Regulation of otic vesicle and hair cell stereocilia morphogenesis by Ena/VASP-like (Evl) in *Xenopus*

Sarah J. Wanner and Jeffrey R. Miller*

Department of Genetics, Cell Biology and Development and Developmental Biology Center, University of Minnesota, Minneapolis, MN 55455, USA

*Author for correspondence (e-mail: mille380@umn.edu)

Accepted 23 May 2007

Journal of Cell Science 120, 2641-2651 Published by The Company of Biologists 2007
doi:10.1242/jcs.004556

Summary

The inner ear is derived from a thickening in the embryonic ectoderm, called the otic placode. This structure undergoes extensive morphogenetic movements throughout its development and gives rise to all components of the inner ear. Ena/VASP-like (Evl) is an actin binding protein involved in the regulation of cytoskeletal dynamics and organization. We have examined the role of Evl during the morphogenesis of the *Xenopus* inner ear. Evl (hereafter referred to as Xevl) is expressed throughout otic vesicle formation and is enriched in the neuroblasts that delaminate to form the vestibulocochlear ganglion and in hair cells that possess mechanosensory stereocilia. Knockdown of Xevl perturbs epithelial morphology and intercellular adhesion in the otic vesicle and disrupts formation of the vestibulocochlear ganglion, evidenced by reduction of ganglion size, disorganization of the ganglion, and defects in neurite outgrowth. Later in embryogenesis, Xevl is required for development of mechanosensory hair cells. In Xevl knockdown embryos, hair cells of the ventromedial sensory epithelium display multiple abnormalities including disruption of the cuticular plate at the base of stereocilia and disorganization of the normal staircase appearance of stereocilia. Based on these data, we propose that Xevl plays an integral role in regulating morphogenesis of the inner ear epithelium and the subsequent development of the vestibulocochlear ganglion and mechanosensory hair cells.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/15/2641/DC1

Key words: Otic vesicle, Morphogenesis, Inner ear, Stereocilia, Mechanosensory hair cell, Vestibulocochlear ganglion

Introduction

The vertebrate inner ear is a sensory organ important for balance and hearing (Tilney et al., 1992). It develops from a thickening in the embryonic ectoderm adjacent to the neural plate called the otic placode (Barald and Kelley, 2004; Riley and Phillips, 2003; Schlosser, 2006; Torres and Giraldez, 1998). The inner ear is a unique sensory structure in that nearly all cells derive from the otic placode itself, with the exception of neural crest-derived pigment cells and the secretory epithelium of the cochlea (Noden and Van de Water, 1992; Torres and Giraldez, 1998). Once the placode has been induced, the thickened placodal ectoderm then undergoes morphogenetic movements to invaginate and form the otic vesicle, which will give rise to the inner ear and neuronal precursors. Neuroblasts differentiate within the ventromedial otic ectoderm, delaminate, and migrate to form the vestibulocochlear ganglion that innervates the inner ear (Fritzsch, 2003; Fritzsch et al., 2002). Subsequently, some cells within the ventromedial region are specified to become hair cells of the saccular maculae, the acoustico-vestibular sensory epithelium of the inner ear that is important for both equilibrium and hearing (Bever et al., 2003). The apical aspects of the mechanosensory hair cells develop an actin rich cuticular plate that anchor highly organized bundles of actin filaments, called stereocilia, which are integral to the detection of motion and sound (Tilney et al., 1992).

Multiple regulators of otic induction have recently come to light, but little is known about the molecular events that control the complex morphogenetic processes required for inner ear development and stereocilia formation. To better understand these processes, we have studied the role of the actin regulatory protein Ena/VASP-like (Evl) during the development of the inner ear and its neuronal derivatives. Evl is a member of the Ena/VASP family of actin regulatory proteins, which have been shown to modulate dynamic actin processes including cellular adhesion and migration (Krause et al., 2003). Ena/VASP family members, which include Enabled (Ena), vasodilator stimulated phosphoprotein (VASP) and Evl, share a highly conserved domain structure including an amino-terminal Ena/VASP homology 1 (EVH1) domain, followed by a proline-rich domain, and a carboxy-terminal EVH2 domain. The EVH1 domain binds proteins containing a F/LPPP amino acid motif and serves to localize Ena/VASP proteins to various subcellular locations. The EVH2 domain functions to bind both G-actin and F-actin, allowing for the enrichment of Ena/VASP proteins at sites of dynamic actin reorganization. The EVH2 domain is also responsible for multimerization with other Ena/VASP proteins. Knockout studies in mouse have shown that Ena/VASP proteins play important roles in regulating multiple actin-dependent processes during development including axon guidance and neural tube closure (Lanier et al., 1999; Menzies et al., 2004), platelet aggregation (Aszodi et al., 1999; Halbrugge and Walter, 1989) and T cell activation and phagocytosis (Coppolino et al., 2001; Krause et al., 2000). In
addition, recent work shows that Ena/VASP proteins regulate integrin-based cell adhesion and motility during somitogenesis in Xenopus (Kragtorp and Miller, 2006). In vitro studies have shown these proteins regulate actin dynamics and migration in fibroblasts by controlling the amount and persistence of actin filament polymerization (Bear et al., 2001; Bear et al., 2002). Together, these studies point to an important role for Ena/VASP proteins in the regulation of morphogenetic processes dependent upon dynamic actin reorganization and cell adhesion.

