ROLE OF THE FRIZZLED SIGNALLING PATHWAY IN CONTROL OF HAIR CELL PRODUCTION AND POLARITY IN THE DEVELOPING INNER EAR

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Abstract

The sensory patches of the inner ear consist of two types of cell: sensory hair cells and supporting cells. The pattern is such that supporting cells surround each hair cell and no two hair cells touch each other. Hair cells also possess planar cell polarity; their stereociliary bundles are precisely oriented in the plane of the epithelium. How hair cells are determined and then oriented is unknown.

The mechanosensory bristles of the fruit fly *Drosophila melanogaster* can be thought of as structures analogous to the sensory patches of vertebrate inner ears. Genetic analysis has shown that several intercellular signalling pathways are required for bristle formation, including those mediated by Notch and Frizzled receptors, and Notch signalling appears to have an analogous function in ear development.

I have explored the possibility that homologues of Frizzled signalling pathway genes may be involved in hair cell production and polarity in the inner ear. I have shown, by in situ hybridization, that several Frizzled pathway genes are expressed in the developing sensory patches of the chick embryonic ear concomitant with hair cell birth and differentiation and, subsequently, become restricted to the hair cell or supporting cell subpopulation. Further, I have used retroviral vectors to misexpress full-length chick frizzled-1, chick frizzled-7 and human dishevelled-1, as well as various truncated (dominant-negative) Dishevelled-1 constructs. The results obtained suggest that a more complex situation may exist in vertebrates than in the fruit fly, and that the Frizzled signalling pathway may have numerous roles in inner ear development.
Acknowledgements

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I dedicate this thesis to the memory of my grandfather, Dewi.
CHAPTER 1: INTRODUCTION

1.1 PLANAR CELL POLARITY REQUIRES THREE LEVELS OF CONTROL

1.2 FRIZZLED SIGNALLING GOVERNS PLANAR CELL POLARITY IN DROSOPHILA

1.2.1 Frizzled signalling has several potential outcomes

1.2.2 PCP genes all relate to Frizzled signalling

1.3 PCP SIGNALLING IN THE DROSOPHILA WING

1.4 FRIZZLED SIGNALLING IS CONSERVED IN VERTEBRATES

1.4.1 PCP genes control convergent extension movements during vertebrate gastrulation

1.5 THE VERTEBRATE INNER EAR ORIGINATES AS ECTODERM

1.5.1 Hair cells are mechanosensory

1.5.2 Supporting cells surround hair cells

1.6 SENSORY PATCHES DIFFER IN CHARACTER, BUT SHARE THE SAME TWO CELL TYPES

1.6.1 PCP is most easily visualised in the basilar papilla

1.6.2 Sensory patches are defined by early expression of regulatory genes

1.6.3 How PCP first emerges in hair cells is unclear

1.7 MUTATIONS IN HOMOLOGUES OF DROSOPHILA PCP GENES DISRUPT HAIR BUNDLE INTEGRITY

1.8 DROSOPHILA SENSORY BRISTLES AS A PARADIGM FOR HAIR CELLS

1.8.1 Frizzled signalling has several distinct functions during sensory organ development

1.8.2 Might Frizzled signalling in sensory patches act in an analogous manner to its role in sense organ development?

1.9 AIM AND SCOPE OF THIS WORK

CHAPTER 2: MATERIALS AND METHODS

2.1 SECTIONING

2.2 IN SITU HYBRIDISATION OF SECTIONED EMBRYOS

2.2.1 Hybridisation

2.2.2 Antibody staining of DIG-labelled probes
3.1 Introduction ................................................................. 65
   3.1.1 Little is known about Frizzled signalling in the inner ear ........................................ 65
   3.1.2 Hair cells are born in a scattered pattern, identified by expression of c-Delta-1..... 67
   3.1.3 Hair cells acquire PCP before the stereociliary bundle matures ...................... 68
   3.1.4 Expression data can be suggestive, but not predictive of gene function .......... 69
   3.1.5 Other Frizzled signalling pathway components may show informative expression patterns... 70
3.2 Results ........................................................................... 72
   3.2.1 Chick has at least ten frizzled genes, but no direct d-frizzled-1 homologue ........ 72
   3.2.2 In situ hybridisation patterns for Chick frizzled genes ........................................ 77
3.3 Discussion .................................................................... 108
3.3.1 Expression of several frizzled genes is consistent with a role in PCP determination........108
3.3.2 Expression of frizzled genes indicates a role in hair cell vs. supporting cell specification in
the maculae and the basilar papilla..................................................................................................................109
3.3.3 C-Celsr-1 subcellular distribution may be indicative of a role in hair cell PCP .............111
3.3.4 C-iroquois-1 and c-iroquois-2 expression patterns suggest redundant roles in delaminating
neurons of the inner ear ....................................................................................................................................112
3.3.5 The expression data predict that interfering with Frizzled signalling would affect sensory
patch development................................................................................................................................................113

4CHAPTER 4: FUNCTIONAL ANALYSIS OF FRIZZLED RECEPTORS DURING SENSORY
PATCH DEVELOPMENT..........................................................................................................................................114

4.1 INTRODUCTION..................................................................................................................................................114
4.1.1 In Drosophila, disruption of PCP signalling can be accomplished by misexpression of
Frizzled-1 alone ....................................................................................................................................................115
4.1.2 The RCAS system drives high-level, ectopic expression of a chosen transgene .............116
4.1.3 RCAS virus can be delivered by injection of virions or electroporation of viral DNA........119
4.2 RESULTS .............................................................................................................................................................122
4.2.1 Both Frizzled RCAS constructs had been validated already .............................................122
4.2.2 Electroporation gives RCAS transgene expression up to, but not beyond, E8 ..........123
4.2.3 Injection of virus particles gives longer-lived infections than electroporation ...............132
4.2.4 Effects of c-frizzled-1 and c-frizzled-7 misexpression in the inner ear at E10 and E16....136
4.3 DISCUSSION ........................................................................................................................................................147
4.3.1 Electroporation of viral DNA fails to establish long-lived gene misexpression..............147
4.3.2 Injecting viral particles gives long-lived and heavy viral infections............................148
4.3.3 C-frizzled-1 and c-frizzled-7 overexpression fails to disrupt sensory patch development....149

5CHAPTER 5: DISRUPTION OF FRIZZLED SIGNALLING BY MODULATING
DISHEVELLED FUNCTION....................................................................................................................................153

5.1 INTRODUCTION ..................................................................................................................................................153
5.1.1 Misexpression of full-length Frizzled receptors is a limited approach to disrupting wild type Frizzled signalling ................................................................. 154

5.1.2 Dishevelled constructs can specifically inhibit canonical or PCP signalling ................ 155

5.1.3 How Dishevelled constructs elicit their effects depends on interacting partners ........ 157

5.1.4 Vertebrate dishevelled homologues operate in the Frizzled signalling pathway .......... 159

5.1.5 Subcellular localisation, a key factor in PCP signalling, can be visualised using a tagged transgene 160

5.2 RESULTS ........................................................................................................................................................................ 162

5.2.1 RCAS viruses carrying tagged versions of Dishevelled were successfully produced ...... 162

5.2.2 Dishevelled viruses successfully infect the inner ear ................................................. 169

5.2.3 Dishevelled constructs do not alter cell fate in the chick inner ear ......................... 175

5.2.4 Dishevelled constructs do not alter PCP determination in the basilar papilla .......... 177

5.2.5 DSH(DEP) infected hair cells and c-Celsr-1 expression ........................................... 178

5.2.6 Dishevelled constructs and other Frizzled signalling functions ............................... 179

5.3 DISCUSSION .................................................................................................................................................................... 199

5.3.1 Is dishevelled expressed in the inner ear? ................................................................. 199

5.3.2 Did we chose the correct Dishevelled deletion constructs? ...................................... 201

5.3.3 Can dishevelled work in a heterologous system? ....................................................... 201

6 .................................................................................................................................. CHAPTER 6: DISCUSSION

6.1 SUMMARY ........................................................................................................................................................................ 205

6.2 WHAT HAS BEEN ACCOMPLISHED ........................................................................ 207

6.2.1 A description of wildtype PCP patterns in the basilar papilla ................................ 207

6.2.2 Expression patterns of Frizzled signalling pathway genes ....................................... 211

6.2.3 A comparison of electroporation and virus injection ............................................... 216

6.2.4 Misexpression of Frizzled signalling pathway components ..................................... 217

6.3 WRONG HYPOTHESIS, WRONG APPROACH OR NEITHER? ................................. 217

6.3.1 Frizzled signalling might not control hair cell PCP .................................................. 218
6.3.2 Frizzled signalling might control hair cell PCP ................................................................. 219

6.3.3 Could a different Frizzled signalling system explain our results? ........................................ 227

6.4 Future work .......................................................................................................................... 228

7 ........................................................................................................................................................ REFERENCES

230
Chapter 1: Introduction

Cells adopt a bewildering number of different shapes and sizes. Some are structurally polarised, whereas others have no clear polarity or only acquire it in response to extracellular cues. An epithelial cell, with its well-defined apico-basal axis, is a good example of the former. A macrophage, which only adopts a polarised structure in response to chemotactic signals, illustrates the latter. In some cases, polarity along two axes is apparent. Epithelial cells, in particular, can be polarised in a plane orthogonal to the apico-basal axis. This type of asymmetry is called Planar Cell Polarity, or PCP. Examples include the hairs and bristles on the body and appendages of the fruitfly *Drosophila melanogaster* (Nubler-Jung and Mardini 1990), the cilia lining the respiratory tract (Satir 1980) and the hair cells of the vertebrate inner ear (Tilney et al. 1987). A common feature of all these examples is that they involve the coordinated planar asymmetry of broad fields of epithelial cells; each cell is polarised in its own right, and is also aligned with its nearest neighbours and, ultimately, the overall body plan. This thesis is primarily concerned with one particular instance: the sensory hair cells of the ear, and the problem of how their planar polarity is controlled.

1.1 **Planar cell polarity requires three levels of control**

At the single cell level, structural asymmetry is a readout of the cytoskeleton. Control of cell shape ultimately resides in the distribution of actin filaments and microtubules,
though the relative contribution of each is unclear, and may vary between different
developmental contexts. For example, epithelial cells of the *Drosophila* wing each sprout
an individual hair. This hair is initiated as a single asymmetrically placed focus of F-actin
dpolymerisation (Turner and Adler 1998). Through a mechanism that also involves
microtubules, actin assembly is biased towards this site and stabilised within the cell,
resulting in the emergence of an asymmetric structure on the apical cell surface (Turner and
Adler 1998). Maintenance of asymmetry is an important aspect of the process; the
unpolarised macrophage can also bias actin assembly, but does so in a dynamic way, as it
moves. It does not maintain the asymmetry, but rather constantly destroys and replaces sites
of cytoskeletal growth. In contrast, epithelial cells possessing planar cell polarity maintain
their cytoskeletal asymmetry.

Individual cells must be asymmetric for planar cell polarity to be significant at the
tissue level, but this is not sufficient. Cells polarised individually, but not correlated with
one another, would form a tissue with a disordered and chaotic pattern. Instead, one finds
that all tissues displaying PCP show a good correlation in the PCP vectors of cells that
neighbour one another. This is as true for cells of the *Drosophila* wing, where all hairs
point in the same direction, as for the hair cells of the vestibular patches in the vertebrate
inner ear, where the overall PCP pattern is more complex (see figure 1.1).

The third tier of PCP control is the coordination of the polarity of the multicellular
tissue with the body plan as a whole. For example, *Drosophila* wing hairs all point in a
proximal to distal direction. That is, they are not only aligned with one another, but
consistently point in the same direction with respect to the body of the insect. Similarly, the
Figure 1.1: A comparison of invertebrate and vertebrate PCP. (A) is an adult Drosophila wing. Each hair, or trichome, points from proximal (left) to distal (right). (B) is a saccule, viewed by scanning electron microscopy. Each hair bundle has a PCP vector, with the kinocilium (the tallest projection) heading an array of stereocilia.
fly has predictably oriented hairs and sensory bristles on its thorax and abdomen. These point from anterior to posterior. Cells many cell diameters apart all possess the same PCP vector. This reflects a level of control distinct from coordination of neighbouring cells, and ensures that an entire tissue can correctly fulfill its function in detecting directional signals or performing oriented actions in proper relation to the body as a whole.

To understand PCP signalling, therefore, it is necessary to understand several processes. How does an individual cell acquire polarity? In most cases the planar axis, unlike the apico-basal one of an epithelial cell resting on a basal lamina, has no obvious structural features that would neatly explain how a vector could emerge. If an extracellular cue of some description is responsible, it is not a priori obvious what it should be. If there is an intracellular symmetry-breaking mechanism by which a cell will spontaneously develop planar polarity (with potentially random orientation) even in the absence of an external cue, we need to discover how it works. Further, how do neighbouring cells coordinate their vectors? Once a cell has acquired asymmetry, how does it relay this information to surrounding cells? And, finally, how is the entire process coordinated at the tissue level, so that PCP is correctly correlated with the body plan?

Implicit in the questions asked is a need to identify the molecules which control PCP signalling at its various levels, to identify their functions and interrelationships. Although there is no model system in which all this has been done, the molecular basis of PCP signalling has been at least partly elucidated in *Drosophila*, and a key signalling pathway identified.
1.2 Frizzled signalling governs planar cell polarity in Drosophila

Fruit fly genetics has long been a powerful means of studying developmental genes. For nearly a century, since long before the molecular nature of any lesion could be assessed, flies with mutant phenotypes have been identified and propagated. One such mutation, in a gene called dishevelled, is characterised by an abnormal patterning of the hairs that cover the body and limbs of the fly (Theisen et al. 1994). Normally, hairs on the appendages point in a proximal to distal direction, while bristles on the body point anterior to posterior. In flies homozygous for the dsh' allele, these hairs and bristles are misoriented, forming swirls and whorls instead of the regular, wildtype pattern. Similar types of polarity disorder are seen in flies with mutations in frizzled (Vinson and Adler 1987) and a handful of other genes. Molecular studies have identified the protein products of these genes and have begun to show how they function in PCP. Dishevelled and Frizzled both turn out to be components of the same signalling pathway— the Frizzled signalling pathway— which is thus central to the control of PCP.

1.2.1 Frizzled signalling has several potential outcomes

Frizzled signalling is also referred to as Wnt signalling, Frizzled proteins being transmembrane receptors, and proteins of the Wnt/Wingless family being the extracellular ligands that bind to them (Bhanot et al. 1996; Tomlinson et al. 1997). The founder member of the Wnt family, the Drosophila gene wingless, codes for a secreted glycoprotein and first came to notice for its fundamental role in the organisation of the body plan (Lawrence et al.
1996; Cadigar and Nusse 1997). It is essential, for example, for outgrowth of the wings, and for the construction of the body segments. These functions depend on the so-called canonical Wnt signalling pathway, which acts through β-Catenin/ Armadillo and members of the TCF/ LEF/ Pangolin family of transcription factors to control gene transcription. PCP, however, is governed by a separate branch of the pathway, not involving β-Catenin and TCF proteins, but sharing with the canonical pathway the receptor Frizzled and the immediate intracellular effector Dishevelled (see figure 1.2). It is still not clear whether PCP depends on the Wnt proteins (Drosophila has another six putative Wnt family members in addition to Wingless itself), but it certainly depends on the receptor Frizzled and components both upstream and downstream from it, which I will briefly review.

1.2.2 PCP genes all relate to Frizzled signalling

Frizzled proteins are seven-pass transmembrane receptors of the G-protein coupled superfamily. However, it is unclear whether G-proteins are involved in all signalling through these receptors. Evidence for their involvement is, at present, scant (Slusarski et al. 1997), (Schaefer et al. 2001). What is known is that not all Frizzled receptors are equivalent. In Drosophila, there are four Frizzled receptor genes. The best studied, frizzled-1 and frizzled-2, have overlapping but distinct functions. Both gene products will bind Wingless, but Frizzled-2 does so with a much higher affinity and appears to be the primary transducer of canonical signalling. Frizzled-1—the original Frizzled—can signal via the canonical pathway, but most prominently activates the PCP pathway. Frizzled-2 cannot activate the PCP pathway (Boutros et al. 2000).
Figure 1.2: the Frizzled signalling pathway

Shown is a simplified version of the Frizzled signalling pathway, with emphasis on the canonical and PCP components.

Canonical signalling is shown in green. In response to a Frizzled-transduced Wnt signal, Dishevelled antagonises the degradation of β-catenin mediated by the Axin/APC/GSK-3β complex. This allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus, where it complexes with transcription factors of the TCF/LEF family and activates transcription of target genes. In the absence of Wnt signalling, TCF/LEF actively repress target genes (Brantjes et al. 2002).

PCP signalling is shown in red. Frizzled, Dishevelled and Flamingo form an interdependent triumvirate, each relying on the others for correct PCP function. Downstream lie JNK signalling, through which gene transcription can be controlled, and small GTPases, particularly Rho A, mediating direct effects on the cytoskeleton.

The Ca2+ signalling pathway is not shown in full (due to space constraints). Also, it is unclear exactly what components are part of this signalling cascade. However, it is thought that G-proteins serve to transduce a signal from Frizzled, leading to release of intracellular calcium ions (Slusarski et al. 1997). This in turn activates PKC, which activates unknown downstream effectors.
Wnt
LRP5/6
cell
Frizzled
membrane
PDZ DEPDIX
Dishevelled
Axin
APC
Rho AJNK
cytoskeleton
nucleus
Transcription of target genes

Ca\(^{2+}\) pathway
flamingo

designation of components
The immediate downstream cytoplasmic effector of Frizzled signalling is Dishevelled, and the ways in which the different Frizzled family members interact with Dishevelled may in part determine how they signal (Axelrod et al. 1998), (Boutros et al. 1998). In contrast with wingless and frizzled, there is only one dishevelled gene in the Drosophila genome. Frizzled-1, when activating the PCP pathway, causes Dishevelled to relocate to the cell membrane. Frizzled-2, although it requires Dishevelled for canonical signalling, does not change the subcellular distribution of Dishevelled (Axelrod et al. 1998). In fact, Dishevelled appears to lie at the branching of the ways between canonical and PCP signalling.

Dishevelled is a modular protein, with three well defined domains, called the DIX, PDZ and DEP domains respectively (and a number of other conserved regions) (Axelrod et al. 1998). The DIX domain is required for canonical signalling; it interacts with downstream effectors such as axin and Shaggy, thereby regulating the activation of β-catenin (Rothbacher et al. 2000).

The DEP domain is required for PCP signalling (Axelrod et al. 1998), (Boutros et al. 1998). The venerable dsh\(^{1}\) mutation, which causes PCP defects but has no effect on processes requiring canonical signalling, is caused by a single base pair change in this domain (Axelrod et al. 1998). When the DEP domain is deleted or mutated, Dishevelled loses the ability to relocate to the cell membrane and to activate downstream components of PCP signalling.

The PDZ domain is assigned different functions by different studies, which possibly reflects multiple roles. Taken together, these studies indicate that the PDZ domain may
contribute to both canonical and PCP pathways (Rousset et al. 2001), (Rothbacher et al. 2000). Notably, it binds both Naked cuticle and Strabismus/ Van Gogh, which appear to promote PCP signalling and abrogate canonical signalling (Yan et al. 2001), (Park and Moon 2002). This finding reinforces the theory that Dishevelled acts as the switch control, determining which downstream strand of the Frizzled signalling pathway will become activated. (I will discuss the functions of the different domains of Dishevelled in more detail in chapter 5, where I will describe experiments using different portions of the Dishevelled protein).

