 (HH17) and collected at E6.25 (HH29), stained for the viral epitope 3C2. The virus infected the otic epithelium broadly. (C,D) Paintfills of uninjected (C,C') and cNtn2-myc RCAS injected (D,D') inner ears from E7 (HH30/31) embryos, viewed laterally (C, D) or top down (C', D'). Overexpression of cNtn2-myc caused a low incidence of fat canals (arrowheads) (n=4/26 ears), consistent with inhibition of fusion.



**Figure S2: Fusion defects in mice with altered levels of Ntn1 or its receptors.** In control *Ntn1*<sup>CE/CE</sup> (A) and *Ntn1*<sup>CE/+</sup> embryos (see Fig. 8A), all three canals are well-formed. In *FoxG1*<sup>Cre</sup>; *Ntn1*<sup>CE/CE</sup> embryos, the lateral canal was truncated in both ears from one animal (n=1/6 total animals; 2/10 ears), with no other obvious canal defects. (C) All three canals were present in mice homozygous for both a null allele of *DCC* (Fazeli et al., 1997) and a severely hypomorphic gene trap mutation of *Neogenin* (*Neo1*<sup>Gt</sup>) (Bae et al., 2009). This phenotype contrasts with the severe arrest of canal development observed in *Ntn1* null mutant animals (D). As in *Ntn1* hypomorphs (Abraira et al., 2008), fusion is severely blocked in *Ntn1* null mutants (Yung et al., 2015), with no fusion in the posterior or lateral canals (n=8/8 ears) and either no fusion (n=4/8 ears) or partial fusion (n=4/8 ears) in the anterior canal. Across animals, one showed no fusion in either ear, two showed some fusion in one ear, and one showed some fusion in both ears, indicating that the variation in phenotype is unlikely to be tied to genetic background.



**Figure S3: Expression of Ntn receptors in chick inner ears.** *In situ* hybridization for chicken Ntn receptors on E6.25 (HH29) tissue. Low (A – D) and high (A' – D') power images show that while many receptors are present in the developing ear (outlined in dashed yellow lines, A-D), none are enriched at the fusion plate (yellow arrow, A'-D'). hb, hindbrain; sag, statoacoustic ganglion.

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