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## Cross-repressive interactions between *Lrig3* and *Netrin1* shape the architecture of the inner ear

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### SUMMARY

The sense of balance depends on the intricate architecture of the inner ear, which contains three semicircular canals used to detect motion of the head in space. Changes in the shape of even one canal cause drastic behavioral deficits, highlighting the need to understand the cellular and molecular events that ensure perfect formation of this precise structure. During development, the canals are sculpted from pouches that grow out of a simple ball of epithelium, the otic vesicle. A key event is the fusion of two opposing epithelial walls in the center of each pouch, thereby creating a hollow canal. During the course of a gene trap mutagenesis screen to find new genes required for canal morphogenesis, we discovered that the Ig superfamily protein *Lrig3* is necessary for lateral canal development. We show that this phenotype is due to ectopic expression of the axon guidance molecule *Netrin1* (*Ntn1*), which regulates basal lamina integrity in the fusion plate. Through a series of genetic experiments, we show that mutually antagonistic interactions between *Lrig3* and *Ntn1* create complementary expression domains that define the future shape of the lateral canal. Remarkably, removal of one copy of *Ntn1* from *Lrig3* mutants rescues both the circling behavior and the canal malformation. Thus, the *Lrig3/Ntn1* feedback loop dictates when and where basement membrane breakdown occurs during canal development, revealing a new mechanism of complex tissue morphogenesis.

### Keywords

inner ear; morphogenesis; vestibular system; *Netrin*; *Lrig*; basement membrane

### INTRODUCTION

Precise spatiotemporal regulation of intercellular signaling is critical for molding tissues into three-dimensional structures during organogenesis. One of the most striking examples of tissue morphogenesis is the development of the three-dimensional architecture of the inner ear, which houses the sensory organs for hearing and balance. Angular acceleration is detected by three fluid-filled semicircular canals that are oriented with respect to the three dimensions of space. Changes in head position result in fluid movement within the canals, thereby activating specialized hair cells in the sensory epithelia located at the base of each canal. Subsequently, vestibular ganglion neurons convey signals from the sensory epithelium to the central nervous system. The inner ear is a small, intricately-shaped organ that does not tolerate even subtle changes to its structure; indeed, even the slightest perturbations in the structure of the vestibular canals can result in debilitating dizziness, vertigo, and abnormal posture in humans (Sando et al., 2001; Sando et al., 1984).

Morphogenesis of the inner ear is set in motion by early patterning events, which result in the expression of key cell fate determinants within discrete domains of a primordial structure. The inner ear is sculpted from a simple ball of epithelium called the otic vesicle (Fig. 1) (Fekete, 1999). The semicircular canals are derived from two outpocketings, the canal pouches, which are specified by transcription factors such as *Otx1* and *Dlx5* (Merlo et al., 2002; Morsli et al., 1999). A critical event in canal development is the formation of the fusion plate in the center of the pouch. During this process, the basement membrane breaks down, allowing signaling molecules to induce proliferation in the surrounding mesenchyme and the two epithelial walls to come together (Martin and Swanson, 1993; Pirvola et al., 2004; Salminen et al., 2000; Streeter, 1907). At the same time, fusion plate cells lose their columnar morphology and intercalate to form a single layer of cells (Martin and Swanson, 1993). Importantly, these changes in cell morphology and basal lamina integrity occur only in the vicinity of the fusion plate. In contrast, the epithelium in the perimeter of the pouch remains intact and will eventually form the walls of the mature canal. Hence, the final shape of each canal is determined by when and where fusion occurs.

A key regulator of fusion is the laminin-related molecule Netrin1 (*Ntn1*). In *Ntn1* mutant mice, the inner ear fails to acquire its mature shape due to an arrest in canal morphogenesis (Salminen et al., 2000). Although best characterized for its function as a secreted chemoattractant for axons during neuronal development (Moore et al., 2007), *Ntn1* is also critical for many aspects of tissue morphogenesis, including cell migration and local cell adhesion (Srinivasan et al., 2003; Yebra et al., 2003). In the inner ear, *Ntn1* is required for breakdown of the basement membrane surrounding the fusion plate (Salminen et al. 2000). *Ntn* proteins are localized to the basal lamina (Salminen et al., 2000; Schneiders et al., 2007) and can bind to collagen and laminin *in vitro* (Schneiders et al., 2007; Yebra et al., 2003), but how *Ntn1* signaling promotes basal lamina breakdown remains unclear (Matilainen et al., 2007).

Despite abundant evidence that *Ntn1* is a powerful morphogen, little is known about the pathways that restrict *Ntn1* expression to highly limited spatiotemporal domains in any developing system (Kennedy, 2000). In addition to its functions during development, *Ntn1* is overexpressed in several human cancers (Fitamant et al., 2008; Link et al., 2007), underscoring the importance of understanding how expression of *Ntn1* is regulated.

Here, we demonstrate that *Ntn1* expression is controlled by cross-repressive interactions with the Ig superfamily protein *Lrig3* that define the boundary between the fusing and non-fusing regions of the lateral canal pouch. This novel feedback loop dictates when and where basement membrane breakdown occurs, thereby ensuring that the inner ear acquires its precise three-dimensional shape.

## MATERIALS and METHODS

### Mice

The *LST016* gene trap line was previously reported (Leighton et al., 2001; Mitchell et al., 2001). The mice have been maintained for >5 generations on the C57Bl6/J background. Genotyping was performed using the following primers: **LST016F** (GAGGTGCCTGATGCTTAAGTT TCG); **LST016R** (TTCAACCTTGGCTTCCAATGTCCA); and **GTR7** (CAAGTCTATCCTAGGGAAAGGGTC) which is specific to the *Lrig3<sup>LST016</sup>* gene trap vector *pGT2TMPFS*. The *Lrig3<sup>fllox</sup>* conditional allele was produced via homologous recombination using a targeted construct with LoxP sites surrounding the ATG-containing exon1. Heterozygous mice were produced by germline Cre-excision using a global deleter line (Schwenk et al., 1995). Genotyping was performed using the following primers

**VEA303** (CGGAATTTCTACAATCTCAGC); **VEA304** (GTGCTCCTGGTGGCTCAGT); **VEA305** (CCCCCTCCAATTTTAACAAA). The *Ntn1* gene trap line was previously reported (Serafini et al., 1996). The mice have been maintained for >5 generations on the C57Bl6/J background. Genotyping was performed using the following primers: **pGT1\_8TM3021R** (GTTGCACCACAGATGAAACG); **pGTEn2\_1723** (TCCCGAAAACCAAAGAAGAAG); and **pGT1\_8TM1743** (GAACCTAACAAGAGGACAAG). The described genotyping screen allows for the detection of the *Ntn1*-specific gene trap vector *pGT1.8TM*. Heterozygous and mutant genotypes were confirmed by evaluating the thickness of the ventral commissure in the neural tube following BIII-Tubulin and Neurofilament immunostaining on cryosectioned embryos. These two gene trap lines are annotated as *Lrig3*<sup>+/-</sup> or *Ntn1*<sup>+/-</sup> for the heterozygous condition and *Lrig3*<sup>-/-</sup> or *Ntn1*<sup>-/-</sup> for the mutant condition. *Unc5hb* mutant mice were maintained on a CD1 background and genotyped as described (Lu et al., 2004). The *Integrin a6* gene trap mice (Mitchell et al., 2001) were maintained on a C57Bl6/J background and were genotyped using the following primers: **LST045F5** (GCCCAAATCCCTTGTGTATG); **LST045R1** (ACCCACAGCAACCTTTGTTC); and **GTR3** (TCTAGGACAAGAGGGCGAGA). The mice were maintained in accordance with institutional and NIH guidelines approved by the IACUC at Harvard Medical School and the University of Virginia.

