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Vertebrate Lrig3-ErbB Interactions Occur In Vitro but Are Unlikely to Play a Role in Lrig3-Dependent Inner Ear Morphogenesis

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Abstract

**Background:** The Lrig genes encode a family of transmembrane proteins that have been implicated in tumorigenesis, psoriasis, neural crest development, and complex tissue morphogenesis. Whether these diverse phenotypes reflect a single underlying cellular mechanism is not known. However, Lrig proteins contain evolutionarily conserved ectodomains harboring both leucine-rich repeats and immunoglobulin domains, suggesting an ability to bind to common partners. Previous studies revealed that Lrig1 binds to and inhibits members of the ErbB family of receptor tyrosine kinases by inducing receptor internalization and degradation. In addition, other receptor tyrosine kinase binding partners have been identified for both Lrig1 and Lrig3, leaving open the question of whether defective ErbB signaling is responsible for the observed mouse phenotypes.

**Methodology/Principal Findings:** Here, we report that Lrig3, like Lrig1, is able to interact with ErbB receptors in vitro. We examined the in vivo significance of these interactions in the inner ear, where Lrig3 controls semicircular canal formation by determining the timing and extent of Netrin1 expression in the otic vesicle epithelium. We find that ErbB2 and ErbB3 are present in the early otic epithelium, and that Lrig3 acts cell-autonomously here, as would be predicted if Lrig3 regulates ErbB2/3 activity. However, inhibition of ErbB activation in the chick otic vesicle has no detectable effect on Netrin gene expression or canal morphogenesis.

**Conclusions/Significance:** Our results suggest that although both Lrig1 and Lrig3 can interact with ErbB receptors in vitro, modulation of Neuregulin signaling is unlikely to contribute to Lrig3-dependent processes of inner ear morphogenesis. These results highlight the similar binding properties of Lrig1 and Lrig3 and underscore the need to determine how these two family members bind to and regulate different receptors to affect diverse aspects of cell behavior in vivo.

Introduction

The mammalian genome contains an expanded repertoire of transmembrane proteins that carry both leucine-rich repeats (LRR) and immunoglobulin (Ig) domains in their extracellular domains [1]. Within this LRR-Ig superfamily, only the Lrig subfamily contains both invertebrate and vertebrate orthologs, represented by Dlig1 (also called Lambik) in flies and Lrig1, Lrig2, and Lrig3 in vertebrates. Lrig proteins have large extracellular domains with either sixteen (Lrig1 and Lrig3) or fifteen (Lrig2) LRRs and three Ig domains (Fig. 1). Based on a distant similarity to Kekkons, which are the only large family of LRR-Ig proteins in flies, Lrig1, Lrig2, and Lrig3 in vertebrates. Lrig proteins have been identified for both Lrig1 and Lrig3, leaving open the question of whether defective ErbB signaling is responsible for the observed mouse phenotypes.

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Lrig3 mutant mice, on the other hand, exhibit obvious developmental defects that manifest as abnormal morphogenesis of the inner ear and craniofacial structures [27]. The inner ear defect is caused by expanded expression of the secreted protein Netrin1, a known regulator of inner ear development [20]. How the Lrig3 transmembrane molecule ultimately induces changes in gene expression is not known. Hence, it remains unclear whether the varying collection of defects observed in Lrig1 and Lrig3 mutant mice are due to changes in ErbB receptor signaling.

There are several reasons to believe that like Lrig1, Lrig3 could also regulate ErbB signaling. First, Lrig1 is known to interact with ErbB family members through its extracellular domain, which is highly conserved in Lrig3. In addition, the intracellular tail of Lrig3 contains putative SH2 and SH3 domains that have the potential to bind to activated ErbB receptors. Second, Lrig1 and Lrig3 expression overlap extensively during development, suggesting a possible functional overlap [27]. Third, despite this broad expression, both Lrig1 and Lrig3 mutant mice display relatively mild phenotypes. Strikingly, the Lrig3 mutant phenotypes occur in regions where Lrig3 is expressed, namely the lateral canal epithelium of the inner ear and the branchial arches. Thus, Lrig1 may compensate for Lrig3 to regulate ErbB signaling in other regions of the animal. Indeed, like Lrig1 and Lrig3, ErbB2-4 are also expressed during cochlear maturation [29,30]. Finally, ErbB signaling plays a crucial and well-established role in complex morphogenesis of other organ systems, suggesting a possible role for the ErbB receptors in canal formation [8,10,31].

Based on the close homology between Lrig1 and Lrig3, their overlapping expression patterns, and the known importance of ErbB signaling during development, we hypothesized that Lrig3 modulates the Neuregulin pathway in the inner ear. To test this idea, we performed experiments that address three basic questions: 1) Can Lrig3 interact with ErbB receptors in vitro? 2) Are ErbB receptors expressed during inner ear development? and 3) Is ErbB signaling necessary for canal morphogenesis? Our results indicate that although Lrig3 can interact with ErbB receptors in vitro, reduction of Neuregulin signaling in vivo does not cause any detectable changes in Netrin gene expression or in the structure of the inner ear. These results add to a growing body of evidence that Lrig proteins exert their effects through multiple signaling pathways with diverse roles in development and disease.