Previously, we have shown that Xenopus Evl (hereafter referred to as Xevl) is expressed strongly in the otic placode and vesicle throughout early otic development (Wanner et al., 2005). The known role of this protein in regulating actin dynamics and cell adhesion coupled with its expression in the otic tissues suggest that Xevl might play an important role in the formation of the otic vesicle and inner ear components. To better understand the role of Xevl in inner ear morphogenesis, we have more precisely defined Xevl expression within the otic vesicle and have determined the requirement for Xevl function during otic development. We show that Xevl is expressed throughout the otic placode and is later enriched at the ventromedial region of the otic vesicle that gives rise to neuroblasts of the vestibulocochlear ganglion and the mechanosensory hair cells. Xevl protein is enriched at apical cell-cell junctions in the otic vesicle epithelium, in delaminating neuroblasts and neurons of the vestibulocochlear ganglion, and at the cuticular plate of mechanosensory hair cells underlying the actin-rich stereocilia. Using a morpholino to knockdown Xevl protein production we provide evidence that Xevl is required for multiple facets of inner ear morphogenesis including establishment of epithelial morphology and cell-cell adhesion in the otic vesicle. In addition, Xevl is necessary for development of the vestibulocochlear ganglion and proper stereocilia formation in mechanosensory hair cells. Together, these data establish an important role for Xevl in the morphogenetic mechanisms that regulate vertebrate inner ear development.

Results
Xevl expression in the developing inner ear
To analyze the precise expression of Xevl throughout otic development we performed in situ hybridization analyses on sections of embryos at various stages. Xevl transcripts are first observed after neurulation (stage 20) throughout the thickened ectoderm of the otic placode (Wanner et al., 2005). As the otic placode undergoes invagination, Xevl is expressed throughout the placode with the strongest expression in the ventromedial region (Fig. 1B). Between stages 25 and 30, cells in the ventromedial region of the otic vesicle undergo a change from an epithelial fate to a neuroblast fate, delaminate from the otic epithelium, and migrate to form the vestibulocochlear ganglion (Fig. 1A, vg), located between the otic vesicle and the neural tube. At these stages, Xevl is expressed most intensely in the ventromedial region (arrow in Fig. 1C). By stage 35, Xevl continues to be expressed in the ventromedial region of the otic vesicle as well as in cells delaminating from this region (arrow). (D) At stage 35, Xevl expression is found in the ventromedial region of the otic vesicle that will give rise to the sensory epithelium of the saccular maculae (arrowhead) as well as in the vestibulocochlear ganglion (arrow). (E) At stage 45, Xevl is enriched at the presumptive sensory epithelium (arrowhead) and is weakly expressed in the vestibulocochlear ganglion (arrow). ov, otic vesicle; vg, vestibulocochlear ganglion. Bar, 100 μm.

Xevl depletion causes defects in otic vesicle morphology
To address Xevl function in otic development we utilized a morpholino antisense oligonucleotide strategy to block synthesis of Xevl protein (Heasman et al., 2000). A translation-blocking antisense morpholino (XevlMO) was designed against the 5’UTR of Xevl upstream of the translational start site. To verify inhibition of Xevl protein translation by the XevlMO, we generated an affinity-purified peptide antibody that recognizes all three Xevl isoforms by western blot analysis (Fig. 2A). Injection of XevlMO caused a marked reduction in production of all Xevl isoforms compared with control morpholino injected (coMO) embryos (Fig. 2A). Levels of β-tubulin were unaffected by injection of the XevlMO (Fig. 2A).

In addition, injection of two additional non-specific morpholinos did not have an affect on Xevl levels (data not shown). Together, these data demonstrate that injection of XevlMO specifically depletes Xevl protein during development.

The effect of Xevl depletion on otic development was first examined by in situ hybridization analysis using the panplacodal marker XEya1 (also known as eya1) (David et al.,
Regulation of inner ear development by *Xenopus* Evl

2001). For these studies, XevlMO was injected unilaterally at the 4-cell stage in a region fated to contribute to the otic vesicle. Co-injection of XevlMO with GFP mRNA was performed to assure specific targeting to the head region (data not shown). The uninjected contralateral side served as a control (Fig. 2D). This analysis revealed that expression of XEya1 was unaffected by Xevl depletion at stage 20 indicating that Xevl is not required for induction of the otic placode. Analysis of XEya1 expression at stage 35 revealed that 86% of embryos injected with XevlMO (n=121) with 72% exhibiting a strong reduction in size and 14% displaying a mild reduction. Otic vesicle size was unaffected in 14% of injected embryos. (F) XEya1 expression on the uninjected side of a rescued embryo. (G) XEya1 expression with 43% displaying normal otic vesicles, 21% exhibiting a mild phenotype and 36% exhibiting a strong phenotype (n=77). Arrow marks the otic vesicle in D-G.