Apart from frizzled-1 and dishevelled, there are a number of other PCP genes. These fall into two different categories. One class includes PCP genes whose products are required for correct PCP determination in all adult cuticular tissues. They form the basic machinery and are termed the core PCP genes. A second class includes tissue-specific components that interpret the asymmetric outcome of the activity of the core genes and give rise to the appropriate PCP structure. These genes will differ, for example, between the eye and the wing, where the read-out of PCP signalling is different (oriented clusters of cells versus oriented hairs). Table 1.1 summarises the main characteristics of both classes of PCP gene.

I do not propose to deal exhaustively with all the genes listed. However, the more important members of this group deserve consideration, and I will describe them briefly here.

Prickle is thought to be an antagonist of Frizzled-1 signalling (Tree et al. 2002). A cytoplasmic protein, it has conserved PET and LIM domains but, these apart, shares no significant homology with known protein families. The prickle genetic locus is complex;
three differentially spliced isoforms of prickle exist, but each includes the PET domain.

Prickle has been shown to bind the DEP domain of Dishevelled, and block its ability to associate with the membrane (Tree et al. 2002). This inhibits PCP signalling by Frizzled-1, which requires Dishevelled membrane accumulation.

Flamingo is an atypical cadherin, with a large extracellular domain consisting of many cadherin repeats, and a 7-pass transmembrane domain like that of a G-protein coupled receptor. In S2 cell aggregation studies, Flamingo has been shown to interact homotypically (Usui et al. 1999). It is unclear if it does so in the embryo, however. Flamingo has several distinct functions. Flamingo null flies are embryonic lethal, but lethality can be rescued by driving expression of a flamingo transgene in the CNS (Usui et al. 1999). This fits with a proposed role for Flamingo in axon guidance (Gao et al. 2000). These rescued embryos, which lack flamingo expression in epithelia, exhibit a PCP defect consistent with flamingo being a core PCP gene. Flamingo has been proposed to serve as a scaffold for signalling by other PCP proteins (such as Frizzled-1, Dishevelled and Prickle), as well as signalling in a manner antagonistic to Frizzled-1 itself (Tree et al. 2002).

Flamingo is not the only cadherin to be involved in PCP. Mutations in fat and dachsous (Adler et al. 1998) give PCP phenotypes in both the wing and eye (Yang et al. 2002). Both encode cadherin molecules but, unlike Flamingo, do not possess G-protein coupled receptor homology (Clark et al. 1995). Recent work in the Drosophila eye suggests they may act upstream of Frizzled signalling. Dachsous, in concert with the secreted molecule Four-jointed, sets up a gradient of Fat expression, which is highest at the dorsal and ventral poles of the eye. This,
Table 1.1: Genes implicated in establishment of planar cell polarity in *Drosophila*

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein type encoded</th>
<th>Site of function</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prickle</td>
<td>Cytoplasmic, PET domain</td>
<td>All PCP sites</td>
<td>Blocks Dishevelled membrane binding</td>
<td>(Tree et al. 2002)</td>
</tr>
<tr>
<td>Flamingo</td>
<td>Atypical cadherin</td>
<td>All PCP sites</td>
<td>Multiple.</td>
<td>(Usui et al. 1999)</td>
</tr>
<tr>
<td>Van gogh</td>
<td>TM protein</td>
<td>All PCP sites</td>
<td>Upregulates PCP signalling/abrogates canonical signalling.</td>
<td>(Adler et al. 2000), (Taylor et al. 1998)</td>
</tr>
<tr>
<td>Dachsous</td>
<td>Cadherin</td>
<td>Eye, wing, abdomen.</td>
<td>Controls Fat activity</td>
<td>(Adler et al. 1998), (Yang et al. 2002)</td>
</tr>
<tr>
<td>Fat</td>
<td>Cadherin</td>
<td>Eye, wing, abdomen.</td>
<td>Controls Frizzled-1 activity.</td>
<td>(Yang et al. 2002)</td>
</tr>
<tr>
<td>Rho A</td>
<td>Small GTPase</td>
<td>Eye, wing.</td>
<td>Control of cytoskeleton</td>
<td>(Strutt et al. 1997)</td>
</tr>
<tr>
<td>Four-Jointed</td>
<td>Secreted or TM type II protein</td>
<td>Eye, wing, abdomen.</td>
<td>Potential Factor X Extracellular signal</td>
<td>(Yang et al. 2002), (Zeidler et al. 2000)</td>
</tr>
<tr>
<td>Diego</td>
<td>Ankyrin repeat protein</td>
<td>All PCP sites</td>
<td>Scaffolding factor. Localises with Flamingo.</td>
<td>(Feiguin et al. 2001)</td>
</tr>
<tr>
<td>Widerborst</td>
<td>PP2A catalytic subunit</td>
<td>Wing</td>
<td>Multiple.</td>
<td>(Hannus et al. 2002)</td>
</tr>
<tr>
<td>Jun kinase</td>
<td>MAP Kinase</td>
<td>Eye</td>
<td>Downstream of Dishevelled</td>
<td>(Fanto et al. 2000)</td>
</tr>
<tr>
<td>Multiple wing hairs</td>
<td>Required to limit number of trichomes</td>
<td>Wing</td>
<td>Trichome formation.</td>
<td>(Lee and Adler 2002)</td>
</tr>
<tr>
<td>Fuzzy</td>
<td>Required to limit number of trichomes</td>
<td>Wing</td>
<td>Trichome formation.</td>
<td>(Lee and Adler 2002)</td>
</tr>
<tr>
<td>Inturned</td>
<td>Required to limit number of trichomes</td>
<td>Wing</td>
<td>Trichome formation.</td>
<td>(Lee and Adler 2002)</td>
</tr>
<tr>
<td>Drok</td>
<td>Negative regulator of actin polymerisation</td>
<td>Wing</td>
<td>Trichome formation.</td>
<td>(Winter et al. 2001)</td>
</tr>
<tr>
<td>Myosin VIIa</td>
<td>Motor protein</td>
<td>Wing</td>
<td>Trichome formation.</td>
<td>(Winter et al. 2001)</td>
</tr>
<tr>
<td>Mirror</td>
<td>Iroquois family transcription factor</td>
<td>Eye</td>
<td>Required for border formation. Repressed by mirror.</td>
<td>(Yang et al. 1999)</td>
</tr>
<tr>
<td>Fringe</td>
<td>Glycosyl transferase</td>
<td>Eye</td>
<td>Border formation. Repressed by mirror.</td>
<td>(Cho and Choi 1998)</td>
</tr>
</tbody>
</table>
in turn, controls differential Frizzled-1 activity in the R3 and R4 cells, which defines the polarity of each ommatidium (Yang et al. 2002). The roles of Fat and Dachsous in the wing are less well understood, but again are thought to be upstream of Frizzled signalling (Adler et al. 1998).

Downstream of Frizzled-1, Rho A is thought to have a critical role in all PCP signalling (Strutt et al. 1997). Rho A is a small GTPase, and is thought to signal both to the cytoskeleton and to the nucleus. In the wing, Rho A signalling both promotes and limits actin polymerisation, ensuring that structural asymmetries (such as hairs) arise at the correct site, and not in ectopic locations (Winter et al. 2001). Downstream effectors of Rho A include Drok (Drosophila rho-associated kinase) and Myosin VIIa. These proteins act to limit actin assembly to one site only; in their absence in the wing, hairs are formed ectopically or in excessive numbers. Rho A also signals via the Jun Kinase pathway, leading to effects on gene transcription (Fanto et al. 2000). The nuclear targets are unknown, however.

1.3 PCP signalling in the Drosophila wing

As noted above, all PCP signalling in Drosophila requires a core set of genes, including frizzled, dishevelled, flamingo, prickle and rho. Although PCP was originally thought of as an identical process in all tissues, it now seems that divergent sets of additional genes are required in harness to these core genes: PCP signalling in the wing, for example, may differ substantially from PCP signalling in the eye. I will therefore avoid mixing examples from different tissues, and instead concentrate here on the action of the
core genes in a well-studied and straightforward example, the wing hairs (a more complex example, the sensory bristles, will be discussed later in this chapter).

The *Drosophila* wing possesses hairs, termed trichomes, individually produced by each epidermal cell (Wong and Adler 1993). The trichome originates on the apical surface, distal to the body, and also points in a distal direction. All trichomes point the same way, and therefore the wing has a single PCP vector, pointing proximal to distal.

Within each developing wing cell, Frizzled-1 is at first expressed uniformly at the cell surface. In response to an unknown signal (candidates have been advanced, but not proven) the pattern of Frizzled-1 changes, and from approximately 20 hours apf (after puparium formation) Frizzled-1 becomes concentrated at the distal surface (Strutt 2001). It is not alone in changing its subcellular distribution. At the same time, Dishevelled also becomes localised to the distal surface (Axelrod 2001). Flamingo localises to both distal and proximal surfaces (Usui et al. 1999). These three genes form a triumvirate; removal of any of them abrogates both PCP signalling and the subcellular localisation of the other two proteins. Their interdependence suggests a positive feedback loop is required to maintain localisation and correct PCP.

Other gene products also become redistributed within the cell. Prickle localises to the proximal cell surface, and is required for correct PCP signalling (Tree et al. 2002). When overexpressed, it causes a cell non-autonomous phenotype (as does Flamingo). Other genes induce non-autonomous phenotypes by their absence. For example, *van gogh* clones in the wing cause PCP defects in cells proximal to the clone (Adler et al. 2000). Conversely, when *frizzled-1* clones are created, genetically normal cells distal to the clone are disturbed in
their PCP (Vinson and Adler 1987). These effects imply that cells communicate with their neighbours, and that this communication is essential for coordinating their orientation.

The ultimate role of the core PCP genes role is to establish a single focus at the distal surface of the cell, from which the trichome will emerge. Active Frizzled signalling is required for the trichome to emerge in the correct position and nowhere else (Strutt 2001). The readout of signalling is a remodelling of the actin and microtubule cytoskeletons (Turner and Adler 1998). Downstream of Frizzled-1, it is thought that Dishevelled activates Rho A which, in turn, promotes actin polymerisation at the correct site. The pathway appears to branch below Rho A, however, with different components regulating the orientation, number and site of establishment of the hairs. For example, recent studies have shown that Drok (Drosophila rho-associated kinase), which acts downstream of Frizzled-1 and Rho A, is required to ensure that only one trichome emerges (Winter et al. 2001). In drok mutant clones in the wing, most cells produce several hairs, although they all emerge at the distal surface and are correctly oriented. A target of Drok is non-muscle myosin II which, along with myosin VIIa, is responsible for directing the specific site of F-actin assembly that prefigures trichome emergence at a single location.

1.4 Frizzled signalling is conserved in vertebrates

The Frizzled signalling pathway, like so many invertebrate signalling cassettes, has been both conserved and embellished in vertebrates. Frizzled homologues have been discovered in every vertebrate system explored, from the frog *Xenopus laevis* through fish, chick, mouse and man (Slusarski et al. 1997), (Sumanas et al. 2002), (Winklbauer et al. 2001).
The Drosophila genome contains four frizzled genes. The human genome contains ten, with similar numbers found in other species. The number of Wnt genes has increased in similar measure. Even before considering increases in the numbers of conserved downstream components, as well as new vertebrate-specific components, it is clear that potential pathway complexity has increased enormously. Nevertheless, clear conservation of function has emerged, which hints at similar mechanisms being employed in similar developmental contexts.

A well-studied example of conserved canonical function is axis formation in vertebrate embryos. In Xenopus, ectopic expression of dishevelled, activated β-catenin or certain Wnts (such as Wnt5a) leads to formation of a second axis (Rothbacher et al. 2000). Canonical signalling is necessary and sufficient for axis formation in vertebrate embryos.

Another notable example is the role of canonical signalling in the mammalian gut epithelium. In over 80% of human colorectal cancers, the APC gene is deleted or mutated (Rowan et al. 2000). APC is a negative regulator of canonical signalling; it targets β-catenin for degradation, thereby preventing its accumulation in the nucleus (where it acts in concert with TCF transcription factors to transcribe growth-promoting genes, such as cyclin D1). In some colorectal cancers, mutations in β-catenin are seen, which prevent its degradation (and therefore act in much the same way as APC deletion). The Frizzled signalling pathway is known to play a mitogenic role in numerous contexts, although this is the clearest human example.

Until recently, the healthy number of reports of canonical pathway signalling in vertebrates contrasted starkly with the dearth of reports on potential conservation of the PCP pathway. However, a number of papers have now been published on a PCP-like
process in vertebrates: convergent extension movements during gastrulation. Further, mutant analyses suggest that many of the vertebrate genes involved are direct orthologues of *Drosophila* PCP genes.

1.4.1 PCP genes control convergent extension movements during vertebrate gastrulation

Convergent extension refers to the process whereby cells of the ectodermal and mesodermal layers of the gastrula intercalate in a lateral to medial fashion, narrowing the embryo and elongating it along the anterior-posterior axis as gastrulation proceeds. By analogy with PCP, the cells show behaviour that is polarised in a plane orthogonal to the plane of the cell sheet— that is, they show a directed motility along the medio-lateral axis.

The first report on potential Frizzled signalling involvement in this developmental process identified a zebrafish mutant, *silberblick*, in which convergent extension fails (Heisenberg et al. 2000). It is ironic, when one considers the vain search for a PCP ligand in *Drosophila*, that the gene responsible should turn out to be *wnt-11*. Further reports identified a need for *frizzled-7*, as well as homologues of *dishevelled, naked cuticle, strabismus* and *Jun N-terminal kinase (JNK)* in this process (Wallingford et al. 2000), (Winklbauer et al. 2001), (Yan et al. 2001), (Darken et al. 2002), (Yamanaka et al. 2002). Absence or misexpression of any of these genes led to aberrant convergent extension. These papers provide a clear demonstration that the PCP branch of Frizzled signalling is conserved in vertebrates, and plays an analogous role to that in *Drosophila*. However, the depth of understanding at the subcellular level, as well as the inventory of the genes involved, lag well behind. It is not known if the protein products are asymmetrically...
localised, rely on feedback loops, are connected to the actin cytoskeleton by small GTPases or how many additional genes are required. Also, it has not been shown that the pathway identified in convergent extension mutants is also required for PCP in other vertebrate tissues. Notably, from the point of view of this study, a resounding silence surrounds PCP determination in the inner ear (at least in terms of Frizzled signalling). Proof of principle has been established, but a clear picture of the vertebrate pathway and its developmental contexts is yet to emerge.

1.5 The vertebrate inner ear originates as ectoderm

The inner ear of vertebrates is a remarkable organ, responsible for the detection of sound, gravity and acceleration. It arises from a patch of epithelium that is initially on the surface of the embryo. In the chick, ectoderm adjacent to rhombomeres 5 and 6 is the founder tissue (Ladher et al. 2000). Inductive molecules, including several from the FGF (fibroblast growth factor) and Wnt families, signal from adjacent deeper tissues, causing the overlying epithelium to invaginate, forming the otic cup (Ladher et al. 2000). Over several days, the otic cup pinches off to become a vesicle, then undergoes the morphological changes necessary for production of the mature, convoluted structure.

The epithelium becomes specialised over time. Initially uniform, it gives rise to such diverse structures as semicircular canals (fluid-filled structures required for sensation of rotational acceleration), the endolymphatic duct and sac (for resorption of the endolymph, which bathes the hair cells) and the neurons of the cochleo vestibular ganglion (which innervates the inner ear). These components are all essential for correct function, but
mechanosensation— that is, mechanosensory transduction— itself occurs elsewhere. It is the sensory regions of the epithelium, consisting of hair cells and supporting cells, which directly perform this function.

1.5.1 *Hair cells are mechanosensory*

Hair cells transduce shear motion into an electrical signal. They are the effectors of inner ear function: some of them detect gravity and acceleration, while others detect sound (Hudspeth 1997). Each hair cell possesses a hair bundle, composed of stereocilia and a single kinocilium, which projects into the lumen of the ear. The stereocilia are arranged in precise rows, graded in height, like organ pipes. It is the interaction of the bundle of stereocilia with an overlying layer of extracellular matrix which provides the basis of mechanosensation. Tilting of the bundle through shear displacement of the matrix relative to the hair cell body causes the opening of ion channels and an influx of positive ions into the hair cell (Hudspeth 1997). At its basal surface, the hair cell synapses with a dendrite of a sensory neuron. Depolarisation of the hair cell by the influx of ions causes release of neurotransmitters, generating a nerve impulse, and the signal is propagated to the brain.

A notable feature of the transduction mechanism, crucial for any discussion of hair cell PCP, is that hair cell activation only occurs when the bundle is displaced in a specific direction (from the shortest towards the tallest stereocilium). The orientation of each hair cell within the sensory epithelium dictates when it will become activated. Extrapolated to the organ as a whole, this means that correct function relies on the correct orientation of all the hair cells, relative to one another and the body plan. To understand how a functional ear
is created, therefore, we have to discover how the pattern of hair cell planar polarity is set up.

1.5.2 Supporting cells surround hair cells

Supporting cells, as their name implies, assist hair cell function in a number of ways. They provide physical support; their presence is essential for the integrity of the sensory patch (Haddon et al. 1999). In their absence, as seen in the zebrafish mind bomb mutant, regions consisting entirely of hair cells become excluded from the inner ear epithelium (Haddon et al. 1998). In addition, supporting cells are a source of new hair cells. Hair cell production is continuous in some patches, whilst in others it can be triggered by damage to existing hair cells (Stone and Rubel 2000). In both cases, support cells can reenter the cell cycle and divide to produce hair cells in the required numbers. In effect, they behave as stem cells.

Supporting cells also secrete molecules required for correct hair cell function— in particular, components of the extracellular matrix that overlies the hair cells (Cotanche 1987). Further, they play a vital role in maintaining the high concentration of potassium ions in the inner ear lumen. In mice lacking the kcc-4 potassium/chloride co-transporter gene, potassium ions cannot be transported through supporting cells, and hair cells subsequently die (Boettger et al. 2002).

1.6 Sensory patches differ in character, but share the same two cell types
The hair cells and supporting cells in the ear are grouped in a number of discrete sensory patches. In every mature sensory patch the basic topology of the multicellular pattern is similar: no two hair cells abut, each being surrounded by supporting cells (see figure 1.3).

The simplicity of this scheme is appealing: two cell types generated in a strict pattern, with well-defined functions. However, sensory patches are neither all identical nor, indeed, homogeneous. Differences in morphology, hair cell type, supporting cell type, overlying matrix and planar cell polarity reflect the unique function of each patch, and complicate the task of unravelling the molecular basis of sensory patch formation. Further complications arise when one considers that hair cells within the same sensory patch differ markedly from one another, most notably in their stereociliary arrays.

