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The vertebrate Planar Cell Polarity pathway regulates Convergent Extension and Hair Cell Polarization independently in the Cochlea

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Doctor of Philosophy

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B.S./B.A., East Carolina University, 2004

Advisor: Ping Chen, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Graduate Division of Biological and Biomedical Sciences
Biochemistry, Cell, and Developmental Biology

2011

## Abstract

The vertebrate Planar Cell Polarity pathway regulates Convergent Extension and Hair Cell Polarization independently in the Cochlea

By Maria Fernanda Chacon Heszele

The planar cell polarity (PCP) signaling pathway consists of conserved PCP and ciliary genes and is required for the apparent convergent extension (CE) and coordinated cell polarization of the sensory hair cells in the mammalian cochlea. However, it is unknown whether the PCP pathway regulates these two diverse morphogenesis processes through the same molecular mechanism. We found that the sensory epithelium in the mouse cochlea undergoes dramatic changes in cell geometry and cellular boundary remodeling during CE. Furthermore, this cellular morphogenesis is accompanied by dynamic expression of adhesion components N-cadherin and E-cadherin, implying a role for cellular adhesion molecules in CE. Supporting this idea, a conditional knockdown mouse model of a component of adherens junctions, p120-catenin, shows altered or diminished expression of N-and E-cadherins in the cochlea and phenocopies the defects in cochlear extension observed in PCP and ciliary mutants. Interestingly, the p120 knockout mice do not display defects in cell polarization, indicating a specific role for p120 in CE but not hair cell polarization. Conversely, two mouse models of the Usher syndrome, the most common genetic cause of human deafness, show defects in cell polarization but no defects in cochlear CE. These results indicate that the PCP signaling pathway regulates cell polarization and CE of the cochlea independently via distinct molecular mechanisms. The cochlear CE is driven by a p120-dependent mechanism while hair cell polarization is regulated by a machinery involving Usher genes.

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**Acknowledgements**

First and foremost I want to thank my mentor, **Ping Chen** for being willing to take a chance in me when few others would have done so. Ping welcomed me into her lab at a time when my confidence in my abilities (and future) as a scientist was shaken, to say the least. Over the last couple of years, Ping has introduced me to a beautiful and engrossing area of research and, at the same time, has helped me regain my confidence as a scientist and significantly improve in my thinking, writing and presentation skills. Ping, I cannot thank you enough for the trust you placed in me and the time and energy that you've taken to guide me through graduate school. I feel honored and privileged to be able to say that I've been your graduate students. Thanks!

I want to continue by thanking my committee, **Tamara Caspary, Victor Faundez, Andy Kowalczyk, Sho Ono,** and **Win Sale** for their help and guidance throughout graduate school. It's been fun to work with and learn from you guys! Additionally, I want to give Win special thanks for guiding me to the Chen Lab when I was choosing a new home at Emory and to Victor for his help during the writing of this dissertation.

I am grateful to my fellow Chen Lab members (past and present) for their everyday help and support in the lab. In particular, Richard Anderson, Jack Etheredge, Michael Kelly, Amy Li, Dongdong Ren, Padmashree Rida, and Dong Qian. You guys make it fun to come to work every day!

I am grateful to the **Department of Cell Biology** and the **Biochemistry Cell and Developmental Biology Graduate Program.** Graduate school doesn't

go well without the assistance of an army of people who work behind the scenes and make our everyday lives easier. Thank you!

A special thank you goes to the **Department of Physiology** and **Doug Eaton** and **Eric Eaton.** Most of the confocal images presented in this manuscript were taken in the confocal microscope that they graciously granted me use of.

I would also like to thank my fellow BCDB graduate students. In particular, I want to thank **Jennifer Holzman** for countless hours of science talk and her never-ending guidance on how to become a better teacher. Thanks Jen!

I would be remiss not to mention **Gary and Barbara Ratner**. I am lucky to have you in my life. At this point, you aren't just friends, you're family.

On that note, I want thank my parents, **Manuel Chacon** and **Ana Heszele** for their unconditional love and unquestioning support in my choice to become a scientist. I also want to thank my grandmother **Amparo Gil** for being my first role model in science.

Last, but certainly not least, I want to thank my fiancé, **Ivan Mihailov.** Your constant support and encouragement throughout my graduate experience has been fundamental in helping me reach this goal. I am truly blessed to have you in my life.

## List of Abbreviations

**AC:** Anterior cristae

**AJ:** Adherens junction

**CE:** Convergent extension

**DC:** Deiters' cell

**DS:** Donkey serum

**Fz3:** Frizzled3

**GFP:** Green fluorescent protein

**GS:** Goat serum

**IFT:** Intra flagellar transport

**IHC:** Inner hair cell

**IPC:** Inner pillar cell

**IPH:** Inner phalangeal cell

**L:** Lateral (side of the cochlea/organ of Corti)

**LC:** Lateral cristae

**M:** Medial (side of the cochlea/organ of Corti)

**OC:** Organ of Corti

**OHC:** Outer hair cell

**OPC:** Outer pillar cell

**PC:** Posterior cristae

**Pcdh15:** Protocadherin-15

**PBS-T:** Phosphate-buffered saline with 0.1% Triton-X

**PCP:** Planar cell polarity

**SA:** Saccule

**Sans:** Scaffold Protein Containing Ankyrin repeats and SAM domain

**uL:** micro liter

**UT:** Utricle

**Vangl2:** Van Gogh-like 2

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# Chapter 1 : Introduction

### *Overview and Significance:*

Vertebrate planar cell polarity (PCP) signaling is essential for diverse developmental and physiological cellular processes, including coordinated polarization of epithelial cells (Guo, Hawkins, & Nathans, 2004; Montcouquiol et al., 2003), convergent extension (CE) (Wallingford & Harland, 2002), oriented cell division (Gong, Mo, & Fraser, 2004; B. Lu, Usui, Uemura, Jan, & Jan, 1999), and growth cone guidance (Shafer, Onishi, Lo, Colakoglu, & Zou, 2011). The overall goal of this dissertation is to understand how various forms of PCP in vertebrates are regulated via a common set of genes. In particular, we use the auditory sensory organ in the mouse cochlea as a model system to address whether the PCP processes of CE and coordinated sensory cell morphological polarization in the cochlea (P. Chen, Johnson, Zoghbi, & Segil, 2002; McKenzie, Krupin, & Kelley, 2004; Montcouquiol, et al., 2003) are regulated via the same or different molecular modules downstream of common PCP regulatory genes.

Although much has been learned about PCP since the initial characterization of genes that control PCP in *Drosophila* almost 25 years ago (Vinson & Adler, 1987) the molecular basis for how these common regulatory genes control various forms of PCP is largely unknown. The morphological manifestations of PCP in vertebrates differ drastically, implicating cell-context-dependent regulation by the common PCP genes. The lack of careful characterization of morphogenesis itself and the complexity of regulatory network

involved in morphogenesis, however, have hindered the understanding of how PCP signaling is transduced into cells to affect cellular polarity in a coordinated manner among neighboring cells. The dissection of the vertebrate PCP pathway in various cellular processes will further illustrate how this pathway controls various aspects of development and provide insights to related developmental diseases. In addition, such studies will help to address fundamental issues in cell biology regarding cell shape and tissue morphogenesis.

In this dissertation we have focused on the PCP processes of CE and coordinated polarization of epithelial cells using the mouse inner ear as a model system. We explore the molecular machineries that act with common PCP genes to regulate these two forms of PCP. The PCP processes of CE and coordinated sensory cell morphological polarization occur concurrently during terminal differentiation and are required for proper development of the cochlea,(Figure 1.3) (P. Chen, et al., 2002; McKenzie, et al., 2004) . Disruptions of common PCP genes cause defects in apparently in both CE and coordinated sensory cell orientation in the cochlea (Curtin et al., 2003; McKenzie, et al., 2004; Montcouquiol, et al., 2003; Y. Wang, Guo, & Nathans, 2006). This model system, therefore, provides a unique opportunity to dissect the morphological and molecular regulation of two diverse cellular processes by the common PCP genes. In particular, we tested our hypothesis that, CE and coordinated sensory cell morphological polarization are controlled by separate molecular modules downstream of the common PCP genes.

Using a number of different mutants, which will be described in detail later in this thesis, we show data in support of the hypothesis that the PCP processes of CE and coordinated sensory cell morphological polarization are controlled by separate molecular modules downstream of vertebrate PCP signaling. We demonstrate that even within the same tissue different forms of PCP can be regulated *independently* of one another.

***Planar Cell Polarity***

PCP refers to the phenomenon in which cells within a tissue are polarized or oriented along the plane of a tissue in a coordinated manner. In order for tissues to be able to accomplish this feat, two different but interrelated processes have to take place. (1) Individual cells within the tissue must become intrinsically polarized. (2) The direction of intrinsic cell polarization has to be coordinated among neighboring cells throughout the tissue (Figure 1.1).

Intrinsic Cell Polarization

What is intrinsic planar cell polarization? The process of intrinsic or morphological cell polarization involves the differential distribution of cellular components or organelles within a single cell. For instance, during (1) Cell division: during cell division the centrioles/mitotic spindle are oriented along one axis of the cell; (2) Cell movements/cell migration: during movement cells can develop polarized protrusions that allow them to move one direction; (3) Morphological polarization of sensory cells: in the sensory cells of the inner ear a primary cilium and a group of microvilli-based filaments called stereocilia, are arranged towards one side of the apical surface of the cell, this morphological polarization of the cell is important for its sensory function. Both mesenchymal and epithelial cells can be intrinsically polarized. Intrinsic polarization of mesenchymal cells generally involves the distribution of cellular components or organelles on opposite or different sides of the cell while intrinsic polarization of epithelial cells generally involves the differential distribution of cellular

components or organelles on an axis perpendicular to the basal-apical polarity axis (illustrated on Figure 1.1).

## Coordinated Cell Polarization

Although different cellular and developmental processes can be regulated by PCP these different forms of PCP share a requirement for intrinsic polarization of individual cells that must be coordinated throughout the plane of the tissue. In other words, in tissues that display PCP, the direction of intrinsic cell polarization is *coordinated* among neighboring cells throughout the tissue. Coordinated cell polarization/orientation allows for important developmental and morphogenetic processes to take place. For instance, during (1) Oriented cell division, (2) convergent extension (CE) and, (3) coordinated morphological polarization of sensory cells. (1) Oriented cell division: is required for proper morphogenesis of the kidneys (Fischer et al., 2006; Luyten et al., 2010; Nishio et al., 2010) and the regulation of cell fate and maintenance of stem cell populations during asymmetric cell divisions (Albertson & Doe, 2003; Bellaiche, Beaudoin-Massiani, Stuttem, & Schweisguth, 2004; Segalen et al., 2010). Oriented cell division takes place when the centrioles/mitotic spindles are aligned along one axis (the PCP axis) allowing all the cells to divide in a common direction [reviewed in (Segalen & Bellaiche, 2009)]. (2) CE: is an important developmental process required during gastrulation (Carreira-Barbosa et al., 2009; Wallingford, Goto, Keller, & Harland, 2002) and neurulation (Ciruna, Jenny, Lee, Mlodzik, & Schier, 2006; Doudney & Stanier, 2005; Wallingford & Harland, 2002) and it takes place when coordinately polarized cellular protrusions along one axis (the PCP axis) allow for

cellular intercalation along that axis and the subsequent extension of the tissue on the perpendicular axis (Keller et al., 2000) [reviewed in (Lawrence & Morel, 2003; McNeill, 2009; Tada & Kai, 2009; Wada & Okamoto, 2009)]. (3) Morphological polarization of sensory cells: for example, in the inner ear the coordinated morphological polarization of sensory cells in the cochlea along the PCP axis is required for hearing (Dabdoub et al., 2003; Montcouquiol, et al., 2003; Y. Wang, et al., 2006) [reviewed in (Jones & Chen, 2008; Kelly & Chen, 2007; Rida & Chen, 2009).

The fact that PCP is observed in diverse cellular processes and can involve the differential arrangement of many different cellular structures and organelles (mitotic spindles, the actin cytoskeleton, hairs, etc) highlights the universal importance of PCP. However, the diverse cellular manifestations of PCP also make the study of PCP a complex process. How do cells know to orient these different structures in a specific direction? Are the instructions the same in different tissues/organs? How do cells sense the “correct” direction of orientation? How is this processes controlled? In other words, what are the molecular mechanisms that control PCP in different tissues and how do they work? Is the role of PCP controlled in different cellular processes directed by the same mechanisms or are they controlled by separate molecular modules? These questions have driven the study of PCP for nearly 25 years and have allowed for the identification of genes that collectively regulate diverse forms of vertebrate PCP.

***Vertebrate PCP Signaling***

In order for tissues to display PCP (1) individual cells within the tissue must become intrinsically polarized and (2) the direction of intrinsic polarization must be coordinated among different cells along the plane of the tissue. During this process, general/global polarity signals must be presented to/by the tissue for the establishment of tissue polarity. Additionally, individual cells within the tissue must be able to understand global polarity cues in order to carry out the morphological changes that mark their intrinsic polarization in a in a specific, coordinated direction. In vertebrates, different genes have been identified to play a role in the establishment of tissue polarity (also referred to as “active PCP signaling”) and in the regulation of intrinsic or morphological cell polarization. In the following sections the identity of these genes and their specific roles in the regulation of PCP will be explained.

Establishment of Tissue Polarity and PCP Signaling

PCP was first characterized in the late 1980's in studies on the uniform orientation of bristles in the *Drosophila* wing, a prime form of epithelial PCP (Vinson & Adler, 1987). This and other early studies in *Drosophila* allowed for the identification of a set of genes that not only regulates uniform orientation of cells in the wing, but also other epithelial tissues in the fly that display coordinated cell polarization. This set of the genes required for all the epithelial PCP features of the fly was designated as “core” PCP genes. The core PCP genes encode for a set of trans-membrane proteins and their immediate downstream effectors which have been implicated in the regulation of PCP (Klingensmith, Nusse, & Perrimon,

1994; Taylor, Abramova, Charlton, & Adler, 1998; Theisen et al., 1994; Usui et al., 1999; Vinson & Adler, 1987; Zeidler, Perrimon, & Strutt, 1999) [reviewed in (Axelrod, 2009; McNeill, 2010; H. Strutt & Strutt, 2009)]. The known *Drosophila* core PCP proteins include the transmembrane proteins Van Gogh (Vang) (Taylor, et al., 1998; Wolff & Rubin, 1998), Flamingo (Usui, et al., 1999), Frizzled (Fz) (Vinson & Adler, 1987; Vinson, Conover, & Adler, 1989), and the cytoplasmic proteins Dishevelled (Klingensmith, et al., 1994; Theisen, et al., 1994) and Prickle (Gubb et al., 1999; Heitzler et al., 1993). Subsequent studies have allowed for the identification of vertebrate homologues of all known *Drosophila* core PCP genes (Bekman and Henrique, 2002; Darken et al., 2002; Formstone and Little, 2001; Katoh, 2003; Kibar et al., 2001; Klingensmith et al., 1996; Lijam and Sussman, 1995; Montcouquiol et al., 2003; Park and Moon, 2002; Sussman et al., 1994; Tissir et al., 2002; Tsang et al., 1996; van Gijn et al., 2001; Wallingford et al., 2002; Wallingford et al., 2000; Winklbauer et al., 2001; Yang et al., 1996) as well as one additional vertebrate membrane core PCP protein, PTK7 (X. Lu et al., 2004). A multitude of studies have implicated core PCP proteins in the regulation of all known PCP processes, including oriented cell division in a variety of tissues (Gong, et al., 2004; B. Lu, et al., 1999; Segalen, et al., 2010); coordinated cell polarization in the skin (Guo, et al., 2004), inner ear (Davies, Formstone, Mason, & Lewis, 2005; Montcouquiol, et al., 2003; Y. Wang, et al., 2006) [reviewed in (Chacon-Heszele & Chen, 2009)] and the embryonic node (Antic et al., 2010; Song et al., 2010; Zhang & Levin, 2009); and CE during gastrulation (Carreira-Barbosa et al., 2003; Veeman, Slusarski,

Kaykas, Louie, & Moon, 2003; Wallingford et al., 2000; Winklbauer, Medina, Swain, & Steinbeisser, 2001) and neurulation (Curtin, et al., 2003; Wallingford, et al., 2002; Wallingford & Harland, 2002; J. Wang et al., 2006).Thus, defects in core PCP proteins lead to a variety of developmental phenotypes in vertebrates including (but not limited to) misorientation of cells in the skin; defects in body axis elongation and neural tube closure; defects in the orientation of sensory cells in the inner ear; and defects in left-right patterning caused by abnormal organization of *ciliated* cells within the embryonic node.

How do core PCP proteins work in the regulation of PCP? Core PCP regulate “active PCP signaling” which involves the asymmetric localization of core PCP proteins along the PCP axis throughout the plane of the tissue (Bastock, Strutt, & Strutt, 2003; Das, Jenny, Klein, Eaton, & Mlodzik, 2004; del Alamo & Mlodzik, 2006; Montcouquiol et al., 2006; Shimada, Usui, Yanagawa, Takeichi, & Uemura, 2001; J. Wang et al., 2005; Y. Wang, et al., 2006). This asymmetric localization is both required for the establishment of tissue polarity by core PCP proteins as well as one of the clearest readouts of core PCP protein function. Mutations in one core PCP protein disrupt the asymmetric localization of other core PCP proteins within the same tissue in a non-cell autonomous manner (Adler, Taylor, & Charlton, 2000; Vinson & Adler, 1987; Y. Wang, et al., 2006) [reviewed in (Montcouquiol, Jones, & Sans, 2008; D. I. Strutt, 2002) This phenomenon helps identify whether a disruption of PCP occurs at the level of core PCP proteins or whether a disruption in PCP is taking place downstream (or

parallel) of core PCP signaling. Overall, the distinctive, asymmetric distribution of core PCP proteins is a key factor in their regulation of PCP.

Numerous studies have shown that, in addition to the disruption in the asymmetric localization of other core PCP proteins, mutations in PCP proteins cause defects in the *coordinated* orientation cells along the PCP axis but that individual cells within the tissue remain intrinsically polarized. In other words, in core PCP mutant tissue, the morphological polarization of individual cells is maintained but the direction of polarization is no longer coordinated throughout the tissue (Curtin, et al., 2003; X. Lu, et al., 2004; Y. Wang, et al., 2006). Combined, these data suggest that core PCP proteins play a role in the establishment of tissue-wide polarity but not intrinsic or morphological cell polarization. Additional data that supports this idea come from the inner ear vestibular organs where a line of reversal in the intrinsic polarization of cells is found within the tissue in spite of the fact that the localization of core PCP protein Vangl2 the same across the line of reversal (Deans et al., 2007).

## Morphological or Intrinsic Polarization and PCP Signaling

The establishment of intrinsic planar polarization within individual cells is a complex process that requires the differential distribution of proteins and organelles to different parts of a cell (illustrated in Figure 1.1). This process of morphological polarization likely involves a tightly controlled regulation of protein transport and localization. How the cells controls changes of several different cellular components during the establishment of intrinsic cell polarization remains

to be elucidated, however, recent work has suggested that this process requires the involvement of different ciliary genes (Aigouy et al., 2010; Antic, et al., 2010; Borovina, Superina, Voskas, & Ciruna, 2010; Cao, Park, & Sun, 2010; Guirao et al., 2010; Heydeck, Zeng, & Liu, 2009; Jones et al., 2008; Marshall, 2010; Song, et al., 2010).

A number of studies have shown a connection between ciliary genes and PCP. Like mutations in core PCP proteins, mutations in ciliary genes lead to defects multiple PCP processes for example, defects in CE (Ferrante et al., 2009; Jones, et al., 2008); oriented cell division (Jonassen, San Agustin, Follit, & Pazour, 2008), and defects in morphological polarization of sensory epithelial cells in the inner ear (Jones, et al., 2008).

Although it is not yet completely clear if of how primary cilia and core PCP proteins interact during the establishment of PCP, the majority of the data supports a model where cilia play a role in the regulation of intrinsic cell localization downstream or parallel to the asymmetric distribution of core PCP proteins [for a lucid and through review on this subject, see (Wallingford & Mitchell, 2011)]. Work from our laboratory has shown that conditional knockout of intraflagellar transport proteins (IFT proteins), which are required for the buildup and maintenance of primary cilia, leads to the disruption of PCP in the auditory epithelia within the inner ear (Jones, et al., 2008). Not only is PCP disrupted in the IFT conditional knockout tissue but in contrast with core PCP mutants, where individual cells remain intrinsically polarized, in IFT mutant tissue, some cells within the tissue are morphologically apolar (they have failed to intrinsically

polarize). Also in contrast with core PCP mutants, in IFT knockout tissue the asymmetric localization of core PCP proteins is maintained (Jones, et al., 2008) which suggests that the role for primary cilia in the regulation of cell polarization takes place downstream of core PCP signaling. Consistent with this idea, recent work suggests that the initial apical docking and formation of primary cilia takes place randomly but that during development, in tissue that undergoes PCP, cilia become polarized along the PCP and that this polarization requires fluid flow and the asymmetric distribution of core PCP proteins (Guirao, et al., 2010). Further evidence in support of this idea comes from findings that show that the positioning of primary cilia is disrupted in various core PCP mutants (Borovina, et al., 2010; Song, et al., 2010). However, the position of the core PCP proteins does not seem to be the ultimate determinant of ciliary positioning or cilia-dependent morphological cell polarization. As previously mentioned, the morphological polarization of the vestibular organs in the inner ear provides compelling evidence in support of this idea, where a line of reversal in the position of primary cilia within the cells (which marks the intrinsic or morphological polarity of these cells) is found within some of the vestibular organs. However, the localization of core PCP protein Prickle2 remains the same across the line of intrinsic or morphological polarity reversal (Deans, et al., 2007).

### *Outstanding Questions in PCP Signaling*

In spite of the fact that PCP has been studied for almost 25 years, the molecular mechanisms that control vertebrate PCP are not well understood. Work during all these years has show that disruptions in PCP signaling, either by mutations in core PCP or ciliary genes lead to a variety of cellular and developmental defects (illustrated on Figure 1.2). However, many questions about PCP regulation remain unanswered. This is particularly true in regards to the molecular mechanisms that regulate PCP downstream of core PCP and ciliary genes. In order to be able to start addressing detailed mechanistic questions about how PCP is regulated, we must first determine whether different PCP processes are controlled via the same or different molecular modules downstream of the known common regulatory genes. How does PCP signaling regulate so many different processes through common core PCP and ciliary genes? What are the downstream pathways/signals that regulate different PCP processes such as intrinsic or morphological polarization and CE of a tissue? Are all of these vertebrate PCP processes regulated by the same molecular machinery or do they branch off downstream of core PCP and ciliary genes?

In this thesis we have focused on coordinated cell polarization and CE. We have chosen the mouse inner ear as a model system to explore the question of how are these two PCP processes regulated downstream core PCP and ciliary genes. Normal development of the cochlea within the inner ear requires both CE and the coordinated morphological polarization of sensory cells (Figure 1.4). Therefore, we use the embryonic mouse cochlea as a model system for the

characterization of the molecular mechanisms that regulate these two vertebrate PCP processes downstream of core PCP and ciliary genes.

## *PCP Signaling During Development of the Mammalian Inner Ear*

### The Cochlea Undergoes CE during Development

The auditory sensory cells are situated within the cochlea, one of six sensory epithelia found in the mammalian inner ear (Figure 1.3A). During development the cochlea undergoes a dramatic elongation process. Cochlear elongation can be easily observed looking at mouse cochlear samples between embryonic days 14.5 (e14.5) to 18.5 (e18.5) (Figure 1.4 A-C). Cells within the pro-sensory region of the cochlea exit cell division between embryonic days 12.5 (e12.5) and 14.5 (e14.5) (P. Chen, et al., 2002; P. Chen & Segil, 1999; McKenzie, et al., 2004). Thus, the exit from cell cycle takes place well before the extension of the cochlear epithelium is completed. After e14 and throughout the end of embryonic development, the elongation of the nascent sensory region of the cochlea takes place in the absence of cell division (Figure 1.4 D). This process of cochlear elongation takes place concomitantly with the narrowing the pro-sensory epithelium during cochlear development when the post-mitotic pro-sensory cells converge along the planar polarity axis of the cochlea (show in Figure 1.5), allowing for the extension of the cochlear epithelium on the perpendicular axis (J. Wang, et al., 2005). Between e14-e18, cochlear elongation takes place in the absence cell division or cell death within this region (P. Chen, et al., 2002; P. Chen & Segil, 1999; McKenzie, et al., 2004) which suggests that the total number of cells within this growing region does not change during cochlear extension. Thus, the developing cochlea displays CE, a PCP process that is regulated by PCP signaling in vertebrates.

In order to better understand how cochlear CE is regulated we have found it useful to study data of how CE is regulated in *Drosophila.* A series of seminal studies looking at CE during *Drosophila* germband extension showed that the regulation of cell-cell contacts, including the polarized expression of contractile and cell-junction proteins plays a key role in the control of CE. These studies found that during CE in *Drosophila* cell-cell contacts rapidly contract along one axis (the axis of convergence) in a process that requires the polarized distribution of contractile proteins (e.g. non-muscle Myosin II) (Zallen & Blankenship, 2008; Zallen & Wieschaus, 2004). This rapid contraction of cellular contacts leads to the formation of a transitional of cell-cell junction that is characteristic of tissue undergoing CE and allows for the rapid morphological changes that take place during CE while maintaining tissue integrity. These transitional states are multicellular junctions called rosettes. Rosettes are cell-cell junctions involving five or more cells sharing a single vertex (Zallen & Blankenship, 2008). Rosettes are subsequently resolved by the elongation of cell-cell contacts on the perpendicular axis (the extension axis) via the polarized localization of junctional proteins (e.g. E-cadherin) (Rauzi, Lenne, & Lecuit, 2010) in along the axis of tissue extension. This dynamic process of cell-cell contact contraction and expansion requires a balanced control in the expression and *localization* of both contractile proteins and cell-cell adhesion proteins.