![Fig. 2. Xevl knockdown disrupts otic vesicle development.](image)

(A) Western blot analysis using a Xevl polyclonal antibody shows a marked reduction in Xevl protein production in embryos injected with XevlMO compared with embryos injected with control MO (coMO). Levels of β-tubulin are unaffected by injection of the XevlMO. Numbers on right indicate molecular mass markers (kDa). (B) Quantitative analysis of Xevl knockdown and rescue experiments indicating the percentage of embryos displaying perturbed otic vesicle development. (C-G) Head region of *Xenopus* embryos at stage 35 (C) Diagram showing the olfactory placode (ol), lens placode (lens), otic vesicle (ov), epibranchial placodes (epi), and lateral line placodes (unlabeled). (D) XEya1 expression on the uninjected side of the embryo. (E) XEya1 expression on the XevlMO-injected side of the embryo. Otic vesicle size is reduced in 86% of Xevl-depleted embryos (n=121) with 72% exhibiting a strong reduction in size and 14% displaying a mild reduction. Otic vesicle size was unaffected in 14% of injected embryos. (F) XEya1 expression on the uninjected side of a rescued embryo. (G) XEya1 expression on the uninjected side of a rescued embryo injected with Xevl-GFP mRNA and XevlMO. Expression of Xevl-GFP results in rescue of XEya1 expression with 43% displaying normal otic vesicles, 21% exhibiting a mild phenotype and 36% exhibiting a strong phenotype (n=77). Arrow marks the otic vesicle in D-G.

Xevl is required for epithelial morphology and cell adhesion in the otic vesicle

In our initial analysis of cell number, we observed that otic vesicles from Xevl-depleted embryos appeared misshapen and many cells failed to adopt a columnar morphology suggesting that Xevl may function to regulate cell shape and adhesion during otic vesicle formation. To analyze whether Xevl plays a role in the formation and maintenance of the columnar epithelium of the otic vesicle, we analyzed the presence and persistence of cell adhesion markers α-catenin, vinculin and occludin in control and Xevl-depleted embryos. α-catenin
localizes to adherens junctions (Scott and Yap, 2006), vinculin is found in both adherens junctions and focal adhesions (Ziegler et al., 2006), and occludin is a major component of tight junctions (Furuse et al., 1993). In control embryos, α-catenin outlines the cells of the otic vesicle and is enriched apically at adherens junctions (Fig. 3A,A’). Similarly, vinculin (Fig. 3B,B’) and occludin (Fig. 3C,C’) are also enriched apically in control embryos. In Xevl-depleted embryos, cell shape was perturbed such that cells of the otic epithelium fail to establish a columnar morphology (Fig. 3D-F). Along with perturbation of cell shape, Xevl depletion correlated with decreased levels of α-catenin at adherens junctions in 69% of injected embryos (Fig. 3D’,D’; n=29). Likewise, Xevl depletion reduced the levels vinculin (Fig. 3E,E’) and occludin (Fig. 3F,F’) at adherens junctions and tight junctions, respectively. These phenotypes can be rescued by injection of Xevl mRNA (data not shown), indicating that the loss of epithelial integrity is specific to knockdown of Xevl in the otic vesicle.

The observed defects in cell morphology and localization of junctional proteins suggest that Xevl may play a role in regulating cell adhesion during otic vesicle morphogenesis. To test this idea we examined the localization of Xevl protein upon formation of the otic vesicle at stage 35. We found that Xevl localizes to sites of cell-cell contact (Fig. 4A) and is enriched apically in the otic vesicle epithelium (arrow in Fig. 4A).

Additionally, Xevl staining is enhanced in the ventromedial region of the otic epithelium where mechanosensory hair cells later form (arrowhead in Fig. 4A). Since Xevl localizes to cellular adhesions and Ena/VASP proteins have been shown to be important factors in cellular adhesion (Krause et al., 2003), we performed an aggregation assay to directly examine whether Xevl regulates cell-cell adhesion in the otic epithelium. We found that dissociated otic vesicles from uninjected embryos were able to form large aggregates, demonstrating their ability to form strong intercellular adhesions (Fig. 4B). By contrast, cells from Xevl-depleted otic vesicles formed small loosely adherent aggregates (Fig. 4C). Together, these data indicate that Xevl is required for establishment of epithelial morphology and cellular adhesion in the otic vesicle.

Xevl is required for vestibulocochlear ganglion formation

In situ hybridization also detected strong Xevl expression in neuronal precursors, the vestibulocochlear ganglion and mechanosensory hair cells, suggesting that Xevl may play a role in controlling the development of these components of the inner ear later in development. To identify potential roles for Xevl in later developmental events, we analyzed the requirement for Xevl in development of the vestibulocochlear ganglion and mechanosensory hair cells. In these experiments, we limited our analyses to embryos displaying mild defects in establishment of epithelial morphology and cellular adhesion in the otic vesicle.

First, we analyzed in detail the distribution of Xevl protein in the otic vesicle during development of the vestibulocochlear ganglion and mechanosensory epithelium. We found that Xevl protein is strongly expressed in the vestibulocochlear ganglion and is enriched at the apical aspect of cells in the otic vesicle sensory epithelium (Fig. 5A, red in C). These sensory regions of the developing inner ear were coabeled with islet-1, a transcription factor that is one of the earliest markers of inner ear neural progenitors and sensory neurons (Fig. 5B, green in C). Xevl is also found in the cell bodies and projections of sensory neurons of the vestibulocochlear ganglion that
Regulation of inner ear development by *Xenopus* Evl

The localization of Xevl to cells of the vestibulocochlear ganglion and otic vesicle sensory epithelium are consistent with a role for Xevl in regulating the formation of sensory structures of the inner ear and neural innervation of the otic vesicle.