### Quantitative PCR

cDNA was made from total mouse E11.5 embryonic RNA using random primers. *Lrig3* was amplified as described (Abraira et al., 2007) with SYBR Green Supermix (BioRad) and primers flanking the *Lrig3* exon3/4 boundary: **VEA48** (GAACAACAATGAATTAGAGACCATTC) and **VEA49** (AGGGTGGAAAGGCA-GTTCTC). Levels were normalized based on amplification of GAPDH from the same samples: **GAPDHe-F** (CTCATGACCACAGTCCATGC), **GAPDHe-R** (GCACGTCAGATCC-ACGAC). Loss of *Lrig3* message from *Lrig3* null mice was confirmed by RT-PCR amplification of E12.5 embryonic cDNA using primers **VEA48** and **VEA49**.

### Paintfilling

E12–E14 embryos were fixed overnight at 4° C with Bodian's Fix, dehydrated overnight at RT with 100% EtOH, then cleared overnight at RT with methyl salicylate. Heads were hemisected, and white latex paint (Benjamin Moore) diluted to 0.025% in methyl salicylate was injected into the cochlea with a pulled glass pipette and a Hamilton syringe filled with glycerol.

### In situ hybridization

Non-radioactive *in situ* hybridization was performed on 10–12 μm cryosections using the following probes: *Ntn1* (NM\_008744), *Otx1* (NM\_011023), *Otx2* (NM\_144841), *Dlx5* (NM\_010056), *Hmx3* (NM\_008257). A detailed protocol is available at <http://neuro.med.harvard.edu/site/goodrichweb/>.

### X-gal and PLAP staining

Staining for β-galactosidase and alkaline phosphatase activity was performed as described (Leighton et al., 2001) except that 10–20 μm frozen sections were used, and the tissue was fixed for 1 hour at 4° C.

## Immunohistochemistry

E12 embryos were collected and fixed for 1–2 hrs at 4° C in 4% PFA/PBS and then dehydrated in 30% sucrose/PBS overnight at 4° C. Embryos were then embedded in Neg50 (Richard-Allan Scientific). 5–10 µm cryosections were blocked and permeabilized in 5% Normal Donkey Serum + 2% BSA + 0.1% Triton X-100 for 1 hour at RT. Primary antibodies were added in above block, without Triton X-100, overnight at 4° C at the following concentrations: CollagenIV (1:200, Abcam, ab6586); Pan-mouse laminin (1:250, Chemicon, AB2034); BIII-Tubulin (1:1000, Covance, PRB-435P); and Neurofilament (1:500, DSHB, SH3). The following day, the sections were incubated in secondary antibody (1:2000, Alexa Fluor488 or 568, Jackson Immunoresearch) in block, without Triton X-100. All sections were counterstained with DAPI (1:10,000).

## Histology and Electron Microscopy

Plastic semi-thin (1 µm) and ultra-thin (90 nm) sections for light and electron microscopy studies were obtained with the help from the Harvard Medical School Electron Microscopy Facility ([www.cellbio.med.harvard.edu/research\\_facilities](http://www.cellbio.med.harvard.edu/research_facilities)).

## RESULTS

### Identification of *Lrig3* as a new regulator of inner ear morphogenesis

To gain insight into the molecules that drive inner ear morphogenesis, we took advantage of the fact that even subtle changes in the structure of the inner ear cause dramatic behavioral defects. Candidate genes were identified by screening a large set of mice generated by gene trap technology (Leighton et al., 2001; Mitchell et al., 2001). Upon insertion into the intron of a gene, the gene trap vector simultaneously blocks transcription and reports the normal expression pattern of that gene through a  $\beta$ -galactosidase reporter (Fig. 2A). X-gal-stained heterozygous embryos were examined to find genes expressed in restricted domains of the otic vesicle prior to morphogenesis, and then homozygous mutants for the best candidate genes were assessed for defects in hearing and balance.

In this screen, we identified a strain of mice, *LST016*, with  $\beta$ -galactosidase activity in the lateral wall of the otic vesicle (Fig. 2B,C), a region fated to give rise to the lateral semicircular canal (Fekete and Wu, 2002). Homozygous mutants exhibit circling and head tossing behaviors, consistent with the presence of an inner ear defect (Fig. 2D). Visualization of the three-dimensional structure of the vestibular apparatus revealed a fully penetrant truncation of the lateral semicircular canal in homozygotes (Fig. 2G-J). Histological studies confirmed that the canal epithelium is missing as early as E13 (data not shown). The lateral ampulla is unaffected, and the sensory epithelium is properly innervated, as determined by Myosin VIIa and Neurofilament immunostaining (Fig. 2I,J and data not shown). Although the reporter is also active in the developing cochlea (Supp. Fig. 1H), the mice develop normal hearing as assessed by auditory brainstem response (ABR) assays (Supp. Fig. 2). All mutants also display craniofacial deformities and a dramatically shortened snout (Fig. 2E,F).

*LST016* mice carry an insertion of the *pGT2TMPFS* gene trap vector in the third intron of the *Lrig3* gene (Fig. 2A), causing a truncation of the wild-type transcript and fusion of the  $\beta$ -galactosidase reporter protein to *Lrig3* at amino acid 126 (Mitchell et al., 2001). *Lrig3* is one of three members in a family of single-pass transmembrane proteins containing 15 leucine rich repeats (LRR) and three immunoglobulin (Ig) domains in the extracellular domain and intracellular tails that vary in length and composition (Guo et al., 2004; Hedman and Henriksson, 2007). Quantitative RT-PCR of homozygous *Lrig3* tissue (n=4 embryos) detected only  $3.05 \pm 1.59$  % of the wild-type transcript, indicating that the phenotype is

severely hypomorphic. To confirm that the phenotype is due to a loss of *Lrig3*, a null allele was made by flanking the ATG-bearing exon of *Lrig3* with LoxP sites (Fig. 2A). After germline Cre-mediated excision, no *Lrig3* messenger RNA remained (data not shown). Null mutant mice exhibit the same lateral canal and craniofacial defects evident in the gene trap allele (data not shown), demonstrating that the phenotypes reflect a complete loss of *Lrig3* function.