Based on these findings from *Drosophila*; we hypothesized that the regulation of cell-cell contacts might also play a role in the regulation of CE in vertebrates. Based on this hypothesis, we first analyzed the morphological

changes that take place during cochlear CE, and determined that regulation of cell-cell contacts is indeed a likely mechanism for the regulation of CE in the mouse cochlea. Subsequently, we determined that disruption of components of the adherens junctions (AJs) using conditional knockout mouse of p120-catenin (Davis & Reynolds, 2006) (which stabilizes cadherin at the membrane and thus AJs) [reviewed in (Reynolds, 2007)] leads to defects in cochlear CE but not to defects in coordinated sensory cell polarization.

Additionally, we found that in vertebrate PCP mutants, the expression patterns of the AJ proteins N-cadherin and E-cadherin is abnormal. Overall, these results, presented in detail in Chapters 2 and 3 suggest that the regulation of cell-cell contacts plays a role in the regulation of CE in the mouse cochlea downstream of vertebrate PCP signaling. Our results also suggest that CE in the cochlea is regulated independently of coordinated cell polarization. Coordinated cell polarization is another PCP process that, as will be explained in the next section, is both required for normal cochlear development and regulated by vertebrate PCP signaling.

## Sensory Hair Cell Coordinated Polarity

The mechanosensory cells that allow for perception of sound are localized within the cochlear spiral (Figure 1.3A). These mechanosensory cells are found within a highly organized structure called the organ of Corti (OC) (Figure 1.3 B). The arrangement of the sensory cells within the organ of Corti showcases a

distinctive and beautiful example of PCP (more specifically, coordinated cell polarization). Furthermore, during development, the process of coordinated sensory cell polarization takes place concomitantly with the aforementioned process of CE (Figure 1.5)

The cells within the organ of Corti are arranged in a highly organized mosaic pattern of sensory hair cells (so called due to the hair-like, actin based protrusion on their apical surface) intercalated by non-sensory supporting cells (Figure 1.3 B). On the apical surface of a mature organ of Corti, no two sensory hair cells are in direct physical contact with one another. There are two types of sensory hair cells within the organ of Corti: the inner hair cells (IHCs) and the outer hair cells (OHCs).The IHCs are one row of sensory cells found towards the innermost side of the cochlear spiral. Separated from the inner hair cells by one row of supporting cells (called the inner pillar cells (IPC)), three rows of OHCs are arranged towards the outer side of the cochlear spiral. In a mature organ of Corti, the sensory hair cells are coordinately polarized on their apical surface along the epithelial plane. This polarization takes place along the axis defined by the innermost side of the cochlear spiral (medial side - M on Figure 1.3 A) and the outermost side of the cochlear spiral (lateral side - L on Figure 1.3 A). The mediolateral axis is also referred to as the PCP axis.

On their apical surface, mature hair cells are adorned with hair like, actin-based protrusions called stereocilia (not to be confused with microtubule-based primary cilia). These protrusions are arranged in "V"-shaped bundles. In a mature hair cell, the vertex of the "V" points towards the lateral side of the cochlea

(Figure 1.3 C). Each stereociliary bundle is composed of multiple rows of stereocilia arranged on a height-graded pattern that resembles a staircase. The tallest stereocilia are located on the vertex of the V-shaped bundle (on the lateral side of the hair cell), and the shortest stereocilia within the V-shaped bundle are located towards the opposite (medial) side of the cochlea. During the formation of the stereociliary bundles, a microtubule-based primary cilium, known as the kinocilium, is adjacent to and physically attached to the tallest stereocilia and thus is found at the vertex of the V-shaped stereociliary bundle (Figure 1.3 C). At early stages of the differentiation of sensory hair cells, the kinocilium is found at the center of the developing hair cell. As the sensory hair cells mature, the kinocilium migrates towards the lateral side the cell.

How this process of lateral migration of the kinocilium takes place is not well understood. We hypothesized that the alignment of the centrioles along the PCP axis provides a directional cue that directs the lateral migration of the kinocilium.

In Chapter 2 we show data refuting this hypothesis. We found that the lateral migration of the kinocilium starts at a time when the orientation of the centrioles with respect to the PCP axis is random. However, we also found that the centrioles eventually do become aligned along the PCP axis. Although this alignment isn’t required for the lateral migration of the kinocilium (and thus for intrinsic cell polarization) it coincides with the coordination of the orientation of the V-shaped stereociliary bundles along the PCP axis.

So far we have mentioned that the planar polarization of sensory hair cells can easily be distinguished by either the orientation of the V-shaped stereociliary bundle or the localization of the kinocilium (which is found at the vertex of the V-shaped stereociliary bundle). In addition to these two clear indicators of HC polarization, the staircase-like pattern of the stereociliary bundles also showcases the planar polarity sensory hair cells. The height-graded organization of the stereociliary staircase is oriented towards opposing sides of the PCP axis, another manifestation of PCP within the apical surface of sensory hair cells (Figure 1c). Thus, it stands to reason that the machinery that is in charge of building up these polarized stereociliary bundles during development must be able to interpret and carry out planar polarity cues during the buildup of stereociliary bundles. Previous studies have determined that the Usher proteins are key components of the apical machinery that is responsible for the construction and maintenance of the stereociliary bundles.

## The Usher Proteins Regulate the Morphological Polarization of Sensory Hair Cells

The Usher proteins were first identified as mutant genes in the Usher Syndrome, which is the most common genetic disorder leading to deafness and blindness in humans (Boughman & Fishman, 1983; Boughman, Vernon, & Shaver, 1983; Kimberling et al., 1989). In the cochlea, mutations in Usher proteins primarily lead to defects in the structure of stereociliary bundles (Lefevre et al., 2008). To date 11 Usher genetic loci and nine Usher genes have been

identified, reviewed in (Petit, 2001; Reiners, Nagel-Wolfrum, Jurgens, Marker, & Wolfrum, 2006). The Usher proteins identified to this date vary greatly in their molecular structure and function. They include molecular motors such as Myosin VIIa (Weil et al., 1995), trans-membrane cadherin and cadherin-like proteins such as Cadherin-23 (Oshima et al., 2008) and Protocadherin-15 (Ahmed et al., 2001; Alagramam, Yuan, et al., 2001), scaffolding proteins such as Sans (Weil et al., 2003), and trans-membrane receptors such as Clarin-1 (Adato et al., 2002). To this date the exact molecular mechanisms through which Usher proteins direct stereociliary buildup are not fully understood. However, despite their morphological heterogeneity, a multitude of biochemical, genetic, and cell biological studies have identified the Usher proteins as components of a complex protein network localized in the apical surface of hair cells. This protein network (referred to as the Usher Complex) is required for the proper buildup and maintenance of the stereociliary bundles [reviewed in (El-Amraoui & Petit, 2005; Leibovici, Safieddine, & Petit, 2008; Petit, 2001; Reiners, et al., 2006; Wallingford & Mitchell, 2011)]. Different components of the Usher complex are found on different parts of the apical region of the HCs and through different types of protein-protein interactions, some with actin and some with other Usher proteins, they carry out different functions that allow for the formation and maintenance of stereocilia. Overall, the Usher complex (1) connects the kinocilium to adjacent stereocilia; (2) connects stereocilia the neighboring stereocilia; (3) contributes to the buildup and integrity of individual stereocilia by bundling actin filaments within each stereocilia. Given that, during normal development, vertebrate PCP

signaling controls both the *intrinsic* and *coordinated polarization* of sensory hair cells via signals from ciliary and core PCP genes it follows that, as part of the machinery in charge of the buildup and maintenance of *polarized* stereociliary bundles, the Usher complex must be able to understand polarity cues from vertebrate PCP signaling. Based on this hypothesis, in Chapter 4, we explore the roles of two different Usher proteins, Protocadherin-15 and Sans, in the regulation of hair cell polarization.

Protocadherin-15 (Pcdh-15) is localized to the apical surface of sensory hair cells and mediates linkages between stereocilia as well as between the kinocilium and the adjacent stereocilia (Kazmierczak et al., 2007). In Chapter 04, we show that Pcdh15-functionally null mice have defects in the intrinsic or morphological polarization of sensory hair cells while the asymmetric distribution of core PCP proteins and the extension of the cochlea remain normal.

Additionally, we used mice with a truncation in the scaffolding protein Sans to determine whether this Usher protein could also regulate hair cell polarization. Sans mutant cochlea have subtle defects in coordinated sensory hair cell polarization. These results combined support the idea that cochlear CE and coordinated polarization are regulated via separate molecular modules downstream from vertebrate PCP signaling.

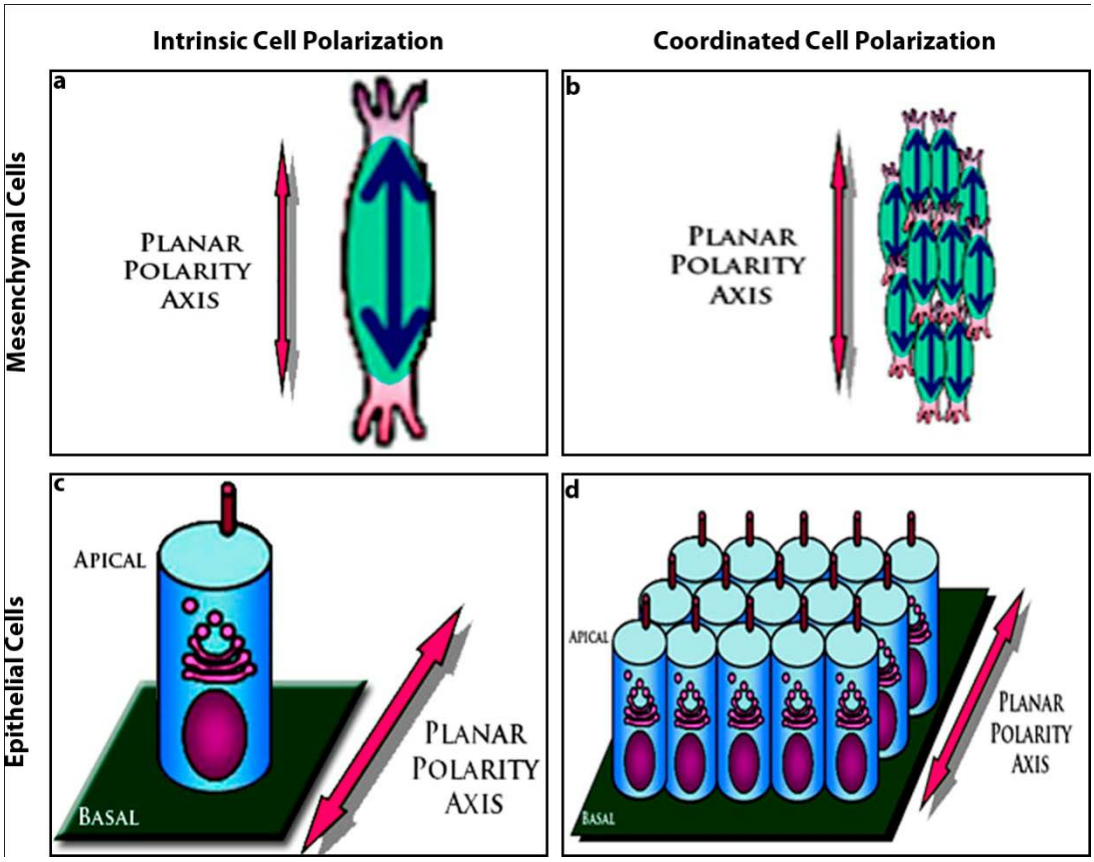
***Summary***

Although much has been learned about the regulation of PCP signaling during the last 25 years, to this date it isn't known whether downstream of common PCP genes, different PCP processes or forms of PCP are regulated via the same or different molecular modules. Using the mouse cochlea as a model system for the study of different forms of PCP we determined that different PCP processes are regulated *independently* of one another. In this thesis we will present evidence showing that the PCP processes of cochlear CE and coordinated sensory hair cell orientation/polarization are regulated via independent mechanisms. Using a p120-catenin conditional knockout and two Usher protein mutant mouse lines, we show that these different mutants have defects in either cochlear CE **or** coordinated hair cell polarization but not both. These phenotypes differ from the results found in core PCP and ciliary mutant mice, which have both defects in cochlear CE **and** coordinated sensory cell polarization.

Thus, in this thesis we have addressed the question of whether different PCP processes are controlled via the same or different molecular modules downstream of vertebrate PCP signaling molecules. By focusing on the study of CE and coordinated sensory cell polarization in the inner ear we determined that these PCP processes are regulated via independent molecular modules downstream of common PCP genes (Figure 1.6). The regulation of cell-cell contacts plays a role in the regulation of CE in a p120-catenin dependent manner

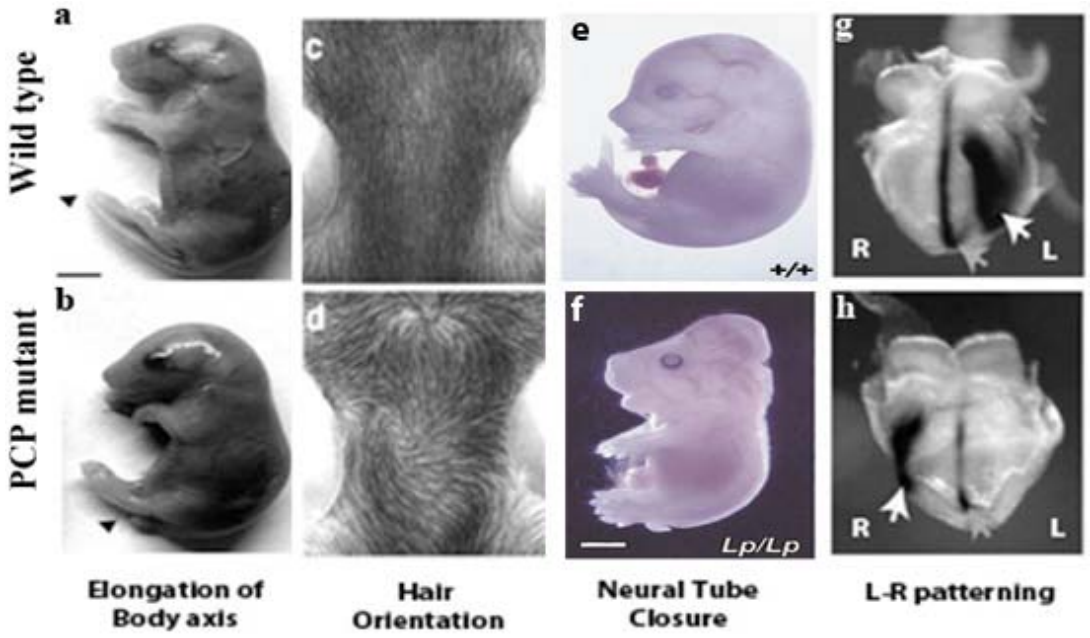
independently of coordinated hair cell polarization while Usher proteins play a role in the regulation of hair cell polarization ***independently*** of cochlear CE.

**Figure 1.1. Planar Cell Polarity**




Illustrations showcasing the process of intrinsic cell polarization as can be observed in mesenchymal cells (A) and epithelial cells (C). Illustrations showing how intrinsic polarization of these cells can be coordinated throughout the tissue during PCP for mesenchymal cells (B) and epithelial cells (D).

**Figure 1.2. Disruptions in Vertebrate PCP Signaling Cause a Variety of Developmental Defects**




Mutations in PCP genes can cause a multitude of developmental defects. A few of these defects are illustrated in this figure.

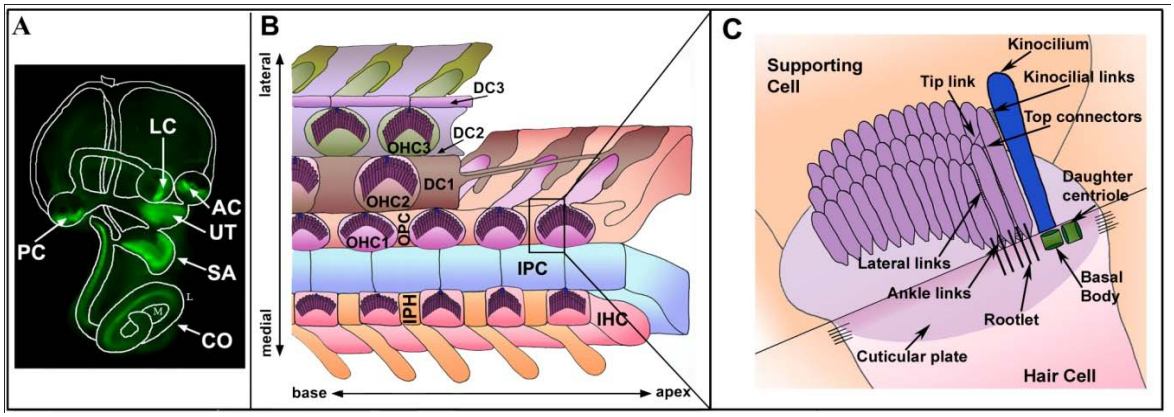
Defects in body axis elongation found in a mouse were core PCP signaling is disrupted (b) as compared with control littermate (a) (Wen et al., 2010)

Defects in coordinated cell polarization can be easily observed in the disorganization of hair cells within the skin illustrated in a core PCP mutant mouse (d) as compared to a control littermate (c) (Klein & Mlodzik, 2005).

Some PCP mutant mice also display varying degrees of neural tube closure defect, for example, mice carrying the *looptail* mutation (lp) in the e core PCP protein Vangl2 (f) display *craniorachischisis*; a complete failure in neural tube closure as compared to a control mouse (e) (Murdoch et al., 2001).

Defects in core PCP proteins can also cause L-R patterning defects; in a Vangl1-/-,Vangl2-/- mouse (h) the L-R axis, as determined based on the expression of *lefty,* is inverted as compared to control (g) due to defects in arrangement of ciliated cells in the embryonic node (Song, et al., 2010).

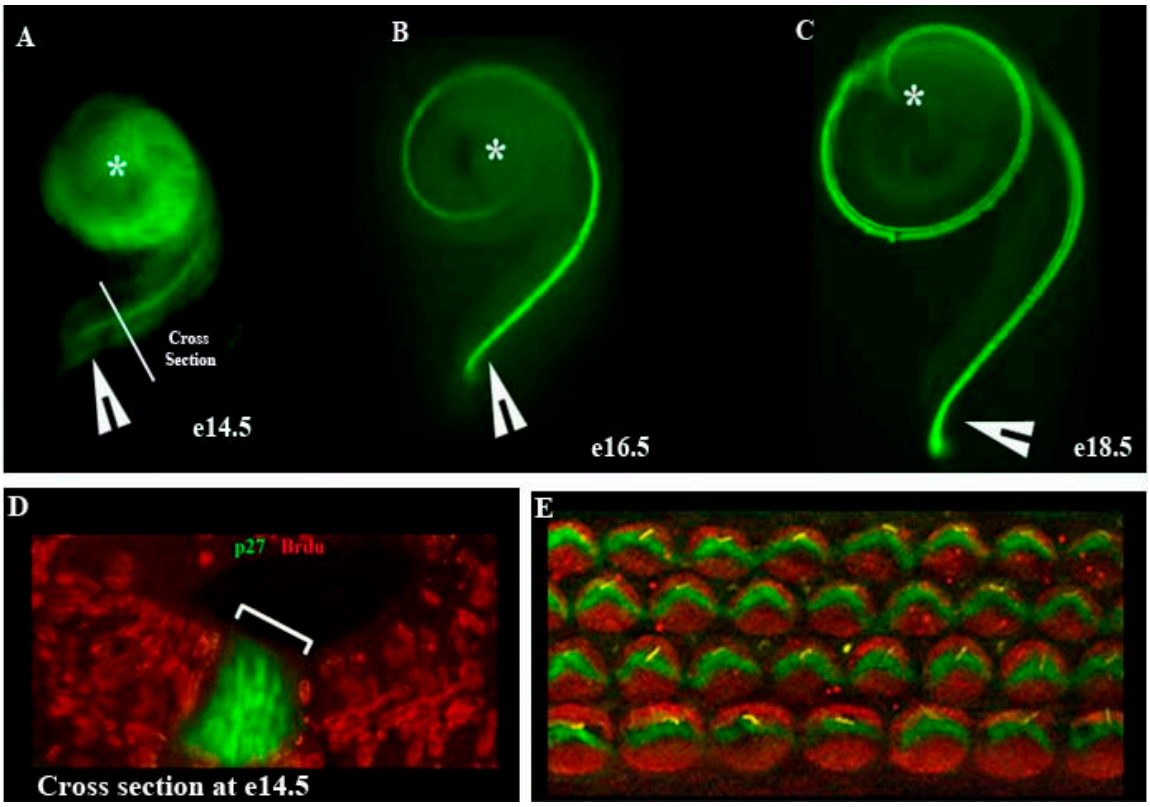
**Figure 1.3. Structure of the Mammalian Inner Ear and Diagrams of the Organ of Corti and the Apical Surface of a Cochlear Hair Cell**




**A.** *Outline of the structure of a mouse inner ear*. The six sensory epithelia in the inner ear are labeled with GFP. *PC:* posterior cristae; *LC:* lateral cristae; *AC:* anterior cristae; *UT:* utricle; *SA:* saccule; *CO:* cochlea; *M:* medial side of the cochlea; *L:* lateral side of the cochlea. **B.** *Diagram of the organ of Corti*. OHC: Outer Hair Cell; IHC: Inner Hair Cell; IPC: Inner Pillar Cell; IPH: Inner Phalangeal Cell; OPC: Outer Phalangeal Cell; DC: Deiter Cell. **C.** *Diagram of the apical surface of a cochlear hair cell*. Different parts of the hair cell are labeled within the image.

Reprinted from (Chacon-Heszele, Rida, & Chen, 2011). Thank you goes to Dr. Padmashree Rida for the artwork on panels B and C.

**Figure 1.4. CE and Coordinated Cell Polarization During Normal Cochlear Development in the Mouse**

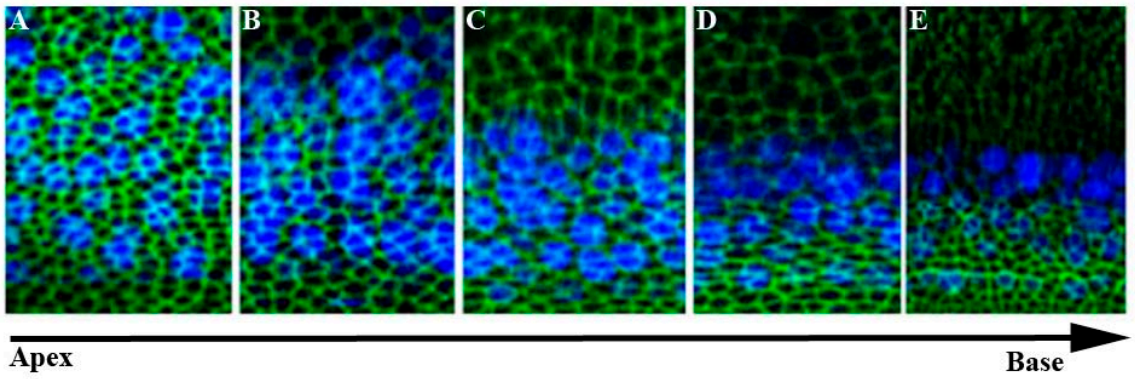



A-C. Extension of the mouse cochlea illustrated from e14.5 to e18.5: The sensory hair cells are labeled with GFP expressed under Math1 promoter. In all stages the basal region of the cochlea is marked with an arrowhead and the apical region with an asterisk.

D. The pro-sensory region of the cochlea is post-mitotic at e14.5: a cross section from cochlea at e14.5 (basal region, position illustrated with a while line on (A)) shows that the p27 (green) pro-sensory cells are negative for BrdU incorporation (red) and thus post-mitotic.

E. During development the sensory hair cells are coordinately polarized: A z-projection of a confocal micrograph of the apical surface of the sensory region of the cochlea at e18.5 illustrates the coordinated polarization of the sensory hair cells of the cochlea. These cells have actin-based V-shaped protrusions (stereocilia) on their apical surface. As readily visible in this image the stereocilia in all the sensory cells point in the same direction. During development, the formation and the formation of coordinately orientated stereocilia as well as the extension of the cochlea are coordinated during development. Stereocilia are labeled in green, primary cilia are labeled in yellow, and the apical surface of the cells is labeled in red.