Neuroblasts are first observed in the otic epithelium during invagination and later delaminate and migrate medially and dorsally to form the vestibulocochlear ganglion (Liu et al., 2000; Van de Water, 1988). To determine whether neuroblast differentiation or migration is affected by loss of Xevl, we examined expression of XNeuroD (also known as neuroD), a factor that controls neuronal differentiation and survival in the inner ear (Kim et al., 2001; Lee et al., 1995; Liu et al., 2000). XNeuroD is expressed in the vestibulocochlear ganglion and in the neuronal precursors in the region of the developing ventromedial sensory epithelium of the otic vesicle at stage 25. Neither XNeuroD expression in the otic vesicle epithelium nor delamination of differentiated neuronal precursors was affected by Xevl depletion since the number of XNeuroD-positive cells found in the otic vesicle epithelium in both control and Xevl-depleted embryos were similar during these stages (Table 1).

Table 1. NeuroD-positive cells during neuroblast delamination

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>XevlMO</th>
<th>n</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>1.5±1.5</td>
<td>1.5±1.5</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>30/31</td>
<td>3.3±2.1</td>
<td>2.6±1.7</td>
<td>7</td>
<td>0.92</td>
</tr>
<tr>
<td>35</td>
<td>7.0±1.7</td>
<td>5.6±1.6</td>
<td>10</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Average number of NeuroD-positive cells (mean ± s.d.) observed during (stage 28-31) and after (stage 35) neuroblast delamination from the otic vesicle epithelium are similar between controls and Xevl-depleted otic vesicles. n denotes number of embryos analyzed at each stage. P values were obtained by Student’s t-test.

Xevl to cells of the vestibulocochlear ganglion and otic vesicle sensory epithelium are consistent with a role for Xevl in regulating the formation of sensory structures of the inner ear and neural innervation of the otic vesicle.

Neuroblasts are first observed in the otic epithelium during invagination and later delaminate and migrate medially and dorsally to form the vestibulocochlear ganglion (Liu et al., 2000; Van de Water, 1988). To determine whether neuroblast differentiation or migration is affected by loss of Xevl, we examined expression of XNeuroD (also known as neuroD), a factor that controls neuronal differentiation and survival in the inner ear (Kim et al., 2001; Lee et al., 1995; Liu et al., 2000). XNeuroD is expressed in the vestibulocochlear ganglion and in the neuronal precursors in the region of the developing ventromedial sensory epithelium of the otic vesicle at stage 25. Neither XNeuroD expression in the otic vesicle epithelium nor delamination of differentiated neuronal precursors was affected by Xevl depletion since the number of XNeuroD-positive cells found in the otic vesicle epithelium in both control and Xevl-depleted embryos were similar during these stages (Table 1). However, compared with control embryos (Fig. 6A), the vestibulocochlear ganglion in Xevl-depleted embryos is smaller and expression of XNeuroD in the ganglion is markedly reduced (Fig. 6B). TUNEL experiments indicate that the

Fig. 5. Xevl localizes to sensory structures of the inner ear. Confocal imaging of frozen sections of stage 35 embryos. Dorsal is up and the otic vesicle is to the right in all images. (A) Xevl is present in the vestibulocochlear ganglion (arrow) as well as in cells within the otic vesicle sensory epithelium (arrowhead). (B) Islet-1 localization in the vestibulocochlear ganglion (arrow) and within the otic vesicle sensory epithelium (bracket). (C) The merged image reveals Xevl (red) is more strongly expressed in the cells that also have strong islet-1 staining (green). DAPI staining is in blue. (D-F) Xevl (D, red in F) colocalizes with neural neurofilament (nf; E, green in F) in neurons of the vestibulocochlear ganglion and the neurites that extend to innervate the otic vesicle epithelium (arrowhead in F) and the hindbrain (arrow in F). Bar, 20 μm.

Fig. 6. Vestibulocochlear ganglion size and organization is perturbed by Xevl depletion. Dorsal is up and the otic vesicle is to the right in all images. (A) Expression of XNeuroD in the vestibulocochlear ganglion (arrow) and ventromedial sensory epithelium (arrowhead) of control stage 35 embryos. (B) Xevl depletion diminishes the size of the vestibulocochlear ganglion (arrow) and reduces the amount of XNeuroD-positive cells in the sensory epithelium and the vestibulocochlear ganglion. The Xevl-depleted otic vesicle also exhibits a loss of columnar morphology in the thickened sensory epithelium compared with the control otic vesicle (arrowhead, 100% affected; n=9). (C-H) Confocal images of vibratome-sectioned embryo at stage 42 showing islet-1 (C,F; red in E,H) and laminin (D,G; green in E,H) immunostaining of the vestibulocochlear ganglion. (F,H) Xevl depletion causes a reduction in the number of islet-1-labeled cells and an overall reduction in the size of the vestibulocochlear ganglion (arrow). (G,H) In addition, the laminin-rich matrix surrounding the vestibulocochlear ganglion (arrow) is disrupted in Xevl-depleted embryos. Bar, 20 μm. A longer exposure time was necessary for the Xevl-depleted otic vesicle to show islet-1 in the vestibulocochlear ganglion, making islet-1 levels appear higher in the neural tube and otic vesicle of Xevl-depleted embryos compared with controls.
reduced size of the vestibulocochlear ganglion is not due to increased cell death of neuronal precursors or cells of the vestibulocochlear ganglion (Table S1 in supplementary material).