Sensory patches can be classified as auditory or vestibular, according to their function. Of the vestibular patches in the chick inner ear, three are termed cristae (anterior, lateral and posterior), and four maculae (saccular, utricular, lagenar and neglecta). The cristae, for example, are located at the ends of the semicircular canals. Movement of fluid through the canals, when the head is rotated, displaces the cupulae – the masses of extracellular matrix-located in the lumen above the cristae, activating hair cells. Similarly, gravity or linear acceleration causes displacement of otoconia embedded in the matrix overlying the maculae, activating the macular hair cells.
Figure 1.3 Sensory patches share the same basic morphology

Shown opposite are a schematic representation of a sensory patch (top) and an image of the chick basilar papilla (bottom). Together they demonstrate the cellular organisation within sensory patches.

The schematic shows a sensory patch in transverse section, adapted from (Adam et al. 1998). Hair cells (red) project their bundles into the lumen of the inner ear. They are located apically, surrounded by supporting cells (yellow) which span the entire thickness of the epithelium. Each hair cell contacts a neuron (green).

The image, taken at E9, shows the chick basilar papilla en face. Hair bundles (red) are shown with an anti-HCA antibody. Phalloidin highlights the actin cytoskeleton (green), outlining each cell. Note that each hair cell is separated from its neighbouring hair cells by supporting cells (which lack a hair bundle).
EAR SENSORY PATCH

- hair cell
- supporting cell
- neuron

E9
The auditory patch of birds, the basilar papilla, contains hair cells possessing bar-shaped hair bundles, with many parallel staggered rows of stereocilia (Cotanche 1987). Although these hair cells all share the same basic structure, there are large variations in stereocilia height, width and number, according to location in the basilar papilla. It is thought these differences reflect the different frequency ranges over which the hair cells operate.

Sound vibrations are delivered to the cells via the tectorial membrane, the mass of extracellular matrix overlaying the hair bundles of the basilar papilla: the tectorial membrane undergoes a shearing movement relative to the hair cells, tilting the hair bundles back and forth along an axis perpendicular to the long (proximo-distal) axis of the basilar papilla. To respond to this stimulation, the hair cells all have their planar polarity vectors aligned similarly, parallel to the direction of the shear.

1.6.1 PCP is most easily visualised in the basilar papilla

By virtue of the bar-shaped hair bundle, and because all hair cells orient their bundles in the same direction, PCP is particularly easy to see in the basilar papilla. PCP in the maculae and cristae is also tightly defined and invariant. However, it is also more complicated. The maculae, in particular, have a complex PCP pattern. Swirls, not unlike the pattern of bristles seen in certain Drosophila PCP mutants, are common (Haddon et al. 1999). Nearest neighbours are closely coordinated, but greater deviation from a single vector is tolerated than in the basilar papilla. Also, the utricular and saccular maculae
contain striola regions where polarity is abruptly reversed (similar to the equator of the *Drosophila* eye) (Dennan-Johnson and Forge 1999).

The tall, tapering shape of vestibular hair bundles is such that PCP is hard to visualise by light microscopy. For this reason, most studies of vestibular PCP have exploited the greater resolving power of SEM to analyse the PCP pattern. Figure 1.4 illustrates the PCP patterns of vestibular and auditory patches.

1.6.2 *Sensory patches are defined by early expression of regulatory genes*

The events that give rise to the ordered PCP patterns occur against a backdrop of other developmental processes, governed by a variety of signalling mechanisms. It is helpful to review briefly these other aspects of sensory patch development before examining the development of PCP in greater detail.

In the developing inner ear, expression of a number of genes defines regions of the epithelium which are competent to form sensory patches. From E3, *c-Serrate-1*, a Notch ligand, is expressed in several domains from which the mature, discrete, patches later emerge (Adam et al. 1998). All cells of the patch express Serrate-1 at equal levels (as judged by immunostaining), both prior to and during the period of hair cell birth (E3-E8). Only when hair cells differentiate does *Serrate-1* expression change, becoming restricted to supporting cells.

*Serrate-1* protein expression in sensory patches can be inhibited using either a *c-Delta-1* dominant negative construct (DI-1DN) or a dominant-negative form of Suppressor of Hairless, suggesting that blocking Notch signalling downregulates Serrate-1 expression.
(Eddison et al. 2000). This implies that Serrate-1 expression may be maintained by a lateral induction mechanism, where cells of the patch signal to each other and mutually reinforce Serrate-1 expression via Notch activation.

Mouse mutants provide an insight into the functional significance of Serrate-1 expression. Serrate-1 knockout mice die before E12, however, which is before sensory patch formation. Viable mutants, in which the activity of Serrate-1 is reduced but not absent, have therefore been used instead. The headturner mouse, for example, carries a missense Serrate-1 mutation. A headturner heterozygote shows a partially penetrant phenotype which, at its most severe, includes complete loss of cristae and a reduction in outer hair cell numbers in the organ of Corti (although, curiously, an additional row of inner hair cells is sometimes also observed) (Kiernan et al. 2001). These observations suggest that Serrate-1 expression is needed to induce and/or maintain sensory patch identity.

Serrate-1 is not the only Notch pathway component known to be expressed in the developing sensory patches. Notch-1 is also broadly expressed, in regions which encompass and extend beyond the sensory regions from E3 onwards. Lunatic fringe, which can potentially glycosylate (and modify the activity of) both Notch and its ligands Delta and Serrate (Panin et al. 2002), is also expressed in sensory patches. Finally, there are two Notch ligands expressed in the hair cells themselves: Delta-1 (discussed in chapter 3) and, later, Serrate-2. It is not clear what roles these additional ligands individually have in terms of hair cell specification, although the obvious suggestion is that they mediate lateral inhibition to control the choice of hair cell fate.
Another gene expressed in developing patches is bone morphogenetic protein-4 (bmp-4) (Wu et al. 1998), (Cole et al. 2000). BMPs are part of the TGF-β superfamily of signalling molecules, which play multiple roles during development, including dorsal-ventral patterning, neural induction and the control of cell growth.

Like Serrate-1, Bmp-4 is expressed in all cells of all sensory patches. Unlike Serrate-1, it is also expressed in some non-sensory regions of the ear (e.g. in mesenchyme adjacent to the site of semicircular canal outgrowth). Also, later expression differs between patches: cristae maintain strong Bmp-4 expression up to E12 throughout the patch, maculae express it more transiently and, in the basilar papilla, expression becomes restricted to hair cells.

Although the Bmp-4 knockout mouse dies too early for analysis of the inner ear phenotype, other studies have been undertaken to address the functional significance of BMP-4. Using ectopic expression of Noggin, an inhibitor of BMP-4 signalling, a number of phenotypes have been observed (Chang et al. 1999), (Gerlach et al. 2000). These include loss of one or more semicircular canal and, sometimes, their associated cristae, general morphological defects and abnormally shaped sensory patches. Cristae apart, however, sensory patch formation proceeds normally in the presence of ectopic Noggin. The functional significance of Bmp-4 expression in sensory patches is therefore unclear. It is worth noting that, in the headturner mouse, Bmp-4 expression is strongly reduced, implying that Bmp-4 expression relies, at least indirectly, on Serrate-1.

The genes expressed from early stages throughout sensory patches establish several signalling pathways in hair cell development, notably the Notch and BMP pathways. There are also roles for FGF, Hedgehog and EGF signalling pathways at various stages of inner ear development (Ladher et al. 2000), (Riccomagno et al. 2002), (Jaszai and Brand 2002).
However, little is known about the role, if any, that Frizzled signalling might have, and how it may interact with other signalling cassettes in sensory patch formation.

1.6.3 How PCP first emerges in hair cells is unclear

Although PCP is clearly visible in mature hair cell bundles, its earliest manifestations are more subtle. When hair cells are born their apical surfaces are covered in microvilli, not stereocilia. The microvilli, which are indistinguishable from those found on other epithelial cell types, are symmetrically distributed across the hair cell surface; no visibly asymmetric structure exists (Stone and Cotanche 1991).

In the chick, the experimental organism investigated in this thesis, most electron microscopy studies have been done on the basilar papilla. In this patch, it appears that the first asymmetric marker is the position of the kinocilium, rather than the graduation of heights of stereocilia (Cotanche 1987). When stereocilia first emerge on the apical surface of basilar papilla hair cells they are of equal length and not obviously polarised. Stereocilia only become different in size and location after the kinocilium has emerged. Conflicting studies suggest either that the kinocilium emerges centrally on the hair cell surface, later migrating to one side, or that the kinocilium emerges already polarised, on one side of the cell (Cotanche and Corwin 1991), (Cotanche 1987), (Stone and Cotanche 1991). Figure 1.5 illustrates the two different theories about kinocilium emergence.
**Figure 1.4** PCP in auditory and vestibular sensory patches

Two SEM images are shown. The top image, taken of the chick basilar papilla at E12, shows the bar-shaped hair bundle characteristic of hair cells in this patch. All the bars are aligned, giving the patch a uniform PCP vector.

The lower image is of a saccular macula. Here the bundles are thinner, with a long, prominent kinocilium. In this case, the dotted line indicates a reversal of polarity. Hair cells point away from this striola region.
Figure 1.5 Two theories of kinocilium emergence in hair cells

a) A single hair cell is shown (shaded grey). On its apical surface are stereocilia (red) and a single kinocilium (green), in the centre of the cell. Initially, according to this theory, neither the stereocilia nor the kinocilium are asymmetrically localised. As the kinocilium relocates, the cytoskeleton will necessarily become polarised. External influences must guide which way the kinocilium moves, if neighbouring cells all move their kinocilia in a similar direction, even if the cells individually have an intrinsic, spontaneous symmetry-breaking mechanism.

b) According to an alternative theory, the kinocilium emerges on one side of the cell because the cytoskeleton is polarised in advance.
What is agreed upon, however, is that once the kinocilium has relocated to one side of the hair cell the stereocilia nearest the kinocilium begin to elongate faster than those further from the kinocilium, and the staircase begins to form (Cotanche 1987).

In the basilar papilla, the first hair cells displaying an asymmetry on their apical surfaces (i.e. asymmetric positioning of the kinocilium) are seen at around E8.5 to E9 (Stone and Cotanche 1991). Initially, neighbouring hair cells are not well aligned with one another. Over time, alignment becomes more precise and, eventually, the entire patch adopts a uniform PCP vector. This process is largely complete by E12 (see figure 1.4), even though the hair cell bundle itself continues to grow (Tilney et al. 1988). Figure 1.6 shows some of these key stages in the emergence of PCP in the basilar papilla.

It is not known how PCP is controlled in the ear. In this thesis, I propose that Frizzled signalling may play an important role in the process. However, other models have been invoked, which do not rely on a molecular cue for correct hair cell orientation. One theory proposes that the tectorial membrane, which overlies hair cells of the basilar papilla, shifts relative to the patch as it grows, and thereby physically reorients hair cells, so that they all point in the same direction (Cotanche and Corwin 1991). The mature stereocilia have extensive contacts with the tectorial membrane, making this an attractive model. However, electron microscopy studies suggest that the timing is awry; PCP is visible in hair cells prior to overgrowth of the tectorial membrane (Denman-Johnson and Forge 1999). Therefore, if the kinocilium does relocate, it is likely to be in response to intracellular cytoskeletal rearrangements, not extracellular physical forces.
Figure 1.6 PCP emergence in the chick basilar papilla

(A) TEM image of an oblique section through an E8.5 basilar papilla. Stereocilia are recognisable on apical hair cell surfaces by their darker appearance as compared with microvilli of neighbouring supporting cells. Kinocilia are indicated (*). Note that, although the location of the kinocilium is not random, the five hair bundles marked are not well aligned with one another.

(B) TEM image of a tangential section of an E9 basilar papilla. Shown, at high power, is a single hair cell bundle. The kinocilium, displaced to the right of the stereocilia, is recognisable by the array of microtubules within it.

(C) SEM image of the surface of an E11 basilar papilla. The majority of hair bundles shown have displaced their kinocilia in the same direction (to the left, in this image).

(D) TEM oblique section of an E11 basilar papilla. The position of the kinocilia (*) indicates that hair cells have well aligned PCP vectors by this stage.
1.6.3.1 *Intracellular asymmetries do not prefigure emergence of hair cell PCP*

Transmission electron microscopy studies (Denman-Johnson and Forge 1999), (Pickles et al. 1991) have not revealed any physical internal structure that prefigures the displacement of the kinocilium to one side of the cell. Nevertheless, relocation of the kinocilium to one side of a hair cell must require some molecular cue. If the cytoskeleton becomes polarised before the kinocilium emerges, one has to ask how early PCP is defined, compared to the visible markers on the apical surface. How long before the kinocilium emerges does the cell ‘know’ where it will be located? In the *Drosophila* wing, PCP is apparent in the subcellular localisation of PCP determinants such as Frizzled-1 and Dishevelled, many hours before actin outgrowth marks the site of trichome emergence (Strutt 2001), (Axelrod 2001). If the Frizzled pathway is performing an analogous function in vertebrate hair cells, it may be that visible, discrete structures such as the kinocilium tell us about PCP long after it has been defined. Perhaps subcellular localisation of signalling molecules marks the initiation of the process in vertebrates, as it does in invertebrates (see below).

1.7 Mutations in homologues of *Drosophila* PCP genes disrupt hair bundle integrity

There are a number of mouse mutants in which the hair bundle is incorrectly assembled. These shed light on the molecular mechanisms controlling hair bundle formation. Pertinently, a number of the mutations identify homologues of genes involved in
PCP determination or PCP expression in *Drosophila*, providing evidence that vertebrate cells may control cytoskeletal asymmetry in a similar manner to invertebrate cells.

Two cadherin genes have been identified which, when mutated in the mouse, perturb the hair cell bundle. *Cadherin 23*, a homolog of the *Drosophila* PCP gene *fat*, is expressed in hair cells. In mice lacking this gene (called *waltzer* mice), the stereocilia are disorganised. Stereocilia are splayed and disordered, but the polarity of the stereociliary array appears to be intact (Di Palma et al. 2001), (Wada et al. 2001). Strikingly, however, the kinocilium is often mislocalised, and its location is out of kilter with the polarity of the bundle.

Mutations in *protocadherin-15*, another cadherin gene with homology to *fat* and *dachsous*, produce a more overt PCP phenotype (Alagramam et al. 2001). The bundle is disorganised, as it is in CDH-23 mutants, but the kinocilium is correctly positioned with respect to the stereocilia. However, the bundle is clearly incorrectly oriented. In a null mutant mouse (the *Ames waltzer* mouse), many hair cells of the organ of Corti have completely misaligned hair bundles, indicating a severe perturbation of PCP signalling. It is unclear, however, if the vestibular patches are similarly affected.

The phenotype of these cadherin mutants is striking, but the affected genes are not thought to be direct targets of Frizzled signalling. Rather, invertebrate studies suggest they may function upstream of Frizzled-1 (Yang et al. 2002). To date, the only known downstream component of the Frizzled signalling cascade to give a hair bundle phenotype is myosin VIIa (Hasson et al. 1995), (Weil et al. 1995). In mice lacking the *Myosin VIIa* gene, the polarity of the bundle appears normal, but the bundle itself is composed of discrete clumps of stereocilia (Self et al. 1998). By analogy with the role myosin VIIa plays...
in *Drosophila*, it is as though nucleation of actin is no longer restricted to a single site: in the fly wing, loss of myosin VIIa gives a multiple wing hair phenotype (Winter et al. 2001). It is tempting to surmise the disorder seen in the mutant mouse has a similar basis. However, this is conjecture at present.

### 1.8 *Drosophila* sensory bristles as a paradigm for hair cells

I have described PCP in the inner ear, and dealt at length with the *Drosophila* PCP pathway, about which much is known, using the wing hairs as an example. This laboratory has drawn parallels between the inner ear and *Drosophila* before, and has concentrated on an analogy between the sensory patches of the ear and the mechanosensory bristles of the insect (Adam et al. 1998), (Eddison et al. 2000). In the context of Notch signalling and cell fate determination, it has been shown that parallels between the mature structures can be extended to their development, even to the extent of identifying orthologous genes playing corresponding roles in vertebrates and invertebrates. The approach has proved successful, and it is perhaps worth extending the comparison to encompass Frizzled signalling. Using the *Drosophila* sense organ as a paradigm for inner ear sensory patch formation allows testable hypotheses to be formulated, a boon when tackling a developmental process about which little is known.

The sensory bristles of *Drosophila* are part of the peripheral nervous system. Mechanosensory (or, sometimes, chemosensory) in function, each bristle emerges from the epithelium and points anterior to posterior (on the thorax and abdomen). The bristle emanates from a shaft cell, and is physically supported by the socket cell. The shaft cell
contacts a neuron, surrounded by a neural sheath cell; the neuron transmits the signal to the CNS. The mature structures' function is strikingly similar to that played by the sensory patch of the inner ear and, also, relies on correct PCP: the bristle has to be properly oriented with respect to the body plan.

When frizzled-1 is mutated, sensory bristles are misoriented (Vinson and Adler 1987). Frizzled-1 is thought to be required to interpret an anterior to posterior gradient to direct PCP in the thorax and abdomen, much as it is required for proximal-distal patterning in the wing (Shulman et al. 1998). However, Frizzled-1 has another important role in sensory organ development; it directs a crucial asymmetric cell division in the plane of the epithelium (Lu et al. 1999). Without Frizzled-1 function, cell fate determination as well as PCP is aberrant (Bellaiche et al. 2001).

1.8.1 Frizzled signalling has several distinct functions during sensory organ development

Each sensory organ arises from a single cell, termed the Sensory Organ Precursor (SOP). The SOP follows a stereotyped series of divisions to generate the four cells of the bristle plus one glial cell that migrates away (Schaefer et al. 2001). All the divisions are asymmetric; the daughter cells adopt different fates from one another. The first division produces one anterior and one posterior daughter, termed pIIb and pIIa respectively. pIIb is the precursor of the neuron, sheath cell and glial cell; pIIa is the precursor of the shaft and socket cells. The key to the difference between pIIb and pIIa can be visualised at mitosis of the SOP; protein determinants are located at specific poles of the SOP, and preferentially inherited by one daughter only (Schaefer et al. 2001). A fate determinant, the Notch
inhibitor Numb, is localised in an anterior crescent, and segregates into the pIIb daughter (Frise et al. 1996), (Knoblich et al. 1995). In numb mutant embryos, both cells lack Numb, and both adopt the pIIa fate (Rhyu et al. 1994). Conversely, Numb overexpression in the SOP causes both daughters to become pIIb cells (Guo et al. 1996).

The location of the Numb crescent is determined, at least in part, by Frizzled signalling (Gho and Schweisguth 1998). In frizzled mutants, the Numb crescent still forms, but is mislocalised, leading to an altered pattern of cell fates. It appears that Frizzled is required to define anterior in the SOP, and its absence leads to an asymmetric cell division out of kilter with the body plan. Frizzled is providing a planar signal; the SOP is an epidermal cell with normal apico-basal polarity, and relies on this Frizzled signal to define its anterior-posterior axis. However, it appears that Frizzled involvement in this asymmetric cell division is distinct from its role in PCP. It has been shown that Gα proteins operate downstream of Frizzled in the control of asymmetric cell division (Schaefer et al. 2001). However, depletion of Gα does not lead to PCP phenotypes, either in bristles or anywhere else. Therefore Frizzled is playing several roles in sensory organ development; it regulates PCP signalling, and also directs asymmetric cell division, by distinct intracellular routes.