**Results**

**Lrig3 Can Bind ErbB Receptors In Vitro**

Lrig1 and Lrig3 are closely related proteins, sharing the same number and arrangement of LRRs and Ig domains in the ectodomain (Fig. 1). The composition of these domains is also strongly conserved, with 53% identity between the LRRs and even higher homology in the Ig domains, which are 72% identical. Lrig2, on the other hand, has one fewer LRR and is rarely expressed in the same tissues as either Lrig1 or Lrig3 [27]. Moreover, Lrig2 shares much less homology in the Ig domains (Fig. 1). We therefore focused on comparisons of Lrig1 and Lrig3, and asked whether Lrig3 behaves like Lrig1 with respect to ErbB binding and protein distribution.

First, we tested whether the homology between Lrig1 and Lrig3 is sufficient to mediate interactions with ErbB receptors. A series of co-immunoprecipitations were conducted by expressing EGFR, ErbB2, or ErbB4 together with epitope-tagged constructs of Lrig1 or Lrig3 in HEK293T cells, and in the absence or presence of the appropriate ligand. Indeed, like Lrig1, Lrig3-flag co-immunoprecipitates with EGFR, ErbB2 and ErbB4 in lysates from transfected cells (Fig. 2A). ErbB3 was not tested as this family member is kinase-dead and functions as an obligate heterodimer with ErbB2 [7].

### Figure 1. Lrig family protein structures

Alignment of Lrig1, Lrig2, and Lrig3 shows that Lrig1 and Lrig3 share the same number and spacing of Leucine Rich Repeats (LRRs, green) and Immunoglobulin domains (Ig, yellow). In contrast, Lrig2 lacks an N’ terminal LRR (blue) and has a different kind of Ig repeat, as reflected in the lower degree of identity with Lrig1. In the extracellular domains, all three family members share a highly conserved (48% identical) stretch of 48 amino acids (bracketed). Apart from this motif and some putative SH2 and/or SH3 domains, the cytoplasmic tails vary widely both in length and composition. Lrig1 is the only family member with a confirmed ability to bind c-Cbl. Arrows indicate degrees of identity between the LRR domain-containing regions and the Ig domain-containing regions. The amino acid (aa) length of each protein is also indicated. Domain structure was obtained with SMART protein (http://smart.embl-heidelberg.de/) and putative intracellular domains were defined by Scansite (http://scansite.mit.edu/).

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Since Lrig3 can to bind to ErbB receptors in vitro, we next asked whether, like Lrig1, Lrig3 has the same subcellular distribution as ErbB receptors. We focused on ErbB4 because it is the only NRG receptor that functions as a homodimer. Lrig1-flag, Lrig3-flag, and ErbB4 constructs were expressed either alone or in combination in HEK293T cells; protein localization was determined by immuno-}

**Figure 2. Lrig1 and Lrig3 bind to and co-localize with ErbB receptor tyrosine kinases in vitro.** (A) Like Lrig1, Lrig3 can co-immunoprecipitate with multiple ErbB receptors in HEK293T cells. Lysates were immunoprecipitated with anti-flag antibodies and blotted with anti-ErbB (top) and anti-flag (bottom) antibodies. Both Lrig1-flag and Lrig3-flag can bind to EGFR (left), ErbB2 (middle), and ErbB4 (right) in the absence (−) or presence (+) of ligand (EGF or NRG). No ErbB receptors were precipitated in the absence of epitope-tagged Lrig (vector). Western blotting confirms the presence of each ErbB receptor (ErbB), and epitope-tagged Lrig family member (flag) in total lysates (bottom). Actin was used as a loading control. (B–D) Flag and ErbB4 immunocytochemistry on HEK293T cells transfected with ErbB4, Lrig1-flag, and/or Lrig3-flag alone (B) or in combinations (C–D). When expressed on their own, Lrig1, Lrig3 and ErbB4 are detected at the cell surface (B). When either Lrig1 (C) or Lrig3 (D) is expressed in combination with ErbB4, the proteins co-localize in intracellular compartments (arrowheads in C and D). doi:10.1371/journal.pone.0008981.g002
(Fig. 2C, arrowhead), with much less protein on the cell surface than when they are expressed independently (Fig. 2A). Similarly, Lrig3 and ErbB4 appear to be enriched in the intracellular pool compared to the cell surface under these conditions (Fig. 2D); however, this effect was not as dramatic as for Lrig1. Although more experiments are needed to elucidate the consequences of Lrig3-ErbB4 co-expression, these results suggest that Lrig1 and Lrig3 share an ability to interact with ErbB receptors in vitro.