Because of the reduced size of the vestibulocochlear ganglion, we examined in more detail the effects of Xevl depletion on the development of the vestibulocochlear ganglion and inner ear sensory neurons. Islet-1 is expressed in the delaminating cells of the otic ectoderm and vestibulocochlear ganglion cells (arrow in Fig. 6C, red in E) (Li et al., 2004a). Upon Xevl depletion, the vestibulocochlear ganglion is markedly smaller with fewer islet-1-labeled cells and the islet-1-positive cells of the ganglion are more disorganized than in controls (arrow in Fig. 6F, red in H). Furthermore, we found that Xevl depletion also affects the distribution of the extracellular matrix protein laminin. In control embryos, a laminin-rich extracellular matrix surrounds the vestibulocochlear ganglion (arrow in Fig. 6D, green in E). By contrast, Xevl depletion resulted in disruption of laminin matrix deposition around the ganglion (arrow in Fig. 6G, green in H).

Neurofilament expression was also examined to determine the effect of Xevl knockdown on the number and distribution of sensory projections innervating the inner ear. In control embryos, strong neurofilament staining is seen in association with sensory projections (Fig. 7A, green in B). Xevl depletion resulted in a reduction in the number of neurites within the vestibulocochlear ganglion and surrounding the otic vesicle near the ventromedial sensory epithelium (Fig. 7C, green in D). In addition, the remaining projections in Xevl-depleted embryos were shorter than controls. Together these experiments reveal a role for Xevl in the formation and organization of the vestibulocochlear ganglion as well as the subsequent development of neurites that emanate from the ganglion.

**Xevl regulates sensory epithelium and stereocilia formation**

To analyze the role of Xevl in the development of the sensory epithelium of the inner ear, we studied the precise localization of Xevl within the otic vesicle at stages when specialized mechanosensory hair cells and stereocilia have formed. These analyses showed that Xevl is enriched apically within the cell body (Fig. 8A,C, red in B and D) and at the cuticular plate that lies at the base of the stereocilium (Fig. 8E, red in F). The apical location of Xevl in mechanosensory hair cells is the same as that of the membrane cytoskeletal protein spectrin (Fig. 8G, red in H), which is an integral component of the stereocilia.

**Fig. 7.** Neurite outgrowth is reduced in Xevl-depleted otic vesicles. Vibrotome sections of the otic vesicle region of stage-35 embryos with dorsal oriented up and the otic vesicle to the right in each image. (A,B) Uninjected embryos exhibit extensive neurofilament-positive projections into the vestibulocochlear ganglion (arrowhead) and the ventromedial sensory epithelium (arrow) of the otic vesicle. (C,D) Xevl-depleted otic vesicles exhibit shorter and fewer sensory projections in both the vestibulocochlear ganglion (arrowhead) and the sensory epithelium (arrow). Neurofilament is shown in green in B,D. Preparations were co-stained with spectrin (red in B,D) to outline cell boundaries. Bar, 50 μm.

**Fig. 8.** Xevl depletion in hair cells disrupts spectrin localization and stereocilia formation. (A-D) Images of frozen sectioned embryos with dorsal oriented up and the otic vesicle on the right of each image. (A) Xevl is present in hair cells of the ventromedial sensory epithelium and is enriched at the apical aspect of these cells (arrow). (B) Xevl (red) and actin (phalloidin; green) colocalize at the apical region of hair cells (arrow) at the base of stereocilia. (C,D) Higher magnification view of Xevl localization in hair cells (arrows) showing the colocalization of Xevl (red) and actin (green) at the apical margin of the hair cells. (E,F) Image of a dissected otic vesicle showing that Xevl (E, red in F) localizes to the cuticular plate at the base of each actin-rich stereocilium (green in F). (G) Images of frozen sectioned embryos showing that spectrin is localized to the cuticular plate at the apical region of hair cells (arrow). (H) Spectrin (red) is at the base of each stereocilium and colocalizes with actin (green) at the apical membrane of hair cells in uninjected embryos. (I) In XevlMO-injected embryos, spectrin levels at the cuticular plate are markedly reduced and hair cells lose their columnar shape (arrowhead). (J) Xevl depletion also results in a reduction in actin staining (green) at the apical portion of hair cells and few stereocilia are present. Spectrin is shown in red in the merged image. Bars, 20 μm, except for E and F where bar, 5 μm.
of the cuticular plate (Drenckhahn et al., 1991; Raphael et al., 1994). Previous studies have demonstrated a direct interaction between Evl and spectrin (Bournier et al., 2006; Rotter et al., 2005) suggesting that Xevl and spectrin may interact at these sites. In support of this idea, hair cells in XevlMO-injected embryos showed a marked reduction in both spectrin and actin at the cuticular plate (Fig. 8I,J, Fig. 9C-E). Furthermore, loss of spectrin at the cuticular plate correlated with defects in stereocilia (Fig. 8I,J, Fig. 9C-E). Thus, Xevl is enriched at the cuticular plate and its function is required for the proper localization of spectrin and actin to this structure.

Actin filaments provide the structural framework for stereocilia and the length of actin filaments in stereocilia is tightly regulated, building a staircase-like pattern of progressively longer stereocilia (arrows in Fig. 9A). In Xevl-depleted embryos, however, we observed a range of phenotypes from absence of stereocilia bundles to stereocilia that appeared disorganized, displaying a ‘splayed’ morphology (Fig. 9B). Stereocilia in Xevl-depleted embryos were also significantly shorter than controls and all of them were approximately the same length, lacking the normal staircase appearance. Stereocilia in control embryos are on average 6.95±0.73 μm in length (n=18) whereas stereocilia in Xevl-depleted embryos are on average 3.60±0.72 μm in length (n=13; P<0.0001, Student’s t-test). Loss of spectrin at the base of Xevl-depleted stereocilia correlated with the splayed and lack of staircase appearance of stereocilia (Fig. 9D,E), suggesting that there may be a link between defects in the cuticular plate and stereocilia formation.