1.8.2 Might Frizzled signalling in sensory patches act in an analogous manner to its role in sense organ development?

I have discussed the issue of PCP in inner ear development, and advanced a case for the involvement of Frizzled signalling in PCP control. Might Frizzled signalling play other roles too, by analogy with its dual role in sensory organ development in Drosophila?
Sensory patch formation may involve asymmetric cell divisions. Hair cells and supporting cells arise from a common progenitor (Fekete et al. 1998), (Stone and Rubel 2000). Since the final ratio of hair cells to supporting cells is tightly regulated, such that the mature patch is always composed of hair cells surrounded by supporting cells, asymmetric divisions may help produce these cell types in the correct number. How these asymmetric divisions might be controlled is unknown. By observation, however, it appears that many cells orient their spindles such that division occurs orthogonal to the apico-basal axis (personal observation, unpublished). The inference is that they would require a planar signal to divide asymmetrically. If Frizzled signalling controls planar asymmetric cell divisions in *Drosophila* sense organ development, might it operate in a similar fashion in the inner ear? In *Drosophila*, Frizzled signalling directs asymmetric segregation of the Notch antagonist, Numb. It is intriguing that, in the chick, Numb is expressed in inner ear progenitor cells, but later restricted to the hair cell lineage (Eddison et al. 2000). Whether this redistribution of Numb occurs by asymmetric localisation at mitosis, and what might control the process, remains to be determined.

### 1.9 Aim and scope of this work

The working hypothesis to be explored and tested in this work is that Frizzled signalling in the vertebrate inner ear controls PCP, as it does in *Drosophila* sensillum development. The *Drosophila* system requires a single, anterior-posterior PCP vector in the thorax and abdomen, and a proximal-distal PCP vector in the wing. Of the sensory patches, only the basilar papilla has a similarly simple PCP pattern. Therefore, our hypothesis
applies best to the basilar papilla, and I have concentrated on PCP determination in this patch.

However, if one also considers Frizzled signalling in the insect eye, where a polarity reversal occurs at the equator, the striola regions of vestibular patches could also, conceptually, be explained in similar terms, although away from the striola the vestibular patches contain PCP patterns that are more complex and less easily reconcilable with the current model of PCP specification in *Drosophila*.

I have sought to explore the function of the Frizzled signalling pathway in the sensory patches of the chick inner ear. Specifically, I have attempted, by in situ hybridisation, to identify frizzled genes that could be considered candidates for controlling PCP and, to a lesser extent, asymmetric cell division. And I have examined the inner ear expression of an orthologue of another PCP gene, *flamingo*, that collaborates with frizzled in control of PCP in the fly. Further, I have used virally-mediated misexpression of both full-length forms of *c-frizzled-1* and *c-frizzled-7* and dominant-negative forms of Dishevelled-1 to investigate the significance of functionally disrupting Frizzled signalling in the inner ear. Although problems of genetic redundancy make the conclusions tentative, the results suggest that the hypothesis is either wrong or simplistic: these types of disruption of Frizzled signalling that I have performed in the inner ear do not appear to alter PCP determination. The expression patterns of the genes and proteins that I have studied nevertheless still suggest that at least some components of the *Drosophila* PCP machinery have a conserved role in PCP in the vertebrate inner ear.
2 Chapter 2: Materials and Methods

2.1 Sectioning

Embryos were part dissected (an incision was made along the dorsal midline, and all tissue anterior to the eyes/posterior to the neck was removed) to aid penetration of fixative, then fixed for a limited time (to avoid destroying the serrate-1 antigen). Briefly, E3-E6 embryos were fixed for no longer than 90 minutes at room temperature in 4% PFA, with E7-E10 embryos fixed for no longer than 150 minutes.

After fixation, embryos were washed 3x in PBT (PBS and 0.1% Tween) for a minimum total time of 1 hour. Embryos were embedded in 5% sucrose, 1.5% lennox agar, equilibrated overnight in 30% sucrose, and cut into 15 or 30um sections using a cryostat. Sections were stored at -20°C in slide boxes for up to 3 months before use.

All sectioning was done by Jenny Corrigan, Department of Zoology, Oxford.

2.2 In situ hybridisation of sectioned embryos

Gene expression patterns were determined by in situ hybridisation. Riboprobes were synthesized using a Boehringer Mannheim DIG RNA labelling kit (catalogue number 1277 073), according to manufacturers instructions, using template DNA at 1ug/ul. Template
DNA was linearised (either by restriction digest or PCR amplification of the probe sequence), phenol-chloroform extracted and ethanol precipitated prior to use.

**Table 2.1** Riboprobes used

<table>
<thead>
<tr>
<th>Probe</th>
<th>Vector</th>
<th>Linearising enzyme</th>
<th>Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick Frizzed-1</td>
<td>PBK-CMV</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>Chick Frizzed-2</td>
<td>Unknown</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Chick Frizzled-4</td>
<td>Bluescript (SK+)</td>
<td>Hind III or Xho I</td>
<td>T3</td>
</tr>
<tr>
<td>Chick Frizzled-5</td>
<td>Bluescript (SK+)</td>
<td>Hind III or Xho I</td>
<td>T3</td>
</tr>
<tr>
<td>Chick Frizzled-6</td>
<td>PBK-CMV</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>Chick Frizzled-7</td>
<td>PBK-CMV</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>Chick Frizzled-9</td>
<td>PBK-CMV</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>Chick Frizzled 10</td>
<td>PAD-GAL4-2.1</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>Chick Celsr-1</td>
<td>TOPO</td>
<td>Not I</td>
<td>T3</td>
</tr>
<tr>
<td>Chick Iroquois-1</td>
<td>Bluescript</td>
<td>Not I</td>
<td>T3</td>
</tr>
<tr>
<td>Chick Iroquois-2</td>
<td>Bluescript</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
</tbody>
</table>

NB Template DNA for both *c-frizzled-6* and *c-frizzled-10* was also prepared by PCR. Using T7 and T3 primers, the *frizzled* sequence was amplified, the fragments gel-purified and used at 1ug/ul for the riboprobe synthesis reaction. All the riboprobes were between 500bp and 1500 bp in length.
All the probes used derive from partial, 3' end cDNA's. The c-celsr-1 and both c-iroquois cDNA's were provided with accompanying sequence, and subsequently checked by restriction digests. All the frizzled probes were sequenced using T3 and T7 primers, and the sequence checked against Genbank, to determine which frizzled gene was encoded.

The in situ protocol described below is based on the procedure outlined in (Strahle et al. 1994).

2.2.1 Hybridisation

Slides were defrosted for 30 minutes at room temperature, then incubated overnight with 75ul of riboprobe (diluted 1/100) in hybridisation mix (1x salts, 50% formamide (fluka), 5% dextran sulphate, 1mg rRNA (Sigma), 1x denhardt's in ddH2O) at 65°C in a sealed box on Whatman paper, wetted with 1x salts/ 50% formamide.

Slides were washed at 65°C in washing solution (1x SSC, 50% formamide, 0.1% Tween in H2O) for 15 minutes and 2 x 30 minutes, then washed at room temperature in TBST (0.01M Tris pH 8.0, 0.15M NaCl, 0.01% Tween in H2O) 2 x 30 minutes.

2.2.2 Antibody staining of DIG-labelled probes

Sections were marked out with wax pen, blocked for one hour (minimum) at room temperature with 20% heat-inactivated sheep serum and 20% Boehringer Block
(Boehringer) in TBST, then incubated overnight at 4°C with 1/1000 anti-DIG Fab fragments (Alkaline phosphatase conjugated, from Boehringer).

2.2.3 Fast red colour development

Slides incubated with anti-DIG antibody were washed twice for 10 minutes in 0.1M Tris pH8.0 in H<sub>2</sub>O. One Fast Red tablet (Boehringer) was dissolved in 2ml 0.1M Tris pH8.0, vortexed and passed through a 0.45um filter. 75ul of the filtered solution was applied to each slide, and colour development allowed to proceed for between 30 minutes and 3 hours at room temperature. The reaction was stopped by repeated washes in PBT (0.1% Tween in PBS).

2.2.4 Serrate-1 antibody staining

After in situ hybridisation and fast red colour development, slides were immunostained with anti c-Serrate-1 rabbit polyclonal antibody to visualise sensory patches. Slides were incubated for either 1 hour at room temperature or overnight at 4°C in 1/200 dilution of antibody serum in 3% BSA, 10% FCS, 0.1% Triton in H<sub>2</sub>O. After 3x washes for 5 minutes in PBT, slides were incubated with 75ul 1/500 secondary antibody in 3% BSA, 10% FCS, 0.1% Triton in H<sub>2</sub>O (the secondary antibody was goat anti-rabbit tagged with Alexa 488, from Molecular Probes).

Slides were washed 3x in PBT and mounted using Citifluor (Mount). Sections were analysed using a Zeiss LSM 510 confocal microscope.
### 2.3 Antibody stains on sections

Sections to be antibody-stained were prepared in exactly the same way as those processed by in situ hybridisation (see above).

Frozen sections were defrosted for 40 minutes at room temperature, then incubated with 75ul primary antibody (see table for dilutions) in 3% BSA, 10% FCS, 0.1% Triton in H2O for either 2 hours at room temperature or overnight at 4°C.

**Table 2.1 primary antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species raised</th>
<th>Dilution used</th>
<th>Reference (where available)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick Serrate-1</td>
<td>Rabbit (polyclonal)</td>
<td>1/200</td>
<td>(Adam et al. 1998)</td>
</tr>
<tr>
<td>Hair cell antigen (HCA)</td>
<td>Mouse monoclonal (IgG1)</td>
<td>1/200</td>
<td>(Goodyear and Richardson 1997)</td>
</tr>
<tr>
<td>GAG (retroviral p27)</td>
<td>Rabbit (polyclonal)</td>
<td>1/500</td>
<td></td>
</tr>
<tr>
<td>GAG</td>
<td>Mouse monoclonal</td>
<td>1/5</td>
<td>(Potts et al. 1987)</td>
</tr>
<tr>
<td>Haemagluttinin tag (HA)</td>
<td>Mouse monoclonal (IgG1)</td>
<td>1/300</td>
<td>(Kolodziej and Young 1991)</td>
</tr>
<tr>
<td>Calbindin</td>
<td>Mouse monoclonal</td>
<td>1/200</td>
<td></td>
</tr>
<tr>
<td>Calretinin</td>
<td>Rabbit polyclonal</td>
<td>1/200</td>
<td>(Rogers 1987)</td>
</tr>
<tr>
<td>Chick Celsr-1</td>
<td>Rabbit polyclonal</td>
<td>1/300</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Beta-tubulin</td>
<td>Mouse monoclonal</td>
<td>1/500</td>
<td>(Banerjee et al. 1988)</td>
</tr>
<tr>
<td>Acetylated tubulin</td>
<td>Mouse monoclonal</td>
<td>1/500</td>
<td>(Piperno and Fuller 1985)</td>
</tr>
</tbody>
</table>
2.4 ToPro staining

A nuclear stain was used on both sections and whole-mount cochleas and maculae. After antibody stains had been completed, specimens were washed once in Buffer C (Slow-Fade kit from Molecular Probes) then incubated with 1/500 ToPro in Buffer C for 10 minutes minimum at room temperature. Slides were mounted using Slow-Fade in glycerol and whole mount specimens were mounted in Citifluor.

2.5 Phalloidin staining

Phalloidin was used to mark the actin cytoskeleton. Fluorescently labelled Phalloidin (Molecular Probes Alexa 488, 594 or 660) was used at approximately 1/50 dilution, and added at the same time as the ToPro nuclear stain described above.

2.6 Electron microscopy

All electron microscopy work was undertaken with the kind assistance of the EM unit at 44 Lincolns Inn Fields, and in particular by Steve Gschmeissner. Both TEM and SEM were carried out on cochleae, which were initially prepared in the same fashion, as follows: Embryos were sacrificed between E8 and E14, and fixed in 4% paraformaldehyde and 2.5% glutaraldehyde overnight at 4°C (except where specimens were first analysed by
confocal microscopy, in which case glutaraldehyde was not used, since it destroys epitopes).

After repeated washes in PBS, cochleae were dissected out by opening the dorsal midline and removing cochleae from their cartilaginous surrounding. For TEM, cochleae were embedded in resin and cut into ultrathin sections. For SEM, cochleae were further dissected to reveal the surface of the basilar papilla.

2.7 Constructs for gene misexpression experiments

2.7.1 Frizzled Constructs for gene misexpression

Both c-frizzled-1 and c-frizzled-7 full-length constructs were provided, already cloned into the RCAS (B) viral vector, as a kind gift from Cliff Tabin, Harvard University, USA. Information provided with them indicated that they were cloned into the RCAS system in a similar way to the Dishevelled constructs described in detail below. Sequence of the transgenes was checked by sequencing, and the viral construct was checked by restriction digests.

2.7.2 Dishevelled constructs

Full-length human dishevelled-1 cDNA in pcDNA3 (cloned into the EcoRI site) was a kind gift of Dr M. Snyder, Yale University, USA (Semenov and Snyder 1997). All dishevelled constructs were derived from this plasmid.
The design for the constructs is based on (Axelrod et al. 1998) and (Boutros et al. 1998). The scheme was as follows:

Fragments of human dishevelled-1 were amplified by PCR (using dishevelled-1 in pcDNA3 as template) and cloned into a TOPO vector. Primers contained restriction sites for subsequent cloning steps (NcoI at the 5’ end and EcoRI at the 3’ end).

Each fragment was sequenced to ensure no errors occurred during the PCR reaction. Fragments were excised from TOPO vectors (using the restriction sites in the primers) and, where applicable, ligated together. Fragments were ligated into SLAX 12, a shuttle vector containing sequences necessary for high level expression of transgenes in the RCAS expression system. The constructs were excised from SLAX 12 using Cla I, and ligated into the ClaI site of RCAS A, the viral vector.

All Dishevelled constructs were tagged with either FLAG or HA epitope tags (or both). All tags were inserted at the C terminus of the protein. Where a FLAG tag was inserted, this was contained in the 3’ primer sequence. The HA tag, a kind gift of Trevor Duhig, (Cell Cycle laboratory, Cancer Research UK) was ligated into SLAX 12, to create SLAX HA, which was subsequently used to tag the dishevelled fragments by subcloning. The HA sequence is described in (Struhl and Greenwald 1999), and codes for three copies of the Haemagglutinin epitope, recognised by the HA.11 mouse monoclonal antibody (see table of primary antibodies used).

The table below lists all the PCR primers used, as well as a description of the dishevelled constructs they were used to make.
Table 2.3 Dishevelled deletion constructs

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Primers used (FLAG version)</th>
<th>Primers used (HA-tag version)</th>
<th>Construct description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSH (FL)</td>
<td>D5.1</td>
<td>D5.1</td>
<td>Full-length dishevelled-1.</td>
</tr>
<tr>
<td></td>
<td>DFLAGSTOP</td>
<td>DHA</td>
<td></td>
</tr>
<tr>
<td>DSH (DIX)</td>
<td>D5.1-D7</td>
<td>D5.1-D7</td>
<td>DIX domain only.</td>
</tr>
<tr>
<td></td>
<td>D8-DFLAGSTOP</td>
<td>D8-DHA</td>
<td></td>
</tr>
<tr>
<td>DSH (PDZ)</td>
<td>D5.1-D2</td>
<td>D5.1-D2</td>
<td>PDZ domain only.</td>
</tr>
<tr>
<td></td>
<td>D9.1-D10FLAG</td>
<td>D9.1-D10HA</td>
<td></td>
</tr>
<tr>
<td>DSH (DEP)</td>
<td>D5.1-D2</td>
<td>D5.1-D2</td>
<td>DEP domain only.</td>
</tr>
<tr>
<td></td>
<td>D3-DFLAGSTOP</td>
<td>D3-DHA</td>
<td></td>
</tr>
<tr>
<td>DSH (-)</td>
<td>D5.1-D2</td>
<td>D5.1-D2</td>
<td>Non-conserved regions (i.e.</td>
</tr>
<tr>
<td></td>
<td>D8-DFLAGSTOP</td>
<td>D8-DHA</td>
<td>lacking all three known</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>domains).</td>
</tr>
</tbody>
</table>

Primer sequences:

(restriction site sequences in italics, dishevelled-1 sequences in bold, FLAG tag
sequences underlined).

D2: \textit{gcagcggccggttgctga}
2.8 RCAS Virus

2.8.1 Chick embryonic fibroblast revival

For all RCAS production and titring, Line O chick embryonic fibroblasts were used. Frozen aliquots of the cells had been prepared by Mark Eddison, as described in (Morgan and Fekete 1996).

Individual vials of frozen cells were thawed at 37°C, added to 10ml culture medium (10% Fetal Calf Serum, 2% Chick Serum in E4 medium, passed through a 0.2um filter) and spun down at 800rpm for 5 minutes. The supernatant was removed, and the cells were resuspended by pipetting ten times with 5ml fresh culture medium, and then plated onto a 24cm² tissue culture flask. Cells were incubated at 37°C, at 5% CO₂.
2.8.2  RCAS Virus Production

All RCAS viral production techniques were based on (Morgan and Fekete 1996). Briefly, chick embryonic fibroblasts were passaged to 40-80% confluence on 30mm tissue culture plates and transfected using the Superfect Transfection Reagent (Qiagen, 301305). Transfected cells were then incubated at 37°C until confluent, at which point virus was harvested in one of two ways:

1) At confluence, cells were passed onto a 25 cm\(^2\) flask, then a 75cm\(^2\) flask, a 175cm\(^2\) flask and, finally 3x 175cm\(^2\) flasks. At confluence, cells were incubated overnight in harvesting medium (2% fetal calf serum, 0.2% chick serum in E4) for three consecutive days, and the supernatants pooled for virus collection (stored at −80°C).

2) At confluence, cells were passed onto a 100mm tissue culture plate. Cells were grown until superconfluent, then harvested (as in (1)) for four consecutive days. Again, supernatants were pooled for virus collection.

Pooled supernatants were passed through a 0.45um filter, then ultracentrifuged at 22000 rpm for 3 hours at 4°C using a SW40 Beckman swing rotor. The supernatant was poured off, and the pellet was gently resuspended at 4°C by rocking in the remaining liquid. 10ul aliquots were snap frozen in liquid nitrogen and stored at −80°C, until use for injection into embryos (see below).
2.8.3 RCAS Titre

Titres were determined by making serial dilutions of a single aliquot of concentrated virus (as described in (Morgan and Fekete 1996)), infecting 30mm plates of chick embryonic fibroblasts and counting infected cells (or clones of infected cells) by GAG and (where applicable) HA staining. Titres obtained are shown in the results chapters.

2.8.4 Virus Injection

Chick eggs were incubated at 37°C to between stages 10-14, windowed and injected with RCAS virus (with a trace of fast green added to aid visualisation). While the embryo remained on its ventral surface, virus was injected above the head, and allowed to stream into both otic cups. Once the embryo had turned, one cup only was injected.

After injection, windows were taped up and the egg returned to the incubator until a suitable time point.

2.8.5 Electroporation of RCAS Viral DNA

The electroporation technique described below is based on (Momose et al. 1999). Embryos were incubated until stages 12-14, windowed and electroporated with RCAS viral DNA (at 1ug/ul in PBS, 1mM MgCl₂ with a trace of fast green). For this, the DNA was injected into the otic cup and the injection needle withdrawn. In place of the injection
needle, a tungsten needle was inserted into the otic cup (and wired up as the cathode). The anode was placed adjacent to the neural tube and a 7V pulse was applied across the electrodes (three times, at 50 millisecond intervals). Successful electroporation led to a colour change of the fast green (from green to blue) and bubbles appearing on the cathode.