### Does Lrig3 Act Through ErbB Pathways in Vivo?

Although Lrig3 can bind to ErbB receptors through a highly conserved ectodomain, the cytoplasmic domain of Lrig3 bears little homology to Lrig1, leaving open the question of how Lrig3 activity might ultimately influence NRG signaling. To gain insight into this question, we took an in vivo approach to determine whether the phenotypes observed in Lrig3 mutant mice could be due to misregulated ErbB signaling. The most salient defect in Lrig3 mutant mice occurs in the vestibular apparatus of the inner ear, which normally consists of three semicircular canals oriented with the three dimensions of space. In the absence of Lrig3, the lateral semicircular canal is truncated due to increased expression of Netrin1, which encodes a secreted extracellular matrix molecule required for canal development [27,28]. A role for ErbB signaling during canal development has not yet been examined. Therefore, we sought to determine whether Lrig3 acts through ErbB pathways to orchestrate canal morphogenesis.

### ErbB Receptors Are Expressed in the Otic Vesicle Epithelium

In order to uncover whether Lrig3 acts through ErbB receptors, we first asked whether ErbB receptors are present during inner ear development. The vestibular canals are sculpted from the otic vesicle, a hollow ball of epithelial cells that is formed by invagination of ectoderm at the level of the hindbrain. The lateral canal develops from an epithelial outpocketing of the lateral wall of the otic vesicle at E12 (Fig. 3A). In situ hybridization for ErbB receptors in E12 otic vesicles revealed that EGFR cannot be detected in the inner ear but that ErbB3 is broadly expressed throughout the epithelium (Fig. 3B,C). In situ probes for other receptors proved to be unreliable and inconclusive. Therefore, to test for the presence of other ErbB receptors, we microdissected otic vesicle epithelia and performed a Western blot analysis with antibodies to all four ErbB receptors (Fig. 3D). As seen by in situ hybridization, ErbB3 protein is present in abundance, with only trace amounts of EGFR. ErbB2 protein is also present at high levels, consistent with the fact that ErbB2 and ErbB3 nearly always function as a heterodimer. ErbB4 is not detected. Therefore, we conclude that ErbB2, which cannot bind ligand, and ErbB3, which is kinase-dead, act together to receive NRG signals in the otic epithelium.

### Lrig3 Acts within the Otic Vesicle Epithelium to Control Fusion Plate Formation

In our model, Lrig3 acts cell-autonomously to control ErbB receptor signaling and hence expression of Netrin1 in the otic epithelium [27]. In support of this idea, in situ hybridization of ErbB3 reveals strong expression in the epithelium but not the surrounding mesenchyme. To determine whether Lrig3 is required in these ErbB-expressing cells, we generated and analyzed epithelial-specific knock-outs of Lrig3.

Previously, a conditional Lrig3 allele was generated by flanking the ATG-bearing exon of Lrig3 with LoxP sites [27]. Null mutant mice exhibit lateral canal truncations and craniofacial defects, consistent with the complete loss of Lrig3 messenger RNA (Fig. 4C,D and data not shown). To specifically delete Lrig3 from otic epithelium, Lrig3flox/− conditional mice were crossed to mice carrying a Pax2Cre transgene, which induces recombination of a β-galactosidase reporter gene throughout the epithelium (Fig. 4F) [32]. Deletion of Lrig3 from canals epithelial cells results in the same lateral canal truncation evident in hypomorphic and null alleles of Lrig3 (Fig. 4G). Thus, Lrig3 acts within the otic epithelium to coordinate canal morphogenesis.

### Expression of Dominant-Negative ErbB in the Chick Otic Vesicle Has No Effect on Canal Morphogenesis

Our results indicate that Lrig3 can bind to ErbB receptors and that ErbB2/B3 are present in the otic epithelium, where Lrig3 acts cell autonomously to control canal formation. However, while Lrig1 is known to inhibit ErbB signaling, whether or not Lrig3 has the same molecular effects is unclear. If Lrig3 normally inhibits...
ErbB activity, then decreased ErbB signaling is predicted to lead to a loss of Netrin1 expression and hence arrested canal formation, as occurs in Netrin1 mutant mice. Alternatively, if Lrig3 potentiates ErbB activity, we would predict canal truncations similar to what is seen in Lrig3 mutant mice. To distinguish between these possibilities, we performed an unbiased experiment to ask whether ErbB signaling is required for any aspect of canal morphogenesis.

Several different ErbB mouse mutants are available and have been studied extensively [33-38]. However, most mutant mice die at early embryonic stages due to various defects in cardiac development and are therefore not useful for the study of inner ear morphogenesis [39–42]. Therefore, we turned instead to the chick as a model system for canal morphogenesis. The mouse and chicken inner ear develop similarly both at the cellular and molecular level [43,44]. However, the chicken is more accessible and amenable to manipulations than the mouse. Indeed, the chick has been used successfully in the past for the study of both FGFs and BMPs in inner ear morphogenesis [45,46].