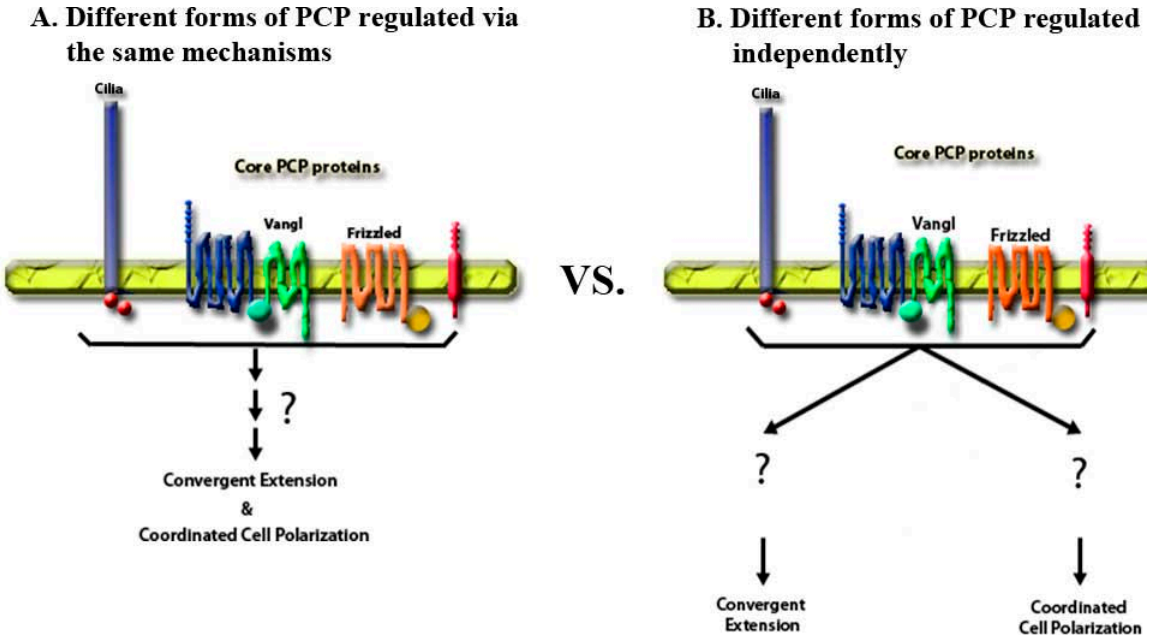
**Figure 1.5. Convergence of the Pro-sensory Cells of the Cochlea along the Planar Polarity Axis During Development**




The "pro-sensory" cells that will make up the sensory region in the mature cochlea (both hair cells and supporting cells) are labeled with Sox2 (blue), the actin cytoskeleton is labeled with phalloidin (green); E14.5.

This image was modified from (Chacon-Heszele*, Ren*, Reynolds, Chi, & Chen). All images used to make this figure were acquired by a former member of the Chen lab, Dr. Dong-Dong Ren (Renee).

**Figure 1.6. Competing models for the Regulation of CE and Coordinated Cell Polarization tested in this Thesis**




In this thesis we have focused on the question of whether different PCP processes are regulated via the same or independent molecular modules downstream of the common regulatory genes that together make up the vertebrate PCP signaling pathway. As explained in the introduction, vertebrate PCP is regulated by conserved "core" PCP genes first identified in *Drosophila* as well as ciliary genes.

We used the PCP processes of CE and coordinated sensory cell polarization in the cochlea to address this question. We hypothesized that, as illustrated in B, CE and coordinated cell polarization are regulated via separate molecular modules downstream of vertebrate PCP signals. In this thesis we will present data supporting this hypothesis and model B.

# Chapter 2 Morphogenesis of the Mouse Inner Ear During Development

## *Introduction*

During development, the sensory region of the mammalian cochlea undergoes both CE and coordinated cell polarization. First, the cochlear spiral elongates in a process that involves the convergence of the post-mitotic pro-sensory cells along the mediolateral axis of the cochlea (Figure 1.5). Second, the highly specialized sensory hair cells within the cochlea build up and display coordinately polarized actin-based stereociliary bundles on their apical surface (illustrated on Figure 1.3). The formation of these polarized stereocilia depends on the accurate and precise positioning of a microtubule-based primary cilium along the mediolateral planar polarity axis within each hair cell. Remarkably, this process is coordinated throughout all the sensory hair cells within the cochlea.

### CE of the cochlea

The cells that give rise to the organ of Corti (OC) exit cell cycle between e12.5 and e14.5 (P. Chen, et al., 2002; P. Chen & Segil, 1999; McKenzie, et al., 2004). These "pro-sensory" cells undergo a graded differentiation process that starts at base of the cochlea and progresses toward the apex of the cochlea. Additionally, the differentiation of the hair cells starts with the innermost (or medial) row of hair cells and progresses towards the outermost (or lateral) hair cell rows (P. Chen, et al., 2002; McKenzie, et al., 2004). During this period of differentiation, the developing OC increases in length 3 to 4 fold and narrows

without increased cell numbers or cell death (P. Chen, et al., 2002; P. Chen & Segil, 1999; McKenzie, et al., 2004). The elongation of the sensory region takes place via the convergence of these post-mitotic pro-sensory cells (which express the transcription factor Sox2) along the mediolateral (PCP) axis of the cochlea, allowing for the elongation of the tissue on the perpendicular axis. This process is illustrated in Figure 1.5 (Chacon-Heszele, Ren et al; *under review*).

As was described in Chapter 01, in vertebrates, the process of CE is regulated by PCP signaling. The requirement for PCP signaling in the regulation vertebrate CE has been extensively characterized in a number of vertebrate model systems [reviewed in (Axelrod & McNeill, 2002; Jones & Chen, 2007; Wallingford & Harland, 2002)]. Mutations in either core PCP proteins or ciliary genes lead to defects in CE in multiple vertebrate tissues, including the mammalian cochlea [reviewed in (Jones & Chen, 2007)].

In recent years, a coherent model of the mechanisms that regulate *Drosophila* CE has come to light. In a series of very elegant studies it was determined that the regulation of CE in *Drosophila* depends on the regulation the contraction and expansion of cell-cell junctions in a process that depends on the polarized distribution of contractile and junctional proteins [reviewed in (Fernandez-Gonzalez, Simoes Sde, Roper, Eaton, & Zallen, 2009; Zallen, 2007; Zallen & Blankenship, 2008)]. These studies identified key transitional cell-cell junctions that allow for the maintenance of tissue integrity during the dynamic cellular processes that take place during CE. These characteristic transitional cell-cell contacts, or rosettes, contain five or more cells sharing a single vertex.

These changes in cellular morphology during CE (which include the formation and resolution of cellular rosettes) involve the rapid contraction of cell-cell junctions along the axis of cell convergence followed by the expansion and stabilization of new cell-cell junctions on the axis of elongation of the tissue [reviewed in (Zallen & Blankenship, 2008)].

The contraction of cell-cell junctions along the axis of cell convergence is mediated by contractile proteins like non-muscle Myosin II. During this process, the polarized localization of Myosin II allows for the rapid contraction or shrinkage of cell-cell contacts along the axis of convergence and leads to the formation of cellular rosettes (Fernandez-Gonzalez, et al., 2009). On the other hand, the expansion and stabilization of newly formed cell-cell junctions is regulated by cell junction proteins such as E-cadherin, a component of adherence junctions (AJ). During this process, the polarized localization of E-cadherin along the axis of tissue elongation allows for the extension of cell-cell borders and the fast resolution of cellular rosettes (Rauzi, et al., 2010). Thus it is easy to visualize that mutations that affect the equilibrium between the counteracting contractile and expansive forces during this highly dynamic process can lead to defects in CE.

Based on the findings from *Drosophila*, we hypothesized that the dynamic regulation of cell-cell contacts might play a role in the regulation of cochlear CE downstream of PCP signaling. Based on this hypothesis, we characterized the morphological changes that take place in the developing cochlea, and examined the expression of adherence junctional components within the developing cochlea. Here, we show that transitional cell rosettes are present early in the pro-

sensory domain of the developing cochlea and that they disappear as differentiation progresses. We also show the normal expression patterns of the adherence junction proteins N- and E-cadherin at different stages of cochlear development. We found that the expression of N- and E-cadherin marks a sharp boundary between the IHC and OHC regions of the nascent organ of Corti, respectively and that the onset of expression of these adhesion proteins coincides with the stabilization of the time at which hair cells within these regions first become discernible.

Formation of Polarized Stereociliary Bundles

The sensory hair cells of the cochlea have two distinctively planar polarized structures on their apical surface: (1) an actin-based, V-shaped stereociliary; and (2) a microtubule-based primary cilium (kinocilium) which is found at the vertex of the V-shaped stereociliary bundle. During hair cell differentiation, the proper orientation of the stereocilia requires accurate and precise positioning of the kinocilium towards lateral side of each hair cell. However, the process through which the kinocilium migrates from the center to the lateral side of the cell during hair cell differentiation and stereociliary bundle formation has not been well characterized.

Given that there are two closely apposed centrioles within the apical surface of differentiated hair cells and that kinocilium stems from one of these centrioles (referred to as the basal body) we hypothesized that alignment of the centrioles along the PCP axis provides a directional cue for the lateral migration

of the kinocilium. In this chapter we will provide evidence that the centrioles do align along the PCP axis. However, contrary to our original prediction, we determined this alignment does not take place until after the lateral migration of the kinocilium has started. Instead of providing a directional cue for the lateral migration of the kinocilium from the center of the cell, the alignment of the centrioles along the PCP axis seems to coincide with the final, alignment of the V-shaped stereociliary bundles along the PCP axis which is coordinated throughout the sensory epithelium.

## *Results*

### Characterization of Cellular Morphology and Expression of Cell Adhesion Proteins during Cochlear Development

#### *The cochlea undergoes dramatic morphological changes during development*

In the mouse cochlea, the precursor cells that give rise to the sensory and supporting cells that make up the mature organ of Corti exit cell cycle between embryonic days 12.5 to 14.5 (e12.5 to e14.5) (P. Chen, et al., 2002; P. Chen & Segil, 1999; McKenzie, et al., 2004). These post-mitotic cells within the pro-sensory domain cells undergo dramatic morphological changes throughout embryonic development. At e14.5 these post-mitotic pro-sensory cells domain of the cochlea are mostly hexagonal in shape on their apical surface, with a centrally positioned basal body/kinocilium; the geometrical long axis of the cells is aligned with the longitudinal (or extension) of the cochlear duct (Figure 2.1A (Chacon-Heszele*, et al.)). In contrast, the cells within a differentiated organ of Corti, at the end of embryonic development (e18.5), display a highly organized mosaic pattern consisting of three rows of outer hair cells (OHC1-3) one row of inner hair cells (IHC) intercalated with several types of non-sensory supporting cells of various shapes (Figure 2.1B). These supporting cells are: inner pillar (Ip) cells (which separate the inner hair cell row from the three outer hair cell rows); outer pillar (Op) and Dieters' cell (Dc) (Figure 2.1B). In contrast with the mostly uniform hexagonal cell shape pattern seen in the pro-sensory cells at e14 (Figure 2.1A), one can readily appreciate that the cells that make up a mature organ of Corti at e18 display a number of different cell shapes and cell-cell contacts on

their apical surface. The sensory hair cells have a round or oval shape, and contact four neighboring supporting cells. The outer pillar cells and the dieter cells that intercalate the second and third outer hair cell rows have an hourglass shape and contact eight different neighboring cells. The inner pillar cells have a rectangular shape and, depending on their position, share cell contacts with 4 to 8 neighboring cells (Figure 2.1B).

Based on the fact that these dramatic changes in cell morphology take place without a disruption in integrity of the epithelium and evidence from *Drosophila* showing that CE in that system is regulated via a mechanism involving the dynamic regulation of cell-cell contacts, we hypothesized that cochlear CE might be regulated via a similar mechanism. Thus, we characterized the changes in cell morphology that take place during the differentiation of the organ of Corti. As explained below, this analysis suggested that indeed there's a dynamic remodeling of cell-cell contacts during the formation of the organ of Corti.

Initially, polygonal-shaped cells have mostly tri-cellular contacts (Figure 2.2A). As the tissue differentiates cell-cell contacts change, in addition to tri-cellular vertices, we find that about 50% of cell vertices are formed by four cells and around 5% of cell vertices are in the shape of cellular rosettes (five or more cells sharing a single vertex; magenta arrowheads, Figure 2.2A). As the tissue differentiates, the number of cellular rosettes diminishes (Figure 2.2B,C) eventually giving way to cell contacts involving mostly tri-cellular vertices in the differentiated organ of Corti (Figure 2.2 D). These dynamic changes in cellular

contacts implicate that controlled shrinkage and extension of cellular contacts is taking place during cochlear development. This finding is consistent with our working hypothesis that cochlear CE is regulated by a mechanism involving the dynamic regulation of cell-cell contacts. Thus, we proceeded to characterize the expression of the AJ proteins N-cadherin and E-cadherin in the cochlea during development.

### *Expression of E- and N-cadherin Demarcates Different Regions in the Developing Organ of Corti*

During CE in *Drosophila* shrinkage of cell contacts is regulated by contractile proteins such as non-muscle myosin II (Fernandez-Gonzalez, et al., 2009; Zallen & Wieschaus, 2004). The counteracting force and stabilization of new cell contacts is regulated by cell adhesion proteins, such as the AJ protein E-cadherin (Rauzi, et al., 2010). Cadherins are key components of the AJs. Our findings on Figure 2.2 suggests that dynamic changes in cell-cell junctions are taking place during cochlear development we decided to o explore whether changes in adherence junctions could play a role in the morphogenesis of the cochlea. To do this we examined the expression patterns of N-cadherin and E-cadherin during development of the organ of Corti.

We found that both E-cadherin and N-cadherin are expressed during differentiation of the organ of Corti. However, their expression is restricted to different regions of the developing organ of Corti. The expression of N-cadherin and E-cadherin demarcates a distinctive boundary between the IHC region and the OHC region of the cochlea.

E-cadherin expression can be first detected at about embryonic day 15.5 where it is found in the nascent OHC region (Figure 2.3 A,E). At this stage, the developing OHCs can start to be identified based on actin enrichment on their apical surface. As early as it can first be detected, E-cadherin expression is restricted to the OHC domain (above the white dotted line on Figure 2.3 A,E). As development progresses, E-cadherin levels within the outer hair cell region increase and clear expression of E-cadherin can be detected throughout the outer hair cell region including the OHCs, the Deiter cells and the row of cells lateral to the third row of Deiter cells (the Hensen cells (bracket)) (Figure 2.3 B,F).

The expression pattern of N-cadherin is complementary to that of E-cadherin. N-cadherin expression can be detected earlier in development than E-cadherin. N-cadherin expression within the developing organ of Corti is first noticeable at e14-e14.5. Interestingly, this stage coincides with the earliest time when the IHCs can be identified by actin enrichment on their apical surface (asterisks, Figure 2.3 C,G). At this early stage, expression pattern of N-cadherin is already restricted to the medial, IHC region (below dotted white line; Figure 2.3 C,G). This expression domain is maintained throughout development of the organ of Corti (Figure 2.3 D,H). Thus the onset of expression of N-cadherin E-cadherin in the different regions of the organ of Corti coincides with the initial stabilization of the hair cells within each region. This complementary expression pattern of N-cadherin and E-cadherin in the organ of Corti at later stages has

recently been reported by others (Yamamoto, Okano, Ma, Adelstein, & Kelley, 2009).

## Characterization of Centriole Orientation and Kinocilia Lateral Migration during Hair Cell Differentiation

### *Migration of the Kinocilium towards the Lateral Side of Differentiating Hair cells Independently of Centriole Orientation*

During cochlear development, while the sensory cells of the developing organ of Corti undergo the dramatic morphological changes previously described, they also become differentiated. This process of hair cell differentiation involves the migration of the kinocilium from the center to the lateral side of the cell and the formation and coordinated alignment of the V-shaped stereociliary bundles throughout the cochlear epithelium on the apical surface of the cell. These nascent arise from an apical meshwork of actin found on the apical surface of hair cells (the cuticular plate, see Figure 1.3 C).

Stereocilia first emerge as short microvilli throughout the apical surface of differentiating hair cells. At this early stage of hair cell differentiation the microvilli surround a centrally localized kinocilium (Lim & Anniko, 1985). As differentiation progresses, the stereocilia elongate and the kinocilium migrates from the center to the lateral side of the cell. During this process, links are formed between adjacent stereocilia and between the tallest stereocilia and the kinocilium. Microvilli that are not part of these cohesive V-shaped stereociliary bundles are

eventually resorbed while the stereocilia that form the stereociliary bundle continue to grow and eventually give rise to the height-graded stereociliary staircase present in mature HCs (illustrated Figure 1.3 C) (Furness, Richardson, & Russell, 1989; Lenoir, Puel, & Pujol, 1987; Pickles et al., 1989; Pickles, Comis, & Osborne, 1984).

Thus, the formation of coordinately polarized V-shaped stereocilia at the apical surface of developing HCs requires the careful placement of the kinocilium along the PCP axis aligned with the center of and on the lateral side of the cell. This process requires the coordination of stereocilia formation and the lateral migration of the kinocilium. Given that precise positioning of the kinocilium on the lateral side of differentiating hair cell defines the direction of hair cell polarization, we hypothesized that orientation of the centrioles would provide a directional cue for the lateral migration of the kinocilium. If so, we predicted that the centrioles would align along the PCP axis prior to the lateral migration of the kinocilium.

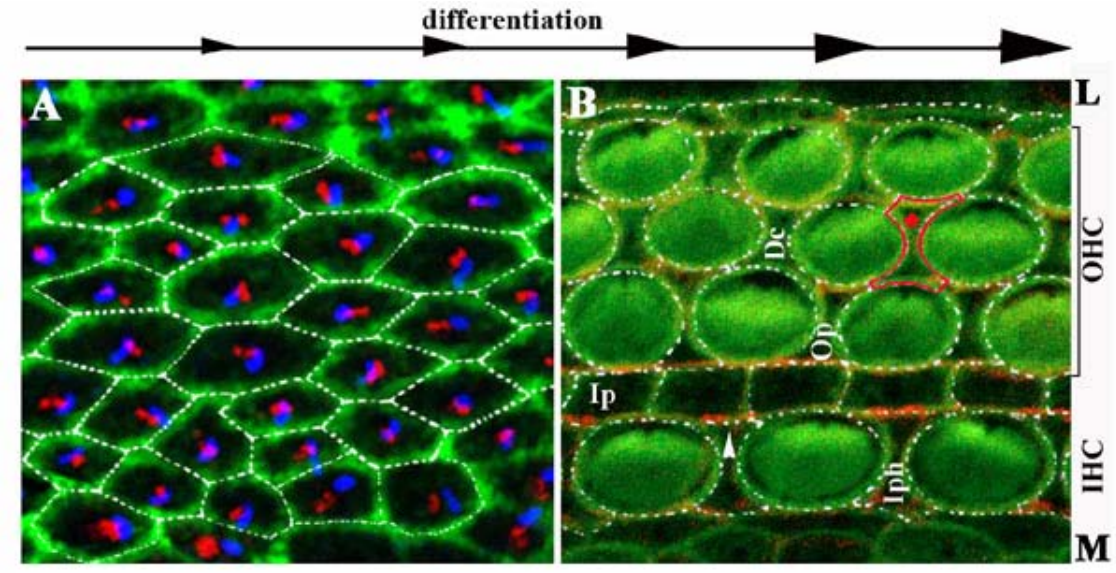
Contrary to our hypothesis, we found that the centrioles are not aligned along the PCP axis at the time of which the lateral displacement the kinocilium begins. At embryonic day 14, the centrioles are randomly oriented (Figure 2.4 A,B, C). Even at embryonic day 16, when (in most IHCs) the kinocilium has clearly already started to migrate towards the lateral side of the cell (Figure 2.4D, E) the centrioles are still randomly oriented with respect to the PCP axis (Figure 2.4F). However, as described below, the centrioles eventually do become aligned along the PCP axis.

***Centriole Alignment takes place late in Embryonic Development Concurrently with Coordinated Orientation of the Kinocilium and V-shaped Stereocilia along the PCP Axis***

We also found that the kinocilium does not move directly from the center towards the lateral side of the cell in a straight line along the PCP axis. Instead, the kinocilium first migrates towards the lateral side of the cell within about 60° from either side of the PCP axis. As individual hair cells mature, the kinocilium is eventually aligned with the PCP axis. Interestingly, even though the centrioles are not aligned with the PCP axis when the kinocilium starts to migrate to the lateral side of the cell in differentiating hair cells they eventually become aligned with the PCP axis. This process of centriole alignment with the PCP axis takes place gradually.

Before e18 and on the less differentiated apical regions at e18, the centrioles of differentiating hair cells are still randomly oriented with respect to the PCP axis (Figure 2.5 A-C;E-F). However, on the more differentiated, basal region of the cochlea, by e18 the daughter centriole is now consistently positioned laterally of the basal body (Figure 2.5I,K,L). By p0, in mature hair cells the kinocilia is consistently positioned along the PCP axis. Furthermore, at this stage in the majority of hair cells the centrioles are also aligned along the PCP axis, with the daughter centriole aligned at 0° from the basal body. It is of note that in most instances, the orientation of the maturing, V-shaped stereociliary bundles coincides with the orientation of the centrioles (Figure 2.5 J,M,N).

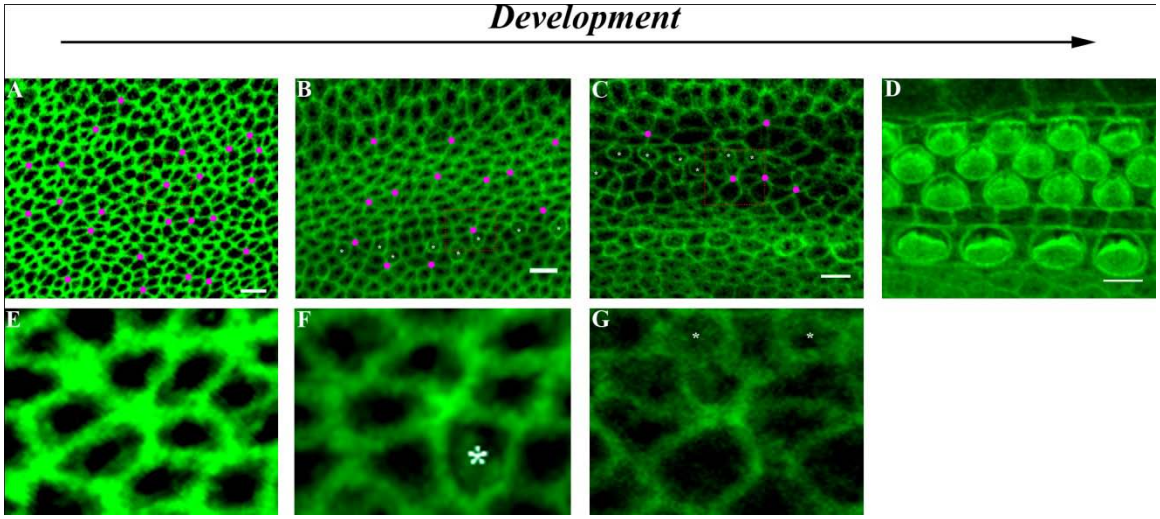
**Figure 2.1. Cells in the Developing Cochlea undergo dramatic morphological changes during Development**




*Confocal micrograph of the apical surface of the cochlea at e14 (A) and e18 (B)*.

**A.** F-actin is stained with Phalloidin (green); centrioles are labeled with gamma tubulin (red), and kinocilia are labeled with Arl13b (blue). The outline of cells is indicated with the dotted white lines. Note the hexagonal shape present in the majority of cells and that the long axis of the cells is oriented along the longitudinal axis of the cochlea (the axis of elongation; perpendicular of the mediolateral axis) **B.** F-actin is labeled with phalloidin (green) and the core PCP protein Frizzled3 is labeled with rhodamine (red). Note the asymmetric localization of Fz3 within the organ of Corti at e18. IHC: Inner Hair Cells; OHC: outer hair cells; Iph: Inner phalangeal cells; Ip: Inner pillar cells; Op: outer pillar cells; Dc: Deiter cells. M: medial side of the cochlea; L: lateral side of the cochlea. This figure was extracted from (Chacon-Heszele*, et al.).

**Figure 2.2. Cell Contacts change during the Gradual Differentiation of the Organ of Corti**




**A.** *Confocal micrograph of the apical surface of the cochlear epithelium at e14*: Note the high frequency of cellular rosettes (cell junctions of 5+ cells sharing a single vertex; magenta circles) throughout the cochlear epithelium at this stage. One of these rosettes is magnified in (E).

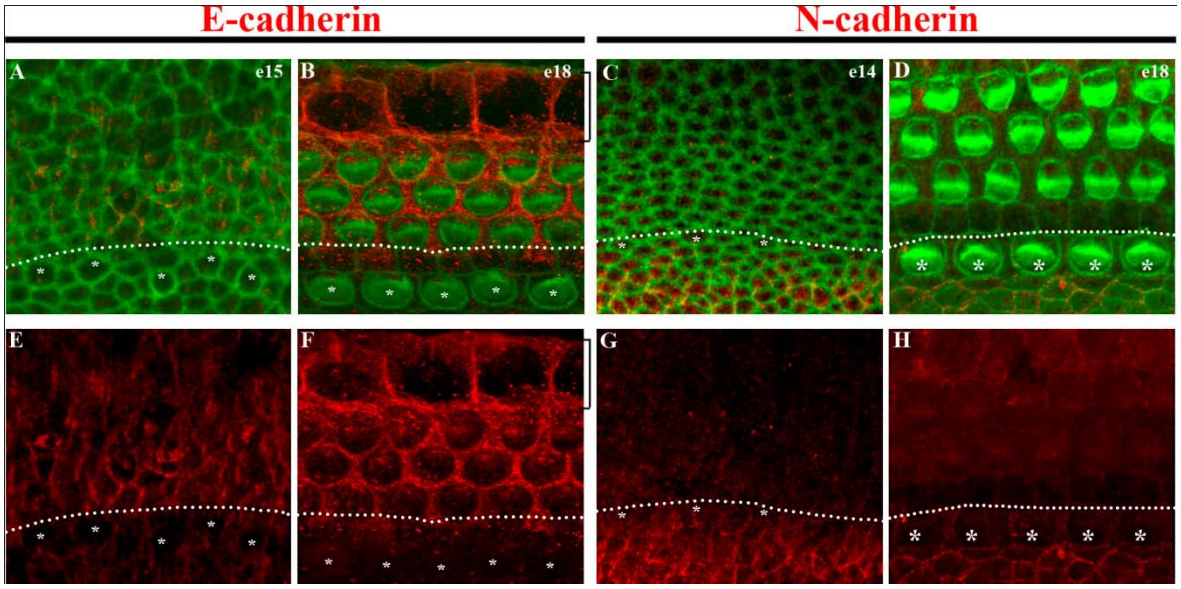
**B.** *Confocal micrograph of the apical surface of the cochlear epithelium at e15*: Note that at this stage the nascent inner hair cells are already visible (asterisks) however, they haven't reached their final configuration (where a single supporting cell separates each inner hair cell). At this stage, cellular rosettes (magenta circles) are present in both the future outer hair cell and inner hair cell region of the cochlea (one of these rosettes involving a developing IHC is shown on F).