After electroporation, windows were taped up and the egg was returned to the incubator until a suitable timepoint for fixation.

2.8.6 Assaying effects of gene misexpression in the inner ear

Embryos fixed before E10 were processed for cryo-sectioning. Above E10, embryos were processed for dissection and analysis of sensory patches in whole mount preparation.

Embryos to be sectioned were sacrificed by decapitation. The top portion of the head was removed (anterior to the eyes), to aid penetration of fixative. Then embryos were incubated overnight at 4°C in 4% paraformaldehyde. Subsequently, embryos were sectioned as described above (section 2.1).

Embryos for ear dissection were sacrificed by decapitation, and the top portion of the head was removed. The head was opened along the dorsal midline, and the remnants of the CNS were removed. Embryos were fixed overnight at 4°C in 4% paraformaledeyhde and washed several times in PBT (PBS and 1% Triton). Sensory patches were dissected on Sylgard dishes in PBT.

Sections and dissected patches were processed in essentially the same manner. Viral infection was assayed by GAG staining (which recognises the p27 RCAS viral epitope) and sometimes, where applicable, HA.11 staining, to identify the transgene. Specimens were
also generally stained with HCA, to identify the hair bundle. Fluorescently-tagged phalloidin (Molecular Probes), which also highlights the hair bundle (as well as cuticular plate and cell outlines) was also sometimes used.
Chapter 3: Expression patterns of Frizzled signalling pathway genes during sensory patch development

3.1 Introduction

There are parallels to be drawn between the patterns of hair cell PCP and the patterns of PCP seen in invertebrate models, notably Drosophila. In order to assess whether the molecular mechanism of PCP is similar between vertebrates and invertebrates, we have looked at the expression patterns of several Frizzled signalling pathway components during chick sensory patch development. We present evidence that several frizzled genes are expressed in sensory patches, during the time of hair cell determination and, potentially, PCP specification. We also show that a flamingo homologue, c-celsr-1, is expressed in hair cells, and that the protein product of this gene has a biased subcellular distribution strikingly similar to Flamingo localisation in the Drosophila wing.

3.1.1 Little is known about Frizzled signalling in the inner ear

To date, studies of Frizzled signalling in the inner ear have been limited. Most of the available data are about expression patterns, but the stages of greatest interest from the point of view of a potential role in PCP determination have not been analysed. For
example, chick cranial placode expression has been reported for c-frizzled-1, c-frizzled-2 and c-frizzled-7 (Stark et al. 2000). These are early stages, however, well before hair cells are born (or even a sensory-competent domain has been specified). It is unlikely that these expression patterns reflect any role at this early stage for these frizzled genes in PCP signalling.

In the mouse, targeted knock out of the frizzled-4 gene leads to progressive deafness (along with other malaises not connected to the inner ear). Frizzled-4 is expressed in inner hair cells of the mouse organ of Corti. Although frizzled-4 absence leads to deafness, why the mice present with this phenotype is unclear (for example, there is no apparent cell death in hair cells lacking frizzled-4). However, there is no indication that the deafness is due to a PCP phenotype: hearing is normal at birth, and is lost only later, and there are no vestibular defects. Since PCP is determined at embryonic stages, one would expect a PCP defect to result in neonatal deafness and vestibular malfunction. Degenerative conditions, as seen in the frizzled-4 mouse, argue against a PCP defect.

Other reports of Frizzled signalling pathway gene expression in the inner ear include x-frizzled-2 (Deardorff and Klein 1999), z-frizzled-7a (Sumanas et al. 2002), wnt-8c (Ladher et al. 2000) and APC (Mogensen et al. 2002). However, none of these are useful for our purposes. The two papers describing Frizzled gene expression show whole-mount figures of early, placodal stages. Again, this is far before sensory patch development and therefore not informative with regard to PCP. Wnt-8c expression is postulated to be involved in early, inductive, signalling and, finally, APC is expressed in adult supporting cells. Nowhere in the literature is there an adequate description of Frizzled signalling pathway components in sensory patches at the time of PCP determination.
A recent report has investigated the role of canonical Wnt signalling in the chick inner ear (Stevens et al. 2002). Overexpression of an activated form of β-catenin gives two striking phenotypes. First, sensory patches are fused or merged, though it is unclear whether this represents conversion of non-sensory into sensory-competent regions, or outgrowth of existing sensory patches. Second, hair cells of the basilar papilla are converted to a vestibular phenotype (as judged by hair bundle morphology) when β-catenin is overexpressed. The implication is that active canonical Wnt signalling is sufficient for a hair cell to adopt a vestibular fate, irrespective of sensory patch identity. These findings are the first functional data on a role for Frizzled signalling in the inner ear at the time of sensory patch development. However, no PCP defects have been observed, consistent with the reported distinction between canonical and PCP signalling.

3.1.2 Hair cells are born in a scattered pattern, identified by expression of c-Delta-1

In order to make sense of the in situ data generated with the frizzled probes, it is necessary to know the timing of hair cell birth and differentiation. When sensory patches are first defined, there are no identifiable differences between the cells within them. By E4, however, the first hair cells are being born (Adam et al. 1998). The earliest marker of nascent hair cells, before any morphological change becomes apparent, is the Notch ligand Delta-1; it becomes expressed shortly after the terminal mitosis, and marks the exit from cell cycle progression and start of differentiation (Adam et al. 1998). Within all patches, Delta-1 positive cells emerge in a scattered pattern and continue to be born for several days.
(In the basilar papilla, hair cells are born in a gradient, emerging first at the proximal end of the patch; in vestibular patches, hair cell production continues throughout life).

Although hair cells are born in a scattered fashion, corresponding roughly to their eventual pattern in the mature patch, cell rearrangements do occur after the terminal mitosis. It has been observed that two hair cells can be born from the same mother cell, but no two hair cells abut in the mature patch. Studies in the chick basilar papilla have demonstrated that hair cells born together move apart in the days after birth, and that substantial cell movements occur throughout the patch (Goodyear and Richardson 1997). What controls these cell movements is unknown. That these movements occur, however, may be significant when considering how hair cells acquire PCP.

3.1.3 Hair cells acquire PCP before the stereociliary bundle matures

As explored in chapter 1, the time at which PCP is determined is still unclear, but must precede hair bundle maturation. Timing of PCP determination is of critical importance when considering genes as candidates to regulate this process; it marks a cut-off point for candidate gene expression. Baldly, a gene can only be considered a candidate if it is expressed in hair cells before or during PCP determination (as judged by the first visible asymmetric marker, on or within hair cells). Though difficult to determine precisely, the cut-off point runs roughly from E7 (in vestibular patches, where the earliest hair cells are born) through E9 (for the basilar papilla). Consequently, my analysis of gene expression patterns does not extend beyond this last cut-off point.
Ideally, an earliest time cut-off point would also be used to assay candidate genes. However, this is not a simple matter. Is it possible to state that a gene is expressed too early to affect PCP signalling? For example, must PCP determination necessarily occur after hair cell birth? If one supposes that asymmetric cell divisions give rise to hair cells, might they already possess an intrinsic polarity which will go on to define their hair bundle orientation? In light of this, I have looked at expression patterns from stage 19 onwards (when sensory patches are first defined), rather than focus on a single specific, later, time point.

3.1.4 *Expression data can be suggestive, but not predictive of gene function*

It is important to bear in mind that Frizzled signalling has numerous roles in development, and is not a PCP-specific pathway. Expression data can rule out frizzled genes as PCP candidates, but not rule them in. Frizzled genes expressed in sensory patches at the time of PCP determination may be involved in entirely separate developmental processes.

As discussed in chapter 1, a key feature of Frizzled expression during PCP in *Drosophila* lies in the intracellular distribution of the protein. Both in the wing (Strutt 2001) and, it now appears, the eye (Strutt et al. 2002), Frizzled distribution bias reflects PCP emergence. Thus, ideally, one would want to study subcellular localisation of candidate Frizzled proteins, rather than just use in situ hybridisation to analyse the mRNA. Immunostaining seems an obvious approach, but is problematic. Subcellular localisation of some PCP components in *Drosophila* cannot be visualised by immunostaining, and was not
detected until overexpressed GFP-tagged versions were introduced into cells (Strutt 2001).

I have attempted to mimic this approach, at least in part, using transgenes coding for tagged versions of Dishevelled, another PCP component which becomes localised to sites of PCP signalling (see chapter 5).

3.1.5 Other Frizzled signalling pathway components may show informative expression patterns

Frizzled receptors are only one component of an elaborate and lengthy signalling pathway. On the basis of the studies in Drosophila, other genes are also of interest as PCP candidates in the sensory patches of the inner ear. Along with frizzled genes, I have also examined the expression of c-celsr-1 (a flamingo homologue) and c-iroquois-1 and 2 (mirror homologues). These genes were selected for both theoretical and practical reasons. Theoretical, because they have restricted expression domains in other organisms tested to date (Formstone and Little 2001), (Bao et al. 1999) and their expression patterns could therefore be expected to be more informative than downstream components such as dishevelled or rho, which are often ubiquitously expressed (Sussman et al. 1994), (Malosio et al. 1997). In the case of c-iroquois-1 and 2, analogy with mirror expression in the Drosophila eye suggests specifically that one might hope to see differential expression adjacent to reversals of polarity, in or next to striola regions (mirror is only expressed in the dorsal half of the eye, and misexpression in the ventral half causes formation of ectopic boundaries of polarity reversal (Yang et al. 1999)).
Practically, only a limited number of chick sequences are available. A recent check of the chick EST database suggests that other PCP candidate genes, including several Wnt genes and c-prickle, might be tested in future, but other components remain to be cloned.
3.2 Results

3.2.1 Chick has at least ten frizzled genes, but no direct d-frizzled-1 homologue

I have investigated the expression patterns of frizzled homologues in the chick inner ear. In particular, I have sought to identify a frizzled gene which might control PCP determination. In Drosophila, PCP signalling is mediated exclusively by d-frizzled-1. There are three other frizzled family member genes in Drosophila, but none of these has been shown to signal via the PCP pathway, and the strong phenotype of frizzled-1 homozygous mutants argues against genetic redundancy at the receptor level for PCP signalling. Therefore I attempted to identify, by sequence analysis, an orthologue (or orthologues) of d-frizzled-1 amongst the chick frizzled clones. If a PCP-specific Frizzled could be identified by homology with known PCP Frizzled proteins, it would eliminate the need to explore the expression patterns of all chick frizzled genes.

Although domain-swapping studies have been undertaken between d-Frizzled-1 and d-Frizzled-2, no single 'PCP domain' has been identified in d-Frizzled-1 (Rulifson et al. 2000), (Boutros et al. 2000). The ability to signal via the PCP pathway depends on d-Frizzled-1-specific properties of extracellular and intracellular regions of the protein. This precludes a simple search for a 'PCP motif' within chick Frizzled proteins. I have therefore used whole-protein alignments to try to identify chick orthologues of d-Frizzled-1.
Also included in the alignments is a known vertebrate PCP frizzled gene, *Xenopus frizzled-7*, which is thought to be involved in convergent extension movements during gastrulation, a PCP-like process (Darken et al. 2002), (Winklbauer et al. 2001).

### 3.2.1.1 Sequence analysis has identified the chick frizzled clones we possess

My starting point was a set of partial chick frizzled clones, which had been isolated by Stefan Heller (Harvard Medical School, USA) from chick cDNA libraries, and which he kindly gave me. Eight clones in total were provided. By blasting the sequences of these clones against Genbank, I was able to identify which frizzled gene they corresponded. Further, aligning the different chick Frizzled protein sequences against each other reveals that there are groups of more closely related frizzled genes (see figure 3.1). For example, c-Frizzled-1, c-Frizzled-2 and c-Frizzled-7 are more closely related to each other than they are to other chick Frizzled proteins.

### 3.2.1.2 Some chick frizzled genes are more related to orthologues in other species than to other chick frizzleds

As well as aligning all chick frizzled sequences against each other, I blasted Frizzled protein sequences for genes expressed in the chick inner ear against Genbank, to ascertain if they were closely related to known PCP Frizzled proteins from other species. C-Frizzled-
Figure 3.1 Chick frizzled gene alignments

Two alignments are shown opposite. Both were generated using Vector NTI Align X. The top alignment is of the protein sequence coded by all ten chick frizzled genes. C-Frizzled-8 is only a partial clone, and its position is only approximately calculated (which is why the line denoting c-Frizzled-8 is reversed compared to all others).

There are several groups of more closely-related Frizzled protein sequences. For example, c-Frizzled-1, c-Frizzled-2 and c-Frizzled-7 form one closely related group, while c-Frizzled-9 and c-Frizzled-10 form another.

The second alignment is of the protein sequences coded by the chick frizzled genes expressed in the inner ear, c-Frizzled-1, c-Frizzled-5, c-Frizzled-7 and c-Frizzled-10, against two known PCP Frizzled receptors; d-Frizzled-1 and x-Frizzled-7. C-Frizzled-7 is more closely related to x-Frizzled-7 than it is to any of the other chick Frizzled proteins, but none are closely related to d-Frizzled-1.
1, c-Frizzled-5, c-Frizzled-7 and c-Frizzled-10 are all expressed in the inner ear (see results below). Of these, c-Frizzled-7 is most closely related to known PCP Frizzled genes. Notably, it is more closely related to x-Frizzled-7 than its closest chick Frizzled homologue, c-Frizzled-2. However, it is not a clear-cut correlation: c-Frizzled-7 is more closely related to d-Frizzled-2, which has no PCP function, than it is to d-Frizzled-1 (data not shown).

C-Frizzled-1 is most closely related to Frizzled-1 proteins from man and mouse, and more related to c-Frizzled-2 and c-Frizzled-7 than it is to any known PCP Frizzled proteins, while neither c-Frizzled-5 or c-Frizzled-10 are closely related to any known PCP Frizzled protein from any species.

3.2.1.3 Homology searches indicate that the best strategy is to test all available frizzled clones

Although the alignments described above suggest that certain frizzled genes might be more closely related than others to known PCP frizzled genes from other species, it was still felt sensible to test all frizzled clones in our possession. It is possible that there is more than one PCP frizzled, and that d-frizzled-1 and x-frizzled-7 do not represent the complete set of candidate PCP genes. Therefore we tested all the frizzled clones for expression in the sensory patches of the chick inner ear.
3.2.2 In situ hybridisation patterns for Chick frizzled genes

In situ hybridisations were performed on sets of sections of at least two embryos per stage per gene. The stages analysed were E4, E7 and E9, for all frizzled clones. Further stages were analysed where genes showed interesting expression patterns. In all cases, immunostaining for c-Serrate-1 was used as a marker of sensory patches.

3.2.2.1 C-frizzled-1 is expressed early in sensory patches, and is later restricted to hair cells of maculae

As stated above, c-frizzled-1 expression has been described during early stages of otic placode formation (Stark et al. 2000). However, at E3, the earliest stage I examined, I could detect no staining in the otic vesicle. It seems, therefore, that the early expression of c-frizzled-1 is distinct from the later expression patterns I describe below.

At E4, c-frizzled-1 is expressed in several broad domains (see figure 3.2), overlapping and extending beyond the sensory patches (as marked by c-Serrate-1). For example, c-frizzled-1 expression can be clearly seen in the region of the future presumptive utricular macula, as well as in the region of the future basilar papilla and saccular macula. In both these regions, c-frizzled-1 expression extends into non-sensory regions of the epithelium, beyond the expression domain of c-Serrate-1.

At later stages, c-frizzled-1 expression resolves into a complex pattern (see figure 3.3). At E8, it is expressed faintly in hair cells of the utricular and saccular maculae (faint by comparison with its expression at E4, which I consider to be strong in all sensory regions).
However, by E9, \textit{c-frizzled-1} is no longer present in the maculae at all. Also at E9, it is expressed in at least part of the lateral and posterior cristae (which cell type is hard to distinguish), as well non-sensory regions. For example, it appears to be expressed immediately adjacent to the anterior crista. \textit{C-frizzled-1} is not expressed in the basilar papilla at E9.

3.2.2.2 \textit{C-frizzled-5} is expressed in hair cells of the maculae at late stages

\textit{C-frizzled-5} expression was not detected at E4 or E7. At E8, however, low level expression was detected in hair cells of the utricular and saccular maculae (figure 3.4). No other patches were found to express \textit{c-frizzled-5} at this stage, nor was there any expression in non-sensory regions. Notably, the other maculae (lagenar and neglecta) did not show any expression of \textit{c-frizzled-5}. Therefore, \textit{c-frizzled-5} appears to have a very restricted temporal and spatial expression pattern, being switched on after hair cell birth in the saccule and utricle.

3.2.2.3 \textit{C-frizzled-7} is expressed early throughout sensory patches, and is later restricted to macular supporting cells

\textit{C-frizzled-7} expression is first seen at E4 (figure 3.5). Similar to \textit{c-frizzled-1}, its expression both overlaps and extends beyond that of c-Serrate-1. However, \textit{c-frizzled-7} is expressed in certain non-sensory areas where \textit{c-frizzled-1} is not. The expression patterns of the two genes share common features, but they do not completely co-localise.
By E7, c-frizzled-7 expression has become more complex (figure 3.6). It is expressed adjacent to the posterior crista, in non sensory regions. In the anterior crista, staining is apparent within the patch. In the utricular and saccular maculae, it is expressed in supporting cells only. This contrasts with c-frizzled-1, which is only expressed in hair cells of these patches. From partial overlap at E4, the two genes now show a complimentary pattern in the saccule and utricle.

In the basilar papilla, c-frizzled-7 is still expressed in some cells, but the pattern is difficult to interpret. There is no clear cell type distinction at this stage. It is also expressed in the non-sensory region between the saccular macula and basilar papilla.

At E9, c-frizzled-7 continues to be expressed in supporting cells of the utricular and saccular maculae (figure 3.7). It is no longer expressed in the basilar papilla, nor between the saccular macula and basilar papilla. It is, however, expressed between the utricular macula and lateral crista. No staining is apparent in the lagenar or neglecta maculae.
Figure 3.2 *c-frizzled-1* expression in the inner ear at E4.

A through D are transverse sections through the hindbrain region of an E4 chick embryo. In all images (both in this figure and all subsequent figures showing sections) dorsal is up and, where appropriate, medial is towards the centre. Red is in situ for *c-frizzled-1*. Green is immunostain for c-Serrrate-1, which marks the sensory patches.*

A shows a broad expression domain for *c-frizzled-1*. The future utricle (u.), which stains weakly for c-Serrate-1, expresses *c-frizzled-1*, and there is also non-sensory expression. *C-frizzled-1* is also expressed in the ventricular zone of the ventral part of the neural tube (nt).

B shows a more posterior section through another E4 embryo. Here, expression of *c-frizzled-1* is seen in the patch which will give rise to both the basilar papilla and saccule (bps). *C-frizzled-1* expression also extends more dorsally than the patch, into non-sensory regions (arrow).

C again shows *c-frizzled-1* expression in both the utricle, basilar papilla and saccule, while D is taken more posterior than B, and shows the *c-frizzled-1* is expressed in the region of the future basilar papilla, again extending beyond where c-Serrate-1 can be clearly visualised.