To further examine the role of Xevl in regulating actin organization in stereocilia we examined espin distribution in control and Xevl-depleted embryos. Espin is an actin-bundling protein that is present in stereocilia bundles where it appears to play an important role in regulating steady state length of actin filaments in stereocilia (Bartles et al., 1996; Bartles et al., 1998; Li et al., 2004b; Sekerková et al., 2004; Rzadzinska et al., 2005; Sekerková et al., 2006). In control embryos, strong espin staining is seen in association with stereocilia (Fig. 9F). In Xevl-depleted embryos, espin staining is greatly diminished within the stereocilia (Fig. 9G,H). Together, these data provide evidence that Xevl is a key factor for the development of mechanosensory hair cells and formation of stereocilia in the inner ear.

**Discussion**

Coordination of molecular events that control cell adhesion, migration and polarity are critical for inner ear development and stereocilia formation. Our results show that Xevl is a factor that is integral to multiple aspects of inner ear morphogenesis in Xenopus embryos. Xevl is expressed throughout inner ear development in both ectodermal and neural lineages and at later stages is enriched in the hair cells of the presumptive saccular maculae. We have shown that Xevl is critical for the regulation of the morphogenetic events that occur throughout otic development. In the absence of Xevl, the formation of the otic vesicle is altered, as seen by defects in cellular adhesion and loss of the columnar morphology of the otic epithelium. The vestibulocochlear ganglion is also affected by loss of Xevl function, evident in the reduction in ganglion size, overall disorganization, and decreased neurite outgrowth. The effects of a Xevl knockdown are also evident later in inner ear development, as the cuticular plate is abnormal and stereocilia are shorter and have a splayed appearance. These findings highlight the importance of Xevl in development of the otic vesicle, vestibulocochlear ganglion and mechanosensory hair cells.

**Epithelial integrity of the otic vesicle is regulated by Xevl**

Our analysis indicates that Xevl plays an integral role in the development of the otic vesicle epithelium. We find that Xevl depletion results in a decrease in the size of the otic vesicle at early stages that, in embryos displaying a strong phenotype, appears to be due to a decrease in cell number. The observed decrease in cell number does not appear to be due to defects in otic placode induction or increased cell death. Instead, our data are consistent with the idea that Xevl depletion disrupts cell adhesion, which may lead to the loss of loosely adherent cells during placode invagination and vesicle formation. This idea is supported by the localization of Xevl to sites of cell-

**Fig. 9.** Xevl is required for stereocilia formation. Confocal immunofluorescence images of dissected Xenopus otic vesicles at stage 45 stained with actin (phalloidin; A,B, red in C-H), spectrin (green in C-E) and espin (green in F-H). (A) Uninjected otic vesicles possess stereocilia with a staircase of actin filaments (arrows). (B) XevlMO-injected otic vesicles have shorter stereocilia that are more uniform in length and exhibit a splayed appearance (arrows). (C) Uninjected otic vesicles possess strong spectrin localization at the cuticular plate (arrow). (D,E) Xevl-depleted embryos display a decrease in spectrin staining (arrows) that correlate with defects in stereocilia morphology. (F) In uninjected embryos, espin staining is strongly associated with stereocilia (arrow). (G,H) Xevl depletion causes a reduction in espin levels in stereocilia (arrow). Bars, 5 μm.
cell contact in the otic epithelium and the requirement for Xevl in promoting optimal cell-cell adhesion of otic epithelial cells. The observed loss of columnar morphology of the otic vesicle epithelium coupled with a decrease in the levels of the junction markers α-catenin, vinculin and occludin further supports this model. Loss of cadherin-2, a component of adherens junctions, also results in reduced otic vesicle size (Babb-Clendenon et al., 2006), underscoring the importance of cellular adhesions in otic vesicle morphogenesis.