First, we confirmed the presence of Lrig3 in the chicken inner ear by in situ hybridization of embryonic day 5 (E5) embryos, which is comparable to E12 in mouse. As predicted, Lrig3 is transcribed in the lateral pouch epithelium of the developing chick as it is in mice, suggesting a common function in both species (Fig 5A,D). Similarly, chicken ErbB2 and ErbB3 are expressed broadly throughout the otic epithelium as in mice (Fig 5B,C). Finally, we confirmed expression of two closely-related Netrin genes, cNetrin1 and cNetrin2, which overlap extensively in the chick embryo [47,48]. Notably, there is no mouse ortholog of Netrin2, but in chicks, Netrin activity appears to reflect contributions from both Netrin1 and Netrin2. Consistent with this idea, we found that both cNetrin1 and cNetrin2 are present in the fusion plate epithelium, with cNetrin1 expressed slightly later and at lower levels (Fig 5E) than cNetrin2, which appears to be the major player in chicken (Fig 5F). As in mouse, this expression is complementary to cLrig3, which is downregulated in the presumptive fusion plate and maintained only in the non-fusing epithelium at E6 (Fig 5D–F).

To block ErbB signaling in the chicken otic vesicle, we used a virus to express a truncated version of the ErbB4 receptor. This construct blocks all Neuregulin signaling by binding NRG ligands and preventing them from binding to endogenous ErbB2, ErbB3, and ErbB4 [49] (Fig. 6A). This same strategy was used to block ErbB signaling successfully in both mice and chickens [30,50,51]. The DNErbB4-flag construct was cloned into the RCAS vector; control viruses consisted of an RCAS virus expressing either alkaline phosphatase or a secreted form of GFP. To confirm that the RCAS(A)/DNErbB4-flag blocks ErbB signaling effectively, chicken DF1 cells were transfected with ErbB4 to make them NRG responsive. The same cells were then infected with increasing amounts RCAS(A)/DNErbB4-flag. As expected, increasing amounts of the dominant negative construct cause a dose-dependent reduction in ErbB receptor phosphorylation in response to NRG (Fig 6B). Hence, NRG signaling is strongly blocked in the presence of RCAS(A)/DNErbB4-flag. Having assessed the efficacy of the virus in blocking ErbB signaling, we next tested the ability of RCAS(A)/DNErbB4-flag to inhibit chicken epithelium in vivo. Both in situ hybridization using a probe specific to the DNErbB4 construct (Fig 6E) and ErbB4 immunostaining of infected chicken otic vesicles sections (Fig 6F) demonstrate that DNErbB4 mRNA and protein are produced in otic epithelium. Therefore, we conclude that RCAS(A)/DNErbB4-flag provides a suitable tool for blocking NRG signaling in vivo.

We next asked whether inhibition of NRG signaling has any effect on Netrin transcription or canal morphogenesis. RCAS(A)/DNErbB4-flag (5.6×10⁸ iu/ml) or control virus (RCAS(A)/GFP at 2×10⁸ iu/ml or RCAS(A)/AP at 5×10⁶ iu/ml) were injected into the otic vesicles of E3 chickens, allowed to infect for 72 hours and then sacrificed to assess the level of infection by immuno-staining against the 3C2-gag viral coat protein (Fig. 7A,C,E,G). Since patterns of RCAS infection vary from embryo to embryo and not all infected cells produce the protein of interest, we also confirmed expression of DNErbB4. Consistent with the broad expression of 3C2-gag in the mesenchyme and epithelium, DNErbB4-flag is widely misexpressed in the lateral pouch epithelium at E5 (Fig. 7C). However, no change in cNetrin1 is detected either at E5 (Fig. 7B,D) or E6 (data not shown). Similarly, high levels of cNetrin2 are maintained throughout the fusion plate at E6 despite abundant expression of DNErbB4-flag throughout the lateral pouch epithelium (Fig. 7F,H) (n = 10 DNErbB4 infected and 7 control infected embryos for cNetrin1 and n = 9 DNErbB4 infected and 8 control infected embryos for cNetrin2). To assess
whether reduced ErbB signaling in the developing otic vesicle may result in canal defects independent of changes in Netrin expression, some injected chickens were allowed to develop until E7, when canal morphogenesis is complete. However, paintfills reveal no differences in the shape or size of the canals in RCAS(A)/DNErbB4-flag infected inner ears (n = 10) when compared to controls (n = 8) (Fig. 7I–L). There are other sites of ErbB/Lrig3 interactions, including canal morphogenesis, which may become apparent in future studies.

Figure 5. Expression of cLrig3, cNetrin1, cNetrin2, cErbB2 and cErbB3 in chick otic epithelium. (A–F) In situ hybridization of cLrig3 (A,D), cErbB2 (B), cErbB3 (C), cNetrin1 (E), and cNetrin2 (F) on transverse sections of E5 (A) or E6 (B–F) chicken heads. Dorsal is up; lateral is right. As in the mouse, chicken Lrig3 is initially present throughout the lateral pouch at E5 (arrows, A), and then sustained in the non-fusing epithelium (arrow, D) but not the fusion plate (arrowhead, D) at E6. Hybridization of adjacent sections confirms that chicken Netrin1 expression is complementary to Lrig3, with transcript detected in the fusing (arrowhead, E) but not non-fusing epithelium (arrow, E). In addition, cNetrin2, which does not exist in mice, is robustly expressed overlapping with cNetrin1 in the fusion plate (arrowhead, F). Both ErbB2 and ErbB3 are transcribed throughout the otic epithelium (B) including the vertical pouch (vp), lateral pouch (lp) and cochlear duct (cd).