* Serrate-1 antibody, while a good marker for sensory patches, can be somewhat capricious. The epitope is sensitive to fixation conditions. Also, green fluorescence shows up less well when it colocalises with a strong in situ signal (as is the case in this figure). These factors combine to make Serrate-1 immunostain particularly weak in the sections shown. The entire specimen appears green because the laser power has been increased to visualise Serrate-1: this background noise is reduced in subsequent figures.
Figure 3.3: c-frizzled-1 expression at E8 and E9

A is a high power view of the utricle at E8. Weak c-frizzled-1 expression (red) can be detected in hair cells (hc) but not supporting cells (sc). This expression disappears by E9.

B through E are images of the three cristae at E9. B and D are of the anterior crista. Little or no c-frizzled-1 expression is seen within the patch. Rather, c-frizzled-1 is expressed immediately adjacent to it, as well as in other non-sensory regions (arrow in D).

C and E show the lateral and posterior cristae, respectively. In both cases, there is a partial overlap between c-frizzled-1 and c-Serrate-1, indicating that c-frizzled-1 is expressed only in part of the patch as well as in adjacent, non-sensory regions.
Figure 3.4 *c-frizzled-5* expression in the inner ear at E8

A and B are high power images of the utricle and saccule, respectively, taken at E8. *C-frizzled-5* in situ is shown in red, and c-Serrate-1 immunostain in green. *C-frizzled-5* is expressed in hair cells of both utricle and saccule (hc), but not in supporting cells (sc). The single cell in the supporting cell layer which does express *c-frizzled-5* (arrow in A) is presumed to be a nascent hair cell.
Figure 3.5: *c-frizzled-7* expression at E4.

A through D are images taken at E4 of *c-frizzled-7* expression in the inner ear. In situ signal is red, c-Serrate-1 immunostain is green.

*C-frizzled-7* expression is observed in both the prospective utricle and prospective saccule/basilar papilla. Expression is strongest in the saccule/basilar papilla (bps in A, B and C). Also, expression extends beyond that of c-Serrate-1 (arrows in A and C). Expression of *c-frizzled-7* is also seen in the utricle (B and D).
Figure 3.6 c-frizzled-7 expression at E7

A through C are images taken at E7, with c-frizzled-7 in situ in red, and c-Serrate-1 in green.

A shows the saccule and proximal region of the basilar papilla. In the saccule (s), c-frizzled-7 expression is seen in supporting cells (arrow) but not hair cells (arrowhead). C-frizzled-7 is also expressed in the basilar papilla (though it is not possible to determine which cell type) and in the non-sensory region between the saccule and basilar papilla (ns).

B shows the anterior crista. C-frizzled-7 expression is seen within the patch. It is not clear why c-Serrate-1 immunostain is not well colocalised with c-frizzled-7 in situ signal. It is possible that this is artifactual, and that the particularly heavy deposit of Fast Red stain obscures the green Serrate-1 fluorescence. The sensory patch almost certainly does extend along the whole ventral portion of the epithelium at this location.

C shows the posterior crista. Here, c-frizzled-7 expression is immediately adjacent to the patch.
Figure 3.7 *c-frizzled-7* expression at E9

A is a low power picture, showing the lateral crista (lc), utricle (u) and saccule (s) of an E9 embryo. *C-frizzled-7* expression is in red, c-Serrate-1 immunostain is in green.

*C-frizzled-7* expression can be seen in the supporting cell layer of the utricle and saccule (arrows). *C-frizzled-7* is also expressed in the lateral crista (arrowhead) and in the non-sensory region between lateral crista and utricle (ns).

B is a higher power picture of the saccule. The arrow shows the strong expression of *c-frizzled-7* in supporting cells, while the arrowhead shows that little or no *c-frizzled-7* is expressed in hair cells.
3.2.2.4 C-frizzled-10 is expressed in the basilar papilla

C-frizzled-10 expression was first detected at E7 (expression was not detected at E4, E5 or E6). It is expressed throughout the basilar papilla at this stage (figure 3.8). Expression is not seen in any other region of the inner ear, sensory or non-sensory. By E9 c-frizzled-10 expression is restricted to supporting cells of the basilar papilla. It has not been possible to specify when, in the intervening 48 hour period, the switch from uniform to supporting-cell specific expression occurs. Nor is it clear whether c-frizzled-10 is ever expressed in nascent hair cells (although it must, at E7, be expressed in hair cell progenitors). It is clear, however, that it is a basilar papilla-specific gene, and not expressed anywhere else in the inner ear from E4 through E9.

3.2.2.5 Several chick frizzled genes are not expressed in the inner ear

No expression was found at E4, E7 or E9 for c-frizzled-2, c-frizzled-4, c-frizzled-6 or c-frizzled-9. In the case of c-frizzled-2, it appears that this is due to the probe not being specific for the gene. Although Stefan Heller (Harvard Medical School, USA), who cloned the frizzled genes, labelled it c-frizzled-2, my sequencing data did not tally with it being a frizzled family member. It is therefore still unclear whether c-frizzled-2 is expressed in the inner ear or not (early placodal expression has been previously reported (Stark et al. 2000)).

Given the data on frizzled-4 expression in the mouse, it was perhaps expected that c-frizzled-4 might also be expressed (the basilar papilla being the chick equivalent of the
cochlea, I paid special attention to this patch). However, no expression was ever found with this probe (sequence data confirms that is a genuine frizzled-4 homologue).

C-frizzled-6 expression in the inner ear has been reported (Stefan Heller, unpublished observations). However, I was unable to replicate these findings, and could not see any expression in the inner ear at E4, E5, E6, E7 or E9 for c-frizzled-6.

3.2.2.6 C-celsr-1 (c-flamingo-1) is expressed in mature hair cells

C-celsr-1 expression was first detected in the inner ear at E9 (figure 3.9). It is also expressed in the ventricular zone of the neural tube from at least E6. In the inner ear, c-celsr-1 is expressed in hair cells of all the sensory patches. Strongest and most consistent expression was observed in the cristae. In the maculae, expression was weaker and not apparent in all hair cells of the patches. However, all staining that was seen was in hair cells. C-celsr-1 expression was not observed in supporting cells (except, possibly, in the basilar papilla. Antibody data (see below) suggests that c-Celsr-1 may be expressed in both hair cells and supporting cells of the basilar papilla). No consistent expression was observed at E6, E7 or E8 (although expression in the neural tube was observed), indicating that E9 is when c-celsr-1 becomes switched on in hair cells. Therefore, it appears that c-celsr-1 is expressed in hair cells somewhat later than the earliest markers of differentiation (such as c-Delta-1).

Expression of c-celsr-1 was also observed adjacent to the sensory patches, in a small number of abutting cells. This expression was apparent around all the sensory patches, but strongest in areas adjacent to the anterior crista and the basilar papilla.
3.2.2.7 A c-celsr-1 antibody correlates well with the in situ data

A c-Celsr-1 antibody, generously provided by Caroline Formstone (Kings College, London), has enabled us to both validate the in situ data and to shed light on the subcellular distribution of c-Celsr-1. As shown in figure 3.10, the c-Celsr-1 antibody generates an identical expression pattern to that generated by in situ against c-celsr-1, confirming that we are looking at the same gene and protein product.

C-Celsr-1 is expressed in the adherens zone of hair cells, consistent with c-Celsr-1 being a member of the cadherin superfamily. In non sensory regions, however, it is expressed evenly over the cell surface. In agreement with the in situ data, c-Celsr-1 is seen in hair cells of all the sensory patches (see figure 3.11). Hair cells of the basilar papilla show weakest expression, compared to the other patches. Also, supporting cells of the basilar papilla appear to express c-Celsr-1 at their basal surfaces. This does not necessarily disagree with the in situ data; the pattern of c-celsr-1 in the basilar papilla was difficult to interpret.

The most striking feature of the c-Celsr-1 expression pattern is the subcellular distribution within hair cells. When images are taken of the sensory patches en face, it becomes clear that c-Celsr-1 is not expressed evenly around the cell periphery. Rather, c-Celsr-1 shows a pattern remarkably reminiscent of the expression of Flamingo in the Drosophila wing, becoming concentrated at opposite ends of one axis of the cell, orthogonal to the apico-basal axis. This is seen most clearly in the cristae and utricle (see figure 3.12).
3.2.2.8 C-iroquois-1 and c-iroquois-2 are expressed in a neurocompetent region of the otocyst and in delaminating neurons

C-iroquois-1 and 2 are expressed from otic cup stages (E2.5 onwards). Their expression domains overlap: c-iroquois-1 is expressed in a broader domain than c-iroquois-2, which is expressed in a subset of c-iroquois-1 positive cells (see figure 3.13). C-iroquois-1 is also expressed more strongly at all stages than c-iroquois-2. At E3, both c-iroquois-1 and 2 are expressed in a neuro-competent ventro-medial region of the otocyst. The expression domain for c-iroquois-1 is slightly larger than for c-iroquois-2. Expression is also seen in delaminating neurons, for both genes.

No expression was seen after E3 for either gene, in any region of the inner ear (in situ were carried out at E5, E7 and E9).
Figure 3.8: c-frizzled-10 expression in the inner ear

A-C are all images of c-frizzled-10 expression (red, in situ) and c-Serrate-1 immunostain (green).

A and B are E7 embryos. A is a low power picture, showing utricle (u), saccule (s), lateral crista (lc), basilar papilla (bp), lagena (la) and neglecta (n). C-frizzled-10 is expressed only in the basilar papilla. It is not expressed in any other sensory or non-sensory region of the inner ear, although c-frizzled-10 expression can be seen in a medial area of the neural tube (nt), in a complimentary pattern to c-frizzled-1 (see figure 3.3).

B is a higher power picture of the basilar papilla. At E7, c-frizzled-10 expression is uniform throughout the patch; no cell types are distinguishable.

C is a picture of the basilar papilla at E9. Now, only supporting cells express c-frizzled-10 (arrow). Hair cells of the basilar papilla do not express c-frizzled-10 (arrowhead).
Figure 3.9 *c-celsr-1* expression in the inner ear at E9

A through E are images of E9 embryos, stained for *c-celsr-1* (in situ, red) and *c-Serrate-1* (immunostain, green).

A through C are images of the anterior crista. *C-celsr-1* is expressed in hair cells of the sensory patch (arrow), as well as in an adjacent, non-sensory region (ns). B shows a more posterior part of the anterior crista. Again, hair cell staining is apparent, and it also shows that the non-sensory region of expression abuts the patch.

C is a high power image of the anterior crista. Hair cell staining (hc) can be clearly seen.

D is a low power image, showing the utricle (u) and saccule (s). Similar to the anterior crista, *c-celsr-1* is expressed only in hair cells.

E is the basilar papilla. The staining pattern is not as clear as in the vestibular patches, but *c-celsr-1* is expressed in hair cells (the mRNA appears to be primarily localised to the basal portion of the hair cells). The staining in the tegmentum vasculosum (tv) is likely to be artifactual (the fast red staining system used often lights up in the tegmentum, even without addition of a riboprobe).
Figure 3.10 Comparison between \textit{c-celsr-1} in situ and a c-Celsr-1 antibody.

A is an image of the anterior crista, (ac) taken at E10. c-Celsr-1 Antibody immunostain is shown in green, while HCA immunostain is shown in red, and marks the hair cell bundles. Within the sensory patch, c-Celsr-1 is localised to the apical cell membrane of hair cells, just below the hair cell bundle (arrow). In the adjacent non-sensory region (ns), c-Celsr-1 expression extends evenly over the entire cell surface.

B is an image taken at an almost identical location to A, of an E9 anterior crista. \textit{C-celsr-1} in situ is in red, and c-Serrate-1 immunostain is in green. The in situ pattern reveals the \textit{c-celsr-1} is expressed in hair cells of the sensory patch (arrow). It is worth noting that, without this information, it would be difficult to prove that the staining shown in A is c-Celsr-1 in hair cells, rather than the apical projections of supporting cells.

The non-sensory expression of \textit{c-celsr-1} is also shown (ns, arrowhead). Note the good overall correlation between the in situ and antibody patterns.
Figure 3.11 C-Celsr-1 antibody patterns.

A through C are images taken at E10 of both auditory (A) and vestibular (B,C) patches of the inner ear. Green is immunostain for c-Celsr-1, while red is immunostain for HCA. In A and B, the patches are seen side-on. The apical distribution of c-Celsr-1 is clearly visible (arrows, A and B). In A, non-sensory (ns) expression can also be seen, immediately adjacent to the basilar papilla (bp).

C shows the posterior crista. Unlike A and B, C is an optical section across the patch; in effect, it shows the patch head-on. The patch is curved; the cells are being shown at different depths. In some cases, where the cells are shown just below the surface of the hair bundle, c-Celsr-1 expression appears to be biased to opposite sides of the cell (arrowheads).
Figure 3.12 c-Celsr-1 subcellular distribution is biased

A through C are images taken en face of sensory patches in the chick inner ear, with c-Celsr-1 in green and HCA immunostain in red. A is an anterior crista (ac), while B and C are images of the utricle (u). In all three images, c-Celsr-1 protein distribution is biased to opposite surfaces of hair cells. The pattern is clearest in C, which is a high power image of the utricle. The enrichment of c-Celsr-1 to opposite ends of the cell can be seen (arrowheads).

D is an immunostain (in white) for Drosophila Flamingo in the wing (adapted from Shimada et al. 2001)). Arrowheads show the proximo-distal accumulation of Flamingo protein. Note the similarities between D and C.
Figure 3.13 *C-iroquois-1* and *C-iroquois-2* expression in the inner ear

In all the images, black is in situ signal for either *c-iroquois-1* (A and B) or *c-iroquois-2* (C and D). A is a whole mount E2.5 embryo. *C-iroquois-1* is expressed in both the neural tube (nt) and the otic vesicle (ov), where it is expressed strongest in a medio-ventral region (arrow).

B through D are sections through E3 embryos. B shows *c-iroquois-1* expression. As at E2.5, *c-iroquois-1* is expressed in both the neural tube and the otic vesicle. Within the vesicle, expression is detected in a neuro-competent region of the ventral epithelium (arrow), as well as in the delaminating neurons that form the cochleo-vestibular ganglion (cvg).

C and D show *c-iroquois-2* expression. Similar to *c-iroquois-1*, *c-iroquois-2* is expressed in both the ventral part of the otic vesicle (arrow, C), in the neural tube and in the cochleo-vestibular ganglion (cvg, D).
3.3 Discussion

3.3.1 Expression of several frizzled genes is consistent with a role in PCP determination.

I have described expression patterns for several frizzled genes in the chick inner ear. Three are expressed in a pattern which is, at least in part, consistent with them being involved in hair cell PCP determination. Whether the patterns are indicative of a role in PCP determination is harder to say, given the difficulty in pinpointing the time window within which the process occurs.

In situ hybridisation shows that two frizzled genes, *c-frizzled-1* and *c-frizzled-7*, are expressed from E4 in all the presumptive sensory patches. This is well before PCP becomes apparent; before the emergence of the first hair cells even. That does not preclude their involvement in PCP signalling, however. Even at this early stage it is theoretically possible that signalling through these frizzled genes could determine PCP of hair cells. Moreover, it is possible that the protein may persist and function in PCP after the mRNA has disappeared.

*C-frizzled-10* is expressed only in the basilar papilla. It is remarkable how dynamic the expression pattern is. From no detectable expression at E6, the entire patch expresses *c-frizzled-10* at E7. This would include nascent hair cells, making *c-frizzled-10* a candidate PCP gene despite its later expression being restricted to supporting cells.

A fourth frizzled gene, *c-frizzled-5*, is also expressed in the inner ear. First detectable at E8, *c-frizzled-5* is expressed in hair cells of the sacular and utricular maculae only.
What conclusions can be drawn from these expression patterns? Overall, I feel they provide support for the hypothesis that Frizzled signalling may be involved in PCP determination in the inner ear. As stated in the introduction, expression patterns can never be more than suggestive, but that frizzled genes are expressed before and during the time window for PCP determination in most sensory patches is encouraging. There are, of course, provisos. We have not identified, by sequence analysis, a candidate ‘PCP Frizzled’. Nor have we shown than one of the frizzled genes we have investigated is expressed in all hair cells of all sensory patches, as one might hope for a strong PCP candidate. Rather, we are, I think, relying on the possibility that several frizzled genes may play a role in PCP. Between them, the frizzled genes examined are expressed in at least the majority of the total hair cell complement. In addition, there may be expression below detectable levels (or expression of frizzled genes we missed) which would improve the contention that frizzled genes are expressed in the right place to influence PCP.

3.3.2 Expression of frizzled genes indicates a role in hair cell vs. supporting cell specification in the maculae and the basilar papilla

As stated above, Frizzled signalling is implicated in numerous developmental processes, in addition to PCP. My expression analysis provides hints as to what other functions frizzled genes might have in the inner ear.

The most striking feature of the frizzled expression patterns is how, from initial uniformity, each gene becomes restricted to either the hair cell or the supporting cell lineage. Both c-frizzled-1 and c-frizzled-7 are expressed in cells of all sensory patches at
early stages. By later stages, however, *c-frizzled-1* becomes restricted to hair cells of the maculae, while *c-frizzled-7* becomes restricted to macular supporting cells. The two genes are co-expressed at an early stage of hair cell production, but by the time the maculae have their full hair cell complement (with the caveat that a small amount of hair cell turnover occurs in the mature patches) *c-frizzled-1* and *c-frizzled-7* are expressed in complementary patterns.

Similarly, *c-frizzled-10* is expressed uniformly at first, throughout the basilar papilla. By the time hair cell production is complete, however, it is only expressed in supporting cells.

*C-frizzled-5* is expressed in macular hair cells, similar to *c-frizzled-1*, but its expression is not apparent before E8. It is tempting to speculate that it too shows uniform expression at first (below the detection threshold for in situ hybridisation), but no evidence in favour of that assertion exists. Nevertheless, it fits with the general finding for all frizzled genes after E8, namely that they are all expressed in one cell type only, be it hair cell or supporting cell.

An obvious suggestion from these patterns is that Frizzled signalling is involved in specifying cell types within the sensory patches, or in controlling some very early event in the programme of cell differentiation. Functional data would be required to prove this to be the case, but the expression patterns make it a strong possibility.
3.3.3 C-Celsr-1 subcellular distribution may be indicative of a role in hair cell PCP

While the frizzled genes are expressed in complicated, temporally dynamic patterns, c-celsr-1 expression is refreshingly straightforward. C-celsr-1 is expressed in hair cells of all sensory patches. Expression is first seen at E9, when the full complement of hair cells has already been born. This makes a role for c-celsr-1 in hair cell specification unlikely: the divisions that give rise to the correct numbers of hair and supporting cells have already occurred before c-celsr-1 expression can be detected. This stands in contrast with the observations for frizzled gene expression. It is worth bearing in mind, however, that there are another two flamingo homologues, c-celsr-2 and c-celsr-3, whose expression patterns have not been analysed. Therefore, a role for flamingo-like genes in cell fate determination in the inner ear cannot be entirely discounted. The timing of c-celsr-1 mRNA expression is more consistent with a role in hair cell PCP, analogous to the role of its homologue, flamingo, in PCP in Drosophila. This possibility of a role in PCP is supported by my observations using the antibody against c-Celsr-1 to visualise the distribution of the protein product. It has confirmed the finding of the in situ experiments, that c-celsr-1 is expressed in all hair cells. Over and above this, however, it has demonstrated that c-Celsr-1 has a biased subcellular distribution. Apically located at the hair cell membrane (as expected for a cadherin superfamily member), c-Celsr-1 preferentially accumulates on opposite sides of hair cells. This pattern is strikingly similar to that shown by Flamingo in Drosophila wing cells, where Flamingo has a proximal and distal accumulation. It is striking also that c-Celsr-1 is concentrated at the apical ends of the hair cells, in the region where PCP makes itself manifest.
A curious feature of the c-celsr-1 expression pattern is the expression immediately adjacent to sensory patches. This is clearest at the edges of the basilar papilla. In non-sensory regions, c-Celsr-1 is expressed equally on the whole of the cell surface, unlike its apical distribution in hair cells. It is unclear what the significance of this expression is. Its appearance, at E9, is far later than the patches become specified (c-Serrate-1, for example, delineates the patches as early as E3). C-celsr-1 is therefore not likely to be involved in controlling the size or borders of the sensory patches, which would otherwise be an obvious suggestion. Whatever function it may serve, it is at least consistent with part of the expression patterns for c-frizzled-1 and c-frizzled-7, which are also expressed adjacent to some patches at E9. One would hope to see such colocalisation, if c-Celsr-1 is part of a Frizzled signalling system, as its homologue is in Drosophila.