### Truncation of the lateral semicircular canal in *Lrig3* homozygotes is due to early and ectopic fusion

The semicircular canals develop from two epithelial outpocketings called canal pouches, with the anterior and posterior canals arising from the dorsal pouch and the lateral canal forming from the lateral pouch (Fig. 1). Early patterning events that define the axes of the otic vesicle result in restricted expression of transcription factors that specify the two pouches. Because *Lrig3* expression is restricted to the lateral pouch during these early patterning stages, it seemed possible that *Lrig3* acts in a signaling pathway that ensures restricted expression of transcription factors required for specification of the lateral canal. To test this, we examined the expression patterns of *Otx1*, *Otx2*, *Hmx3*, and *Dlx5*, transcription factors required for normal development of the vestibular system (Merlo et al., 2002; Wang et al., 1998). However, *Lrig3* mutant embryos exhibited no obvious changes in early gene expression, indicating that the lateral pouch is specified in the right place and at the right time (Supp. Fig. 3).

Since canal patterning was unaffected, we asked whether subsequent morphogenesis events proceed normally in *Lrig3* mutant embryos. We visualized pouch outgrowth and fusion by paintfilling inner ears between E11.5 and E12.5 (Fig. 3). The precise stage of canal development was determined by evaluating the extent of fusion in the anterior and posterior canals, which develop earlier than the lateral canal (Martin and Swanson, 1993). In control embryos, the lateral pouch grows out at E12 and fusion begins at E12.5 (Fig. 3A-C). No changes in the size or shape of the early canal pouch occur in E12 *Lrig3* mutant embryos, consistent with correct patterning of the otic vesicle (Fig. 3D). However, fusion in the lateral pouch begins several hours earlier than normal and over a larger area, extending to the perimeter of the lateral pouch epithelium (Fig. 3E). The lateral canal is truncated by E12.5, when fusion is just beginning in wild-type littermates (Fig. 3F). Thus, fusion is expanded and accelerated in *Lrig3* mutants.

### *Lrig3* and *Ntn1* act in complementary domains to regulate basal lamina integrity

While fusion occurs prematurely in *Lrig3* mutants, fusion is arrested in *Ntn1* mutants, raising the possibility that these two genes cooperate to determine the timing and extent of fusion. To gain more insight into the relationship between *Lrig3* and *Ntn1* during canal morphogenesis, we compared their expression patterns on adjacent sections from E12 to E12.5. We found that *Lrig3* expression is enriched in the lateral pouch epithelium during the canal pouch outgrowth stage, when *Ntn1* is not yet expressed (Fig. 3G,G'). Then, just before fusion begins, *Lrig3* expression becomes reduced in the center of the pouch (Fig. 3H), concomitant with the initiation of *Ntn1* transcription in the nascent fusion plate (Fig. 3H'). As fusion progresses, the domain of *Ntn1* transcription expands (Fig. 3I'), while *Lrig3* is downregulated in *Ntn1*-positive cells (Fig. 3I) but is maintained in the surrounding, non-fusing epithelium. After the canal is fully formed, *Lrig3* and *Ntn1* continue to be expressed in non-overlapping domains of the canal epithelium (data not shown). Hence, consistent with their opposing activities during canal development, *Lrig3* and *Ntn1* are expressed in complementary domains of the lateral pouch.



These results suggest that *Lrig3* is required in the non-fusing epithelium to prevent fusion from occurring by balancing the activity of *Ntn1* in the fusion plate. Malformation of the semicircular canals in *Ntn1* mutant mice is preceded by a failure in basement membrane breakdown (Salminen et al., 2000). Therefore, we asked whether the early and ectopic fusion event in *Lrig3* mutant mice is also accompanied by changes in the integrity of the basal lamina that normally separates the epithelium from the surrounding mesenchyme. We found that dramatic changes occur in the basal lamina of *Lrig3* mutant embryos. In control embryos, laminin and collagen networks are intact prior to fusion plate formation, as shown by immunostaining and electron microscopy analysis (Fig. 4A-E). In contrast, in *Lrig3* mutants, the basal lamina is missing or disrupted throughout the lateral pouch, including regions where fusion normally never occurs (Fig. 4F-J). Moreover, the otic epithelium throughout the lateral pouch is abnormally thin, such that even cells in the perimeter of the pouch resemble fusion plate cells (Fig. 4A',F'). Neither Caspase-3 immunostaining (Supp. Fig. 4) nor electron microscopy analysis (data not shown) revealed an increase in apoptosis, suggesting that these morphological changes are not associated with cell death. Thus, *Lrig3* inhibits basement membrane breakdown in the non-fusing epithelium.

### ***Ntn1*-dependent basal lamina breakdown does not require known receptors**

To understand the molecular basis of the basement membrane phenotype, we explored the possibility that *Lrig3* modulates *Ntn1* activity by regulating one of its known receptors. This hypothesis is supported by the facts that *Lrig3* is expressed complementary to *Ntn1* and that *Lrig* proteins regulate degradation of many different transmembrane receptors (Hedman and Henriksson, 2007). *Ntn1* is best known as an axon guidance molecule, which signals through the Ig superfamily of receptors DCC (Deleted in Colorectal Cancer) and Neogenin1, as well as the Unc5 family of receptors (Unc5Ha-d) (Moore et al., 2007). Outside of the nervous system, *Ntn1* signals through Neogenin (Srinivasan et al., 2003), Unc5Hb (Lu et al., 2004), and Integrin  $\alpha$ 3/6 receptors (Yebra et al., 2003) to regulate cell adhesion, migration, and other aspects of tissue morphogenesis.

To identify relevant receptors, we asked whether any of the known *Ntn1* receptors is required for inner ear morphogenesis. *In situ* hybridization screens by our lab and others revealed *Unc5hb*, *Neogenin*, and *Integrin  $\alpha$  3/6* as potential candidates (Matilainen et al., 2007). However, although *Unc5hb* is expressed together with *Lrig3* in non-fusing epithelium, the inner ear forms normally in *Unc5hb* mutant mice (Supp. Fig. 5). Moreover, none of the known *Ntn1* receptors that are expressed in the developing otic epithelium (Neogenin, Integrin  $\alpha$ 3/6) or surrounding mesenchyme (*Unc5hc*) are required for canal morphogenesis (Supp. Fig. 5) (Matilainen et al., 2007).

Interestingly, the pro-angiogenic activities of *Ntn1* have been proposed to be independent of known receptors, suggesting an alternative binding partner in this system (Wilson et al., 2006). Although *Lrig3* is a cell surface protein, *Lrig3* does not appear to be the missing receptor, as tagged versions of *Lrig3* and *Ntn1* do not co-localize in cultured cell lines (Supp. Fig. 6). Moreover, equal amounts of *Ntn1* are secreted from cells in the presence and absence of *Lrig3* (Supp. Fig. 6). Thus, *Ntn1* appears to act through a non-canonical pathway in the inner ear to control basal lamina integrity, either via a novel receptor or a receptor-independent mechanism.