**C.** *Confocal micrograph of the apical surface of the cochlear epithelium at e16.5:* Note that at this stage cellular rosettes are no longer visible throughout the inner hair cell region (in which most hair cells are now separated by a single supporting cell). However, cellular rosettes are noticeable in the developing OHC region. One of these rosettes near nascent OHCs (asterisks) is zoomed in G.

**D.** *Confocal micrograph of the apical surface of the cochlear epithelium at e18.5:* Note that cellular rosettes are no longer present in the developed organ of Corti. Instead, in the organ of Corti at this stage, cells form tri-cellular vertices.

In all images F-actin is labeled with phalloidin (green).

**Figure 2.3. The expression of E- and N-cadherin demarcates a Sharp Boundary within the Developing Organ of Corti**

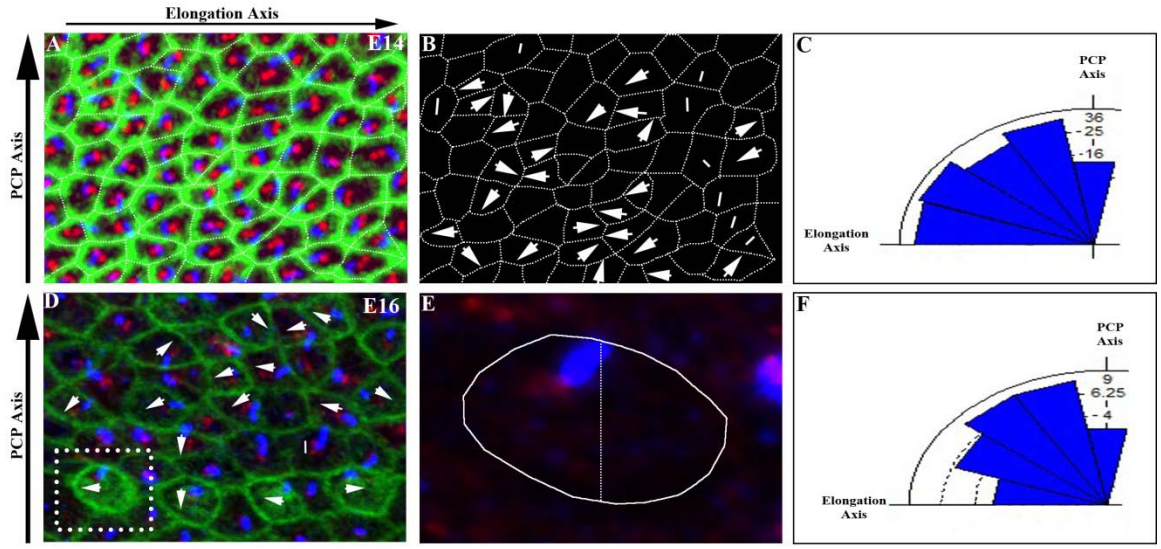



*Confocal micrographs of the organ of Corti at e14 (C,G), e15 (A,E) and e18 (B,D,F,H)*: F-actin is labeled with phalloidin (A-D); E-cadherin (A,B,E,F) and N-cadherin (C,D,G,H) are labeled with rhodamine (red).

*In wild type cells, membrane E-cadherin expression is restricted to the OHC region of the organ of Corti.* Membrane E-cadherin expression is first noticeable in the OHC region at e1e (A,E). Note that maturing IHCs are clearly distinguishable (asterisks) while the OHCs are just starting to become discernible within the OHC region (A). By e18 (B,F) membrane E-cadherin expression is clearly noticeable throughout outer hair cell region including the Hensen cells (black bracket) (B,C).

*In wild type cells, membrane N-cadherin expression is restricted medially of the OHC region of the organ of Corti*. Membrane N-cadherin expression first becomes noticeable at e14 (C,G) as the nascent IHCs (asterisks) first become discernible by F-actin enrichment on their apical junctions. At this stage, membrane N-cadherin expression is limited to the region medial to the OHCs which includes the nascent IHCs. This limited region of membrane N-cadherin expression is maintained at e18 (D,H). The boundary between the lateral OHC region and the medial IHC region of the organ of Corti are indicated in all figures with dotted white lines. Note that N-cadherin membrane expression is first discernible at an earlier stage that E-cadherin (e14 and e15 respectively); and that each membrane cadherin expression becomes discernible as the hair cells within each region start to differentiate.

**Figure 2.4. Centriole Orientation along PCP Axis is not required for Lateral Migration of the Kinocilia during Hair Cell Differentiation**




A. Confocal micrograph of the apical surface of the cochlea at e14 stained with Phalloidin (green), gamma tubulin (red), Arl13b (blue).

B. Panel indicating the orientations of centrioles (arrows) on panel "A." White dotted lines represent outlines.

C. Quantification of centriole orientation (from $0^0$-$90^0$ without distinguishing between the position of each centriole with respect to one another) at e14. In this figure, ninety degrees represents complete alignment along PCP axis while zero degrees represents alignment on the perpendicular axis (or elongation axis).

D. Confocal micrograph of the apical surface of the cochlea at e16 stained with Phalloidin (green), gamma tubulin (red), Arl13b (blue). White dotted box indicates cell zoomed on "E"

E. Single IHC from panel "D" zoomed; phalloidin has been removed. Cell outline: solid white line. PCP plane along the cell center: dotted white line.

F. Quantification of centriole angle orientation at e16.

**Figure 2.5. Centriole Orientation along the PCP Axis takes place late in embryonic development along with the alignment of the Stereociliary Bundles among Hair Cells**

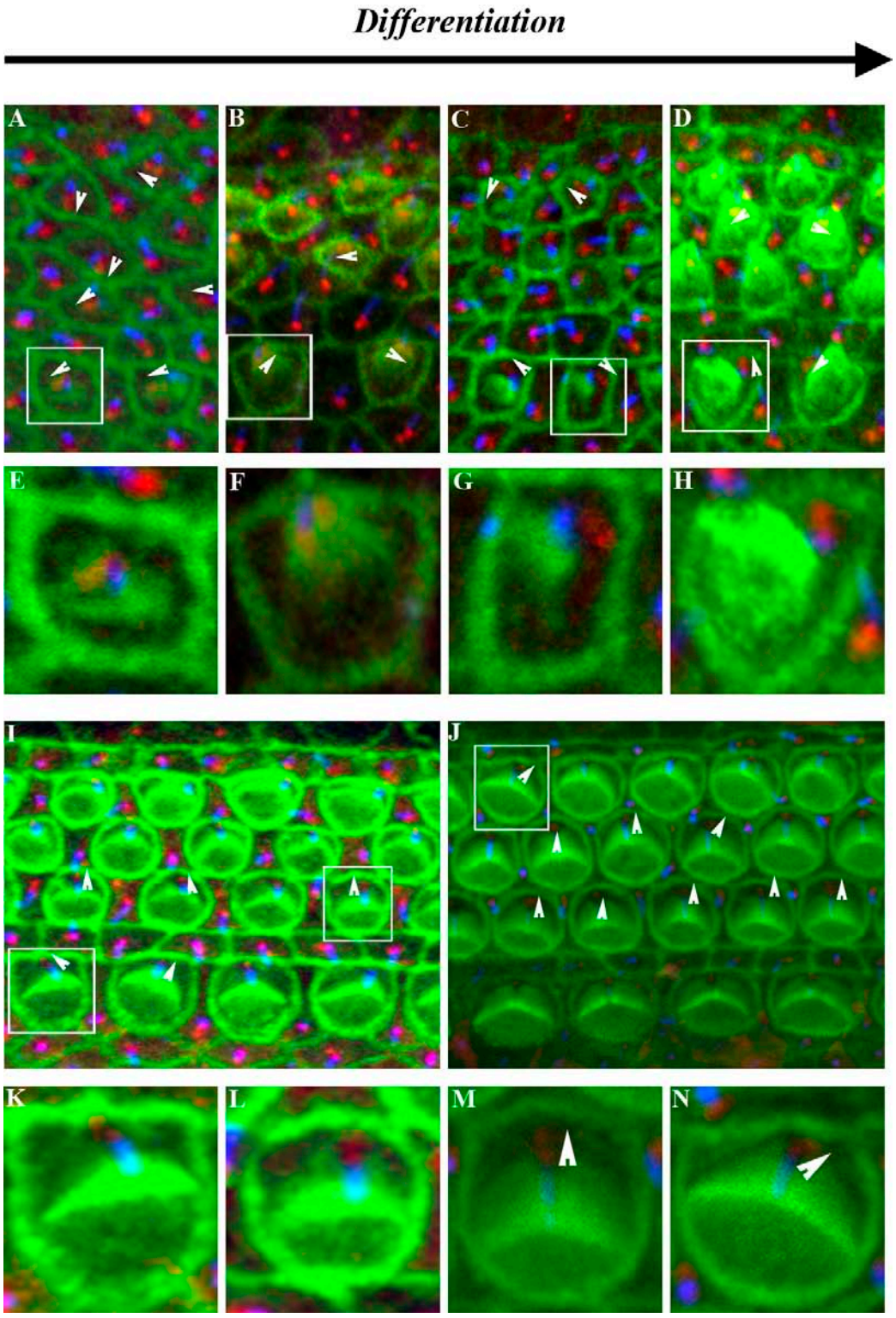


**Figure 2.5. Centriole orientation along the PCP axis takes place late in embryonic development along with the alignment of the stereociliary bundles among hair cells**

*Confocal micrographs of the organ of Corti at different stages of cochlear differentiation.* F-actin is labeled with phalloidin (green); centrioles are labeled with gamma tubulin (red) and kinocilia are labeled with Arl13b (blue). The orientation of the centrioles in individual cells is shown with the white arrowheads, with the base indicating the position of the basal body (where the kinocilium stems from) and the tip indicating the position of the daughter centriole. ***The initial lateral migration of the kinocilium takes place off the cell center***. In maturing hair cells, the kinocilium is first localized at the center of the cell (E). As hair cells differentiate, the kinocilium migrates toward the lateral side of the cell, but this displacement does not take place along in a straight line along the cell center (F-G). Eventually, as the hair cells continue to mature, the kinocilium moves left or right and eventually becomes re-aligned with the center of the cell. ***Centrioles become aligned along the PCP axis late in hair cell differentiation along with the coordinated alignment of the V-shaped stereocilia along the PCP axis***. The orientation of centrioles is random at earlier stages of differentiation (e16-e17; A-C) and remains randomized on the less apical region of the cochlea at e18 (D). However, on the *more differentiated* basal (I) and middle regions of the cochlea (not shown) the orientation of the centrioles starts to become uniform, with the daughter centriole localized laterally of the basal body (arrowheads, I; zoomed in K and L). By p0 (J), in most hair cells the centrioles are aligned along the PCP axis with the daughter centriole aligned at $0^{0}$ (lateral) from the basal body. However, in some hair cells, most of them within the younger outer hair cell rows (OHC2-3), the daughter centriole is not yet aligned with the basal body (N). Note that, in more differentiated regions of the cochlea (e18 middle region and older), the orientation of the V-shaped stereocilia correlates with the orientation of the centrioles in the majority of hair cells (arrowheads I, J; zoomed images K-L).

***Discussion***

The differentiation of the cochlea requires the orchestration of various cellular processes regulated by the vertebrate PCP signaling pathway. These processes include cochlear CE and the formation of coordinately polarized stereociliary bundles in the sensory hair cells within the organ of Corti. In this chapter, we have characterized different aspects of cochlear development including the morphological changes that take place during cochlear elongation and whether the alignment of the centrioles is required for hair cell polarization.

We determined that during the process of cochlear elongation, the post-mitotic pro-sensory cells in the cochlea undergo dramatic morphological changes that allow for the formation of the different of types of cells that make up a mature organ of Corti, with its various distinctive cell shapes, to arise from population of cells that is homogeneous-looking (Figure 2.1). These morphological changes involve changes in cell-cell contacts and include the formation of cellular rosettes, a transitional state required for the maintenance of epithelial integrity in highly dynamic tissue (Figure 2.2). We have also determined that during development, the expression of the adherence junction proteins N-cadherin and E-cadherin demarcate distinctive domains within the developing organ of Corti. Expression of E-cadherin is limited to the lateral region of the organ of Corti (including the OHCs); while the expression of N-cadherin is limited to the medial region where the IHCs are found (Figure 2.3). Furthermore, the membrane expression levels during development leads to an easily distinguishable boundary between the IHC and OHC regions of the organ of Corti based on the

expression pattern of N- and E-cadherin, respectively (Figure 2.3). This observation of differential expression of N-cadherin and E-cadherin within the cochlea during development, lends itself to the idea that differential cell adhesion force could regulate the formation of the cochlear *spiral* during cochlear morphogenesis. An idea that can be further explored in the future using cochlear explants and cadherin inhibiting antibodies to determine if inhibition of N- or E-cadherin alters the spiral shape of the cochlea *in vitro.* This speculation aside, our current observations suggest that the expression of the adherence junctions proteins is strictly regulated during development, which is consistent with the hypothesis that, in an analogous mechanism to the one observed in *Drosophila*, the regulation of cell-cell contacts plays a role in the regulation of cochlear CE. In later chapters we will show further evidence in support of this hypothesis.

At the same time that the aforementioned morphological changes described above are taking place, the sensory hair cells become differentiated. This differentiation process involves the formation of the polarized V-shaped stereociliary bundles on the apical surface of the cells. During this process the cells must be able to interpret polarity cues that allow them (1) form polarized stereocilia and (2) coordinately align the stereocilia along the planar polarity axis throughout the cochlear epithelium. The primary cilium, or kinocilium, is found at the vertex of the V-shaped stereociliary bundles structure in mature sensory hair cells. During HC differentiation the kinocilium migrates from the center to the lateral side of the cell and is physically linked with the nascent stereociliary bundles during hair cell differentiation. These observations suggest that the

positioning of the kinocilium within the apical surface of the hair cells is provides important directional information for the proper polarization of sensory hair cells. This hypothesis is supported by previous work from our laboratory showing that intrinsic hair cell polarization is disrupted in ciliary mutants (Jones, et al., 2008).

The basal body is a modified centriole from which the microtubule-based kinocilium stems from (Marshall, 2008). A second centriole, called the daughter centriole, is also found on the apical surface of the hair cells apposed with the basal body. Given that two points are sufficient to define a vector (and thus provide directional information) we hypothesized that the position of the centrioles would provide directional information for the polarization of hair cells. More specifically, we hypothesized that the centrioles would align along the PCP axis and thus provide a directional cue for the lateral migration of the kinocilium within the apical surface of sensory hair cells. Our results from Figure 2.4 and Figure 2.5 show that this is not the case.

The centrioles are randomly aligned when the kinocilium starts to migrate toward the lateral side of the hair cell (Figure 2.4). However, late in cochlear development, the centrioles do become aligned with the PCP axis (Figure 2.5). Instead of providing a polarity cue for the migration of the kinocilium (and thus the intrinsic polarization of individual hair cells) in the majority of hair cells, the alignment of the centrioles along the PCP axis correlates with the alignment of the V-shaped stereociliary bundles (Figure 2.5). This finding suggests that, perhaps, the process of centriole alignment plays a role in the regulation of the coordinated orientation of the V-shaped stereociliary bundles. However, at this

point whether the alignment of the centrioles is directing the alignment of the stereocilia, or whether the alignment both the centrioles and the stereocilia with the PCP axis are independent processes (perhaps following the same cue) remains an open question.

# Chapter 3 Altered expression of Adherens Junctions proteins correlates with defects in Cochlear Extension in Core PCP and Ciliary Mutants

## *Introduction*

As introduced in Chapter 1 and Chapter 2 of this thesis, the dynamic regulation of cell contacts plays an important role in the regulation of CE in *Drosophila*. This fact, combined with our data from Chapter 2 that shows cell morphological changes taking place during cochlear development, including the formation and eventual resolution of cellular rosettes led us to the hypothesis that dynamic regulation of cell-cell contacts plays a role in the regulation cochlear development.

In a project spearheaded by a former member of the lab, Dr. Dong-Dong Ren (Renee), we characterized the effects of disrupting adherens junctions (AJs) during cochlear development. We used a conditional knockout mouse of the AJ protein p120-catenin (p120) to ablate p120-expresison in the inner ear (Davis & Reynolds, 2006). This protein is required to stabilize AJs; p120 binds to the intracellular domain of cadherins and stabilizes them at the cell membrane by preventing their internalization and eventual degradation (Davis & Reynolds, 2006; Reynolds, 2007). Knockdown of p120 leads to the reduction of the membrane level of cadherins in cultured cells and several tissues (Reynolds, 2007).

Our results show that knockout of p120-catenin in the cochlea causes defects in cochlear CE but not hair cell polarization. Thus, p120-catenin is required for cochlear extension but is dispensable for hair cell polarization. This finding effectively separates two processes regulated by vertebrate PCP in the cochlea, namely cochlear CE and hair cell polarization.

We hypothesized that the requirement for p120-catenin is carried out via its role in the stabilization of cadherins at the cell membrane. Given that disruption of vertebrate PCP signaling causes defects in CE, if the above hypothesis is correct, we expect abnormal cadherin expression in vertebrate PCP mutants. To test this, we examined cadherin expression in the cochlea of two vertebrate PCP mutants which have been previously characterized to have defects in cochlear CE. We used the Vangl2-*looptail* (Vangl2$^{Lp}$) mutant (a well characterized loss-of-function mutation of the vertebrate, core PCP protein Vang-like-2, (Montcouquiol, et al., 2003)) and a conditional knockout mouse for the ciliary gene IFT88/Polaris (Polaris$^{cko}$, (Haycraft et al., 2007)).During this analysis, we found that the membrane expression of N-cadherin and E-cadherin is indeed abnormal in these mice. Thus, our findings suggest that cochlear CE is mediated by a p120-catenin (and perhaps cadherin-dependent) mechanism downstream of core PCP and ciliary signaling.

## *Results*

### Conditional Knockout of p120-catenin leads to characteristic defects in Cochlear Extension but no defects in Coordinated Hair Cell polarization

In a project spearheaded by a former member of the lab, Renee, our lab characterized the effects of disrupting AJs in the cochlea (using a conditional knockout mouse for p120-catenin ($p120^{cko}$). Using the Cre-LoxP system we conditionally knocked out p120 (Davis & Reynolds, 2006) under the control of FoxG1-Cre, expressed in the inner ear during development (Sugiyama, Tsukiyama, Yamaguchi, & Yokoyama, 2011).

The initial characterization of $p120^{cko/cko}$ cochleae showed that, in addition to the expected knockout of p120 (Figure 3.1 A,B), membrane levels of E-cadherin are ablated (Figure 3.1 C,D)while membrane levels of N-cadherin are reduced in the cochlea of $p120^{cko/cko}$ mice. (Figure 3.1 E,F (Chacon-Heszele*, et al.)). Despite this significant reduction in E- and N-cadherin levels, the integrity of the cochlea was maintained in the $p120^{cko/cko}$ mice. Instead of a disruption in the overall integrity of the cochlea, the $p120^{cko/cko}$ mice display defects in cochlear CE (Figure 3.2). However, in contrast with core PCP and ciliary mutants (Figure 3.3) the $p120^{cko/cko}$ mice do not have a defect coordinated hair cell polarization (Figure 3.1). This initial observation of normal polarization in the $p120^{cko/cko}$ mice was later confirmed by quantification of the orientation of the V-shaped stereociliary bundles in the cochlea at e18 (Figure 3.1 I,J). This quantification was performed with images from the basal and middle regions of the cochlea

because, in most e18 cochlear samples, the hair cells in the apical region of the cochlea haven't matured to the point where V-shape of stereocilia is sufficiently defined to allow unequivocal determination of their orientation (Figure 2.2D). Overall, the orientation of stereocilia at e18 isn't significantly different between control and $p120^{cko/cko}$ mice ($p>0.05$ Chi-square analysis and Mardia-Watson Miller tests). Although the $p120^{cko/cko}$ samples have cochlear CE defects, the localization of core PCP protein Vangl2 is not disrupted in the $p120^{cko/cko}$ mice (Figure 3.1 G,H (Chacon-Heszele*, et al.)) suggesting that the CE defects observed in the $p120^{cko/cko}$ mice occur downstream of core PCP signaling.

Core PCP and Ciliary Mutants have abnormal expression patterns of AJ proteins

The findings from the $p120^{cko/cko}$ mice effectively separate two cochlear phenotypes consistently observed in core PCP and ciliary mutants, namely defects in cochlear CE and hair cell polarization (Figure 3.3). While p120-catenin might have multiple roles within the cell, it is likely that its requirement for cochlear CE is mediated by its role in cadherin stabilization on the plasma membrane. If this hypothesis is correct, then we predict abnormal cadherin expression in the plasma membrane of cells in the developing cochlea in vertebrate PCP mutants, which have defects in cochlear CE. To test this prediction, we analyzed the expression of E-cadherin and N-cadherin in the cochlea of a core PCP and a ciliary mutant. The core PCP mutant used was the *looptail* (Lp) mutation of core PCP protein Vangl2. This mutation has been extensively characterized as a loss-of-function allele of Vangl2 (Montcouquiol, et al., 2003). Homozygous $Vangl2^{Lp/Lp}$ mutants have shorter and wider cochlear

ducts and supernumerary hair cells in the organ of Corti (indicating a defect in cochlear CE) and also present with misoriented hair cells within the organ of Corti (Figure 3.3). We also used a conditional knockout mouse of the IFT protein Polaris (IFT88), which is required for the buildup and maintenance of primary cilia (Pazour et al., 2000). Our lab has previously shown that Polaris$^{cko/cko}$ mice present with defects in cochlear CE and defects in coordinated and intrinsic hair cell polarization (Jones, et al., 2008) (Figure 3.3).

The expression of N-cadherin is similarly disrupted Vangl2$^{Lp/Lp}$ and Polaris$^{cko/cko}$ mice. As described in Chapter2, in wild type mice, the expression of N-cadherin is limited to the medial region of the organ of Corti, (including the IHCs but not the OHCs) (Figure 3.4 A, D). In Vangl2$^{Lp/Lp}$ and in Polaris$^{cko/cko}$ mice, the expression of N-cadherin expands past its normal expression domain into the OHC region (Figure 3.4 B,C,E,F). These results support the idea that the regulation of N-cadherin expression to might play a role in the regulation of cochlear CE downstream of core PCP and ciliary signaling.

The results of the analysis of E-cadherin expression are not as straight forward as those from N-cadherin expression. As first characterized by Renee, in Vangl2$^{Lp/Lp}$ mice, E-cadherin expression is still limited the normal expression region within the cochlea (namely OHC region of the organ of Corti). However, in Vangl2$^{Lp/Lp}$ mutants, the levels of membrane E-cadherin expression are drastically reduced as compared to controls. This reduction is particularly noticeable within the cells localized immediately lateral of the OHCs (Figure 3.5, modified from (Chacon-Heszele*, et al.)). In contrast, in Polaris$^{cko/cko}$ mice, the

levels of membrane E-cadherin expression are not reduced in the OHC region (Figure 3.6). E-cadherin levels within this region might perhaps be higher in Polaris$^{cko/cko}$ cochlea than in controls. Although we have not quantified this by western blotting, during confocal imaging the settings (laser power) on the confocal microscope for the imaging of E-cadherin consistently have to be turned down as compared to littermate controls or the images are completely overexposed. It is of note that the levels of membrane E-cadherin were less uniform throughout the Polaris$^{cko/cko}$ samples, suggesting that the regulation of cadherin expression is also disrupted in these mice. Furthermore, in a few cases, the membrane expression levels of E-cadherin seemed reduced in the Hensen cells (the cell row found immediately lateral to the third row of Deiters' cells) (Figure 3.6). However, this last observation wasn't consistent among all samples; in some samples the membrane E-cadherin expression in the Hensen cells looked normal.