3.3.4 C-iroquois-1 and c-iroquois-2 expression patterns suggest redundant roles in delaminating neurons of the inner ear

The reason for testing c-iroquois-1 and c-iroquois-2 was to investigate the possibility that they might have an involvement in PCP similar to mirror, one of three Drosophila iroquois genes, in the Drosophila eye. Mirror is expressed in one half of the eye, and acts to control the reversal of ommatidial polarity that occurs at the eye equator. Reversals of hair cell polarity occur at the striola regions of the utricular and saccular maculae, and the idea that iroquois genes might control this process is attractive. But wrong. At least, c-iroquois-1 and c-iroquois-2 do not have expression patterns consistent with such a role (with the usual proviso that there are other iroquois genes which have not been tested).
Both *c-iroquois-1* and *c-iroquois-2* are expressed early in the inner ear, at the earliest stage of neuronal delamination from the inner ear epithelium (from approximately E2.5), but are not expressed later than this, in any sensory or non-sensory regions. This finding therefore excludes them as PCP candidate genes.

3.3.5 *The expression data predict that interfering with Frizzled signalling would affect sensory patch development*

Frizzled genes are expressed in the sensory patches of the inner ear, from before the birth of the first hair cell onwards. *C-frizzled-1, c-frizzled-5, c-frizzled-7, c-frizzled-10* and *c-celsr-1* are all present in sensory patches of the inner ear at certain periods. This fact alone is enough to justify functional experiments to explore further the role of these genes. As stated in the introduction to this chapter, gene expression data can be, at best, suggestive of gene function. To glean further information about the potential role of these genes, it is necessary to either alter endogenous gene expression or misexpress full-length or mutated forms in the inner ear. The next two chapters deal with two approaches to ascertaining the function of Frizzled signalling in the inner ear: misexpression of full-length c-Frizzled-1 and c-Frizzled-7 and misexpression of dominant-negative forms of Dishevelled, the downstream effector of Frizzled.
4 Chapter 4: Functional analysis of Frizzled receptors during sensory patch development

4.1 Introduction

Our working hypothesis is that Frizzled signalling controls PCP determination and asymmetric cell divisions in the vertebrate inner ear. The analysis of gene expression patterns, described in chapter three, reveals that several frizzled genes and one flamingo-type gene are expressed during sensory patch development in the chick, consistent with these potential developmental functions in the inner ear. In order to pursue the hypothesis, it is necessary to move from descriptive to functional analysis. In this chapter I describe the use of RCAS mediated gene misexpression to analyse the function of \textit{c-frizzled-1} and \textit{c-frizzled-7}. No disruption of sensory patch development was observed with either virus. The conclusion drawn is that either \textit{c-frizzled-1} and \textit{c-frizzled-7} do not function in the predicted manner, or their roles are masked by genetic redundancy. I will also present a comparison of two ways of using the RCAS retrovirus for gene delivery into the inner ear: electroporation of viral DNA and injection of complete virus particles. I show that injection of virus particles is necessary to generate long-lived RCAS infections in the inner ear.
4.1.1 In Drosophila, disruption of PCP signalling can be accomplished by misexpression of Frizzled-1 alone

When testing gene function by overexpression or misexpression, one has to begin by considering what abnormalities one would expect or hope to elicit. The anticipated phenotypes guide the choice of detection methods to be used. An incorrect assessment of what effects gene misexpression might have can lead to the use of inappropriate markers and tests. It is therefore important to consider previously reported uses of the same or similar approaches, in the hope of identifying the most suitable assay methods.

In this case, we are testing the role of Frizzled signalling by overexpressing full-length, presumably functional Frizzled receptors, c-Frizzled-1 and c-Frizzled-7. One can imagine scenarios in which this would not be a good approach. For example, if a ligand or coreceptor is limiting, overexpression of the receptor alone would not be expected to generate ectopic signalling. With regard to the inner ear, little is known about the potential distribution of ligand or coreceptors, and this must be considered a potential drawback. On the other hand, the approach can be informative where gene dosage is important. In fact, experiments from Drosophila support the notion that overexpression of Frizzled-1 can affect PCP signalling. In the wing, driving the frizzled-1 gene under the control of the patched promoter alters the polarity of wing hairs within and adjacent to the clone. The cells realign their PCP vectors to the new gradient of Frizzled-1 signalling (Adler et al. 1997), (Usui et al. 1999).

Vertebrate studies also support this approach. In Xenopus, misexpression of full-length x-frizzled-7 causes defects in convergent extension (Medina et al. 2000). Overall, therefore,
one might expect that overexpressing the correct Frizzled receptor would disrupt normal PCP signalling in the vertebrate inner ear (if, that is, Frizzled signalling is involved in PCP determination at all).

In order to assess a PCP phenotype in the chick inner ear we have stained whole mount cochleae at E14 and E16. Making use of antibodies against components of the hair cell bundle, we have established a technique which allows us to score PCP in the basilar papilla, the chick auditory patch.

In addition, we have used sectioned E10 embryos to assess the overall development of all sensory patches. Mindful of the expression patterns of the frizzled genes outlined in chapter 3, we felt it possible that overexpression of c-Frizzled-1 and c-Frizzled-7 might alter sensory patch size and/or the ratio of hair cells to supporting cells. Using appropriate markers and stages, therefore, we have been able to assess the impact of c-Frizzled-1 and c-Frizzled-7 overexpression on both PCP determination and cell fate specification in the chick inner ear.

4.1.2 The RCAS system drives high-level, ectopic expression of a chosen transgene

As a model organism, the chick is ill-suited for conventional germ-line genetics, but offers exceptional opportunities for reverse-genetic studies of gene function in specific tissues. A key technique in chick reverse genetics is based on the RCAS retrovirus as a vector for gene delivery (Homburger and Fekete 1996), (Morgan and Fekete 1996). RCAS is a replication-competent retrovirus, derived from the Rous sarcoma virus, but with the src oncogene removed, allowing for insertion of a chosen transgene in its place (Morgan and
Fekete 1996). Transgenes of up to 2.2kb in length can be accomodated. The virus, at high
titres, readily infects a large number of different tissues in the developing chick embryo
(including the otic epithelium). Infected cells highly express the transgene as early as 16
hours after virus entry.

There are other advantages to this system, aside from the ease of infection and high
transgene expression. As an established technique, it boasts a large number of viral and
shuttle-vector variants which can be used to further optimise infection rates. Also, since the
virus is replication competent, even a low initial viral load can generate large numbers of
infected cells. In this study, for example, embryos are infected around E2 and analysed
between E10 and E16. The viral load increases considerably during this intervening period,
yielding a larger pool of cells for analysis. Meanwhile, cells that remain uninfected adjacent
to the infected patches provide a convenient control population, helping to highlight any
abnormality due to the infection.

4.1.2.1 Infection-competency can, in some cases, complicate phenotypic analysis

As with any gene delivery system, there are drawbacks. The replication competence of
the virus, for example, is a double-edged sword. Although it results in high viral loads, it
can complicate phenotypic analysis. If the virus is replication incompetent, an infected cell
must have been infected on initial injection (or be a descendant of the originally infected
cell). When RCAS is used, it is not possible to say when infection occurred. It is not clear
in the case of PCP signalling if this is a significant problem. For example, if transgene
expression began after PCP determination had occurred one could envisage obtaining a
false negative result. We do not know enough about the timing of PCP specification to be able to assert how likely this problem would be to emerge, but it must nevertheless be considered a possibility.

4.1.2.2 The RCAS virus does not always faithfully express a transgene

Another drawback with RCAS is that not all infected cells express the transgene. It is unclear why. Late infections and loss of the transgene by selection are potential explanations. There may also be variations in transgene expression as a result of the complex post-transcriptional mechanisms governing production of the different viral proteins at different times in the retroviral life cycle (Morgan and Fekete 1996). Whatever the reason, it is desirable to be able to test for transgene expression, and not rely solely on a viral marker.

The upper limit on transgene size can also cause problems. The closer one gets to the 2.2kb limit, the more often a transgene is lost or mutated, possibly because it is advantageous for the viral life cycle that the viral message be short. The frizzled genes are no longer than 1.8kb, and therefore somewhat less affected than the full-length Dishevelled-1 construct, discussed in chapter 5, which is nearly 2.1kb.

Despite these disadvantages, the RCAS system remains a robust, effective method for gene delivery in chick. It has been used successfully by the laboratory before, notably for misexpression of Delta-1 constructs in both the retina and inner ear (Henrique et al. 1997),(Eddison et al. 2000). These previous successes, along with the advantages listed above, are why I have decided to use RCAS for my functional experiments on PCP
determination in the inner ear, rather than alternative gene-delivery systems such as replication incompetent viruses of the pseudotype class.

4.1.3 **RCAS virus can be delivered by injection of virions or electroporation of viral DNA**

The RCAS system has traditionally relied on transfecting viral DNA into cultured cells, which then produce virus particles that can be harvested from the culture medium. The virus is then concentrated by ultracentrifugation, providing high titre aliquots for injection purposes (Morgan and Fekete 1996). Electroporation of viral DNA directly into cells in embryos has, however, been recently reported as an alternative method for achieving similar infections to those obtained with virions, without the need for laborious production of high titre virus (which typically takes 4-6 weeks) (Momose et al. 1999), (Nakamura and Funahashi 2001), (Yasugi and Nakamura 2000). As I shall describe below, I have attempted both methods, and find that electroporation does not, in my hands, produce a long-lived infection suitable for analysis of PCP determination.

4.1.3.1 **Electroporation of DNA into the chick inner ear has been recently reported**

Electroporation of DNA was originally developed for transforming bacteria with plasmids. It relies on the observation that electric fields can disrupt the phospholipid bilayer and so create transient pores in cell membranes, allowing charged macromolecules such as DNA to be driven by electrophoresis into the cell. Electroporation is now widely used, from bacteria, through cells in culture, to gene delivery directly into embryos. It is this last
application that can be exploited for transgenesis using RCAS. Electroporation of DNA plasmids expressing reporter genes into chick embryos was reported several years ago (Momose et al. 1999), (Nakamura and Funahashi 2001). Because the plasmids did not become integrated into the host cell DNA, reporter gene expression was transient. However, (Momose et al. 1999) suggested that electroporation of RCAS DNA into a cell should lead to integration of the retroviral genome into the host DNA and thus to production of viral particles, which could then go on to infect other cells and establish transgene misexpression beyond the life of the electroporated DNA itself.

Previous workers in the lab had successfully attempted DNA electroporation (though not using RCAS DNA) into chick embryos. With the technique established, I sought to electroporate a number of different RCAS constructs into the inner ear, and compare to the rate of infections with those generated by injection of viral particles. My results, described below, indicate that electroporation successfully produces ectopic expression of viral and transgene markers up to, but not beyond E8. Only injection of viral particles consistently gives infections beyond E8, indicating that electroporation of viral DNA fails to produce replication competent virus.

4.1.3.2 Injected viral particles were successfully used to generate informative infections

Whereas electroporation of viral DNA failed to establish long-lived infections in the chick inner ear, I will present data showing that this can be accomplished by injection of virus particles. This approach has yielded numerous informative patches of infection, and allowed me to test the hypothesis that Frizzled signalling plays a role in PCP determination
and asymmetric cell division in the inner ear. By determining the PCP orientations of infected versus uninfected hair cells, and by counting hair cell to supporting cell ratios in infected and uninfected patches, I have concluded that overexpression of c-Frizzled-1 and c-Frizzled-7 does not affect sensory patch development, at least in these respects.
4.2 Results

First, I will present data from electroporation of \textit{c-frizzled-1} and \textit{c-frizzled-7} RCAS virus DNA into the chick inner ear, concentrating on the extent of infections generated, rather than analysis of any phenotype. I will compare this with the efficacy of generating infections by injecting viral particles. Finally, I will show a phenotypic analysis of the effect of misexpressing \textit{c-Frizzled-1} and \textit{c-Frizzled-7}. I will concentrate primarily on the sensory patches at E10, and counts of hair cells and supporting cells. The amount of data on PCP determination is small, and I have saved the bulk of analysis of this phenotype for chapter 5, where I deal with \textit{Dishevelled} deletion constructs.

4.2.1 Both Frizzled RCAS constructs had been validated already

Before I present my own data, it is worth noting that I already knew that both Frizzled RCAS constructs could successfully generate infections in the chick embryo. Both \textit{c-frizzled-1} and \textit{c-frizzled-7} RCAS DNA were kind gifts of Cliff Tabin. Along with maps for both constructs, he informed me that both viruses had been used to successfully infect chick osteoblasts. Had I not known about these successful experiments, I would have validated the viral constructs myself, using a chick embryonic fibroblast cell line to test infection efficiency.
4.2.2 Electroporation gives RCAS transgene expression up to, but not beyond, E8

The electroporation scheme is illustrated in figure 4.1, and the exact protocol is given in chapter 2. A plasmid coding for the complete RCAS genome is injected into one ear, at approximately E2-E2.5. A tungsten needle, which serves as the cathode, is inserted into the otic cup. The anode, which is a larger electrode, is placed alongside the dorsal neural tube. A small current is applied, which facilitates DNA uptake into cells of the otic cup. Also, any tissue located between the cathode and anode can take up the viral DNA. This explains why the neural tube is often infected, even though that is not the primary tissue of investigation.

Table 4.1 summarises the electroporations undertaken with *c-frizzled-1* and *c-frizzled-7* RCAS virus DNA.

Also, a small number of electroporations were done with c-Delta-1 and c-Delta-1 dominant negative RCAS DNA. The attraction here was that an antibody against the transgene was available, allowing me to check that transgene protein was being produced.

The results obtained indicate that electroporation successfully leads to incorporation of RCAS DNA and transgene expression at early stages. The genome of the *c-frizzled-1* RCAS virus, for example, was strongly misexpressed at E4 in a large region of the otic epithelium, as judged by GAG staining (figure 4.2), in 3 out of 3 surviving embryos. In situ hybridisation with a probe for *c-frizzled-1* confirmed that the virus was also producing *c-frizzled-1* message, again in 3 out of 3 surviving embryos. Similar results were obtained with *c-frizzled-7* RCAS DNA electroporation (3 of 5 surviving embryos showed infections).
Figure 4.1 Electroporation of RCAS viral DNA into the chick inner ear

Shown opposite is a scheme illustrating the electroporation method, alongside a timeline showing some key stages in inner ear development. A simplified diagram shows the embryo in cross section, starting at E1.5. At this stage, the otic placodes have been specified, adjacent to the dorsal neural tube.

Electroporation is carried out at approximately E2 to E2.5. At this stage the otic cup is open and, therefore, accessible to the injection apparatus. A tungsten needle serves as the cathode, and is inserted into the otic cup. The anode is placed adjacent to the neural tube. The viral DNA (blue) is shown present in both the otic cup and the neural tube. Electroporation leads to DNA uptake in cells between the two electrodes.

By E3, cells which have taken up the viral DNA will begin transcription and translation of both viral proteins and the transgene. In theory, this should lead to the generation of viral particles, and the infection will spread.
Virus DNA injected into otic cup. Electroporation causes cells to take up DNA.

DNA within cells codes for viral proteins: spreading infection is generated.

Hair cells born
E4-8, stages 24-33

Hair cells differentiate and mature
E5 - hatching

Embryos sectioned and analysed for presence of viral and transgene markers (E3-E14).
Transgene protein expression could be seen with the c-Delta-1 antibody, as shown in the lower part of Figure 4.2. The c-Delta-1 antibody is not sensitive enough to detect endogenous levels of c-Delta-1 (Henrique et al. 1997), so any expression seen must be from the RCAS transgene which, in this case, was the Delta-1 Dominant negative form (2 out of 2 surviving embryos showed c-Delta-1 expression).

The images shown at E4, therefore, are consistent with electroporation being a highly efficient way of introducing RCAS DNA into the inner ear, with strong transgene expression.

Transgene expression is still visible at E8 (see figure 4.3). In both the ear and the neural tube (which often takes up RCAS DNA during the procedure), one can observe both c-frizzled-1 and c-frizzled-7 message at E8. Again, a strong in situ hybridisation signal correlates well with GAG staining, for both c-frizzled-1 and c-frizzled-7, and is therefore not endogenous mRNA.

After E8, however, no expression of RCAS viral genes or transgenes can be detected by in situ hybridisation, either in section or whole mount specimens. At E10, sections of c-Frizzled-1 RCAS embryos revealed no GAG staining. At E14, whole mount cochleas from embryos electroporated with c-Frizzled-1, c-Frizzled-7, c-Delta-1 and c-Delta1DN RCAS viral DNA were also all negative for GAG expression. If the RCAS virus had successfully been produced from the electroporated DNA, it would be expected to propagate and, therefore, to be detectable at these timepoints. It seems, therefore, that the electroporated DNA gave rise to transcription of both GAG and transgene, but not to new virus particles.
NB this table has exactly the same format as tables 4.2 and 5.3, to facilitate direct comparisons between electroporation of viral DNA and injection of virus particles. The column ‘infections’ refers to viral protein and transgene expression seen in these embryos. It does not, in this case, refer to use of virus particles, but instead to the results obtained from electroporation of viral DNA.
Figure 4.2 Electroporated embryos at E3 and E4

(A) An E3 embryo, stained with Serrate-1 antibody in green, and GAG antibody in red. A small number of infected cells are visible, showing red, in the otic vesicle (ov).

(B) An E4 embryo, with c-frizzled-1 in situ in red and GAG antibody in green. The yellow colour indicates the good colocalisation between c-frizzled-1 message and GAG immunostain in the otic vesicle, although it should be noted that there are regions where GAG is detected but c-frizzled-1 is not.

(C) An E4 embryo, with c-Delta-1 antibody in red and GAG antibody in green. As in B, there is a strong correlation between transgene and GAG expression in the otic vesicle. In this case, the cochleo-vestibular ganglion (cvg) is also strongly infected.
Figure 4.3 Electroporated embryos at E8

All four images are of E8 embryos, with in situ signal in red and GAG immunostain in green.