### ***Lrig3* and *Ntn1* participate in cross-repressive interactions that determine the timing and location of fusion**

Since *Lrig3* does not appear to act through the *Ntn1* pathway, we considered alternative explanations for why basement membrane breakdown is expanded in *Lrig3* mutants. Based on their complementary expression patterns and opposing activities, a simple idea is that the

main function of *Lrig3* is to restrict expression of *Ntn1* to the fusion plate. As hypothesized, we found that *Ntn1* expression is expanded to encompass the entire lateral pouch epithelium in *Lrig3* mutants (Fig. 5A,E). Since *Lrig3* appears to regulate *Ntn1* expression at the transcriptional level, we asked whether the transcriptional downregulation of *Lrig3* in the wild-type fusion plate (Fig. 3) is similarly a result of *Ntn1* activity. To do this, we took advantage of the fact that a placental alkaline phosphatase (PLAP) reporter gene is present in the gene trap vector used to generate *Lrig3* (Fig. 2A) but not in *Ntn1* gene trap mutants (Serafini et al., 1996). As seen by  $\beta$ -galactosidase histochemistry (Fig. 3I), PLAP expression is reduced in the fusion plate in *Lrig3* heterozygous embryos (Fig. 5B). In contrast, in *Ntn1* mutants, PLAP expression persists throughout the lateral pouch (Fig. 5F). Surprisingly, we also observed sustained expression of *Lrig3* itself in *Lrig3* mutants, indicating that *Lrig3* is required for its own downregulation (Fig. 5C,G). Therefore, we asked whether *Ntn1* transcription is also autoregulated. Indeed, *Ntn1- $\beta$  geo* expression is lost from the lateral pouch in *Ntn1* mutants (Fig. 5D,H). *Ntn1* autoregulation appears to be limited to the lateral pouch, since no changes in expression occur in the dorsal pouch. Together, these experiments show that mutually antagonistic interactions between *Lrig3* and *Ntn1* restrict *Ntn1* expression to fusion plate cells and *Lrig3* expression to non-fusing epithelium. This feedback loop seems to be uniquely important for lateral canal development, suggesting that new mechanisms operate in this most recently evolved semicircular canal.

### Loss of one copy of *Ntn1* is sufficient to rescue inner ear defects in *Lrig3* mutants

The presence of cross-repressive interactions between *Lrig3* and *Ntn1* suggests that a slight reduction in *Ntn1* levels might be sufficient to compensate for the loss of *Lrig3*. To test this idea, we reduced the dosage of *Ntn1* in *Lrig3* mutant mice by intercrossing *Ntn1*<sup>+/-</sup>; *Lrig3*<sup>+/-</sup> transheterozygotes and collecting E14.5 embryos (n=81) for paintfilling (Fig. 6). Wild-type (n=4/4) and transheterozygous (n=23/23) embryos developed normal canals (Fig. 6A,B,G), while littermates homozygous for *Lrig3* but wild type for *Ntn1* exhibited lateral canal truncations, as expected (n=11/12 ears) (Fig. 6C,G). In contrast, no truncations occurred in *Lrig3* homozygotes that were also heterozygous for *Ntn1* (n=12/12 ears) (Fig. 6D,G), indicating that the *Lrig3* phenotype is caused by a failure to properly regulate *Ntn1* levels in fusion plate cells. Consistent with the complete rescue, adult *Lrig3*<sup>-/-</sup>; *Ntn1*<sup>+/-</sup> mice did not display circling behavior (n=16). Since the size and shape of the canal pouch is similar in *Ntn1* (Fig. 6E,G) (n=7) and *Ntn1*; *Lrig3* double mutant embryos (Fig. 6F,G) (n=4), *Lrig3* likely acts upstream of *Ntn1*.

We conclude that *Lrig3* plays a pivotal role in lateral canal morphogenesis by restricting *Ntn1* expression to the fusion plate. Cross-repressive interactions between *Lrig3* and *Ntn1* coordinate the timing and location of fusion, thereby determining the shape of the lateral canal.

## DISCUSSION

A central challenge in developmental biology is understanding how signaling pathways cooperate to sculpt tissues with complex three-dimensional shapes. Here, we describe the presence of a novel feedback loop that restricts the expression of two genes with opposing functions to discrete domains of the otic vesicle during inner ear morphogenesis. Because the pattern of *Ntn1* expression determines when and where fusion occurs, the spatiotemporal regulation of *Ntn1* through cross-repressive interactions with *Lrig3* ensures perfect morphogenesis of the elaborate structure of the inner ear.

Historically, much emphasis has been placed on the identification of determinants that define domains within the anlage of a developing structure. More recently, however, it has become clear that an additional level of control is needed to restrict the spatiotemporal

activities of each factor. A common solution to this problem is the production of a feedback-induced antagonist that dampens the activity of a signaling pathway after activation, as is the case for Sprouty and Sef proteins in the FGF pathway (Shim et al., 2005; Tsang and Dawid, 2004). Signaling activity is also modulated by the basal lamina, which can limit dispersal of the protein and its ability to bind to its receptor (Relan and Schuger, 1999). The *Lrig3/Ntn1* feedback loop incorporates both of these features: the induction of an antagonist, *Lrig3*, which controls the timing and extent of *Ntn1*-dependent basal lamina breakdown.

The *Ntn1/Lrig3* feedback loop uncovered by this analysis is a novel mechanism for modulating expression of *Ntn1* during development. In addition to the developing canals, *Lrig3* and *Ntn1* are also expressed in many other regions of the embryo, including the cochlea, neural tube, and somites (Supp. Fig. 1). Although no alterations of these tissues are obvious in *Lrig3* mutant mice (data not shown), this may be due to compensation by the close family member *Lrig1*. Indeed, our expression studies indicate that the only two places where *Lrig1* and *Lrig3* do not overlap are the lateral canal pouch and the branchial arches, consistent with the lateral canal truncation and craniofacial abnormalities evident in *Lrig3* mutant mice. Conversely, psoriasis is the only salient defect reported in *Lrig1* mutant mice (Suzuki et al., 2002). Hence, *Lrig1;Lrig3* double mutants may exhibit additional phenotypes related to misregulation of *Ntn1* and may reveal a general mechanism for *Ntn1* regulation.

Abnormal regulation of *Ntn1* by *Lrig* may have broad implications in disease since disruption of the laminin network is a major factor in tumor invasion (Gupta and Massague, 2006). Consistent with this idea, all three *Lrig* genes are downregulated in certain human cancers, with *Lrig1* studied most extensively (Guo et al., 2006; Hedman and Henriksson, 2007; Hedman et al., 2002; Lindstrom et al., 2007; Ljuslinder et al., 2007). Strikingly, the loss of *Lrig1* correlates with worse outcomes in advanced stage cervical cancer (Lindstrom et al., 2007) and squamous cell carcinomas (Tanemura et al., 2005). In addition, the presence of *Lrig1* is sufficient to inhibit the invasive behavior of bladder cancer cell lines and to prevent breakdown of fibronectin in a cell-matrix adhesion assay (Yang et al., 2006). Conversely, increased *Ntn-1* induces tumor growth and invasion in human colorectal cell lines (Rodrigues et al., 2007) and is associated with poor prognoses for adenocarcinoma (Link et al., 2007). While the effect of *Ntn-1* on tumor behavior is mediated in part by inhibition of apoptosis through the DCC and Unc5h dependence receptors (Fitamant et al., 2008; Mazelin et al., 2004; Rodrigues et al., 2007), our data raise the possibility that in some tumors, *Ntn-1* may act downstream of *Lrig3* to facilitate basement membrane breakdown and tumor metastasis.