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Figure 6. RCAS(A)/DNErbB4-flag produces DNErbB4-flag protein in vitro and in vivo. (A) Diagrams of ErbB2-4 receptors depicting their extracellular domains (blue and yellow), intracellular kinase modules (red), and tyrosine residues (Y). Four Neuregulin ligands (NRG1-4) bind to ErbB3 and ErbB4 as shown. Since ErbB2 cannot bind ligand and ErbB3 is kinase dead, ErbB2 and ErbB3 typically heterodimerize in order to bind and transduce Neuregulin signals. Dominant negative (DN) ErbB4 consists of the extracellular and transmembrane domains of the human ErbB4 receptor (bracketed), which binds to all four NRG ligands and prevents them from interacting with their endogenous receptors. Therefore, DNErbB4 effectively blocks Neuregulin signaling through all ErbB receptors. (B) Western blot analysis of lysates of DF1 chicken cells transiently transfected with ErbB4 and infected with RCAS(A)/DNErbB4-flag in the presence or absence of NRG, as indicated. Production of ErbB4 was confirmed by probing lysates with antibodies to ErbB4 (third row). Antibodies against the flag epitope reveal the relative amount of DNErbB4-flag in cells infected with $5 \times 10^5$ or $5 \times 10^6$ infectious units of RCAS(A)/DNErbB4-flag (second row). NRG stimulation of uninfected cells induces phosphorylation of ErbB4, as detected by blotting with anti-phosphotyrosine (P-Y) antibodies (top). This phosphorylation is reduced in the presence of increased levels of DNErbB4-flag. Actin served as a loading control for the P-Y blot (bottom). (C–F) Adjacent transverse sections through E5 chicken otic vesicles infected with RCAS(A)/DNErbB4-flag 48 hours earlier and processed for DAPI staining (C), anti-3C2 immunostaining (D), DNErbB4 in situ hybridization (E), or anti-ErbB4 immunostaining (F). The DNErbB4 in situ probe and the anti-ErbB4 antibody are specific to human ErbB4. 3C2-gag immunostaining reveals broad infection of the otic epithelium, including the lateral pouch (asterisk). Both DNErbB4 transcript (E) and DNErbB4 protein (F) are produced here (asterisks). In some areas, viral infection does not generate detectable amounts of DNErbB4-flag (arrowhead). Therefore, in all subsequent experiments, the extent of infection was monitored by in situ hybridization for DNErbB4.

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Lrig3/ErbB Interactions

Discussion

Although Lrig proteins harbor common protein-protein interaction motifs in their extracellular domain, their cytoplasmic domains are widely divergent and specific molecular functions have remained elusive. Here, we confirm a general ability for Lrig proteins to interact with ErbB receptors in vitro. However, misexpression of dominant negative ErbB4 has no effect on Netrin expression or the structure of the inner ear, suggesting that aberrant NRG signaling is not a good explanation for Lrig3-dependent aspects of canal morphogenesis. These results add to the mounting evidence that Lrig proteins are not dedicated regulators of ErbB signaling; rather, they may have multiple functions. Our findings highlight the incomplete knowledge of Lrig protein function at present.

Since Lrig proteins have highly conserved extracellular domains with many protein-protein interaction motifs, a better understanding of the nature and variety of bona fide binding partners will provide important insights into Lrig function. Although ErbB proteins were the first binding partners to be identified, Lrig proteins are also able to interact with other receptor tyrosine kinases that share no homology with the ErbB ectodomain. For instance, Lrig1 can also inhibit Met and Ret signaling pathways, while Lrig3 has been shown to bind to the FGF receptor [52–54]. In addition, our own in vitro studies have shown that in addition to the ErbB receptors, Lrig3 is also able to bind to a wide variety of receptors, including the Netrin1 receptors Unc5Ha-c, the Neurotrophin receptor p75 and the axon guidance receptor PlexinA1 (data not shown). Our discovery that the Lrig3 mutant phenotype is apparently unrelated to NRG signaling emphasizes the need to confirm the relevance of each of these in vitro binding interactions for specific in vivo functions.

Although Lrig1 and Lrig3 may share the ability to interact with a wide variety of binding partners in vitro, whether or not this reflects similar cell biological functions in vivo remains unclear. Although the extracellular domains of Lrig3 and Lrig1 are highly conserved, the proteins share very little homology in their intracellular domains. For example, Lrig3 shows no similarity to Lrig1 in the portion of the cytoplasmic domain that binds to c-Cbl. Consistent with this observation, although Lrig3 is able to bind to ErbB receptors, we noticed that unlike Lrig1, Lrig3 does not induce a dramatic downregulation of ErbB receptor levels (see Fig. 2A). Rather, there is a trend towards increased ErbB receptor levels, suggesting that Lrig3/ErbB interactions may not result in efficient degradation at all. Thus, Lrig3’s ability to bind ErbB receptors does not necessarily imply that Lrig3 also induces ubiquitination of ErbB receptors. Indeed, even Lrig1 exhibits c-Cbl independent functions, since Lrig1-mediated degradation of the Met receptor does not involve polyubiquitination [53]. Moreover, Lrig1 inhibits Ret function by preventing recruitment to lipid rafts, with no effect on internalization [52]. Such examples highlight the possibility that individual Lrig proteins may have diverse molecular and cellular functions depending on the context.