**Figure 3.1. Conditional Inactivation of p120-catenin leads to lower levels of membrane E- and N-cadherin but not to defects in core PCP Protein localization or Coordinated Cell Polarization**

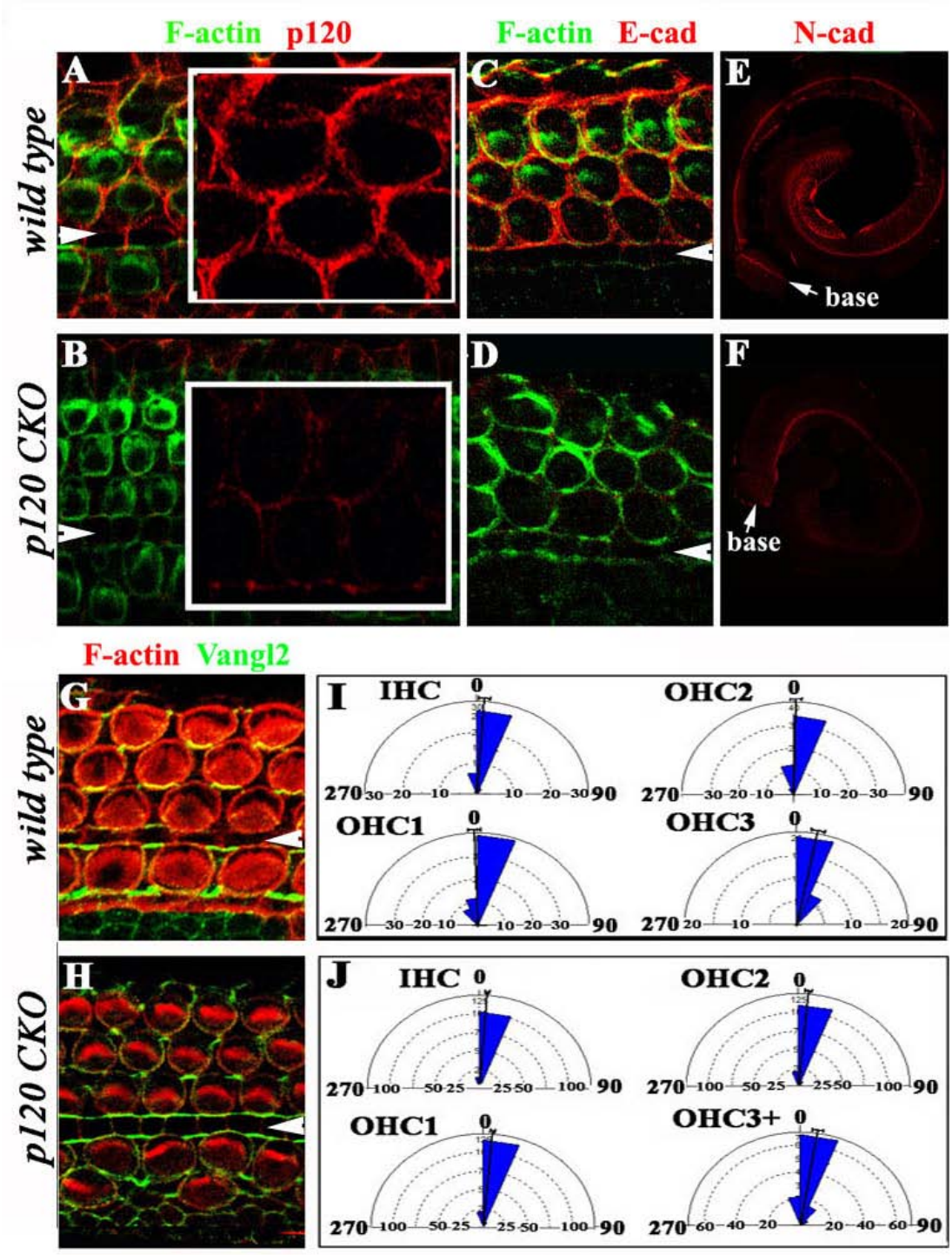


**Figure 3.1. Conditional Inactivation of p120-catenin leads to lower levels of membrane E- and N-cadherin but not to defects in core PCP protein localization or coordinated cell polarization**

p120-catenin protein expression is greatly reduced in $p120^{cko/cko}$ cochlea: The expression of p120-catenin (red) is greatly reduced in the $p120^{cko/cko}$ cochlea (B) as compared with littermate controls (A).

E-cadherin membrane expression is ablated in $p120^{cko/cko}$ cochlea: In p120-cko/cko cochlea (D) the membrane expression of E-cadherin (red) is ablated as compared with littermate controls (C).

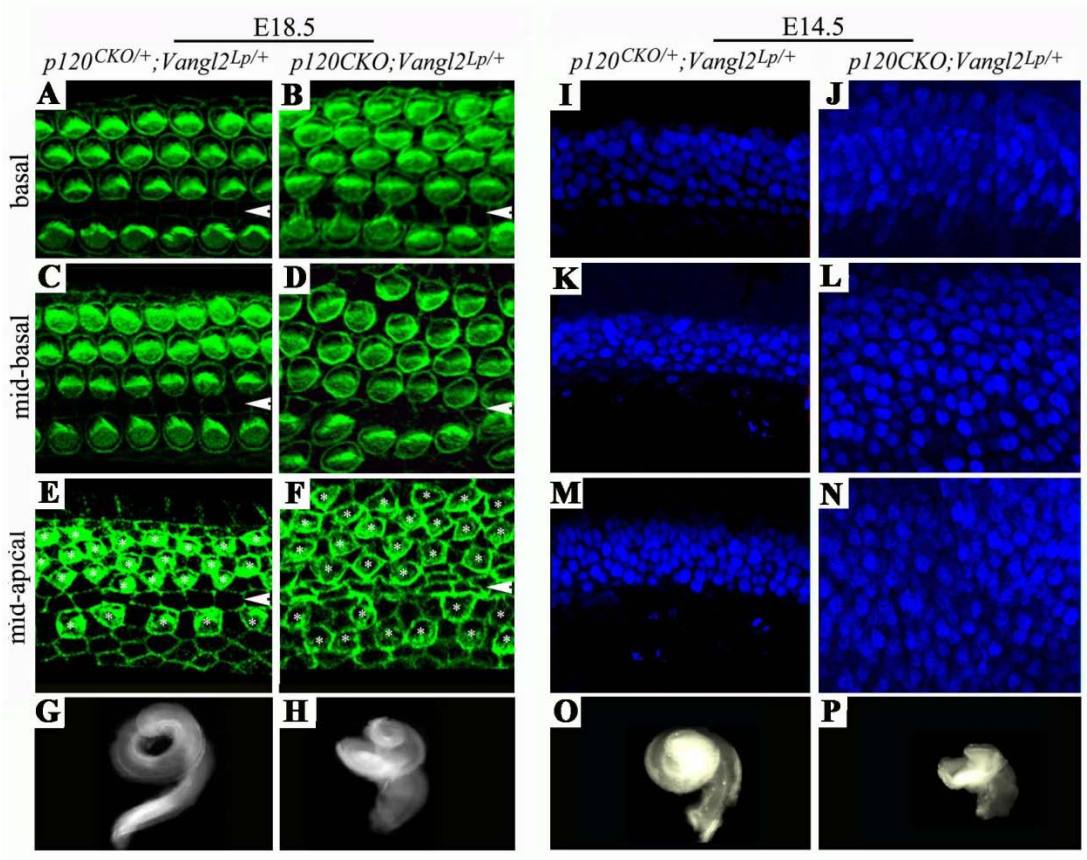
N-cadherin expression is reduced in $p120^{cko/cko}$ cochlea: Although not completely ablated and found within the normal region (medial) region of expression, the membrane levels of N-cadherin (red) are reduced in p120-cko/cko cochlea (F) as compared with littermate control (E).

Asymmetric distribution of core PCP protein Vangl2 is maintained in $p120^{cko/cko}$ cochlea: The asymmetric expression pattern of the core PCP protein Vangl2 (green) is maintained with the $p120^{cko/cko}$ cochlea (H) as compared with littermate controls (G)

The orientation of sensory hair cells along the PCP axis is normal in $p120^{cko/cko}$ cochlea: Using the orientation of the V-shaped stereociliary bundles (where 0 is alignment along the PCP axis pointing towards the lateral side of the cochlea) we quantified the overall orientation of stereociliary bundles in different HC rows in control (H) and $p120^{cko/cko}$ (I) cochlea at e18. We determined that the orientation of the stereocilia (and thus the coordinated polarization) remains normal in $p120^{cko/cko}$ cochlea. $p>0.05$ by Mardia-Watson-Wheeler tests and Chi-square analysis using Oriana3 software.

This figure was generated with data from Dr. Dongdong Ren.

**Figure 3.2. p120-catenin and core PCP Protein Vangl2 genetically interact in the regulation of Cochlear CE**

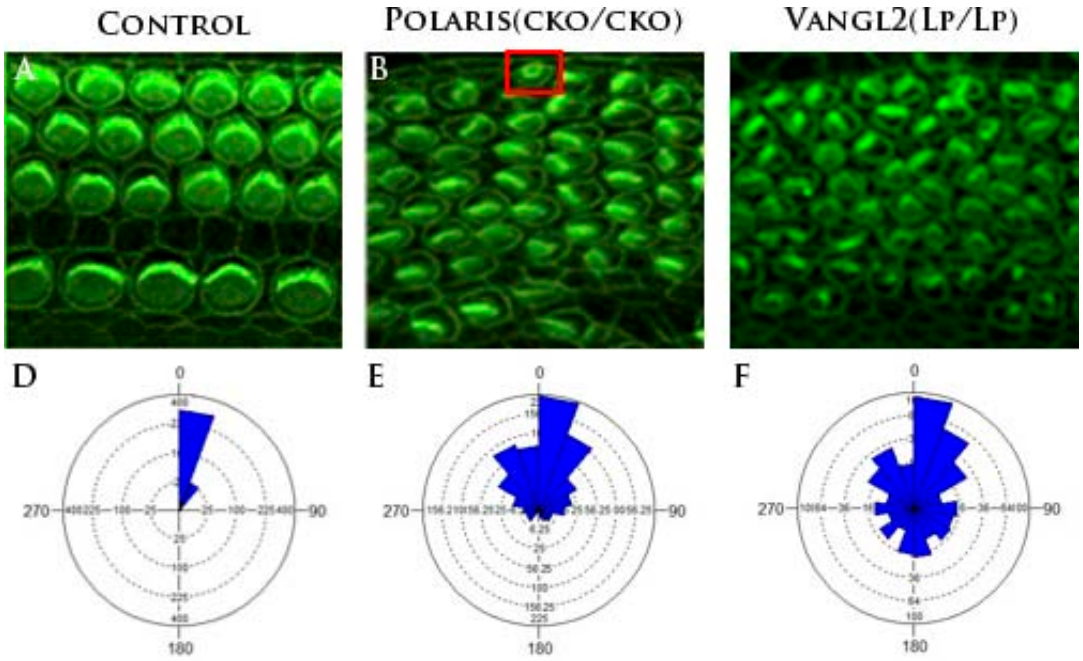



The sensory region is wider in $p120^{cko}$ mice: at e18 in 100% of $p120^{cko/cko}$;$Vangl2^{Lp/+}$ mice (B,D,F) and 50% of $p120^{cko/cko}$ mice (image not shown) the organ of Corti is wider and contains extra sensory hair cells (green) as compared to littermate controls (A,C,E). This defect can also be discerned at e14 by examining the pro-sensory domain using Sox2 (blue) where the developing OC is noticeable wider in 100% of the $p120^{cko/cko}$;$Vangl2^{Lp/+}$ mice (J,L.N) and 50% of $p120^{cko/cko}$ mice (image not shown) as compared with littermate controls (I,K,M).

Shorter cochlea in $p120^{cko/cko}$ mice: both at e14 (P) and at e18 (H) the cochlea is noticeable shorter in 100% $p120^{cko/cko}$;$Vangl2^{Lp/+}$ and 50% of the $p120^{cko/cko}$ mice as compared to littermate controls at e14 (O) and e18 (G).

This figure was generated with data from Dr. Dongdong Ren.

**Figure 3.3. Core PCP and Ciliary Mutants display defects in Cochlear CE and Coordinated Hair Cell Polarization**

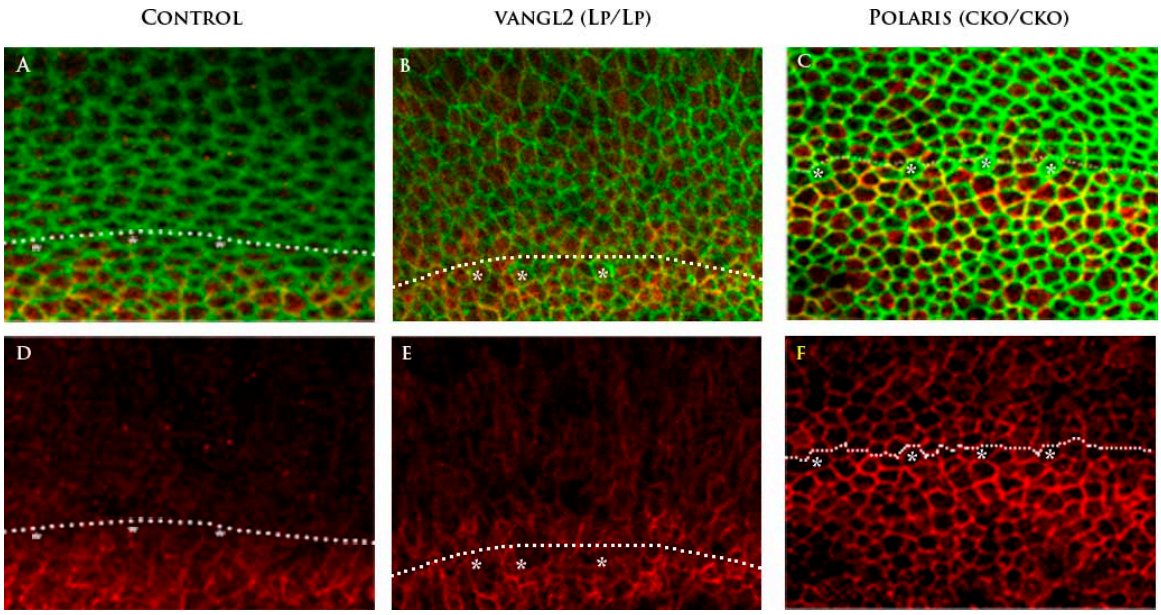



Confocal micrographs of the organ of Corti at e18: Control (A); homozygous Polaris$^{cko/cko}$ (B); and homozygous core PCP mutant Vangl2$^{Lp/Lp}$ (C) mice. F-actin is labeled with phalloidin (green).

Core PCP mutant Vangl2$^{Lp/Lp}$ and Polaris$^{cko/cko}$ mice have defects in coordinated orientation of sensory hair cells: Note the defects in coordinated hair cell polarization in both Polaris$^{cko/cko}$ and Vangl2$^{Lp/Lp}$ mice as visualized by the orientation of the V-shaped stereociliary bundles within each hair cell.

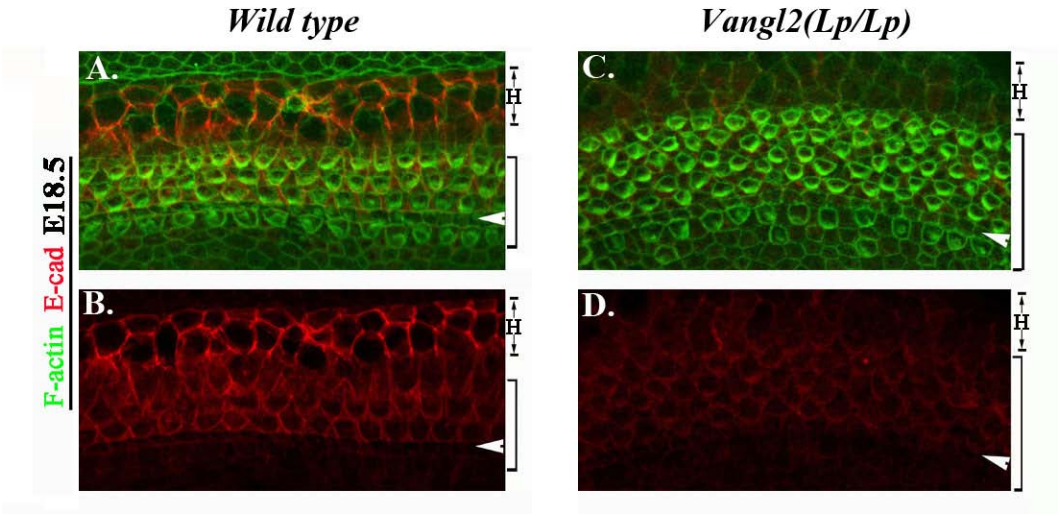
Hair cells in Polaris$^{cko/cko}$ cochlea also display defects in intrinsic polarization: As previously described (Jones, et al., 2008) in Polaris$^{cko/cko}$ mice, some cells lack planar polarization and display non-polar circular stereocilia on their apical surface (red box, B). Rose diagrams displaying quantification of the polarization defects in control and mutant mice are shown on D-F.

**Figure 3.4. N-cadherin Expression Domain is disrupted in Core PCP and Ciliary Mutants**




The expression domain of N-cadherin expands laterally in vertebrate PCP mutants: Control (A,D); core PCP mutant Vangl2$^{Lp/Lp}$ (B,E) and Polaris$^{cko/cko}$ (C,F) mice. Composite images with F-actin (phalloidin, green) and E-cadherin (red) labeling displayed (A-C). E-cadherin only displayed (D-F). White asterisks mark the nascent inner hair cells, noticeable by F-actin enrichment on their apical borders. The line marks the region between the IHC and the OHC regions of the cochlea. Note how membrane N-cadherin staining is absent above the white line in control mice (D) but is clearly present in Vangl2-Lp/Lp (E) and Polaris$^{cko/cko}$ cochlea (F).

**Figure 3.5. Membrane levels of E-cadherin are reduced in the Cochlea of Core PCP Mutant Vangl2$^{Lp/Lp}$**

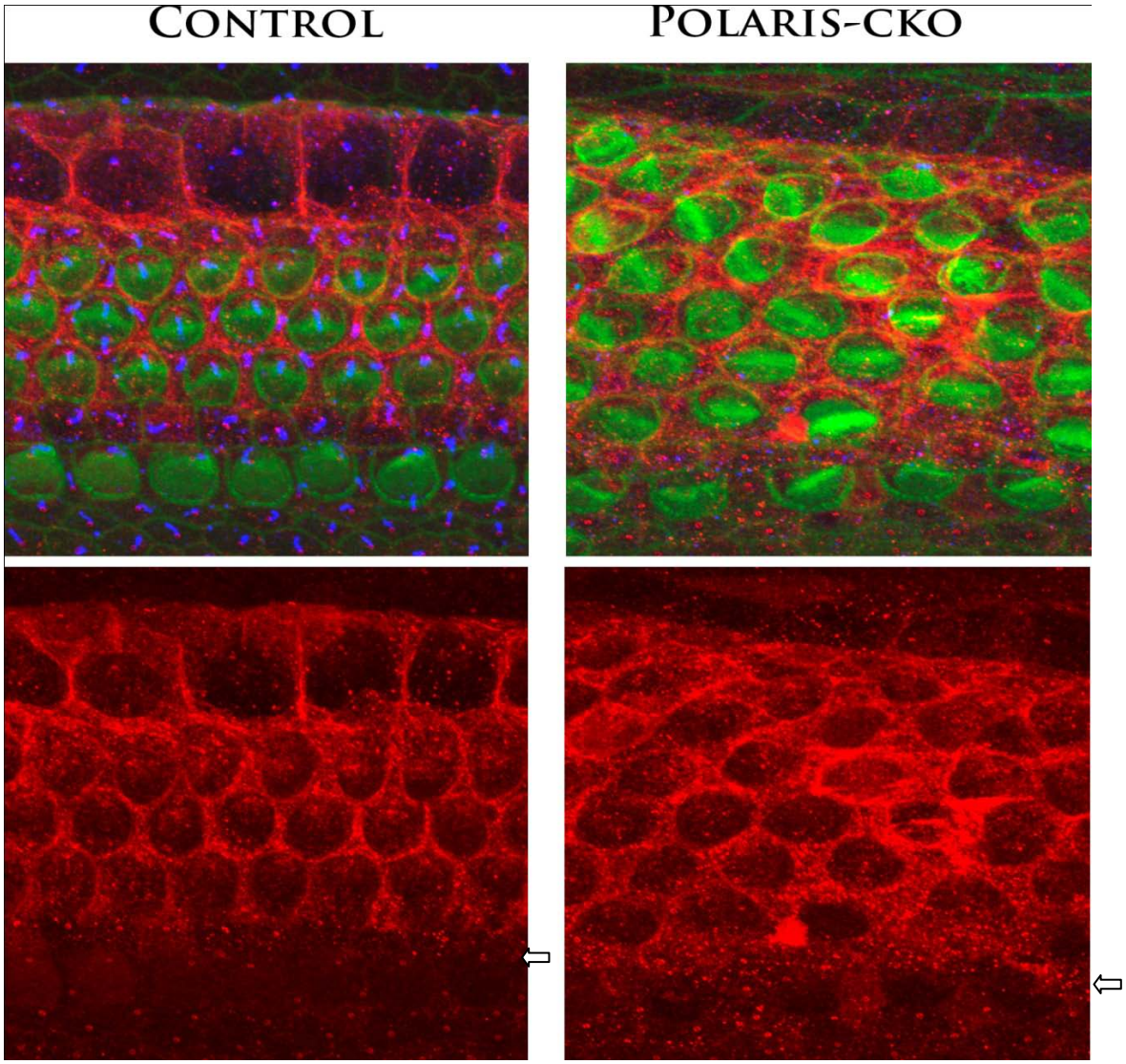



**A,B.** the expression of E-cadherin (red, A,B) is delimited to the outer hair cell region of the organ of Corti at e18 in wild type mice (brackets) including the Hensen cells where it is strongest (H).

**C,D.** In cochlea from core PCP mutant mice, Vangl2$^{Lp/Lp}$, the expression of E-cadherin (red, C,D) is greatly reduced and non-uniform. Additionally, the expression of E-cadherin in the Hensen cells (H) is gone in Vangl2$^{Lp/Lp}$ cochleae.

Data for this figure was obtained by Dr. Dongdong Ren.

**Figure 3.6. Membrane E-cadherin expression is maintained in Polaris$^{cko/cko}$ Cochlea**




Confocal micrographs of the organ of Corti at e18: Control (A,C) and Polaris$^{cko/cko}$ (B,D) mice. F-actin is labeled with phalloidin (green, A,B); the primary cilium is labeled with Aarl13b (A,B). In all images E-cadherin is labeled in red. The location of the inner pillar cells between the IHC and OHC regions are is indicated with the white arrows on the E-cadherin only panels (C,D).

***Discussion***

The PCP pathway was initially characterized in *Drosophila* with the identification of mutations that affected the coordinated polarization of hair bristles in the wing (Vinson & Adler, 1987). The core PCP genes encode a group of trans-membrane proteins and their immediate downstream effectors which regulate the coordinated polarization of cells along the plane of a tissue both in *Drosophila* and in vertebrates (Chacon-Heszele & Chen, 2009). However, studies in vertebrates discovered two key differences between *Drosophila* and vertebrate PCP: (1) vertebrate PCP has a requirement for ciliary genes in addition to core PCP genes and (2) additional processes regulated by PCP signaling in vertebrates as compared to *Drosophila.* Among these additional vertebrate PCP processes, is the regulation of CE (Axelrod & McNeill, 2002). Although CE also takes place in *Drosophila* (e.g. during germband extension), a requirement for PCP signaling in *Drosophila* has not been identified (Bertet & Lecuit, 2009; Rauzi, Verant, Lecuit, & Lenne, 2008).

Examples of vertebrate CE are found in mesenchymal cells (e.g. during gastrulation (Wallingford, et al., 2000)) and in epithelial cells (e.g. during neurulation (Keller, et al., 2000; Keller & Tibbetts, 1989)). However, the molecular mechanisms that regulate CE downstream of PCP signaling are not well understood. To start identifying these molecular mechanisms, we turned to the known molecular mechanisms for the regulation of CE in *Drosophila.* In a series of elegant studies it was determined that CE in *Drosophila* is regulated via the dynamic regulation of cell-cell contacts. More specifically, the polarized

localization of contractile proteins, like non-muscle myosin II (Fernandez-Gonzalez, et al., 2009) allows for the polarized contraction of cell borders along one axis. This process leads to the formation of characteristic rosettes, which consist of 5+ cells sharing a single vertex. These rosettes resolve via the polarized localization of cell junction proteins (such as E-cadherin) on the perpendicular axis. This highly dynamic process permits the rapid elongation of a tissue undergoing CE without disruption of tissue integrity (Bertet & Lecuit, 2009; Kim et al., 2010; Nikolaidou & Barrett, 2005; Rauzi, et al., 2008).

Our study in the mouse cochlea suggests that a similar mechanism is in charge of the regulation of CE in vertebrates downstream of PCP signaling. First, disruption of the AJ protein, p120-catenin, leads to characteristic defects in cochlear extension, without a defect in coordinated cell polarization (Figure 3.1) as is normally observed when vertebrate PCP signaling is disrupted (either through disruptions in core PCP or ciliary genes) (Figure 3.3). Although p120-catenin might have more than one role in the regulation of CE downstream of vertebrate PCP signaling, we postulated that the requirement for p120-catenin in cochlear CE stems from its role in the stabilization of cadherin expression at the cell membrane. If hypothesis is correct, we predicted that the expression of N- and E-cadherin would be disrupted when PCP signaling is disrupted.

Thus we analyzed the expression levels of N- and E-cadherin in PCP mutants. For this purpose, we used a loss-of-function allele of the core PCP protein Vangl2 (Vangl2$^{Lp/Lp}$, (Montcouquiol, et al., 2003)) and a conditional knockout mouse for the ciliary gene Polaris (IFT88) (Haycraft, et al., 2007).