Based on the current understanding of this poorly characterized family of proteins, it is likely that *Lrig3* represses *Ntn1* transcription by regulating activity of a receptor tyrosine kinase signaling pathway. The best studied family member, *Lrig1*, is induced by EGF signaling and antagonizes downstream signaling events by causing degradation of all four ErbB receptors (Gur et al., 2004; Laederich et al., 2004). *Lrig1* has also been implicated as a negative regulator of Met and Ret receptor tyrosine kinases (rTK) (Ledda et al., 2008; Shattuck et al., 2007), raising the possibility that *Lrigs* serve as general antagonists of rTKs. Consistent with this idea, *Lrig3* can bind to the FGF receptor tyrosine kinase receptor *in vitro* and inhibits FGF signaling in the developing neural crest in *Xenopus* (Zhao et al., 2008). This function may be conserved in mice, since *Lrig3* morphant tadpoles exhibit craniofacial defects similar to what is observed in *Lrig3* mutant mice. Since FGF signaling plays a prominent role in canal morphogenesis (Chang et al., 2004; Pauley et al., 2003; Pirvola et al., 2004), the *Lrig3* inner ear phenotype is also likely to be caused by aberrant FGF activity. However, although FGF ligands and receptors have been implicated, the downstream signaling events in the fusion plate remain elusive, with no known target genes and multiple feedback-induced antagonists that are not required for canal development



(Abraira et al., 2007; Shim et al., 2005). Hence, until we have a better understanding of how FGF signaling acts specifically in the fusion plate, it will be difficult to determine whether and how *Lrig3* influences FGF activity.

Together with what is known about *Lrig* functions, the simplest interpretation of our results is that *Lrig3* titrates activity of a receptor tyrosine kinase signaling pathway that normally induces expression of *Ntn1*, as well as *Lrig3* itself (Fig. 7). Prior to fusion, *Lrig3* is expressed throughout the lateral pouch, where it inhibits RTK activity and prevents fusion from beginning (Step 1, Fig. 7). Subsequently, we hypothesize the presence of a fusion plate inducing signal, which overcomes *Lrig3*-mediated inhibition (Step 2, Fig. 7) to activate the RTK pathway and allow expression of *Ntn1* (Step 3, Fig. 7). *Ntn1*, in turn, enhances activity of this same pathway, most likely by promoting breakdown of the basal lamina (Step 4, Fig. 7). Hence, in *Lrig3* mutants, increased RTK signaling results in early and expanded *Ntn1* expression, as well as persistent *Lrig3* expression (Fig. 7A). Conversely, in *Ntn1* mutants, RTK signaling occurs at low levels, both because of the presence of *Lrig3* and the failure to potentiate the pathway through *Ntn1*-mediated breakdown of the basal lamina. Due to the low level of RTK signaling, canal development arrests at the canal pouch stage, such that expression of *Ntn1* is lost and *Lrig3* is never reduced (Fig. 7A).

A key feature of this model is the fact that *Lrig3* is present prior to any of these events, but is also critical for the subsequent emergence of mutually exclusive domains of *Lrig3* and *Ntn1* expression. Since *Lrig3* activity is required for the feedback loop to function properly, the initial *Lrig3* expression also fails to be downregulated in *Lrig3* mutants. Hence, the subsequent interactions between *Lrig3* and *Ntn1* serve to simultaneously reduce *Lrig3* transcription and increase *Ntn1* only in the regions that receive the inducing signal.

The proposed model fits both with the results reported here as well as the known activities of *Lrig3* and *Ntn1*, but also raises several questions for future consideration. Unfortunately, the ability of *Lrig* proteins to bind to widely divergent members of the receptor tyrosine kinase family *in vitro* will make it difficult to pinpoint a single binding partner *in vivo*, especially in a structure as small as the lateral pouch. The best candidate is the FGF receptor, not only because *Lrig3* is known to bind to and inhibit FGF receptor, but also because of the known importance of FGF signaling during inner ear development. In the developing vestibular system, FGF signaling cooperates with the BMP pathway to define sensory and non-sensory domains of the inner ear (Chang et al., 2004; Pauley et al., 2003) and is subsequently required for proliferation of the periotic mesenchyme (Pirvola et al., 2004). Moreover, mesenchymal proliferation is reduced in *Ntn1* mutants, consistent with the idea that *Ntn1*-dependent breakdown of the basal lamina promotes the ability of the FGF ligand to act during canal morphogenesis. Indeed, it is well established that FGF signaling levels are modified by interactions with the basement membrane during other types of tissue morphogenesis (Lonai, 2003; Patel et al., 2007). Although it remains unclear how *Ntn1* mediates its effects, the extent of basement membrane breakdown correlates strongly with the amount of *Ntn1*: breakdown does not occur in *Ntn1* mutants, is expanded in the presence of ectopic *Ntn1* in *Lrig3* mutants, and proceeds normally in rescued embryos that have only one copy of *Ntn1*. Additional experiments will be needed to understand the specific function of *Ntn1* in the basement membrane, as well as how *Ntn1*-induced changes influence the activity of FGF or other signaling ligands in the extracellular matrix.

Because modest perturbations in the structure of the inner ear cause severe behavioral deficits, our genetic studies were able to reveal the consequences of slight changes in signaling activity that may be undetectable by *in vitro* methods. Indeed, lateral canal truncations also occur in *BMP4* heterozygotes, emphasizing the unusual sensitivity of the developing inner ear to modest changes in signaling levels (Chang et al., 2008). Similarly, in

humans, the lateral canal is the most common site of inner ear anomalies (Sando et al., 2001; Sando et al., 1984), emphasizing the importance of identifying the molecular players that make this canal unusually susceptible to developmental insults. Hence, the *Lrig3/Ntn1* feedback loop may provide an additional safeguard for tissues whose function depends critically on the perfect morphogenesis of complex structures.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