To date, investigations of Lrig1 and Lrig3 mutant mouse phenotypes have confirmed that both molecules are important for aspects of development, but have done little to clarify their specific functions [24,27]. Although Lrig1 has been proposed to act through ErbB in the skin, whereas Lrig3 does not seem to function through NRG signaling in the inner ear, our results do not rule out shared functions for Lrig1 and Lrig3 in other tissues. Indeed, based on the broad and overlapping expression patterns overlap outside of the lateral pouch, so it is possible that blocking ErbB signaling could result in other defects of inner ear development that paintfills cannot reveal. In addition, since our approach does not remove all ErbB activity, we cannot rule out that transient and/or low levels of NRG signaling persist and are sufficient for perfect formation of the inner ear. However, since DNErbB4 was broadly expressed (i.e. Fig. 7C,G) and was highly effective in vitro (Fig. 6B), these experiments suggest that misregulation of NRG signaling is unlikely to explain the Lrig3 canal defects.
for Lrig1 and 3 during development, the limited number of phenotypes evident in each mouse mutant suggests that Lrig1 and 3 may function together. Our studies provide additional evidence that Lrig1 and Lrig3 can in fact act through common pathways that have yet to be defined. For instance, Lrig1 and Lrig3 have similar binding properties, raising the possibility that Lrig3 could influence Lrig1’s ability to induce receptor internalization. These similarities with respect to ErbB signaling in vitro could also reflect a basic binding property that enables interactions with another signaling pathway that has yet to be defined. The nature of any additional defects that arise in Lrig1/Lrig3 double mutant mice is likely to provide useful insights into this question in the future.

Since ErbB receptors do not seem to be involved in the Lrig3-Ntrk feedback loop, the identity of the RTK hypothesized to be inhibited by Lrig3 remains unclear. The best candidate is the FGF receptor, not only because Lrig3 is known to bind to and inhibit FGF receptor [34], but also because of the known importance of FGF signaling during canal morphogenesis [45,55–57]. FGF signaling promotes the formation of the fusion plate and the proliferation of the periotic mesenchyme [57]. Although FGF ligands and receptors have been identified in several different regions of the developing inner ear, uncovering a possible Lrig/FGFR interaction in the inner ear will be difficult, as multiple feedback-induced antagonists are present and may mask Lrig activities.

As well as revealing the need to look beyond ErbB receptors as biologically relevant binding partners for Lrig3, our studies provide evidence that NRG signaling is not essential for canal morphogenesis. This result is unexpected given the early and broad expression of ErbB2/B3 in the otic epithelium as well as the prominent role for ErbB activation elsewhere in the embryo. As with any negative result, it is possible that the absence of a phenotype is due to the technical limitations of the experiment, since low level NRG signaling could persist in these conditions. However, we consider this unlikely for the following reasons. First, this virus effectively blocks NRG signaling in chicken cells [Fig. 6]. Second, we achieved broad expression of DNErbB4 throughout the otic epithelium (Fig. 7). Third, since DNErbB4 works by binding to and sequestering the NRG ligand, NRG signaling will be reduced not only in DNErbB4 expressing cells, but also in the surrounding tissue. Fourth, the inner ear is extremely sensitive to even modest changes in signaling, as evidenced by the presence of defects in several heterozygous mouse lines [46,58]. Nonetheless, we did not detect even a subtle change in canal structure or Ntrn expression. Finally, although ErbB2 has been shown to be essential for normal innervation in the inner ear, no canal defects were reported [59]. Thus, we favor the interpretation that NRG signaling is not essential for canal morphogenesis.

Nevertheless, it is possible that NRG signaling is important for other aspects of ear development that may also involve Lrig1 and/or Lrig3. Our assays were designed to detect changes in canal structure and cNtrn1/2 gene expression, and would not have revealed cellular phenotypes in the sensory epithelia or other regions of the inner ear. For example, Lrig1 and Lrig3 appear to overlap with ErbB2+ in the neonatal cochlea [29,60], where they might fine tune NRG-induced production of BDNF and subsequent neuronal ErbB2/4 signaling [4,17,18]. However, our results suggest that this is an oversimplified view of Lrig function. It is possible that other receptor tyrosine kinase pathways are also misregulated in the absence of Lrig1, raising the possibility that ErbB-targeted drugs might not be appropriate in these cases. Conversely, activation of Lrig with new drugs may not be a viable option for slowing tumor development. Through continued dissection of the signaling properties of this intriguing protein family both in vitro and in vivo, the full extent of Lrig’s contribution to development and disease will become clear.