These mice have defects in cochlear extension and in hair cell polarization (Figure 3.3). We found that the expression of N-cadherin was similarly disrupted in both Vangl2$^{Lp/Lp}$ and Polaris$^{cko/cko}$ cochlea. In these mice, N-cadherin expression expands from the medial region of organ of Corti (including the IHCs but not OHCs) into the outer hair cell region (Figure 3.4). This result is consistent with our hypothesis that the regulation cadherin expression would be disrupted when vertebrate PCP signaling is disrupted.

Our results with the analysis of E-cadherin expression suggest that the regulation of E-cadherin expression might be taking place via different mechanisms in Vangl2$^{Lp/Lp}$ vs. Polaris-$^{cko/cko}$ mice. In the Vangl2$^{Lp/Lp}$ mutant mice, the expression of E-cadherin is restricted to the OHC region of the cochlea, but the levels of membrane E-cadherin expression are noticeably reduced (Figure 3.5, (Chacon-Heszele*, et al.)). In the Polaris$^{cko/cko}$ cochlea, membrane E-cadherin expression is maintained in the OHC region (Figure 3.6) but the levels of E-cadherin expression seem much higher than in littermate controls. Although at first puzzling, these results are not inconsistent with our proposal for a role in the regulation of cell-cell contacts in the regulation of cochlear (and potentially vertebrate) CE. In *Drosophila* the regulation of CE via cell-cell contacts is a very dynamic process, requiring a balance and coordination between the localization of contractile proteins and cell adhesion proteins within groups of cells in tissues undergoing CE. Thus, one can understand how disruptions in the overall levels of any of these proteins (either contractile or adhesion proteins) can lead to a disruption of the dynamic cellular interaction that allow for CE to take place.

Consistent with this idea a recent study shows that the genetic or pharmacological disruption of non-muscle Myosin II in the cochlea leads to defects in cochlear CE (Yamamoto, et al., 2009). Another possibility is that E-cadherin does not play as important a role in the regulation of cochlear CE as the restricted localization of N-cadherin to the IHC region. If this is the case, the disruption in the region of expression of N-cadherin (and perhaps the disruption of differential adhesive forces within the developing cochlea) might be sufficient to cause defects in cochlear morphogenesis observed in Polaris$^{cko/cko}$ and Vangl2$^{Lp/Lp}$ mice.

# Chapter 4 Usher Complex proteins are required for Hair Cell Polarization but dispensable for Cochlear CE

## *Introduction*

As has previously been discussed at several points throughout this thesis, disruptions in vertebrate PCP signaling via mutations that affect either core PCP genes or ciliary genes cause defects in both cochlear CE and polarization of the sensory hair cells (Figure 3.3) (Chacon-Heszele & Chen, 2009). However, there are important differences in the polarity defects observed in ciliary mutants when compared to core PCP mutants.

In core PCP mutants, individual hair cells have *intrinsically* polarized stereociliary bundles on their apical surface. However, the coordinated orientation of the stereociliary bundles is disrupted. This loss of coordinated polarization is thought to arise from disruptions in the establishment of tissue polarity by the asymmetric distribution of core PCP proteins, which as previously mentioned is lost in core PCP mutants. In ciliary mutants display a loss of *intrinsic* cell polarization as observed by the fact that in some hair cells the kinocilium and basal body fail to migrate out of the center of the cell leading to the formation of apolar hair cells with centralized basal body/centrioles and circular stereociliary bundles around them. Additionally, in contrast with core PCP mutants, the asymmetric distribution of core PCP proteins is maintained in ciliary mutants (Guirao, et al., 2010; Jones, et al., 2008). These findings combined, suggests that the PCP defects observed in ciliary mutants arise from a disruption

in *intrinsic cell polarization* and not from a disruption in the establishment of tissue polarity signals by core PCP proteins. Additionally, the loss of intrinsic cell polarization observed in ciliary mutants in combination with the observation that the position of the kinocilium marks the orientation of the V-shape stereociliary bundles suggests that communication between the kinocilium/basal body and the nascent stereocilia plays a important role in the formation of the polarized stereociliary bundles and is important for intrinsic hair cell polarization.

One hypothesis as to how the kinocilium directs the orientation of the stereociliary bundles is via the physical links found between the kinocilium and stereocilia. Another hypothesis to explain this phenomenon suggests that signals from the basal body can affect the actin-rich structure on the apical surface of the hair cells (the cuticular plate, Figure 1.3 C) and thus direct the formation of polarized stereocilia from the "ground" up. A comprehensive understanding of the molecular mechanisms that direct the buildup of polarized stereociliary bundles would be immensely helpful to understand how the kinocilium/basal body direct hair cell polarization and rule out or support the hypotheses above. Although the mechanisms that regulate the formation of stereociliary bundles are not completely understood, studies of the Usher syndrome have shed light into part of the molecular machinery responsible for stereociliary bundle buildup and maintenance.

The Usher syndrome is the most common genetic disorder that leads to human deafness (Petit, 2001). Different Usher proteins have been identified due to mutations causing different forms of the Usher syndrome, which in humans

can cause deafness, blindness and vestibular dysfunction (Adato, et al., 2002; Alagramam, Yuan, et al., 2001; Z. Y. Chen et al., 1996; Di Palma et al., 2001; el-Amraoui et al., 1996; Gibert et al., 2005; Schwartz et al., 2005; Siemens et al., 2002; van Wijk et al., 2006; Weil, et al., 1995; Weil, et al., 2003; Weil et al., 1996; Zwaenepoel et al., 2001). The Usher proteins are components of a complex protein network localized in the apical surface of hair cells that is *required* for the proper buildup and maintenance of the stereociliary bundles [reviewed in (Chacon-Heszele, et al., 2011; Reiners, et al., 2006)].

Although all Usher proteins identified to date are apically localized within the hair cells, they vary greatly in their molecular structure and function. Some of the known functions of different Usher proteins include, cross-linking of actin filaments within individual stereocilia (Y. Kikkawa et al., 2005; Li et al., 2004; Volkmann, DeRosier, Matsudaira, & Hanein, 2001); regulating the transport of different proteins into the stereocilia (Senften et al., 2006); mediating physical linkages between the stereocilia and between the kinocilium and the closest stereocilia (Adato, Lefevre, et al., 2005; Ahmed et al., 2006; Kazmierczak, et al., 2007; Michel et al., 2005); and acting as molecular scaffolds that link the actin cytoskeleton with other proteins (including other Usher proteins) (Y. Kikkawa et al., 2003; Reiners, Marker, Jurgens, Reidel, & Wolfrum, 2005; J. Yan, Pan, Chen, Wu, & Zhang, 2010). Given the evidence that Usher proteins are responsible for the formation of stereociliary bundles and that some Usher proteins connect the kinocilium to adjacent stereocilia, we proposed that a careful characterization of different Usher complex mutants might shed light into the question of how the

position of the kinocilium/basal body within the apical surface of the hair cells regulates the formation of ***polarized*** stereociliary bundles. In order to start addressing this question, we decided to examine mutants of two different Usher proteins, Protocadherin-15 and Sans.

Protocadherin-15

As its name suggests, the Usher protein protocadherin-15 (Pcdh-15) is a member of the cadherin superfamily of proteins. Pcdh-15 (along with another Usher protein, Cahderin-23) is a component kinociliary links (between the kinocilium and stereocilia), as well as a component of the tip links and the transient lateral links that are formed between stereocilia (Ahmed, et al., 2006; Ahmed, et al., 2001; Kazmierczak, et al., 2007; Sakaguchi, Tokita, Muller, & Kachar, 2009) (illustrated in Figure 4.4.1 and Figure 4.4.2). Interestingly, it has been previously shown that the distribution of Pcdh-15 and Cadherin-23 in the aforementioned links is polarized along the PCP axis (Kazmierczak, et al., 2007; Sakaguchi, et al., 2009). These findings suggest a role for Pcdh15 in the regulation of the buildup and polarization of the stereociliary bundles.

Pcdh-15 protein has 11 extracellular cadherin repeats (EC), a transmembrane domain, and a unique cytoplasmic domain (Ahmed, et al., 2001) (Figure 4.4.2). Different isoforms of Pcdh-15 have distinctive spatiotemporal distributions within the developing and maturing hair cells and might play different roles in the formation of the stereociliary bundles (e.g. by differential regulation of the formation of tip links vs. kinociliary links) (Ahmed, et al., 2006; Muller, 2008;

D. Yan, Kamiya, Ouyang, & Liu, 2010) (illustrated on Figure 4.4.1). A spontaneous mutation in Pcdh15 (av-*3j)* arose within the *Ames waltzer* mice*,* which are deaf and have vestibular impairment (Alagramam, Murcia, et al., 2001). Pcdh15(av-3j) ($Pcdh15^{3J}$) is a functionally-null mutation of Pcdh-15 which causes an early termination of the protein before its transmembrane domain (Figure 4.4.2). $Pcdh15^{3J/3J}$ mice have been previously described to have a misshapen stereociliary bundles and also present with disruptions in the organization of the cuticular plate (Figure 4.4.3) .

Using the $Pcdh15^{3J/3J}$ mouse model, we will present evidence that functional Pcdh15 is required for the regulation of coordinated and *intrinsic* hair cell polarization independently of CE. We also found that Pcdh15 and the ciliary gene Polaris/IFT88 genetically interact in the formation of polarized stereociliary bundles. Furthermore, consistently with the hypothesis of a role for the regulation of cell-cell contacts in the regulation of cochlear CE (but not hair cell polarization) the expression of N- and E-cadherin is normal in Pcdh15 mutant mice.

Sans

The Usher protein Sans (Scaffold Protein Containing Ankyrin repeats and SAM domain), is a localized at the apical surface of the hair cells and is highly enriched around the basal body (illustrated in Figure 4.4.1). As its name indicates, Sans is a scaffolding protein that contains a number of different protein motifs or domains, including a N-terminal domain with three ankyrin repeats; a central domain; and C-terminal SAM (sterile alpha motif) and PDZ domains

(diagram on Figure 4.4.2) (Weil, et al., 2003). It has been previously determined that Sans binds other Usher proteins within sensory hair cells (Adato, Michel, et al., 2005; Y. Kikkawa, et al., 2003; Weil, et al., 2003). Since there's no evidence of Sans localization within the stereocilia, Sans is thought to be required for regulation of the proper localization of other Usher proteins within developing hair cells (Adato, Michel, et al., 2005; Y. Kikkawa, et al., 2003; Maerker et al., 2008; Weil, et al., 2003; D. Yan, et al., 2010; J. Yan, et al., 2010).

The C-terminal SAM and PDZ domain of Sans are thought to be of particular importance for the interactions between Sans and several other Usher proteins (El-Amraoui & Petit, 2005; Kalay et al., 2005; J. Yan, et al., 2010). The *Jackson shaker* (*JS*) mouse model has a spontaneous, frame-shift mutation in Sans that leads to the truncation of the protein before the SAM motif (illustrated in Figure 4.4.2) (Y. Kikkawa, et al., 2003). These mice ($Sans^{JS/JS}$) are deaf, have vestibular dysfunction and, consistent with the deafness phenotype, have disorganized stereocilia in the cochlear hair cells (Y. Kikkawa, et al., 2003; Lefevre, et al., 2008).

Using the $Sans^{JS/JS}$ mice, we will present evidence of a requirement for full length-Sans protein for the coordinated but not intrinsic polarization of hair cells, independently of cochlear CE.

**Figure 4.1. Diagram of the localization of the Usher Proteins in a Cochlear Hair Cell**

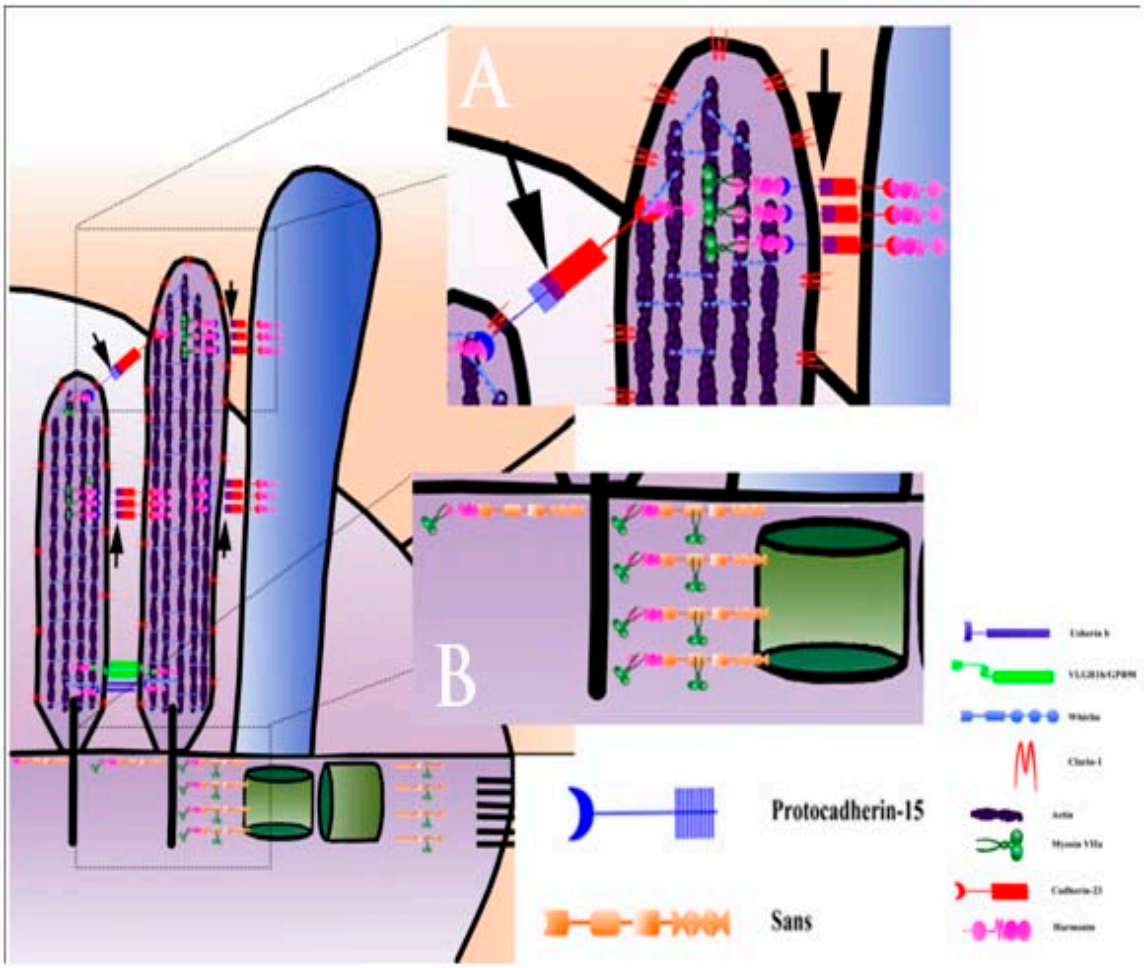


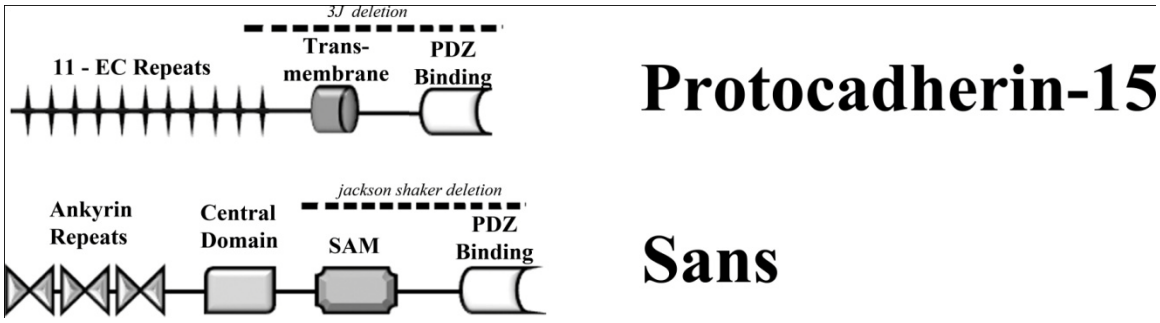
**Figure 4.1. Diagram of the localization of the Usher proteins in a cochlear hair cell**

This figure, adapted from (Chacon-Heszele, et al., 2011), illustrates the known sub-cellular localization of the different Usher proteins in a single sensory hair cell at the end of embryonic development (~e18-p0). The kinocilium (blue); stereocilia (light purple; with actin filaments in dark purple); the centrioles (green) are shown. As the reader can appreciate, members of the Usher complex are localized throughout the apical surface of the sensory hair cell. Usher proteins are found in all types of linkages between the stereocilia and between the kinocilium and stereocilia; within each stereocilia; and on the actin-rich apical surface of the sensory hair cells. Of particular interest is to this work is the localization of the Usher proteins Protocadherin-15 (Pcdh15) and Sans.

Pcdh15 (depicted in bright blue) is a trans-membrane protein whose localization at different links between the stereocilia and between the kinocilium and stereocilia has been extensively characterized (arrows, Box A); reviewed in (Chacon-Heszele, et al., 2011; Muller, 2008).

Sans (depicted in orange) is a PDZ rich protein, localized at the apical surface of the sensory hair cells and highly enriched around the basal body (Box B). Sans acts as a scaffolding protein; its interaction with other members of the Usher complex is thought to play an important role in their localization within hair cells (Adato, Michel, et al., 2005; Weil, et al., 2003; J. Yan, et al., 2010).

**Figure 4.2. Schematic Diagram of Pcdh15 and Sans Protein Motifs including the localization of pertinent mutations**




Pcdh15 contains 11 extracellular cadherin repeats followed by one trans-membrane domain and one PDZ-binding domain. In the A*mes Waltzer* mice, the "av-3J" mutation (used in this study) leads to an early termination codon that causes the deletion of the region indicated by the dotted line (Alagramam, Murcia, et al., 2001).

Sans contains several N-terminal ankyrin repeats, a central domain, a SAM (Sterile alpha motif) domain and a C-terminal PCZ domain. The *Jackson Shaker* "JS" mutation used in this manuscript leads to the deletion of SAM and PDZ binding domains (Y. Kikkawa, et al., 2003).

This figure was modified from (Chacon-Heszele, et al., 2011).

***Results***

The work presented in Chapters 2 and 3 of this manuscript provided evidence that suggests that (1) cochlear CE and coordinated hair cell polarization are regulated *independently* downstream of vertebrate PCP signaling and (2) that the regulation of cell-cell contacts plays a role in the regulation of cochlear extension but not hair cell polarization. These findings, however, leave open the question of what are the molecular mechanisms that control hair cell polarization downstream of core PCP and ciliary genes.

It stands to reason that, in order for hair cells to be able to build ***polarized*** stereociliary bundles on their apical surface, directional information (i.e. polarity cues from core PCP and/or ciliary genes) must be transmitted to the molecular machinery that physically builds up these polarized stereocilia. A multitude of cell biological and biochemical studies have determined that the Usher proteins are important components of this apical machinery in charge of stereociliary buildup (Lefevre, et al., 2008). However, the mechanisms through which polarity cues are interpreted and carried out by the hair cells during the buildup of stereociliary bundles are not fully understood. It is generally accepted that the position of the kinocilium/basal body within the apical surface of sensory hair cells plays an important role in the buildup of polarized stereociliary bundles, an idea that has been supported by defects in stereociliary bundle morphology present in ciliary mutants (Jones, et al., 2008; Ross et al., 2005).

The physical links between the kinocilium and the stereocilia, which are mediated by several Usher proteins, are required for the buildup and orientation

of polarized stereocilia (Lefevre, et al., 2008). However, it is also possible that signals from the basal body within the apical surface of the hair cells directly affect the buildup and/or orientation of the nascent stereociliary bundles from the "ground" up.

In this manuscript we describe that in addition to previously described defects in stereociliary bundle structure and polarization, mutations in two different Usher proteins (Pcdh15 and Sans) affect the localization of the kinocilium within the apical surface of sensory hair cells. The main known role of Pcdh15 involves the formation of kinociliary and stereociliary links (Kazmierczak, et al., 2007; Senften, et al., 2006). Sans is an apically localized scaffolding protein, enriched near the basal body in some cells (Overlack, Maerker, Latz, Nagel-Wolfrum, & Wolfrum, 2008; D. Yan, et al., 2010).

Defects in the Usher Protein Pcdh-15 lead to defects in Coordinated Cell Polarization but not to defects in Cochlear Extension

$Pcdh15^{3J/3J}$ mice have a point mutation that generates functionally null Pcdh15 due to an early termination codon before the transmembrane domain of the protein (Figure 4.4.2) (Alagramam, Murcia, et al., 2001). Mice homozygous for this mutation are deaf and have misshapen/disorganized stereociliary bundles in the cochlea (Alagramam, Murcia, et al., 2001). We show that in addition to having defects in the shape of the stereociliary bundles $Pcdh15^{3J/3J}$ mutants have defects in intrinsic hair cell polarization independently of cochlear CE.

The cochlea of Pcdh15$^{3J/3J}$ mutants are of normal length (Figure 4.4.3B) and the arrangement of three OHC rows and one IHC row is maintained throughout the entire tissue. To determine the polarity of sensory hair cells in the absence of a well organized, V-shaped stereociliary bundle as is the case in the cochlea of Pcdh15$^{3J/3J}$ mice we can use the position of the kinocilium or the position of the fonticulus as markers of hair cell polarity. The fonticulus is area void of actin within the apical surface of sensory hair cells where the centrioles are found. In Pcdh15$^{3J/3J}$ mice because the fonticulus is enlarged and more round that in wild type cochlea (Y. S. Kikkawa, Pawlowski, Wright, & Alagramam, 2008) which makes it a great marker for the orientation of sensory hair cells even when the shape of the stereocilia is disrupted.

As shown in Figure 4.4.3D, in these mice the kinocilium is misoriented within the apical surface of sensory hair cells. Interestingly, in many cases, the kinocilium and basal body fail to migrate out of the center of the cell (yellow arrows). This finding suggests that functional Pcdh15 is required for sensory hair cell polarization. In order to determine whether these polarity defects could be somehow be caused by a defect in the localization of core PCP proteins, we examined the localization of the core PCP protein Frizzled-3 (Y. Wang, et al., 2006). We found that asymmetric core PCP protein localization is maintained in Pcdh15$^{3J/3J}$ mutants (Figure 4.4.3F), suggesting that defects in cell polarity take place downstream of or parallel to core PCP protein asymmetric localization. Taken together, these results show that Pchd15 is required for hair cell polarization but is dispensable for cochlear extension.

Normal expression patterns of E- and N- cadherin in the Cochlea of Pcdh15 mutant mice

The finding that Pcdh15 is required for hair cell polarization but dispensable for CE provided us with another tool to test whether defects in cochlear CE, *but not hair cell polarization*, correlate with abnormal expression of cell-adhesion proteins. If this is the case, in contrast with the Vangl2$^{Lp/Lp}$ and Polaris $^{cko/cko}$ mice, in Pcdh15$^{3J/3J}$ cochlea which have defects in hair cell polarization but not cochlear CE we expected to observe normal expression of N-cadherin and E-cadherin. As shown on Figure, the expression of both N-cadherin and E-cadherin are normal in Pcdh15$^{3J/3J}$ cochlea. This finding supports the idea proposed in Chapter 2 and Chapter 3 cochlear CE *but not hair cell polarization* is regulated via a mechanism that involves the regulation of cell-cell contacts.

Pcdh15 genetically interacts with the ciliary gene Polaris in the formation of stereocilia

Given that the defect in intrinsic hair cell polarization found in Pcdh15$^{3J/3J}$ mice is reminiscent of the intrinsic polarization defect found in Polaris$^{cko/cko}$ mice, we decided to test whether there is a genetic interaction between Pcdh15 and Polaris in the regulation of hair cell polarization in general and intrinsic hair cell polarization in particular. If this is the case, we expected to observe an enhancement of the phenotype observed in these mutants with the introduction of a mutant allele of the other gene. However, in spite of extensive quantification of different combined mutants (including double heterozygous, homozygous

mutants for either gene carrying a single mutant allele of the other gene, and double homozygous mutants) we did not find a significant enhancement of the hair cell polarization defect present in these mice. Neither the overall distribution of the hair cell orientation of the combined mutants nor the number of cells that have a loss of intrinsic polarization suggested a genetic interaction between these proteins in their regulation of hair cell polarity (data not shown).

However, in the course of this study we did find evidence of a genetic interaction between Polaris and Pcdh15.We found that the addition of a single $Pcdh15^{3J}$ allele into either $Polaris^{cko/+}$ (data not shown) or $Polaris^{cko/cko}$ mice led to a noticeable disruption in the organization of stereocilia (Figure 4.4.5), particularly within the IHC row. Although we analyzed the reverse mutants ($Pcdh15^{3J/3J}$ carrying one or two $Polaris^{cko}$ alleles) the strong defect in the shape of stereocilia already present in $Pcdh15^{3J/3J}$ mice made it impossible for use to determine if this interaction is the same in both sets of mutants. Regardless, this observation suggests that the kinocilium (or, IFT) play a role in the construction of stereociliary bundles along with Usher proteins (Pcdh15).

Mutation in Usher Protein Sans leads to *subtle* defects in Hair Cell Polarization and defects in apical actin organization but no defects in Cochlear CE

Thus far, our data using the Usher mutant Pcdh15$^{3J/3J}$ shows that hair cell polarization is regulated in a Pcdh15-dependent manner independently of cochlear CE. Furthermore, the defect in intrinsic hair cell polarization found in the Pcdh15$^{3J/3J}$ mice suggests that, in addition to having a role in the construction of stereociliary bundles, at least one member of the Usher complex also regulates intrinsic hair cell polarization downstream of core PCP signaling.