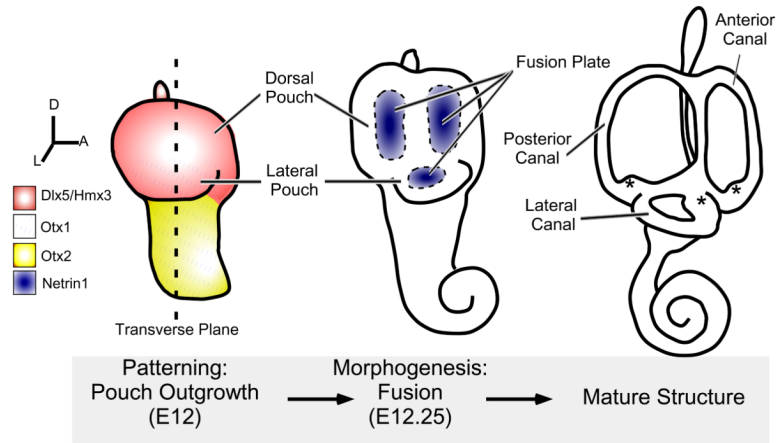
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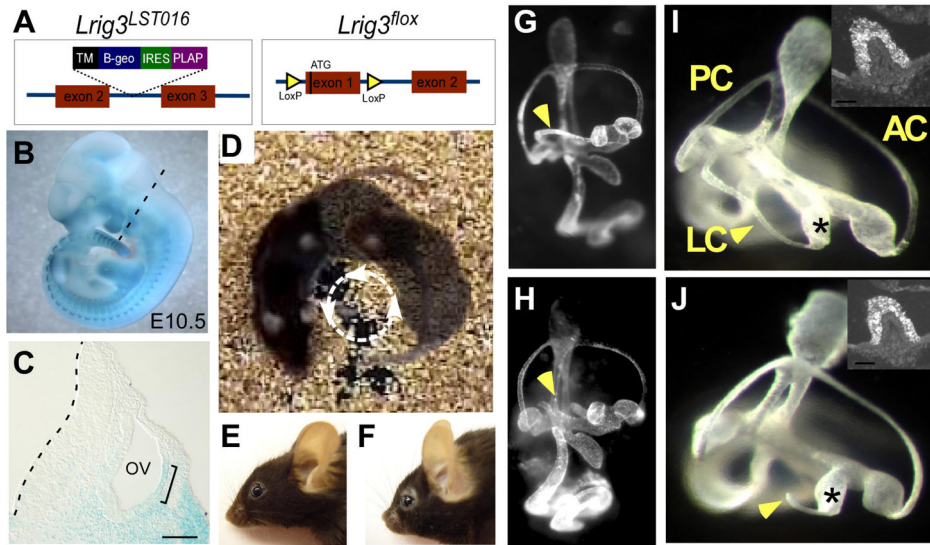
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### Fig. 1. Patterning and Morphogenesis of the Inner Ear

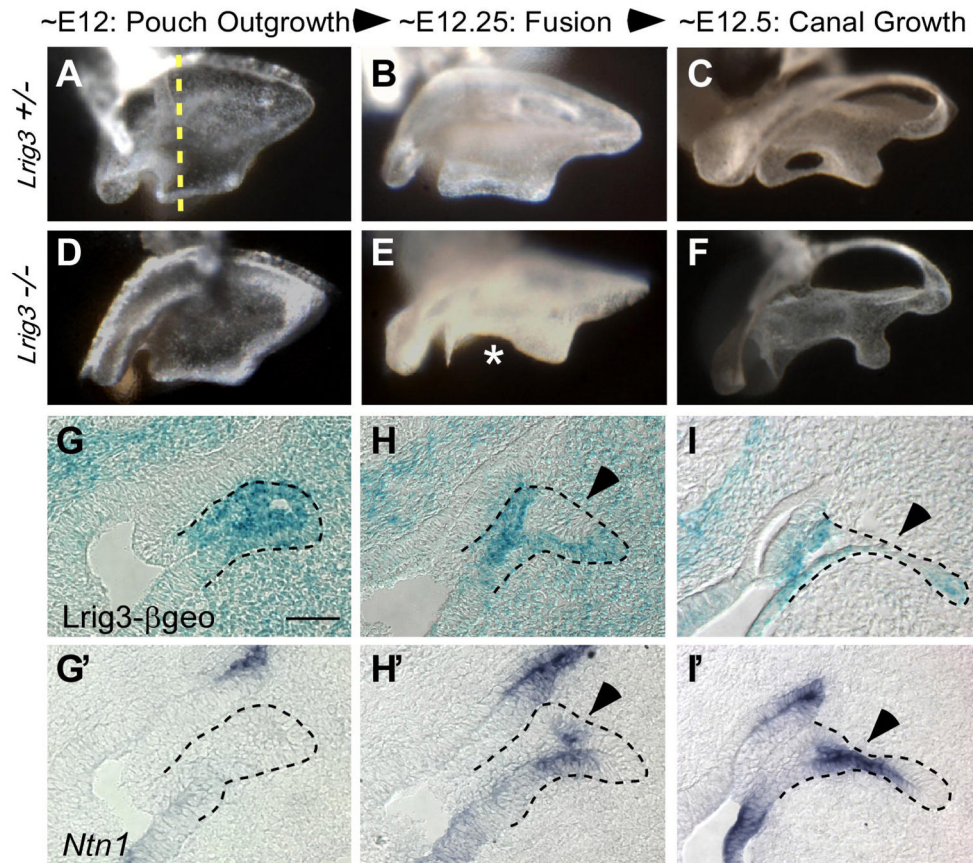
Diagrams of the transformation of the otic vesicle into the mature structure of the inner ear. Early in development (left), the axes of the otic vesicle are patterned, with the presumptive vestibular system expressing *Dlx5* and *Hmx3* (red) and the developing cochlea expressing *Otx2* (yellow). The lateral pouch is defined by expression of *Otx1* (blue dots). A few hours later, during morphogenesis, discrete regions in the dorsal and lateral pouch begin to transcribe *Netrin1* (blue, middle). These regions will subsequently undergo fusion and disappear, leaving the epithelium in the perimeter to form the walls of the mature canals (right). Motion is detected by hair cells housed in swellings at the base of each canal called ampullae (\*). In all of the following figures, paintfilled inner ears are shown looking down onto the lateral canal, with anterior to the right, while sections through the otic vesicle are in the transverse plane (as indicated), with dorsal up and lateral to the right.