Materials and Methods

Plasmids and HEK Transfections

The human Nflag-Lrig1 construct was a gift from H. Hedman (Umea University, Umea, Sweden). The ErbB4 and DNErbB4-flag constructs were kindly provided by G. Corfas (Children’s Hospital, Boston). Full length cDNA from mouse Lrig3 was cloned into pcDNA3.1+ and a flag tag was cloned into the unique Smal site immediately following the signal sequence; the epitope with flanking protein sequence is HGAPGMDYKDDDDKGGQLLD. HEK293T cells were cultured in Dulbecco’s Minimal Essential Medium (DMEM) (Invitrogen) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were plated at 60–70% confluency on six-well or 10 cm plates, transfected using FuGene6 (Roche), and harvested 24 hours later. When indicated, cells were starved in DMEM without serum for 16 hours and treated with 1 nM recombinant human Neuregulin1-b1 (NRG1, R&D Systems) or 200 ng/ml of Human EGF (Protech) for 10 min before lysis.

Co-Immunoprecipitation

Cells were washed with cold phosphate buffered saline (PBS) and lysed in 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, and 1 mM of Pefabloc (Roche). For immunoprecipitations, lysates were pre-cleared with agarse-conjugated normal IgG for 30 min at 4°C and immunoprecipitated with agarse conjugated flag antibody (M2, Sigma) overnight at 4°C. Samples were washed four times with lysis buffer before the beads were suspended in SDS sample buffer and boiled for 5 min. Western analysis was performed using standard protocols and the following antibodies: EGF-R (1:1000, Santa Cruz), ErbB2 (1:1000, Abcam), ErbB3 (1:1000, Santa Cruz), ErbB4 (1:1000, Santa Cruz); flag (1:1000 M2, Sigma), and actin (1:5000, Abcam).

Immunocytochemistry

Cells grown on glass coverslips were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min on ice, permeabilized in 0.1% Triton X-100 for 10 min, blocked with 5% bovine serum albumin (BSA) for 30 min, and then incubated overnight at 4°C with primary antibodies diluted in blocking solution: flag (1:1000, M2 Sigma) and ErbB4 (1:500, Santa Cruz). The coverslips were washed with PBS, and detection was performed using appropriate secondary antibodies (1:2000, Jackson Immunoresearch) for 1 hour at room temperature. Nuclei were counterstained with DAPI for 5 min (1:10,000, Sigma), washed with PBS, and mounted with Vectashield (Vector Laboratories).
Mice

\textit{Lrig3}\textsuperscript{lox/lox} conditional mice [27] and \textit{Pax2Cre} mice [32] have been maintained for over five generations on C57Bl6/J background. \textit{Rosa26} reporter mice (\textit{R26R}) [61] and \textit{Z/EG} reporter mice [62] have been maintained for over five generations on a CD1 background. Genotyping was performed as described or using primers to amplify Cre [297 (ATTTGCTGCAATTACGGGTC) and 298 (ATCAA-GTGTTTCTTGGGGA)] or GFP [7110 (TACGGCAAGCTGACCCCTGAAGTTTC) and 7111 (AAGTCGATGCCCTTCAGCTCTGTTTTCTTTTCGGA)] or GFP [7110 (TACGGCAAGCTGACCCCTGAAGTTTC) and 7111 (AAGTCGATGCCCTTCAGCTCTGTTTTCTTTTCGGA)]. All mice are maintained in accordance with institutional and NIH guidelines approved by the IACUC at Harvard Medical School.

\textbf{In Situ Hybridization}

Non-radioactive \textit{in situ} hybridization was performed on 12–14 \textmu m frozen sections using the following probes: Mouse: \textit{EGFR} (ENSMUST00000020329, nt3816–4429) and \textit{ErbB3} (ENSMUST00000020329, nt3816–4420). Chicken: \textit{Lrig3} (ENSGALG00000009755, nt2931–3643), \textit{ErbB2} (NM_001044661.1, nt2235–2934), \textit{ErbB3} (NM_001044661.1, nt2173–2880), \textit{Netrin2} (L34550, nt2097–2645). The \textit{Netrin2} probe was provided by C. Cepko (Harvard Medical School), and the \textit{DNErbB4} probe was a gift from G. Corfas (Children’s Hospital, Boston). A detailed protocol is available at http://goodrich.med.harvard.edu/.

\textbf{Otic Vesicle Western Blots}

\textit{Pax2Cre/\textit{Z/EG}} otic vesicles were microdissected from E12 embryos and lysed as described above. The samples were homogenized on ice for 10 min, and then centrifuged at 1000 \times g for 10 min at 4°C. Supernatant was either used immediately for Western analysis or frozen in SDS sample buffer at −80°C. Western analysis was performed as described above using the following antibodies: \textit{EGFR} (1:1000, Santa Cruz), \textit{ErbB2} (1:1000, Abcam), \textit{ErbB3} (1:1000, Santa Cruz), \textit{ErbB4} (1:1000, Santa Cruz).

\textbf{X-Gal Staining}

Staining for β-galactosidase was performed as described [63] except that 10–20 \textmu m frozen sections were used, and the tissue was fixed for 1 hour at 4°C.