Xevl controls vestibulocochlear ganglion formation

The otic placode is unique to the cranial placodes as it gives rise to both the neural and ectodermal components of the final structure. In addition to its role in the ectoderm, our studies indicate that Xevl function is critical for development of the vestibulocochlear ganglion. Xevl is enriched in the ventromedial region of the otic vesicle where neuroblasts are specified and delaminate and Xevl depletion causes significant changes in the overall organization and size of the vestibulocochlear ganglion. Additionally, Xevl is highly expressed in the vestibulocochlear ganglion and loss of Xevl results in a reduction in ganglion size and fewer XNeuroD-labeled neuroblasts. Defects in a number of processes including, cell fate specification, cell death, cell migration or cell adhesion could explain these phenotypes. Our experiments show that dejected and control otic vesicles have a similar number of neuroblasts in the epithelium, indicating that neither specification of neuronal fate nor proliferation is altered by Xevl knockdown. In addition, both dejected and control embryos possess a similar number of XNeuroD-labeled cells delaminating from the vesicle. Furthermore, TUNEL analysis indicated that cell death does not account for the decreased size of the vestibulocochlear ganglion. Instead, our data are consistent with the idea that Xevl may be required for the migration of neuronal precursors to their targets and/or the aggregation of cells to form the ganglion. An abundance of studies have demonstrated an important role for Ena/VASP proteins in regulating cell migration (Hoffman et al., 2006; Krause et al., 2003; Kwiatkowski et al., 2003; Menzies et al., 2004; Sechi and Wehland, 2004; Withee et al., 2004), indicating that Xevl may control neuroblast migration by coordinating cytoskeletal dynamics at the leading edge. In support of a role for Xevl in modulating cell adhesion, we have shown that Xevl-depleted otic cells exhibit an impaired ability to aggregate. The importance of cellular adhesions in vestibulocochlear ganglion formation has been demonstrated in a recent study, where loss of cadherin-2 function resulted in a similar reduction in vestibulocochlear ganglion size (Babb-Clendenon et al., 2006). Additionally, Xevl-depleted vestibulocochlear ganglia show a greatly reduced and disorganized laminin-rich matrix surrounding the ganglia suggesting that Xevl may also regulate integrin-based adhesion. A number of studies have shown that inside-out regulation of integrin activity is dependent on an intact actin cytoskeleton (Pankov et al., 2000; Wu et al., 1995; Zaidel-Bar et al., 2003). Since Ena/VASP proteins are thought to modulate the interactions between cell surface receptors and the actin cytoskeleton (Gitai et al., 2003; Hoffman et al., 2006; Jenzora et al., 2006; Kwiatkowski et al., 2003; Scott et al., 2006), Xevl may modulate linkages between the actin cytoskeleton and cell surface adhesion receptors necessary for cell migration, adhesion and matrix assembly during formation of the vestibulocochlear ganglion.

Once the vestibulocochlear ganglion has formed, neurons of the ganglion send out processes to targets in the ventromedial sensory epithelium and to nuclei within the hindbrain (Fritzsch et al., 2002). We found that Xevl is enriched in these sensory projections and Xevl depletion causes a significant reduction in the number and length of neurofilament-labeled projections in the ventromedial sensory epithelium. The reduction in sensory projection number is likely due to a decrease in neuroblasts in the ganglia, which in turn form fewer protrusions. The decrease in sensory projection length can be explained by a requirement for Xevl in modulating actin organization and dynamics in developing neuronal processes. In support of this idea, Ena/VASP proteins have well-established roles in regulating neurite outgrowth and pathfinding (Basan et al., 2000; Gitai et al., 2003; Goh et al., 2002; Lanier et al., 1999; Li et al., 2005; Menzies et al., 2004). An alternative model is that impaired migration of delaminating neuroblasts in Xevl-depleted embryos affects the deposition of extracellular cues that serve to guide neurites back to the ventromedial sensory epithelium. This idea is supported by work in mice which revealed that neuroblasts delaminate from the same region that their projections later innervate, suggesting that neuroblasts deposit guidance cues for neurites to find their proper target (Carney and Silver, 1983; Fritzsch et al., 2002). Together, our data have uncovered a requirement for Xevl in the development of vestibulocochlear neurons, although further studies are required to define the precise role Xevl plays in their development and pathfinding.

Role of Xevl in regulating development of mechanosensory hair cells and stereocilia

Proper stereocilia formation is integral to hearing and balance in vertebrates (Tilney et al., 1992). Stereocilia are unique actin-rich structures with a distinct graded staircase appearance of the bundles and lateral link connections between bundles. The staircase appearance of the stereocilia bundles is controlled by actin incorporation at the stereocilia tip that is scaled to the respective length of the bundles; longer bundles having higher levels of actin treadmilling than shorter bundles (Lin et al., 2005). The organized arrangement of stereocilia is maintained by cadherin-based cross-bridges that link stereocilia bundles together (El-Amraoui and Petit, 2005) and the cuticular plate that anchors the base of the bundles to the apical actin cortex (DeRosier and Tilney, 1989).

In the present study, we found that Xevl is strongly expressed in hair cells of the otic vesicle and is enriched apically at the base of the stereocilium in the cuticular plate. Depletion of Xevl resulted in a significant decrease in stereocilia length, failure of stereocilia to establish a staircase appearance, and disruption of espin localization in stereocilia. One explanation of these findings is that some defects in hair cell and stereocilia formation could be an indirect affect of Xevl depletion on cell adhesion and morphology. However, the localization of Xevl to the base of stereocilia and the known roles of Ena/VASP proteins in the regulation of actin dynamics suggest that Xevl may play a direct role in stereocilia formation. Potential mechanisms by which Xevl could control stereocilia formation include regulation of actin dynamics in stereocilia, modulation of cross-bridges between adjacent
stereocilia, and formation of the cuticular plate. Although a specific function for Xevl in stereocilia is possible, immunolocalization studies failed to consistently detect Xevl protein in stereocilia. Thus, our data favor a model in which Xevl functions at the cuticular plate to promote anchoring of stereocilia in the apical actin cortex. In support of this model, Xevl is enriched at the cuticular plate and Xevl depletion results in a loss of spectrin and actin from this structure. The cuticular plate serves as an anchor for actin filaments in stereocilia and provides support to keep the stereocilia upright (DeRosier and Tilney, 1989; Tilney and DeRosier, 1986; Tilney et al., 1983). Spectrin is thought to form cross-bridges between actin filaments within the cuticular plate, anchoring stereocilia bundles to the apical aspect of hair cells (Drenckhahn et al., 1991; Scarpone et al., 1988; Ylikoski et al., 1992). Thus, the loss of Xevl and spectrin from the cuticular plate may result in a loss of structural integrity of the stereocilia bases causing the actin bundles to be shorter and take on a splayd appearance. Interestingly, spectrin can directly bind Evi though its EVH1 domain and the two proteins colocalize at filopodial tips and the leading edge of lamellipodia (Bournier et al., 2006). Thus, Xevl may act to recruit or maintain spectrin localization at the cuticular plate. Assembly of the cuticular plate would then support proper architecture of the stereocilia bundles. Although this interpretation is consistent with our data, further work is required to determine the precise role for Xevl in regulating stereocilia formation.