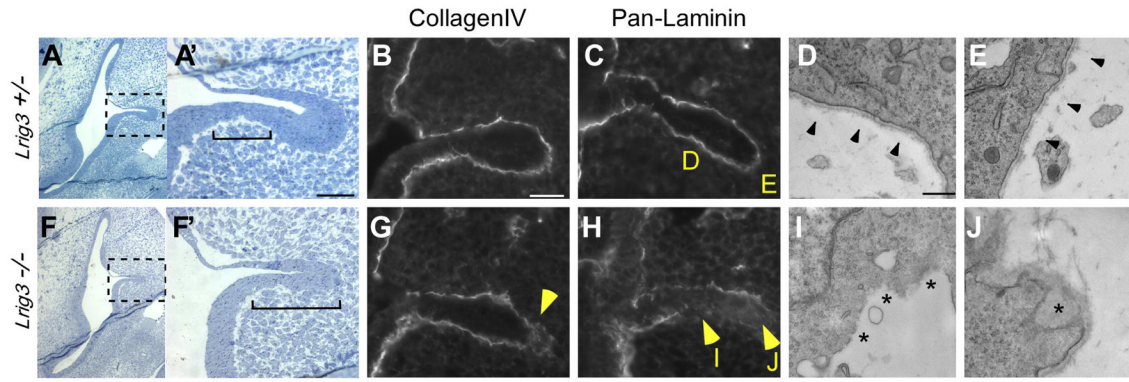


**Fig. 2. *Lrig3* mutant mice exhibit circling behavior due to a truncation of the lateral semicircular canal**

(A) Diagrams of two independent alleles of *Lrig3* illustrating insertion of the gene trap vector in *LST016* mice (left) and the introduction of LoxP sites on either side of the ATG-bearing exon in the conditional *Lrig3<sup>flox</sup>* allele (right). (B,C) X-gal detection of the *Lrig3*- $\beta$ -geo fusion protein in an E10.5 *Lrig3* heterozygous embryo (B) and in sections through the otic vesicle (C) in the plane indicated (dotted line, B).  $\beta$ -galactosidase activity is high in somitic mesoderm, the branchial arches and the limb buds. In the developing inner ear, transcription of *Lrig3* is enriched in the lateral otic epithelium by E10.5 (bracket). Scale bar: 50  $\mu$ m. (D) A single *Lrig3* mutant mouse photographed in three points of its circling trajectory. (E,F) *Lrig3* homozygotes (F) have shortened snouts compared to heterozygotes (E). (G–J) Paintfilled inner ears of E14 *Lrig3*  $+/-$  (G,I) and  $-/-$  (H, J) embryos. Low magnification views of the entire inner ear (G,H) reveal a truncation of the lateral semicircular canal (arrowhead, H). Other structures appear normal in size and shape. High magnification views of the vestibular apparatus confirm truncation of the lateral canal (LC) but not the anterior (AC) or posterior (PC) canals. The lateral ampullae (asterisks) are unaffected, with no change in the number or distribution of MyosinVIIa-positive hair cells in the lateral cristae (insets). Dorsal is up; posterior is to the left.



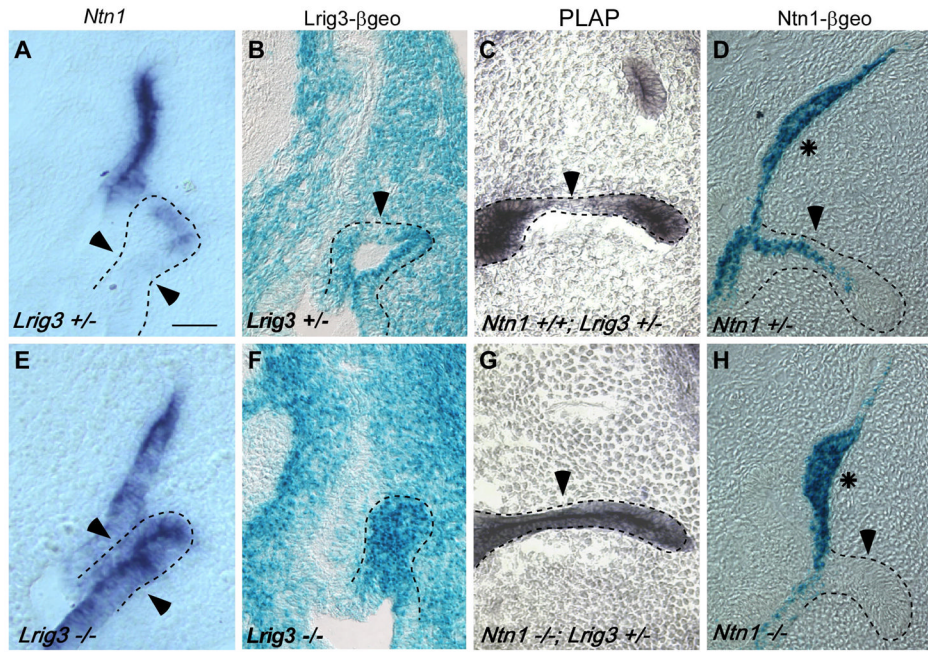
**Fig. 3. *Lrig3* acts in the non-fusing epithelium to prevent premature and ectopic fusion** (A–F) Dorsal views of paintfilled *Lrig3*<sup>+/-</sup> (A–C) and *Lrig3*<sup>-/-</sup> (D–F) inner ears collected at 6 hour intervals from E12 to E12.5. Anterior is to the right. In mutants, early outgrowth is normal (D). However, fusion initiates too early (E) and occurs over a larger area (asterisk) than in control embryos (B,C), resulting in truncation by E12.5 (F). (G–I) Adjacent sections of *Lrig3* heterozygotes through the lateral pouch at the level indicated (dashed line, A) were processed for  $\beta$ -galactosidase histochemistry to reveal *Lrig3*- $\beta$ geo activity (G–I) or for *in situ* hybridization with a probe to *Ntn1* (G'–I'). *Lrig3* is transcribed throughout the lateral pouch prior to fusion (G). Levels become reduced (H) in the nascent fusion plate (arrowhead) just as *Ntn1* expression initiates here (H'). *Lrig3* expression is further diminished (I) as transcription of *Ntn1* expands (I').



**Fig. 4. The basement membrane undergoes early and ectopic breakdown in the inner ear of *Lrig3* mutant mice**

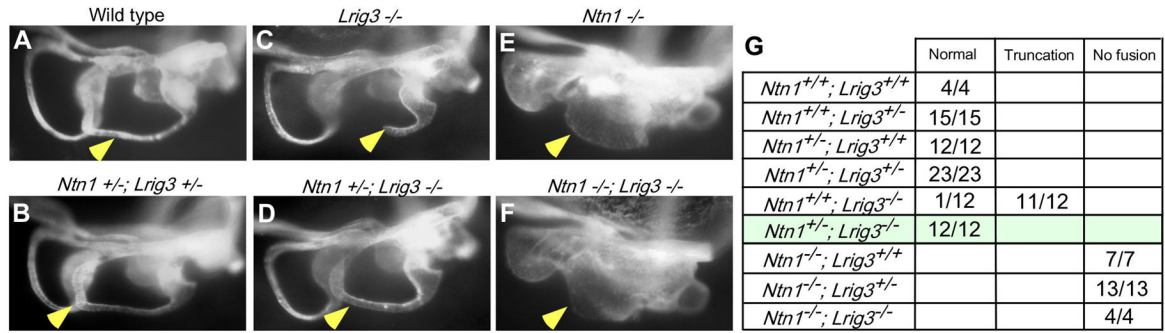
(A,F) Transverse plastic sections through inner ears of E12 *Lrig3*<sup>+/-</sup> (A) and *-/-* littermates (F), with magnified views of the boxed areas (A', F'). Dorsal is up; lateral is to the right. In controls, epithelial cells in the fusion plate intercalate to form a single layer of cells (A'), but in mutants, this region is expanded (brackets, F'). Scale bar: 50  $\mu$ m. (B, C, G, H) Immunofluorescent detection of Collagen IV (B,G) and all laminins (C, H) in E12 *+/-* (B,C) and *-/-* embryos (G,H) sectioned in the transverse plane. In homozygotes, the basal lamina is disturbed by breaks in the laminin network and ectopic accumulation of Collagen IV (arrowheads, G, H). (D, E, I, J) Electron micrographs of the regions indicated in C and H. The basement membrane is continuous in heterozygotes (arrowheads, D, E) but is absent (asterisks, I) or severely disrupted (asterisk, J) in homozygotes. Scale bar: 500 nm.