The migration of the basal body/kinocilium from the center of the cell (which fails in Pcdh15$^{3J/3J}$ and ciliary mutants) is regarded as an important part of the process that regulates hair cell polarization. We hypothesized that another Usher protein, Sans, might play a role in the regulation of this migration process. If so, in the functionally null mutants for Sans$^{JS/JS}$ (Figure 4.4.2, Figure 4.4.6) we expected to find defects in hair cell polarization similar to those observed in Pcdh15$^{3J/3J}$ and Polaris$^{cko/cko}$ cochlea.

In addition to previously described defects in stereociliary bundle structure (Y. Kikkawa, et al., 2003) at first glance it looks like Sans$^{JS/JS}$ mice have also have defects in intrinsic hair cell polarization. Some hair cells display a spot in the center of the apical surface of the cell that looks like a centralized fonticulus (red box - Figure 4.4.6 D). However, close examination of the apical surface of the cells showed that these regions are have lower levels but are not devoid of actin and that centrioles are not localized to these lower actin regions (Figure

4.4.6). This observation suggests that Sans$^{JS/JS}$ hair cells have defects in the organization of the actin-rich cuticular plate but might not have a defect in intrinsic hair cell polarization.

Given that in Sans$^{JS/JS}$ mutants both the shape of the stereociliary bundles and the organization of the apical actin network are disrupted, we could only use the localization of the kinocilium and centrioles to determine whether Sans$^{JS/JS}$ mice have defects in intrinsic hair cell polarization. Careful analysis of the localization of the kinocilium in Sans$^{JS/JS}$ hair cells at the end of embryonic development showed that there is a subtle defect in coordinated hair cell polarization but not in intrinsic hair cell polarization in these mice.

As described in Chapter 2, in wild type mice the migration of the kinocilium from the center to the lateral side of the cell takes place within ~60 degrees on either direction from the PCP axis (arrowheads - Figure 4.4.6C). By e18 in the basal and middle region of the cochlea, in the majority of hair cells the kinocilium (and thus the vertex of the stereociliary bundle) is aligned towards the PCP axis (Figure 4.4.6E). In Sans$^{JS/JS}$ mutants this trend is disrupted. Although localized towards the lateral side of the cell (arrowheads, Figure 4.4.6D) in the majority of hair cells the kinocilium fails to align with the PCP axis (Figure 4.4.6E). It should be noted that just like in Pcdh15$^{3J/3J}$ mice, both the cochlear length and the organization of the hair cell rows are normal in Sans$^{JS/JS}$ mice.

**Figure 4.3. Pchd15 mutant mice have defects in PCP due to defects in *Intrinsic Hair Cell Polarization* downstream of Core PCP Protein asymmetric localization and independently of Cochlear CE**

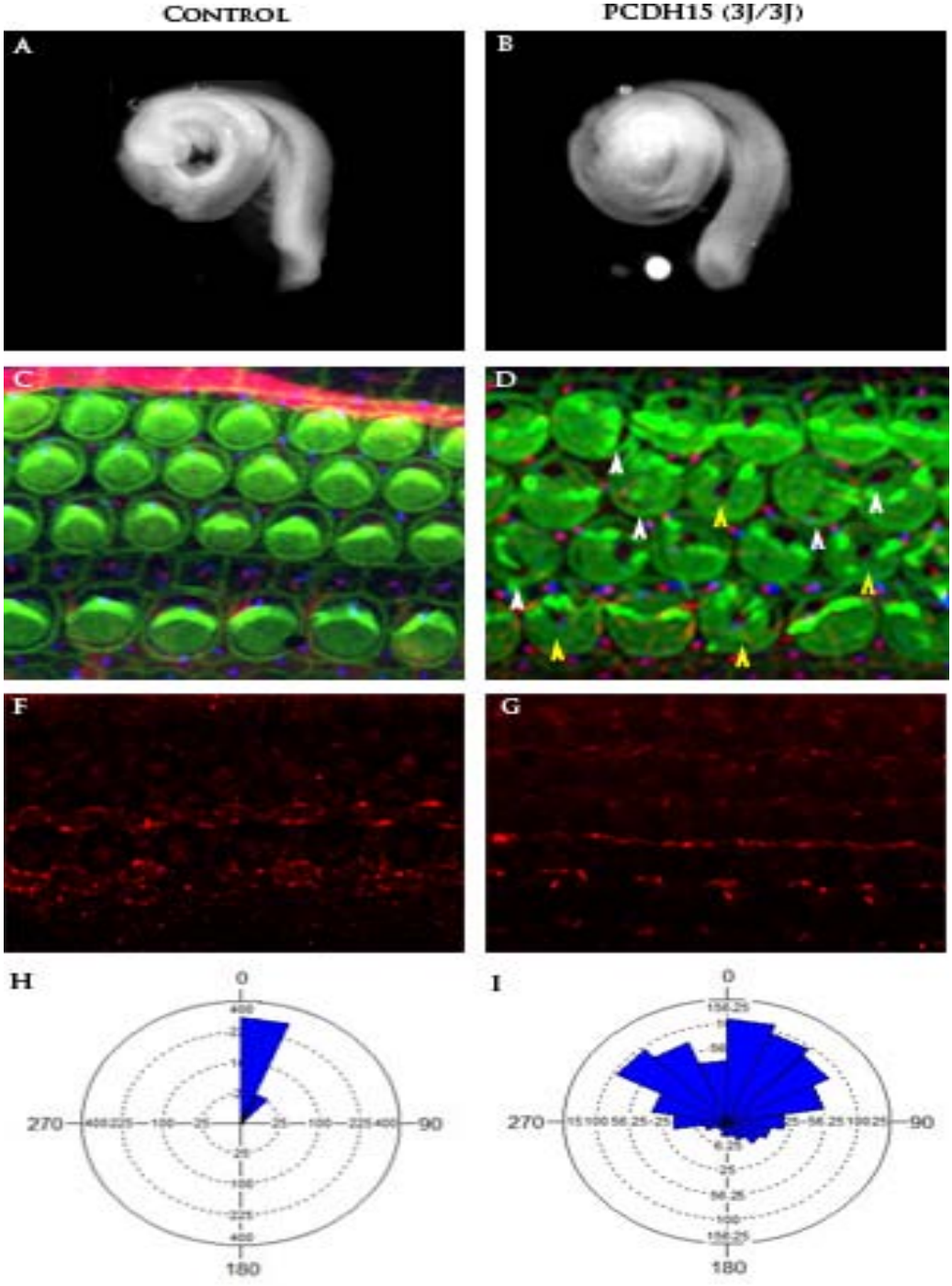


**Figure 4.3. Pchd15 mutant mice have defects PCP due to defects *in intrinsic hair cell* polarization downstream of core PCP protein asymmetric localization and independently of cochlear** CE

***Whole mouse cochleae imaged at e18*.** Wild type (A); Pcdh15-3j (B). Note that Pcdh15 and control cochleae have the same size and number of turns of the spiral.

***Defects in hair cell polarization in Pcdh15$^{3J/3J}$ mice.*** Actin is labeled with phalloidin (green), the centrioles are labeled with gamma tubulin (red), and the kinocilium is labeled with Arl13b (blue). In addition to the defects in stereociliary bundle shape present in the Pcdh15-3j hair cells, these mice have defects cell polarization (white arrows marking the position of the kinocilium). Interestingly, many hair cells have a centrally localized basal body/kinocilium and thus have defects in *intrinsic* cell polarization (yellow arrows). Note that in spite of the polarity defect, the normal organization of the hair cells rows (3OHCs + 1IHC) is maintained (throughout the entire cochlea, not just some regions). Quantification of hair cell orientation is shown for wild type (H) and Pcdh15$^{3J/3J}$ mice (I).

***Normal core PCP protein localization in* Pcdh15$^{3J/3J}$ *cochlea.*** The asymmetric localization of core PCP protein Frizzled 3 (red) is present in wild type (F) and Pcdh15$^{3J/3J}$ mice (G). This result suggests that the hair cell polarization defects observed in Pcdh15$^{3J/3J}$ mice take place downstream of core PCP signaling.

**Figure 4.4. Normal E- and N-cadherin expression pattern in the Cochlea of Pcdh15 mutant mice**

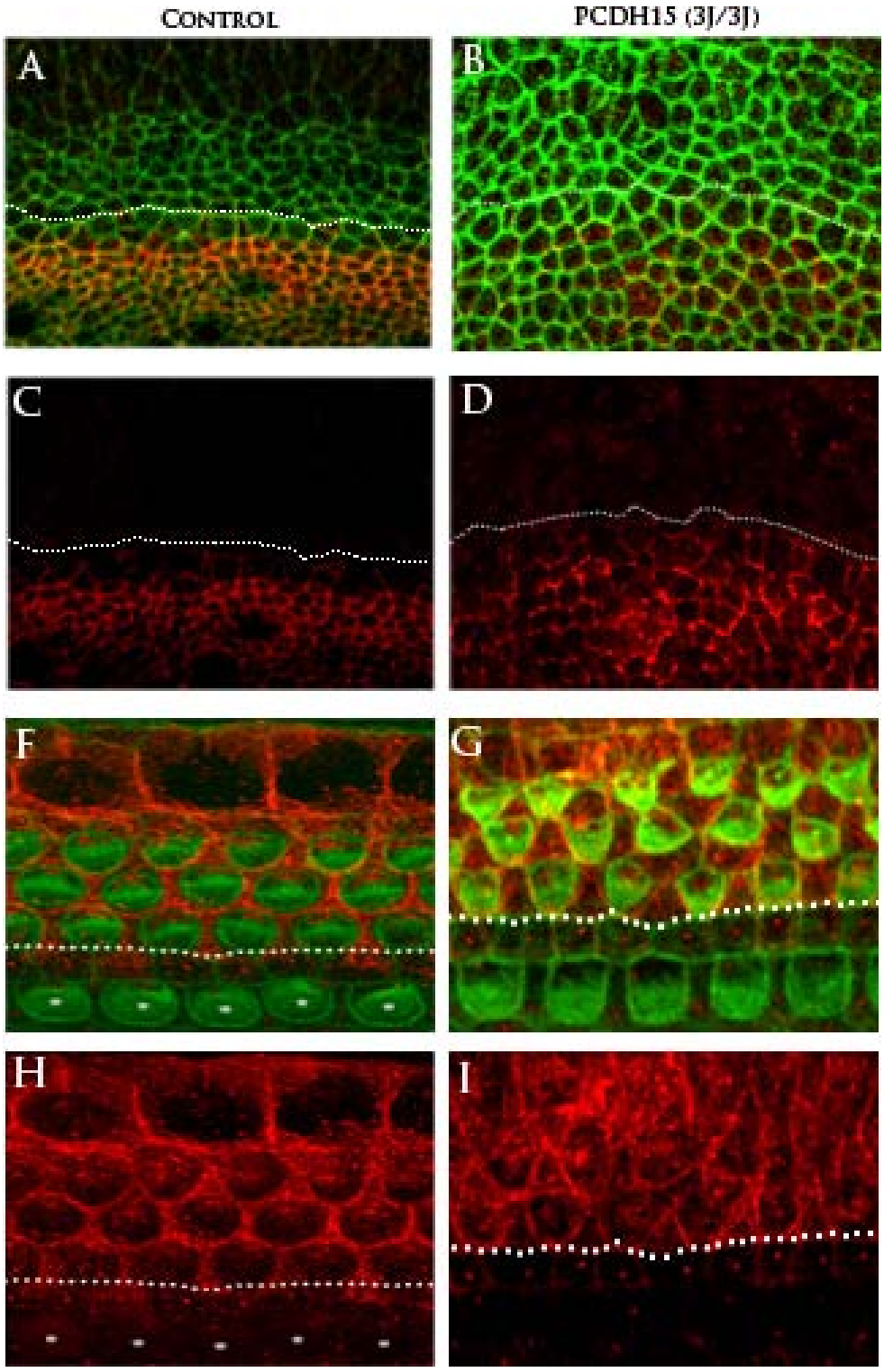
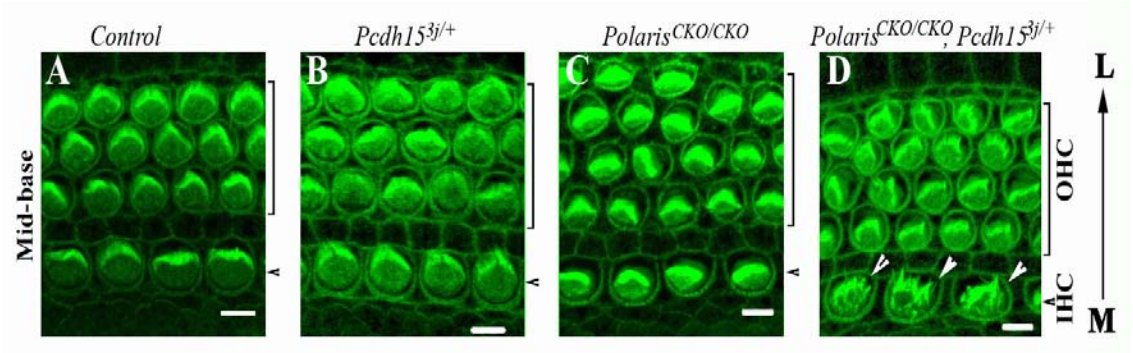


**Figure 4.4. *Normal membrane N-cadherin expression in* Pcdh15$^{3J/3J}$ *cochlea*.**

Phalloidin (green); N-cadherin (red). The membrane expression of N-cadherin in the organ of Corti at e14 is restricted to the IHC region in wild type (A,C) and Pcdh15$^{3J/3J}$ (B,D) cochlea.

***Normal membrane E-cadherin expression in* Pcdh15$^{3J/3J}$ *cochlea*.** Phalloidin (green); E-cadherin (red). The membrane expression of E-cadherin in the organ of Corti at e18 is restricted to the OHC region in wild type (F,H) and Pcdh15$^{3J/3J}$ (G,I) cochlea.

**Figure 4.5. Pcdh15 Genetically interacts with Polaris in the formation of Stereociliary Bundles**




Confocal micrographs of the organ of Corti at e18. F-actin is labeled with phalloidin (green). Wild type(A); $Pcdh15^{3J/+}$ (B); $Polaris^{cko/cko}$ (C); and $Polaris^{cko/cko}$, $Pcdh15^{3J/+}$ (D).

In spite of the hair cell polarization defects observed in $Polaris^{cko/cko}$ cochlea, the V-shape of the stereociliary bundles is maintained in most $Polaris^{cko/cko}$ hair cells (C). In $Pcdh15^{3J/+}$ cochlea, the structure/organization of the hair bundles is also normal (B).

The addition of a single $Pcdh15^{3J}$ mutant allele to $Polaris^{cko/cko}$ mice causes a disruption in the shape of the stereociliary bundles; the cohesiveness of the bundles is disrupted and extra stereocilia are present throughout the apical surface of the hair cells, particularly in the IHCs (white arrows; D).

**Figure 4.6. Mutations in Usher protein Sans lead to subtle defects in Coordinated Hair Cell Polarization but not to defects in Cochlear CE**

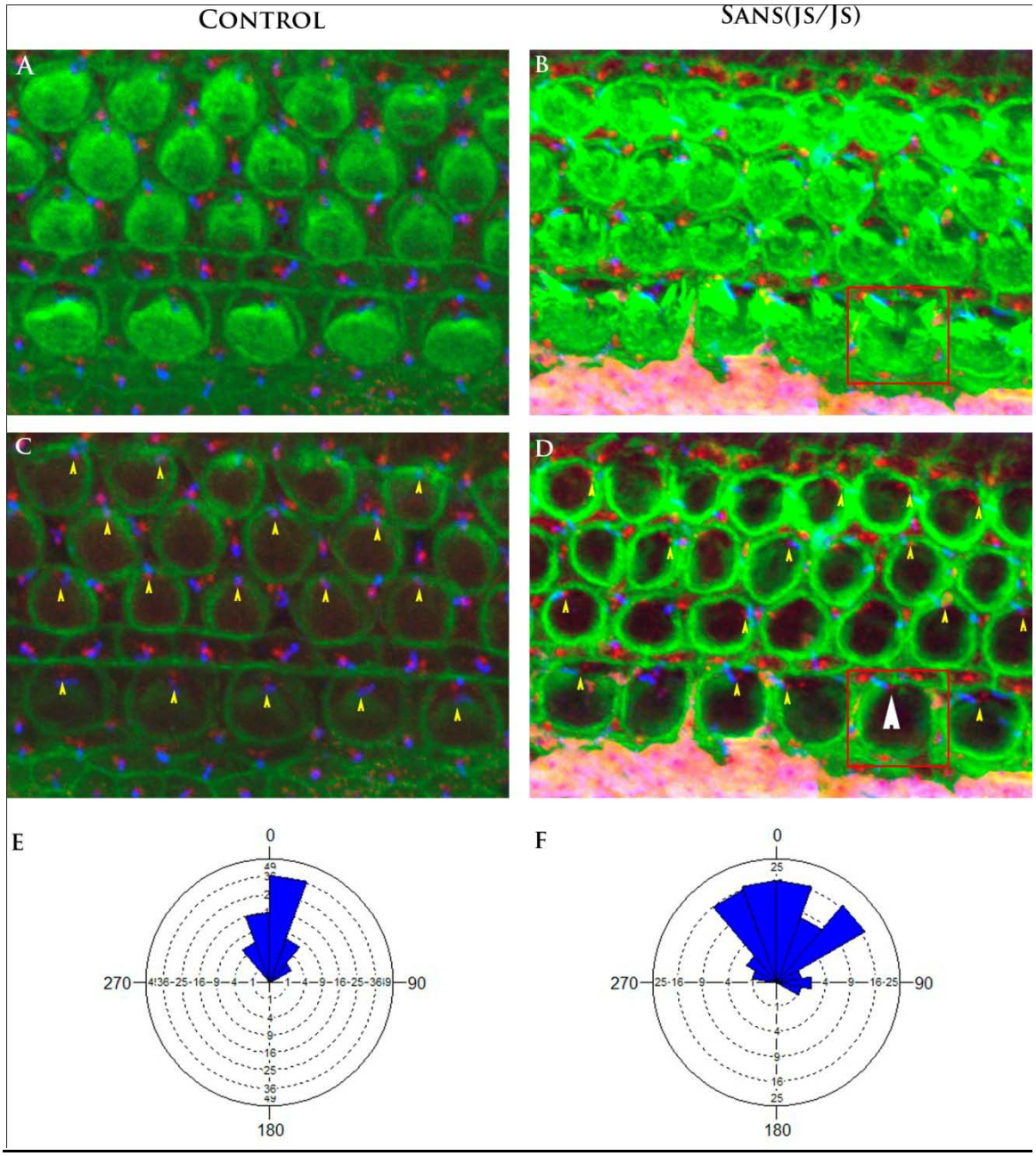


**Figure 4.6. Mutations in Usher protein Sans lead to subtle defects in coordinated hair cell polarization but not to defects in cochlear CE**

***Confocal micrographs of the apical surface of organ of Corti at e18 of wild type* (A) *and* Sans$^{JS/JS}$ *mice* (B).** Actin is labeled with phalloidin (green); the centrioles are labeled with gamma tubulin (red); the kinocilium is labeled with Arl13b (blue). Note the defect in the shape of the stereociliary bundles in Sans$^{JS/JS}$ mice. At first glance it ***seems*** that Sans$^{JS/JS}$ mice display defects in intrinsic cell polarization (red box) similar to those in Pcdh15$^{3J/3J}$mice (Figure 4.4.3).

***Confocal micrographs of the of organ of Corti at e18 of wild type* (C) *and* Sans$^{JS/JS}$ *mice* (D).** To allow for easier visualization of the localization of the centrioles (red) and kinocilium (blue) by the reader, channel containing actin signal (green) has been removed from the uppermost 3-4 Z-slices from the Z-stacks on A and B. Note that in wild type mice the localization the kinocilium (yellow arrows) in most hair cells is aligned with the PCP axis (base, e18). In contrast, although within the lateral side of the hair cells, the orientation of the kinocilium (yellow arrows) fails to be aligned in Sans$^{JS/JS}$ mice (base, e18). Also note the ***lateral*** not central position of the kinocilium (white arrow), showing that this cell (red box) is intrinsically polarized.

***Quantification of the position of the kinocilium with respect to the PCP axis in sensory hair cells in wild type* (E) *and* Sans$^{JS/JS}$ *mice* (F)** [e18, base and middle]. Note that in the majority of wild type cells, the kinocilium is found within $60^0$ of the PCP axis, a trend disrupted in Sans$^{JS/JS}$ hair cells.

***Discussion***

In vertebrates, PCP signaling regulates both cochlear CE and coordinated hair cell polarization. In Chapter 3 we showed evidence suggesting that cochlear extension is regulated independently of hair cell polarization. In this chapter, we have shifted our attention from cochlear extension to hair cell polarization. It stands to reason that the molecular machinery that builds up the (polarized!) stereociliary bundles must be able to interpret polarity cues from PCP signaling during this process. The Usher proteins are critical components of this machinery. A multitude of biochemical and cell biological studies have shown that the Usher proteins are apically localized in hair cells (illustrated in Figure 4.4.1) and that mutations in Usher proteins lead to defects in the formation and maintenance of stereocilia (Adato, Lefevre, et al., 2005; Boeda et al., 2002; Di Palma, et al., 2001; Holme & Steel, 2002; McGee et al., 2006; Michalski et al., 2007). However, the molecular mechanisms that regulate the communication between the Usher complex and PCP signaling during the formation of polarized stereociliary bundles remain unclear.

Using mutant mice of two Usher proteins, Pcdh15 and Sans, we show that these Usher proteins are required for the intrinsic and coordinated polarization of sensory hair cells, respectively.

Protocadherin-15 (Pcdh15) is a trans-membrane protein important for the formation of linkages between stereocilia and between the kinocilium and stereocilia. Using a functionally null allele we have shown that Pcdh15 is required for sensory hair cell polarization (Figure 4.4.3) but is dispensable for cochlear

extension. We have also shown that Pcdh15 genetically interacts with Polaris in the regulation of the formation of stereociliary bundles (Figure 4.4.5). This finding suggests that the kinocilium (or at least IFT) is required along with Pcdh15 for normal morphogenesis of the stereociliary bundles. Pcdh15 mutant mice have a defect in intrinsic hair cell polarization (Figure 4.4.3) similar to the defects observed when ciliary genes are disrupted (Jones, et al., 2008). Given that the migration of the basal body/kinocilium from the center of the cell is required for the intrinsic polarization of hair cells, the finding that Pcdh15 is also required for this process suggests that communication between the basal body/kinocilium and Pcdh15 is taking place and is required for this migration process. The formation of kinociliary and stereociliary links is the most likely mechanism to explain the requirement of Pcdh15 in the regulation of cell polarization. However, there is also evidence that $Pcdh15^{3J/3J}$ have defects in the organization of cuticular plate and other apical structures (Y. S. Kikkawa, et al., 2008) and thus, a role for Pcdh15 in the regulation of hair cell polarization by disruption of the apical surface of the cell can't be ruled out at this point.

Using a mutant of the Usher protein Sans, we found that, in addition to previously characterized defects in stereociliary bundle formation (Y. Kikkawa, et al., 2003; Lefevre, et al., 2008), Sans is required for the proper organization of apical actin on the surface of sensory hair cells. In contrast to our expectations, we did not find a defect in intrinsic hair cell polarization in Sans mutant cochlea.

Instead, we found that Sans mutants have subtle defects in hair cell polarization. By analyzing the localization of the kinocilium in Sans mutants at the

end of embryonic development we were able to determine that the lateral migration of the kinocilium from the center of the cell is disrupted in Sans$^{JS}$ mice. Although in most cases, the kinocilium does migrate towards the lateral side of the cell, in Sans$^{JS/JS}$ mice this process is disrupted. In wild type mice, the kinocilium migrates towards the lateral side of the cell within a 60 degree angle from the PCP axis (in either direction) and by e18 (base and middle region) in the majority of hair cells the kinocilium is positioned along the PCP axis. In Sans mutants, the general angle of lateral migration is wider, and by e18 (in the base and middle regions of the cochlea) the kinocilium fails to be positioned along the PCP axis in the majority of the cells (Figure 4.4.6). This result suggests that Sans is required for the coordinated alignment of the kinocilium along the PCP axis throughout the sensory hair cells in the mouse cochlea. However, given that the fact that in these mice the kinocilium is localized to the lateral side of the cell, further analysis of older (post-natal) Sans$^{JS/JS}$ samples is required to determine whether the coordinated alignment of the kinocilium along the PCP axis eventually resolves in these mice.

Overall, our combined results from Pcdh15$^{3J/3J}$ and Sans$^{JS/JS}$ mice show that functional Usher proteins are required for hair cell polarization but are dispensable for cochlear CE. Furthermore, the fact that the Pcdh15$^{3J/3J}$ mutants had defects in hair cell polarization but no defects in cochlear CE allowed us to test the hypothesis that cochlear CE, but not hair cell polarization, is regulated via a mechanism that depends on the proper localization/expression of cell-junction proteins. Based on our hypothesis, we predicted that the Pcdh15$^{3J/3J}$

mice, in contrast with Polaris$^{cko/cko}$ and Vangl2$^{Lp/Lp}$ mice will have normal expression of N- and E-cadherin. Our results show that expression N-cadherin and E-cadherin is indeed normal in Pcdh15$^{3J/3J}$ mice (Figure 4.4.4) and thus support the idea that vertebrate PCP signaling regulates cochlear CE and hair cell polarization *independently* in the cochlea.