In summary, our data provide evidence that Xevl plays an integral role in the development of the vertebrate inner ear. Xevl is required for establishment of epithelial morphology in the otic vesicle, formation of the vestibulocochlear ganglion, and development of mechanosensory hair cell stereocilia. Our findings together with the known role of Ena/VASP proteins in regulating actin dynamics indicate that Xevl function in inner ear development probably depends on its ability to control actin architecture during morphogenesis of the inner ear. Many genes associated with inner ear development and human deafness function to control actin dynamics, underscoring the importance of regulating cytoskeletal organization for normal inner ear morphogenesis. Future studies will help define how these genes work together to control inner ear development as well as the morphogenesis of other vertebrate sensory cells containing microvilli and how the absence of these genes leads to defects in the detection of chemical and mechanical stimuli.

Materials and Methods

Embryos and microinjections

*Xenopus laevis* embryos were obtained by fertilizing eggs from females injected with human chorionic gonadotropin (Sigma). Eggs were dejellyed with 2% cysteine and rinsed in modified Steinberg’s solution and maintained for up to 5 hours in 1 mM Ca2+/Mg2+-free Ringers solution. Otic vesicles were dissociated using 2 mg/ml collagenase for 20 minutes at room temperature following the manufacturer’s instructions. For histology, stained embryos were dehydrated, embedded in TissuePrep2 paraffin mix (Fisher), and cut into 10 µm sections using a rotary microtome.

**Antibody production and western blot analysis**

Anti-Xevl polyclonal antibodies were raised against a peptide corresponding to amino acids 277-295 (C-ELAKRRKAAASYTDDKGDQKK) conjugated to KLH (Sigma Genosys). Anti-Xevl antibodies were affinity purified using Xevl peptide coupled to SulfoLink beads ( Pierce). For western blot analyses, embryos were injected with morpholinos at the 4-cell stage in both dorsal blastomeres (5 ng/blasto mer). Control and morpholino-injected embryos were collected at stage 35, homogenized in ice cold Triton lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM EDTA, 0.5% Triton X-100), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 10 µg/ml leupeptin and 10 µg/ml apro tin). Approximately one embryo equivalent was loaded per lane on a 12% polyacrylamide gel and blotted to nylon membrane (Bio-Rad). Blots were blocked in 5% milk in TBS + 0.1% Tween 20 and probed with anti-Xevl antibodies at a 1:500 dilution for 5 hours at room temperature, washed, and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:1000; Jackson Immunolabs). Signal was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to X-Omat LS film (Kodak). Blots were probed with anti-β-tubulin antibody for 1 hour (1:200; E7, DSHB, Iowa City, IA) as a loading control.

**Immunofluorescence and subcellular localization studies**

Embryos were fixed at the appropriate stage with MEMFA (MEM salts and 3.7% formaldehyde) and whole-mount in situ hybridization was performed as described previously (Harland, 1991) using DIG-labeled RNA probes and BM Purple (Roche) as an alkaline phosphatase substrate. Antisense DIG-labeled probes were synthesized using cDNA encoding Xevl (David et al., 2001) and the 3’ portion of Xevl (Wanner et al., 2005). Specificity was confirmed by using the corresponding sense riboprobes. MEMFA-fixed embryos were also used for TdT-mediated UTP-biotin nick-end labeling (TUNEL) using TdT purchased from Gibco-BRL, following the manufacturer’s instructions. For histology, stained embryos were dehydrated, embedded in TissuePrep2 paraffin mix (Fisher), and cut into 10 µm sections using a rotary microtome.

**Aggregation assay**

Twenty otic vesicles were dissected from both uninjected and XevlMO-injected embryos anesthetized at stage 35 and collected in agarose-coated dishes containing 1 × Ca2+/Mg2+-free Ringers solution. Otic vesicles were dissociated using 2 mg/ml collagenase for 20 minutes at room temperature followed by manual dissociation with a pipette. The otic cell suspension was then washed three times with 1 × Steinberg’s solution and maintained for up to 5 hours in 1 × Steinberg’s alternating 5 minutes on an orbital shaker and 30 minutes without shaking to allow for reaggregation.


Supplemental Fig. 1. Distribution of Xevl-GFP in the otic vesicle. Xevl-GFP mRNA (500 pg) was injected at the 8-cell stage and embryos were fixed and sectioned at stage 35. Xevl-GFP positive cells (green in merged image) are distributed throughout the otic vesicle. Actin staining (red in merged image) is shown to outline cells in the otic vesicle.
Table S1. Otic vesicle TUNEL staining.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>XevlMO</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>35</td>
<td>0-2</td>
<td>0-2</td>
<td>5</td>
</tr>
<tr>
<td>42</td>
<td>1-5</td>
<td>1-4</td>
<td>8</td>
</tr>
</tbody>
</table>

TUNEL staining in Xevl-depleted embryos is similar to controls throughout otic vesicle formation.