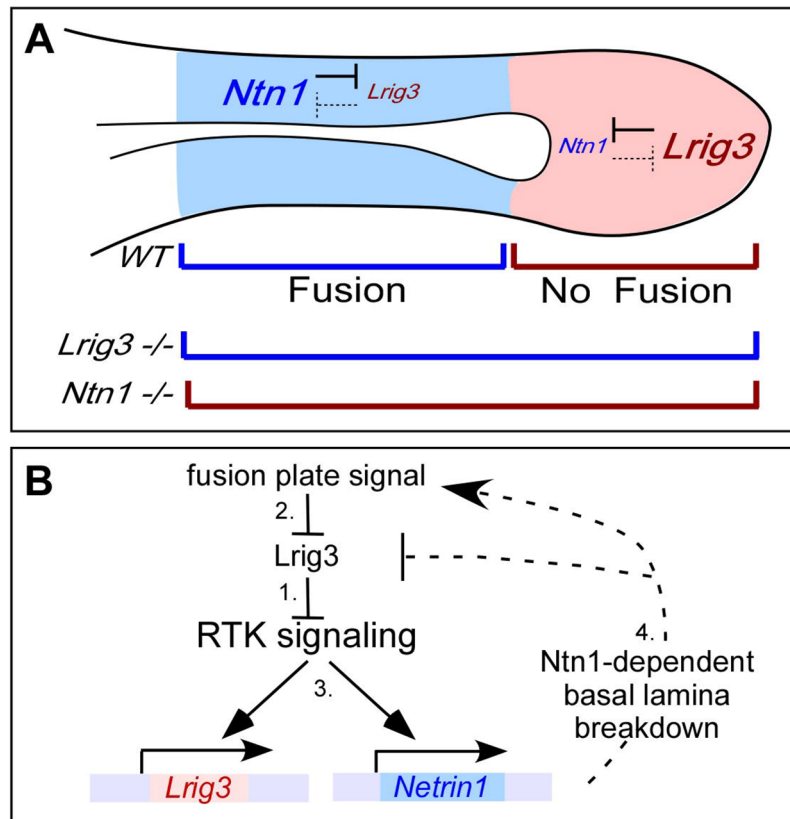
**Fig. 5. *Lrig3* and *Ntn1* participate in cross-repressive interactions that define the fusing and non-fusing domains of the lateral pouch**

(A, E) *In situ* hybridization of *Ntn1* on transverse sections through E12 *Lrig3*<sup>+/-</sup> (A) and *-/-* (E) embryos. In *Lrig3* mutants, *Ntn1* expression is expanded to fill the lateral pouch (outlined). (B, F) *Lrig3* and *Ntn1* mutant mice were generated with two different gene trap vectors, so only *Lrig3*<sup>LST016</sup> mice carry a Placental Alkaline Phosphatase (PLAP) reporter. Hence, PLAP histochemistry reveals *Lrig3* transcription in *Ntn1*<sup>+/+</sup>; *Lrig3*<sup>+/-</sup> (B) and *Ntn1*<sup>-/-</sup>; *Lrig3*<sup>+/-</sup> embryos (F) at E12.5. Like β-geo, PLAP staining of *Lrig3* heterozygotes (B) is absent from the fusion plate at E12.5 (arrowhead). In contrast, *Lrig3* transcription is sustained in the fusion plate of age-matched *Ntn1* homozygotes (F). Note that these embryos are 12 hours older than those in A, C, E, and G. (C, G) β-galactosidase histochemistry of E12 *Lrig3*<sup>+/-</sup> (C) and *Lrig3*<sup>-/-</sup> (G) littermates. As previously demonstrated (Fig. 3), *Lrig3*-βgeo levels are reduced in fusion plate cells (arrowhead, C) compared to the surrounding epithelium. However, in *Lrig3* mutants (G) reporter activity is present at high levels throughout the pouch. (D, H) β-galactosidase histochemistry of E12 *Ntn1*<sup>+/-</sup> (D) and *-/-* (H) littermates. *Ntn1*-βgeo is active in the fusion plate (arrowhead) in heterozygotes (D), consistent with *in situ* hybridization results (see Fig. 3). However, no activity is detected in the lateral pouch of *Ntn1* homozygotes (H). *Ntn1*-βgeo expression is unchanged in the dorsal pouch (asterisks). Scale bar: 50 μm.



**Fig. 6. The *Lrig3* mutant lateral canal truncation is rescued by removal of one copy of *Ntn1*** (A–F) Paintfilled E14 inner ears from transheterozygous intercross littermates. Canals (arrowheads) are normal in wild-type (A) and transheterozygous (B) embryos, while the lateral canal is truncated in *Lrig3* homozygotes (C). The truncation is fully rescued when one wild type copy of *Ntn1* is removed from *Lrig3* homozygotes (D). Fusion does not occur in *Ntn1* mutants (E) or *Ntn1*;*Lrig3* double mutants (F). (G) Table summarizing the proportion of ears with normal, truncated, or unfused semicircular canals for each genotype in offspring from *Ntn1*<sup>+/-</sup>; *Lrig3*<sup>+/-</sup> intercrosses. The rescued population is highlighted in green. Note that the *Ntn1* phenotype is not influenced by the absence of *Lrig3*.





**Fig. 7. Proposed model for the *Lrig3*/*Ntn1* feedback loop**

(A) A diagrammatic view of the lateral pouch during canal morphogenesis. Cross-repressive interactions between *Lrig3* and *Ntn1* define the boundary between fusing (blue) and non-fusing (red) regions of the otic epithelium. When the regulatory loop is interrupted by loss of *Lrig3*, fusion is expanded, while in *Ntn1* mutants, fusion does not occur. Interactions between *Lrig3* and *Ntn1* ensure that these two genes become confined to distinct domains of the lateral pouch. (B) We propose the following model for the *Lrig3*/*Ntn1* feedback loop. *Lrig3* is present throughout the canal pouch before fusion and inhibits activity of a receptor tyrosine kinase (RTK) signaling pathway (1). Subsequently, fusion is initiated by an unknown fusion plate signal, which we hypothesize acts through the RTK pathway by inhibiting *Lrig3* (2), resulting in transcription of *Ntn1* (3). *Lrig3* initially continues to be transcribed, as expected for a feedback-induced antagonist. However, the increased levels of *Ntn1* eventually inhibit *Lrig3* expression, either by inhibiting *Lrig3* or by potentiating activity of the fusion plate signal. For instance, *Ntn1* protein may augment activity of the RTK pathway by promoting basal lamina breakdown (4).