\textbf{Construction, Production and Validation of RCAS(A)/DNERbB4 Virus}

A Replication-Competent Avian sarcoma-leukosis retroviral vector, RCAS(A), containing the DNERbB4-flag construct was built using Gateway recombination cloning into the RCASBP-Y DV destination vector [64]. Specifically, attB1 and attB2 sites were added to the ends of DNERbB4-flag fragment using PCR with primers B1F_DNEB (5’ GGGGACCACTTTGTACAAAAAAG- CAGGCTGAAACCAGTATGAAAGCCCGGACAGGACT 3’) and B2R_DNEB (5’ GGGGACCACTTTGTACAAAAAAGGC- TGGGTCTCAGTTGTCTAGCTATGTTTGG 3’). The PCR fragments were first inserted into pDONR221, using BP Clonase II (Invitrogen), resulting in an entry vector, pME-DNEB. The DNERbB4-flag fragment in pME-DNEB was cloned into RCASBP-Y DV using LR Clonase II (Invitrogen) to create pRCAS-DNERbB4 proviral vector. RCAS/DNERbB4-flag virus stock was generated in UMNSAH/DF-1 chicken fibroblasts (ATCC CRL-12203), concentrated by centrifugation and titrated on these cells as described previously [65].

RCAS(A)/DNERbB4-flag was used at a titer of 5.6 \times 10^9 infectious units/ml (iu/ml). Control viruses included an RCAS(A)/GFP at a titer of 2 \times 10^9 infectious units/ml and RCAS(A)/AP at a titer of 5 \times 10^8 infectious units/ml. To validate that RCAS(A)/DNERbB4-flag blocks NRG signaling, DF1 chicken cells were grown to ~30% confluency and transiently transfected with ErbB4 using FuGene6 (Invitrogen); the next day, each well was infected with either no virus, 5.6 \times 10^5 iu/ml or 5.6 \times 10^6 iu/ml of virus for an hour while rocking at 37°C. 24 hours post-infection, cells were serum starved for an additional 16 hours and then treated with 1 nM recombinant human Neuregulin1-b1 (NRG1, R&D Systems) for 10 minutes before lysis. Western analysis of the cell lysates was performed with the following antibodies: phosphotyrosine (1:1000, Upstate), flag (1:1000, M2, Sigma); ErbB4 (1:1000, C-10, Santa Cruz); and actin (1:5000, Abcam). Chicken DF1 cells were maintained in DMEM (Invitrogen) with 10% FBS, 5% chicken serum, and 1% penicillin/streptomycin.

\textbf{Viral Infection of Chick Otic Vesicles}

White leghorn premium quality chicken eggs were maintained in a 39°C humidified incubator for the duration of the experiment (7 days), according to IACUC guidelines at Harvard Medical School. RCAS(A)/DNERbB4-flag virus or control virus was injected into the right otic vesicle of E3 (stage 17) chick embryos. In order to visualize the amount of virus injected, 1–5 μl of 0.25% Fast Green solution was combined with 10–50 μl of virus. Using a backloading pipette tip (Eppendorf), 5 μl of the virus with the Fast Green solution was loaded into a glass pipette that had been previously prepared by pulling Omega dot capillaries (No. 30-30-0 1.0 mm od; 0.75 mm id; 100 mm long; FHC, Brunswick, ME). To inject the virus, we used a picospritzer needle holder attached to a micromanipulator (Picospritzer: Picospritzer III from Parker Ins; micromanipulator: Siskiyou Instruments; stand: Fisso S-20 from Flexbar). Tungsten needle and forceps were used to open the vitelline membrane that surrounds the head of the embryo. The loaded pipette tip was inserted into the otic vesicle using the micromanipulator, and with the picospritzer, the virus was injected to fill the entire otic vesicle. Following a successful injection the egg was sealed and placed back into the humidified incubator until E5, E6, or E7.

\textbf{Immunohistochemistry}

E5 chick embryos were collected and fixed for 1–2 hours at 4°C in 4% PFA/PBS and then dehydrated in 30% sucrose/PBS overnight at 4°C and equilibrated in Neg50 (Richard-Allan Scientific) for 2–3 hours at 4°C, followed by embedding in Neg50. Frozen sections (10–14 μm) were blocked and permeabilized in 5% normal donkey serum (DakoCytomation) and equilibrated in Neg50 (Richard-Allan Scientific) for 2–3 hours at 4°C and equilibrated in Neg50 (Richard-Allan Scientific) for 2–3 hours at 4°C. Primary antibodies were added into the above block, without Triton X-100 at 1 hour at room temperature. Primary antibodies included: rabbit antibodies: phosphotyrosine (1:1000, Upstate), flag (1:1000, Santa Cruz); and actin (1:5000, Abcam). Chicken DF1 cells were maintained in DMEM (Invitrogen) with 10% FBS, 5% chicken serum, and 1% penicillin/streptomycin.

\textbf{Paintfills}

E7 chick or E14 mouse heads were fixed overnight at 4°C with Bodian’s Fix, dehydrated overnight at room temperature with 100% ethanol, then cleared overnight at room temperature with methyl salicylate. Heads were hemisectioned, 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.
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References


Author Contributions

Conceived and designed the experiments: VEA LG. Performed the experiments: VEA LG. Analyzed the data: VEA LG. Contributed reagents/materials/analysis tools: TS DF. Wrote the paper: VEA LG.