## Chapter 5 Overall Conclusions and Perspectives

Over the last 25 years much progress has been made in the general understanding how PCP is regulated. Genes responsible for the establishment of planar polarity in vertebrates including conserved “core” PCP and ciliary genes have been identified and determined to play a role in the establishment of tissue-wide polarity and intrinsic or morphological cell polarization, respectively [reviewed in (Wallingford & Mitchell, 2011)]. It has also become clear that diverse cellular and developmental processes are regulated by PCP signaling in vertebrates. However, it is not yet clear how these diverse forms of PCP are controlled downstream of the known common regulatory genes that make up the PCP signaling pathway. Thus, we sought to determine whether different forms of PCP are regulated via the same or different molecular modules downstream of conserved PCP signals. .

We used the PCP processes of CE and coordinated sensory cell polarization in the mouse cochlea during embryonic development to address this key question in our current understanding of PCP. We started with the careful characterization of different aspects of the differentiation of the cochlear epithelium during the formation of the auditory sensory organ, the organ of Corti. During this characterization process, we examined morphological changes that take place within the cochlear epithelium during the extension of the cochlea. Furthermore, we examined a potential role for the alignment of the centrioles during the lateral migration of kinocilium that takes place during hair cell

differentiation. Based on the morphological changes we observed in the developing organ of Corti and on data suggesting that *Drosophila* CE is regulated via dynamic control of cell adhesion, we proceeded to characterize the expression pattern of cell junction proteins during cochlear development. First, we examined AJ proteins N- and E-cadherin to determine if their expression correlates with morphological changes observed during CE of the cochlea. Next, we further explored the potential roles for N- and E- cadherin in cochlear CE by disrupting p120-catenin, which is required for stable membrane expression of N- and E-cadherin. Finally, we explored the role of the Usher complex, which builds the hair bundles in the sensory hair cells, in the morphogenesis of the cochlea during terminal differentiation

These approaches, collectively, have allowed us to illustrate the cellular morphogenesis process associated with CE during terminal differentiation of the cochlea, reveal the distinct and dynamic expression of N- and E-cadherins, and identify the specific requirement for p120-catenin in cochlear CE and Pcdh15 for hair cell intrinsic polarity, respectively. Our data leads us to a working model where cochlear extension is regulated via changes in cell-cell adhesion while the polarization of sensory hair cells is regulated in an Usher/Pcdh15 dependent manner downstream of vertebrate PCP signaling (Figure 5.1)

Centriole alignment along the PCP Axis is not required for the initial Intrinsic Polarization of Sensory Hair Cells but might play a role in fine tuning the Coordinated Orientation of Stereocilia throughout the Cochlea

Previous work has suggested that the positioning of the centrioles found at the base of primary cilia (i.e. the mother centriole where the primary cilium stems from and an apposed daughter centriole) plays a role in coordinated cell polarization (Guirao, et al., 2010; Mirzadeh, Han, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2010). Although most of this work has dealt with the coordination of the positioning and beating of motile cilia in ependymal cells, we hypothesized that centriole alignment along the PCP axis might provide a polarity cue for the lateral migration of the kinocilia along the PCP axis in the nascent sensory hair cells. Contrary to what we expected, we determined that the alignment of the centrioles along the PCP axis isn't required for the lateral migration of the kinocilium (Figure 2.4). Instead, in wild type cells, the kinocilium starts migrating from the center to the lateral side of cell at a time when the centrioles are randomly oriented with respect to the PCP axis. Instead of following a straight path from the center of the cell to the lateral side of the cell along the PCP axis, the kinocilium moves toward the lateral side of the cell following a path that falls within $60^0$ of either side of the PCP axis (Figure 2.5, quantified in Figure 4.4.6 E). After it is localized to the lateral side of the cell, the kinocilium eventually becomes aligned with the PCP axis. At the same time that this alignment is taking place, the centrioles also become aligned with the PCP axis. Interestingly, the alignment of the V-shaped stereociliary bundles on the

sensory hair cells throughout the organ of Corti generally *correlates* with the general orientation of the centrioles as they become aligned on the PCP axis (Figure 2.5). This finding suggests that, although not required for the initial polarization of sensory hair cells along the PCP axis, the alignment of centrioles might play a role in the coordinated orientation of the kinocilia and the stereociliary bundles within the sensory hair cells in the organ of Corti.

Cells within the developing OC undergo dramatic morphological changes likely mediated through the ynamic regulation of cell-cell contacts

The terminal differentiation of the cochlea occurs during E14 to E18 in mice. We examined cellular morphogenesis at the apical surface of cells during this process. We determined that the cells within the developing organ of Corti undergo a series of dramatic changes in cellular arrangements, and that these changes involve the formation of cellular rosettes from regularly shaped pentagonal and hexagonal cells, followed by the eventual resolution of these cellular rosettes (Figure 2.2). Rosettes are a transitional cell clusters characteristically found in epithelia undergoing a dynamic regulation of cell-cell contacts; as has been characterized during the CE process observed during dorsal closure in *Drosophila* (Bertet & Lecuit, 2009; Kim, et al., 2010; Nikolaidou & Barrett, 2005; Rauzi, et al., 2008).

Dynamic expression of N- and E-cadherins and their potential role in cochlear CE

The dramatic morphological changes we observed within the developing organ of Corti (Figure 2.1, Figure 2.2) suggested that a dynamic regulation of cell-cell contacts is taking place during cochlear development. Thus we decided to examine the expression pattern of E-cadherin and N-cadherin during cochlear development. Consistent with the idea that there is a dynamic regulation of cell-cell contacts in organ of Corti during development, we found that the expression of the AJ proteins N- and E-cadherin demarcate a sharp boundary between the IHC an OHC regions of the developing organ of Corti (Figure 2.3). Furthermore, we were able to assert that earliest time at which the membrane expression of N-cadherin and E-cadherin is noticeable within the IHC and OHC regions of the cochlea (respectively) coincides with the time at which the sensory hair cells within each region first become identifiable. .These findings suggest that the membrane localization of these proteins is dynamically regulated during cochlear development. Given that classical cell biological studies have shown that differential cell adhesion plays a role in tissue morphogenesis (Steinberg, 2007; Townes & Holtfreter, 1955), these results implicate that the differential expression of N- and E-cadherin in the cochlea could play a role in the cochlear morphogenesis and could regulate cochlear CE. We further speculate that the differential expression of N- and E-cadherin could play a role in the formation of the cochlear *spiral* shape.

AJ protein p120-catenin is required for proper Cochlear CE but dispensable for Coordinated Morphological Polarization of Sensory Hair Cells

To further test the hypothesis that a dynamic regulation of cell-cell adhesion plays a role in during cochlear CE, we decided to disrupt cell-cell contacts by using a conditional knockout mouse of the cell adhesion protein p120-catenin.Our characterization of the effects of the deletion of the AJ protein p120-catenin within the cochlea (a project spearheaded by a former member of the lab, Renee) suggested that the PCP processes of cochlear CE and coordinated sensory hair cell morphological polarization are regulated independently. We found that cells within the cochlea of p120-catenin conditional knockout mice display defects in CE but are normally polarized along the PCP axis (Figure 3.1).

It should be noted that, given that the experimental model we used abrogates the expression of p120-catenin in the cochlea, although unlikely, it is possible that the requirement for p120-catenin in the regulation of cochlear CE could be taking place through a function of the protein other than its role in the stabilization of cadherins at the cell-membrane. Thus, further experiments are required for us to be able to determine whether N-cadherin and/or E-cadherin play a direct role in the regulation of cochlear CE. In order to address this question we have obtained and are currently breeding mice for the conditional knockout of N- cadherin and E-cadherin in the inner ear.

Defects in CE found in core PCP and Ciliary Mutants correlate with disruptions in the expression of E-cadherin and N-cadherin

The analysis of the p120-catenin conditional knockout (cko) mice also suggested that a mechanism involving the stabilization of cell-cell contacts, similar to the CE process in *Drosophila* (Bertet & Lecuit, 2009; Rauzi, et al., 2008) might also play a role in the regulation of vertebrate PCP. Based on this hypothesis, we predicted that mutants that have defects in hair cell polarization and cochlear CE (like vertebrate PCP mutants; Figure 3.3) would also have abnormal expression of cell junctional proteins within the developing organ of Corti. However, mutants that have defects in hair cell polarization but not defects in cochlea CE (like Pcdh15 - Figure 4.4.3) would have normal expression of cell-junctional proteins.

Our work shows that this is indeed the case. The expression pattern of N- and E-cadherin is normal in $Pcdh15^{3J/3J}$ mice (which have defects in hair cell polarization but no defects in CE) (Figure 4.4.4). The expression pattern of N-cadherin is disrupted in the same manner in both core PCP and ciliary mutants (Figure 3.4). The expression of E-cadherin does not change in the same way in the core PCP and ciliary mutants analyzed in this study. Membrane E-cadherin levels in the are reduced in the cochlea of core PCP mutant $Vangl2^{Lp/Lp}$ (Figure 3.5, (Chacon-Heszele*, et al.)). However, in $Polaris^{cko/cko}$ mice, although non-uniform the expression of E-cadherin in the cochlea seems to increase (Figure 3.6). Although perhaps puzzling, the results from E-cadherin expression analysis are not inconsistent with our overall hypothesis. First, it is possible that E-

cadherin does not play as important role in the regulation of cochlear CE as N-cadherin. Second, even if E-cadherin is indeed an important player in the regulation of cochlear CE, the model of the regulation of CE in *Drosophila* suggests that disruptions in the equilibrium of proteins that cell-cell dynamics can lead to defects in CE. Consistent with this idea, a recent publication has shown that disruption of non-muscle Myosin-II leads to defects in cochlear CE (Yamamoto, et al., 2009).

The Usher complex is required for the regulation of Hair Cell Polarization but dispensable for Cochlear CE

As mentioned Chapter 4, the Usher mutant Pcdh15$^{3J/3J}$ displays defects in hair cell polarization but no defects in cochlear CE. Interestingly, hair cells within the cochlea of Pcdh15$^{3J/3J}$ mice display defects in both coordinated and (in a manner reminiscent of Polaris$^{cko/cko}$ mice (Jones, et al., 2008)) many hair cells also display defects in intrinsic cell polarization. These defects in hair cell polarization are independent of cochlear CE (which is normal in Pchd15-3j mice) and take place downstream (or parallel) of core PCP protein localization (Figure 4.4.3). Based on the similarity between the phenotype observed in Pcdh15-3j mutants and the phenotype observed in Polaris$^{cko}$ mice (Jones, et al., 2008) we hypothesize that the physical linkages between the kinocilium and stereocilia mediated by Pcdh15 play an important role in the process that regulates intrinsic hair cell polarization. However the fact that Pcdh15$^{3J/3J}$ mutants also have

disruptions in the organization of the apical surface of sensory hair cells (Y. S. Kikkawa, et al., 2008) make it difficult to ascertain with certainty whether the requirement for Pcdh15 in the regulation of intrinsic cell polarity does indeed depend on these physical links or not.

Interestingly, our work with another Usher mutant ($Sans^{JS/JS}$) provides further evidence that the regulation of hair cell polarization takes place independently of cochlear extension (data not shown). However, our results from the analysis of $Sans^{JS/JS}$ mice suggests that Sans plays a different role in the regulation of hair polarization that Pcdh15. Sans is a scaffolding protein localized on the apical surface of hair cells particularly on the sub-basal body region (Maerker, et al., 2008; D. Yan, et al., 2010). Sans plays an important role in the trafficking of other Usher proteins to stereocilia (Adato, Michel, et al., 2005; D. Yan, et al., 2010). Interestingly we did not find a defect in intrinsic cell polarization in the $Sans^{JS/JS}$ mutant mice. Instead, in addition to previously described defects in the shape of stereociliary bundles (Y. Kikkawa, et al., 2003), we found defects in the apical organization of actin within the sensory hair cells in $Sans^{JS/JS}$ mice. This disruption in apical actin leads to low-actin region within the apical surface of sensory hair cells that do not correspond to the localization of the centrioles/basal body (Figure 4.4.6). This phenotype that at first glance could lead to the mistaken assumption that $Sans^{JS/JS}$ have defects in intrinsic hair cell polarization. We also determined that at e18, $Sans^{JS/JS}$ mice have a subtle hair cell polarization defect. This defect is manifested by the localization of the kinocilium on the lateral side of sensory hair cells, which in many cases, although

laterally positioned, fails to align along the PCP axis. This finding suggests that, in addition to its requirement for the formation of stereociliary bundles, Sans might also be required for the coordinated alignment of the kinocilium along the PCP axis in sensory hair cells throughout the cochlear epithelium. However, examination of older (post-natal) $Sans^{JS/JS}$ required to determine whether this defect in the coordinated positioning of the kinocilium along the PCP axis in throughout the sensory hair cell of the cochlea is eventually resolved or persists later in life in the $Sans^{JS/JS}$ mice.

Cochlear CE and Hair Cell Morphological Polarization are regulated via independent mechanisms downstream of PCP Signaling

The work presented in this thesis provides multiple lines of evidence in support of the hypothesis that the PCP processes of cochlear CE and coordinated sensory hair cell polarization are regulated by distinctive molecular modules downstream of common PCP regulatory proteins.

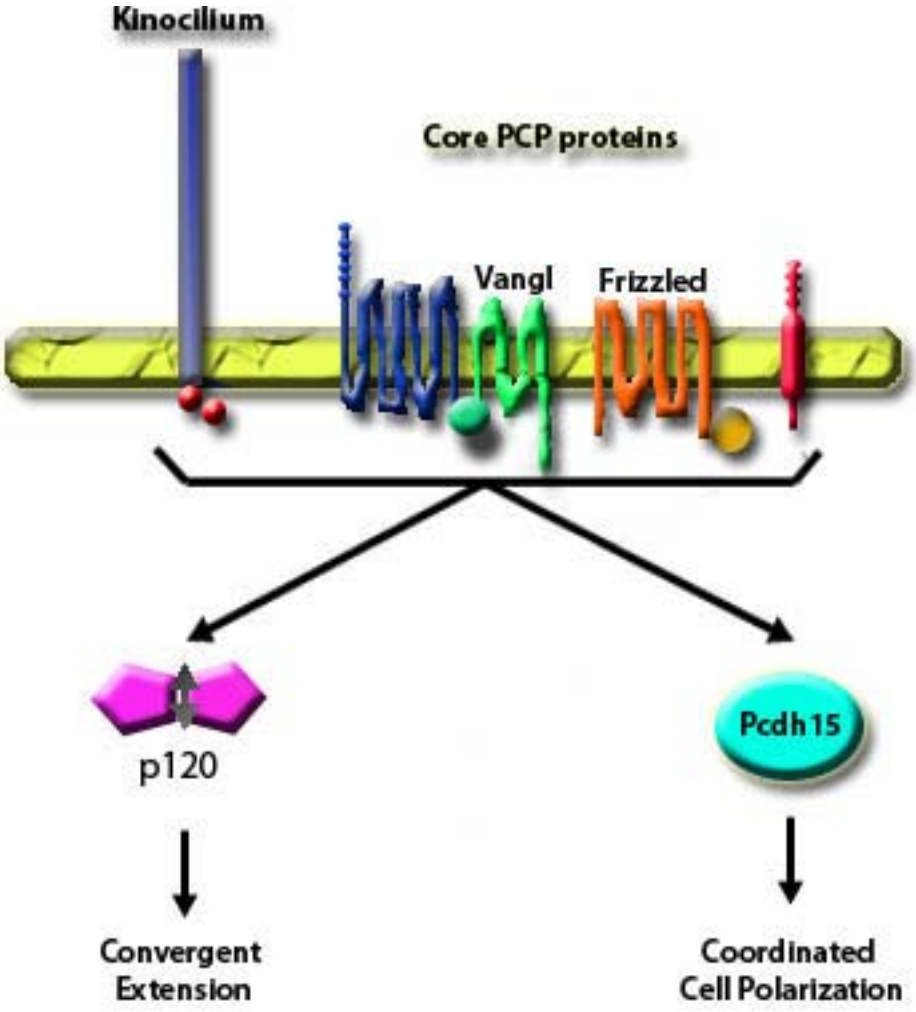
We found that, in a similar process as has been characterized during *Drosophila* CE*,* the dynamic regulation of cellular contacts plays a role in the regulation of vertebrate CE. To be more specific, a p120-dependent (and perhaps cadherin-dependent) mechanism regulates cochlear CE *independently* of hair cell polarization

We also determined that Pcdh15, a member of the Usher complex, plays a role in the regulation of the intrinsic or morphological polarization of sensory

hair cells but is dispensable for the regulation of cochlear CE. Additionally, another member of the Usher complex, Sans, might play a role in the regulation of the coordinated positioning of the kinocilia and stereocilia along the PCP axis but is dispensable for the regulation of cochlear CE. Thus, we found that two different members of the Usher complex, which builds up the stereociliary bundles, are required for proper morphological polarization of sensory hair cells but dispensable for cochlear CE.

Combined, our results support a model where different forms of PCP, namely cochlear CE and coordinated morphological polarization of sensory hair cells are regulated by separate molecular modules downstream of PCP signaling (Figure 5.1). Cochlear CE is controlled via a p120-catenin mechanism while the morphological polarization of sensory hair cells is regulated in an Usher dependent manner.

**Figure 5.1. Working model: Cochlear CE is regulated via a p120-catenin dependent mechanism while Coordinated Hair Cell Morphological Polarization is regulated via an Usher/Pcdh-15 dependent mechanism downstream of Core PCP Signaling**



# Chapter 6 Materials and Methods

Mouse strains and animal care.

Animal care and use was in accordance with US National Institutes of Health (NIH) guidelines and was approved by the Animal Care and Use Committee of Emory University. The following mouse strains were obtained from the Jackson Laboratories: Foxg1tm1(cre)Skm (*Foxg1-Cre*); A*mes Waltzer:*Pcdh15(av-3j) (*Pcdh15*$^{3J}$); *Jackson Shaker:* (Sans$^{JS}$); and LPT/Le (*Vangl2*$^{Lp}$). The Polaris/IFT88-floxed strain (*Polaris*$^{LoxP/LoxP}$) was a gift from B. Yoder (Birmingham, Alabama) (Haycraft, et al., 2007).

To increase the yield of the desired phenotypes, for the conditional knockout experiments, homozygous floxed females were crossed with heterozygous males carrying a Cre allele whenever possible.

For staged embryos, the morning after mating for an overnight mating (or the morning when a plug was observed for multiple-night matings) was designated as embryonic day 0.5 (e0.5).

Inner ear fixation and dissection

Inner ears were fixed using 4% paraformaldehyde (in PBS) overnight at 4C. After fixation embryonic cochleae were dissected using watch-maker forceps following these steps:

1. The cartilage was separated from the sensory epithelia in the inner ear
2. The cochlea was separated from adjacent vestibular organs.
    - During this procedure, particular care must be taken not to break the saccule, which is apposed to the basal region of the cochlea
3. Innervations and mesenchymal tissue is separated from the cochlear epithelium
4. The roof of the cochlea is removed, leaving the sensory hair cells exposed
5. If present after roof removal, membranes found about the sensory hair cells must be removed from the samples to avoid problems with the staining

Note: Biology tip forceps are recommended for dissection of young samples (e14 and younger) and for membrane removal. Number 5 tip forces are preferred for older samples. Do not use biology tip forceps for the removal of cartilage as this will damage the forceps.

Inner ear immunostaining

After dissection cochleae were stained using the procedures described below:

1. Block:
    a. Block in 10% Donkey serum (DS) in 1X PBS-T
    b. 1 hour at room temperature
    c. Goat serum (GS) can also be used if preferred
2. Primary antibody incubation:
    a. Incubate samples in primary antibody in 5% DS, PBS-T
        i. Antibody dilutions listed below
        ii. Overnight at 4 C.
3. Wash 01:
    a. Wash samples in 1X PBS, 3 times.
    b. Wash time depends on antibody:
        i. Short washes: 15 minutes
        ii. Long washes: 2 hours
4. Secondary antibody incubation:
    a. Incubate samples in secondary antibody of choice
    b. 1:1000 dilution in 1X PBS-T
    c. Either 2 hours at room temperature or overnight at 4C
        i. If the primary antibody requires long washes, incubate overnight

5. Wash 02:
    a. Wash in 1X PBS-T, 2-3x
    b. Wash time depends on antibody used and length of secondary antibody incubation
        i. Short wash: 15 minutes at room temperature
            - Use for short wash antibodies only
        ii. Long wash: 2 hours at room temperature
            - Use if antibody is listed as long washes below or
            - If secondary antibody incubation was done overnight
6. Phalloidin incubation:
    a. Use phalloidin to visualize cortical actin, cuticular plate and stereocilia on apical surface of hair cells
    b. Dilute Phalloidin-(flourophore of choice) in 1X PBS-T (1:1000)
    c. Incubate at room temperature 20-40 minutes
    d. Move the samples to PBS until mounting
7. Mounting:
    a. Mount samples using Fluoromount
        i. 25-30uL of Fluoromount are sufficient to mount a cochlea and all sensory organs.
        ii. For confocal imaging it is best to use the 1 ½ coverslips.

For a reference on immunostaining procedures see (J. Wang, et al., 2005).

***Primary antibodies used and wash times used***

1) **Arl13b**, rabbit antibody. (Caspary, Larkins, & Anderson, 2007)
    a) Dilution (1:1,500)
    b) Works with short and long washes
    c) Works well with Cy5 secondary antibody
2) **Frizzled-3**, rabbit antibody (Y. Wang, et al., 2006)
    a) Dilution (1:50)
    b) Long wash required; high background otherwise.
3) **Gamma-tubulin**, mouse monoclonal antibody (Sigma, T6557)
    a) Dilution (1:200)
    b) Best with Rhodamine or AF555 secondary antibodies as the signal is lost very quickly with Cy5 secondary antibody.
    c) Long wash recommended; high background otherwise
4) **p120-catenin**, mouse monoclonal antibody (BD Laboratories, 610133)
    a) Dilution (1:200)
    b) Long wash or short wash
5) **E-cadherin**, mouse monoclonal antibody (BD Laboratories, 610181)
    a) Dilution (1:200)
    b) Long wash is best for this antibody
6) **N-cadherin**, mouse monoclonal antibody (BD Laboratories, 610920)
    a) Dilution (1:400);
    b) Best with short washes but long washes work as long.
        - Long wash required after secondary antibody incubation overnight.

7) **Sox2**, goat antibody (Santa Cruz, sc-17320)
    a) Dilution (1:400).
    b) Do not to use goat serum for block if this antibody is used
    c) Works well with short washes or long washes

Inner ear and sensory hair cell imaging:

For image acquisition the following microscopes were used: Olympus SZX12 upright microscope, Olympus Fluoroview FV-1000 confocal microscope, and Zeiss LSM510 confocal microscope.

- Olympus SZX12: was used to obtain whole cochlea images for comparison of total cochlear length
- Olympus Fluoroview FV-1000: was used to obtain confocal images of the apical surface of the organ of Corti at different developmental stages. The recommended settings for this purpose are:
    - **For visualization of the centrioles:**
        - 100x magnification
        - 2048 resolution (x,y)
        - 0.25uM Z-slice
    - **For imaging of the surface of the organ of Corti:**
        - 40 or 60x
        - 1600 to 2048 resolution (x,y)
        - 0.5uM to 1uM per Z-slice
- Zeiss LSM510 confocal microscope:
    - **For visualization of the centrioles:**
        - 100x not recommended (lower numerical aperture)
        - 63x objective
        - 2048 resolution (x,y)
        - 0.25 uM Z-slice

- **For visualization of the apical surface of the organ of Corti:**
  - 63x objective
  - 2048 resolution
  - 0.5uM to 1uM per Z-slice

Analysis of stereociliary bundle orientation and morphology.

The V-shaped hair bundle orientation was determined using NIH ImageJ software. The angle of orientation was calculated by drawing a line from the position of the kinocilium through the middle of the V-shaped stereocilia (bisecting line). For $Pcdh15^{3J/3J}$ or $Sans^{JS/JS}$ mutants where the shape of the stereocilia is disrupted, the position of the fonticulus and the kinocilia was used, respectively.

The angle of orientation is defined as the angle formed between the bisecting line and the line parallel to the medial to lateral axis, or the mediolateral axis, of the cochlear duct and assigned the negative or positive degree measurements designate orientations to the left or the right side of the mediolateral axis, respectively. In wild-type animals, this angle is between 0 and 30 degrees on either side of the bisecting line.

Each row of hair cells was divided into three groups according to position along the longitudinal axis of the cochlea: base, middle and apex. Due to the differentiation gradient within the single cochlea during development, hair cells in the apex region are less developed. Only hair cells from the base and middle regions of the cochleae were included for polarity quantification. At least 25 hair cells in each row in each region were quantified for each sample, and at least three animals per genotype were analyzed.

The distribution of angles along the length of the cochlear duct was plotted using Oriana3. Oriana3 program automatically converts negative angles to a positive angle in a $360^0$scale. A $-45^0$ degree deviation to the left of the

mediolateral axis would be converted to a $315^0$ at the $360^0$ scale by Oriana3 software. Cells that had a central fonticulus/kinocilium with circular stereocilia were classified as having the maximum deviation from the normal distribution, namely $180^0$. Data are presented as means. Statistical significance was analyzed by Chi-square analysis and Mardia Watson Wheeler tests using Oriana3 software (Kovach Computing)

## Chapter 7 References

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