(A) and (B) Images of the cochlea, with c-frizzled-7 message shown.

(C) and (D) Images of the ventral part of the neural tube, with c-frizzled-1 message shown.

In all four cases, there is strong expression of transgene message in the epithelium, coincident with GAG immunostain.
All embryos up to E10 were sectioned. Only sections including part of the inner ear were analysed. An infection was scored if a viral or transgene marker was observed in these sections. In practice, this meant either the inner ear or neural tube: electroporation of viral DNA never resulted in viral or transgene expression outside these two tissues.

E14 cochleae were dissected out and examined by GAG immunostain, in conjunction with HCA immunostain to visualise hair bundles. Only GAG expression within the cochlea was scored. In situ hybridisation was not done on dissected cochleae because it is incompatible with HCA immunostaining (which is absolutely required to locate the basilar papilla and assess PCP).

Another problem with the electroporation technique was the survival rate. Only a little over 30% of electroporated embryos survived. This was a disadvantage when trying to generate large numbers of infected patches to analyse, and compares unfavourably with the survival rate from injected virus particles (see below).

In the light of the electroporation results, I returned to the original method of introducing transgenes, injecting complete virus particles, and examined the efficiency of infection, relative to electroporated RCAS DNA.

4.2.3 Injection of virus particles gives longer-lived infections than electroporation

Whereas little has been reported about DNA electroporation into the inner ear, much work has been done using injection of virus particles (Homburger and Fekete 1996),
(Morgan and Fekete 1996; Henrique et al. 1997). It did not, therefore, seem necessary to do
an exhaustive analysis of the behaviour of the viral infection at early stages (although, in
chapter 5, I will present data showing that transgene protein expression is detectable as
early as 3 days after viral injection, confirming that virus particles give strong early
transgene expression).

In this series of experiments, therefore, I used injection of RCAS virus particles to
generate patches of \textit{c-frizzled-1} and \textit{c-frizzled-7} misexpression in the inner ear. The
injection scheme is very similar to the electroporation scheme outlined in figure 4.1. Virus
particles were injected at the same stage as RCAS DNA was (from E2 to E2.5). The only
difference, however, was that injection of virus particles does not require a subsequent
electroporation step. Rather, immediately after injection of virus particles, the embryos can
be returned to an incubator until the appropriate age for analysis.

I assayed two timepoints: E10 and E16. At E10, all embryos were fixed and sectioned,
although only those sections containing the inner ear were analysed. At E16, cochleae were
dissected from fixed tissue and analysed as whole-mount specimens.

At both E10 and E16, viral load was assessed by immunostaining against the GAG
viral antigen. The number of embryos showing infections within the inner ear (at E10) and
basilar papilla (at E16) are summarised in table 4.2.

The expression data presented in chapter 3 is consistent with a role for Frizzled
signalling in PCP determination and cell fate specification. E10 and E16 were chosen as
timepoints for analysis of misexpression of \textit{c-frizzled-1} and \textit{c-frizzled-7} because I felt they
offered the best chance of identifying a role for Frizzled signalling in these two
developmental processes.
At E10, the full complement of embryonic hair cells has been born, and any cell fate
determination phenotype (be it due to a defect in asymmetric cell division or any other
process) should be visible. Similarly, PCP specification is clearly visible in the basilar
papilla by E16 using the Hair cell antigen antibody (Goodyear and Richardson 1997),
which marks the hair bundle. We therefore sought to examine infections generated by
injection of viral particles for *c-frizzled-1* and *c-frizzled-7* RCAS at these stages.

Figure 4.4 shows misexpression of *c-frizzled-1* at E10. In contrast with the results
obtained by electroporation, it is clear that there is a substantial virus load, with numerous
informative patches of infection. Even compared to E8 embryos that have been
electroporated and still show expression of viral genes, there is a clear improvement in the
number of cells infected. Thus injection of virus particles is clearly a more effective means
of achieving sustained infection of the otic epithelium.

E16, whole-mount cochleae were also assessed for c-Frizzled-1 and c-Frizzled-7
RCAS infection. There are two sensory patches within the cochlea; the basilar papilla and
the lagena macula. The basilar papilla runs along one side of the cochlea, and can be
viewed face-on, to assess PCP. However, the lagena macular is located at the distal end of
the cochlea, and its horseshoe shape makes it extremely difficult to analyse within the
whole mount specimen. In effect, therefore, analysis of the whole-mount cochleae involved
scoring one sensory patch only; the basilar papilla. Since only one sensory patch was being
assessed (compared to the nine assessed by sections at E10), there was an expected drop in
the percentage of specimens showing infections. Nevertheless, both c-Frizzled-1 RCAS
virus and c-Frizzled-7 RCAS virus were detected at E16, using GAG immunostaining (see
Again, in contrast to electroporation, injection of virus particles was successful in generating long-lived infections, which are indicative of replication-competency.

**Table 4.2:** Injection of c-Frizzled-1 and c-Frizzled-7 RCAS virus particles

<table>
<thead>
<tr>
<th>Inserted transgene</th>
<th>Stage</th>
<th>Number of embryos surviving/ Injected</th>
<th>Infections</th>
<th>Markers used</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Frizzled-1</td>
<td>E10</td>
<td>22/42</td>
<td>16 embryos</td>
<td>GAG showed infections.</td>
</tr>
<tr>
<td></td>
<td>E16</td>
<td>6/12</td>
<td>2 basilar papilla</td>
<td>GAG infections.</td>
</tr>
<tr>
<td>c-Frizzled-7</td>
<td>E10</td>
<td>22/42</td>
<td>14 embryos</td>
<td>GAG showed infections.</td>
</tr>
<tr>
<td></td>
<td>E16</td>
<td>4/12</td>
<td>1 basilar papilla</td>
<td>GAG infection.</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>54/108</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NB This table is essentially identical in format to table 4.1. Embryos at E10 were sectioned, and only sections including the inner ear were analysed. At E16, cochleae were dissected and stained as whole-mount specimens.

Infections were scored as positive if, at E10, GAG expression was seen in the inner ear. At E16, only infections within the basilar papilla were scored.
4.2.4 Effects of c-frizzled-1 and c-frizzled-7 misexpression in the inner ear at E10 and E16

Having established that injected virus particles give strong infections persisting at least up to E16, I asked if misexpression of c-Frizzled-1 and/or c-Frizzled-7 caused disruption of normal sensory patch development, either by altering the proportion of hair cells to supporting cells, altering hair cell PCP or by altering the boundaries of the sensory patches.

The results indicate that no alteration of any of these parameters occurred as a result of c-Frizzled-1 or c-Frizzled-7 misexpression.

4.2.4.1 Misexpression of c-frizzled-1 and c-frizzled-7 does not alter cell fate determination

I made counts of the numbers of hair cells, identified by HCA stain and apical, rounded nuclei, and supporting cells, identified by basal, obliquely spheroidal shaped nuclei, both within infected patches and in the adjacent, uninfected epithelium. Infected patches were identified by GAG immunostaining, and counts were done only where an infected patch appeared to be homogenous (i.e. all cells within the patch were infected). Figure 4.5D illustrates, within the utricular macular, a patch of c-Frizzled-7 RCAS virus misexpression which proved suitable for scoring ratios of hair cells to supporting cells. The results are summarised in table 4.3, which represents data from a total of 4 infected patches, each of at least 20um in length, and adjacent non-infected epithelium. There is no gross alteration of the ratio of hair cells to supporting cells per unit length of epithelium, compared to
adjacent, uninfected control tissue. There are differences in the absolute number of hair and supporting cells, but this is not unexpected. The number of cells does vary across sensory patches, and they are by no means homogenous (Goodyear and Richardson 1997). This means that the control tissue may vary in absolute terms from the infected tissue simply because it is in a different part of the same sensory patch, and does not imply that misexpression of c-Frizzled-1 or c-Frizzled-7 changes the absolute numbers of hair cells.

**Table 4.3** Hair cell and supporting cell counts in c-Frizzled-1 and c-Frizzled-7 infected patches

<table>
<thead>
<tr>
<th>Inserted transgene</th>
<th>Sensory patch (area analysed)</th>
<th>Uninfected hair cells/supporting cells per 10um</th>
<th>Ratio</th>
<th>Infected hair cells/supporting cells per 10um</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Frizzled-1</td>
<td>Utricle (100um)</td>
<td>2.1/7.8</td>
<td>0.24</td>
<td>1.2/5.1</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Anterior crista (50um)</td>
<td>1.25/5.6</td>
<td>0.22</td>
<td>1.7/7.0</td>
<td>0.24</td>
</tr>
<tr>
<td>c-Frizzled-7</td>
<td>Utricle (50um)</td>
<td>2.2/6.2</td>
<td>0.35</td>
<td>2.5/7.0</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Lateral crista (50um)</td>
<td>1.2/5.8</td>
<td>0.21</td>
<td>1.6/6</td>
<td>0.27</td>
</tr>
</tbody>
</table>

NB counts were done on 1 um thick optical slices, with the epithelium seen in transverse section, to ensure all counts were equivalent. Sections were stained with GAG
and HCA immunostains, and a ToPro nuclear dye. Hair cells were counted using both hair bundles and apical nuclei (in the event of a cell possessing one but not the other, presence of a hair bundle was considered as defining a hair cell). Supporting cells were counted using basal, oblique spheriodal nuclei.

Wildtype counts were done on tissue adjacent to infected patches.

4.2.4.2 C-Frizzled-1 and c-Frizzled-7 misexpression did not alter sensory patch integrity

The majority of E10 embryos had infections in only one ear. Using HCA and GAG immunostaining, it was possible to compare the size of individual sensory patches in the infected ear with those in the uninfected ear. I did this for all sensory patches where the majority of cells were infected. In total, I looked at 12 patches for c-Frizzled-1 and 8 patches for c-Frizzled-7 misexpression. I did not observe any differences between infected and uninfected patches. As judged by eye, there was no observable difference in the size of the sensory patch, location of the sensory patch boundaries or position of the relative sensory patch relative to other sensory patches, for either c-Frizzled-1 or c-Frizzled-7 RCAS virus infections.
**Figure 4.4** c-Frizzled-1 misexpression in the inner ear at E10

Images A through D are of E10 embryos, immunostained for GAG (green), HCA (red) and ToPro nuclear stain (blue).

(A) Shown are the utricle (u) and lateral crista (lc). There are two separate patches of viral infection. One is in a non-sensory region, while the other is within the utricle.

(B) Shown is an infected patch within the anterior crista (ac),

(C) An infection that encompasses part of the lateral crista and adjacent non-sensory epithelium.

In A through C, there is no obvious defect in either sensory or non-sensory regions of the otic epithelium, as judged by hair cell bundle stains and nuclear markers.

(D) A high power image, showing that the c-Frizzled-1 virus successfully infects both hair cells and supporting cells (arrow and arrowhead, respectively).
**Figure 4.5** c-Frizzled-7 misexpression in the inner ear at E10

Images A through D are of E10 embryos, immunostained for GAG (green), HCA (red) and ToPro nuclear stain (blue).

A and C show infections predominantly in non-sensory regions, while B and D show infections within sensory patches.

(A) The saccule (s), utricle (u) and lateral crista (lc) are all visible. There are several patches of infection, including two that appear to immediately flank the lateral crista.

(B) The saccule and utricle are shown. It is particularly striking that nearly the entire length of the utricle is infected.

(C) The anterior crista (ac) and utricle are largely devoid of GAG immunostain, but the surrounding non-sensory regions are heavily infected.

(D) Shown, at higher power, is an infection of c-Frizzled-7 virus in the utricle. Note that there are no apparent differences between the infected region, in green, and the uninfected region. This indicates that c-Frizzled-7 misexpression does not alter gross patch morphology.
4.2.4.3 Misexpression of c-frizzled-1 and c-frizzled-7 did not alter wildtype PCP in the basilar papilla

4.2.4.3.1 Hair cell PCP could not be assessed at E10

I attempted to assess PCP at E10 using sectioned tissue. All sections were cut to a thickness of 30μm, which is a depth of approximately 5-6 cell diameters. Using a combination of HCA immunostaining and phalloidin staining of the actin cytoskeleton, I attempted to use confocal microscopy to generate 3D reconstructions of the surface of the basilar papilla from a series of optical slices. However, the resolution proved insufficient to assess PCP by this method.

I also attempted to assay PCP in both auditory and vestibular patches by using a combination of β-tubulin types I and II immunostaining and phalloidin. Staining for β-tubulin is meant to identify the kinocilium (Craig Stevens, personal communication). In conjunction with phalloidin, which marks the actin cytoskeleton, it can be used to assess PCP of hair cells (Haddon et al. 1999). However, I was unable to mark the kinocilium with this, or any other anti-tubulin antibody. Therefore, I was unable to assess the effect on PCP determination of misexpression of c-Frizzled-1 and c-Frizzled-7 at E10.
4.2.4.3.2 Hair cell PCP in the basilar papilla was unaltered by c-Frizzled-1 or c-Frizzled-7 misexpression at E16

I generated a small number of infections in cochlea of E16 embryos with c-Frizzled-1 and c-Frizzled-7. At this stage, HCA immunostaining can be used to accurately assess hair cell PCP, as shown in figure 4.7. Two infections were achieved with the c-Frizzled-1 RCAS virus, and one with the c-Frizzled-7 RCAS virus. The results, which were too small to be considered definitive, indicate that overexpressing c-Frizzled-1 or c-Frizzled-7 does not alter wildtype PCP determination in the basilar papilla. For both c-Frizzled-1 and c-Frizzled-7, hair cells staining positive for the viral marker can be seen to have normal hair bundles which are correctly oriented with respect to their neighbours.
Figure 4.7 Misexpression of c-Frizzled-1 and c-Frizzled-7 in the basilar papilla at E16

In both cases, GAG immunostain is in green, HCA immunostain is in red and phalloidin stain is in blue.

(A) A c-Frizzled-1 RCAS virus infection, at low power. The image is an optical section tangential to the surface of the basilar papilla at E16. Note that hair cells which are GAG positive have hair bundles oriented in a similar direction to adjacent, uninfected hair cells.

(B) A c-Frizzled-7 RCAS virus infection, at high power. The image shows the basilar papilla in transverse section at E16. A single GAG-positive hair cell is visible. Again, its hair bundle is aligned with that of the neighbouring, uninfected hair cells.
4.3 Discussion

4.3.1 Electroporation of viral DNA fails to establish long-lived gene misexpression

The results presented indicate that electroporation of RCAS DNA is not equivalent to injection of virus particles. The key difference was the longevity of transgene expression. In the case of DNA electroporation, transgene expression was lost by E10, which is 8 days after DNA uptake. By contrast, injection of virus particles at E2 gave robust viral expression as late as E16, or 14 days after viral uptake. This time difference may be critical when assaying PCP determination, and I have therefore concluded that, for my purposes, virus particle injection is the method of choice.

There are additional problems with electroporation as a technique, even where transgene expression does not need to be so long-lasting. In embryos that do successfully incorporate the viral DNA, there tends to be a lighter viral load than that achieved by virus injection. A greater number of GAG-positive cells is seen in embryos injected with virus particles than with electroporated viral DNA (although individual cells appear to express similar levels of GAG). This is unsurprising; if the virus is failing to spread, it will necessarily result in fewer infected cells. Also, there is only moderate survival. It is not clear why electroporation should cause such high mortality, but the effect is to increase the numbers of electroporations needed to generate sufficient infections for scoring phenotypes. Finally, electroporating a single embryo can take several minutes more than injecting a single embryo with virus. The difference, though small, becomes significant
when large numbers of specimens are required, and adds to the general inefficiency of
electroporation as a technique.

On initial inspection, a surprising feature of the electroporation results was that, at E8,
there was still strong viral and transgene expression, but by E10 all viral and transgene
markers were lost. Transgene expression at E8 is six days after DNA uptake, and the
original paper on electroporation in chick reported transgene loss within 48-72 hours
(Momose et al. 1999). I therefore originally took the expression at E8 as strong evidence
that new RCAS virus was being produced, and was propagating an infection which would
otherwise be lost as the electroporated DNA was degraded and diluted. However, the lack
of expression at E10 and E14 indicates that RCAS virus particles are not produced from
electroporated DNA. Instead, it appears that electroporated DNA can survive longer than
originally thought.

Recently, evidence to support the idea of electroporated DNA longevity has emerged,
and our observations are consistent with this new data (Groves 2001). Other groups have
reported transgene expression up to 6 days after electroporation, without viral sequences
needed for propagation of an infection. This is consistent with my findings that
electroporation at E2 can result in transgene expression at E8, without a requirement for in
vivo production of infectious virus particles.

4.3.2 Injecting viral particles gives long-lived and heavy viral infections

The key problems with electroporation of RCAS DNA, namely short-lived infections,
moderate viral load and poor survival are all overcome by injection of RCAS virus
particles. With the proviso that a successful viral titre is required, virus particle injection consistently gives strong infection of the otic epithelium, in both sensory and non-sensory regions. Crucially, for my purposes, infections endure until the late stages necessary for assaying transgene effects on cell fate and PCP determination. Overall, therefore, it seemed sensible to pursue the question of c-Frizzled-1 and c-Frizzled-7 function using injection of virus particles.

4.3.3 C-frizzled-1 and c-frizzled-7 overexpression fails to disrupt sensory patch development

Injection of virus particles gave good infections for both c-Frizzled-1 and c-Frizzled-7 RCAS viruses, and allowed me to look for possible effects of misexpressing these Frizzled receptors. Disappointingly, however, I either failed to generate a phenotype, or failed to spot one. In chapter 6, I will deal in more detail with the implications of the negative results from these experiments, as well as the experiments one might consider to resolve the issue of whether or not Frizzled signalling has the roles in inner ear development that we hypothesise. However, there are a number of possibilities which relate specifically to the use of Frizzled receptor overexpression which I will cover here.

4.3.3.1 Overexpression of Frizzled receptors in other systems

In the introduction to this chapter, I described successful uses of Frizzled receptor overexpression to perturb PCP signalling (Medina et al. 2000), (Usui et al. 1999). Do they,
however, represent an exception to the rule? Might it be that Frizzled receptor overexpression will only work in certain developmental circumstances?

In the cases where Frizzled overexpression has been successful, it has already been shown that the mutant case, loss of that particular Frizzled receptor, gives a phenotype. For example, loss of *Drosophila frizzled-1* was shown to perturb PCP signalling in the fly wing (Vinson and Adler 1987) long before overexpression of *frizzled-1* was shown to also have a PCP phenotype (Krasnow and Adler 1994). In essence, the overexpression experiments are conducted in a system where genetic redundancy is not thought to be a concern. In our case, we do not know the mutant phenotype for loss of either *c-frizzled-1* or *c-frizzled-7*. However, we do know that their expression patterns in the inner ear are extremely similar (at least at E4), raising the spectre of genetic redundancy at the receptor level for Frizzled signalling. This must therefore stand as a potential reason behind the failure of overexpression of *c-frizzled-1* and *c-frizzled-7*.

In addition to a role in PCP determination, Frizzled-1 in *Drosophila* is required to orient the asymmetric cell division of the sensory organ precursor cell (SOP) (Lu et al. 1999). In this case, however, there are no reported uses of overexpression of a Frizzled receptor to disrupt this control mechanism. Rather, the reports present loss-of-function data. Again, with regard to a potential role for c-Frizzled-1 and c-Frizzled-7 in cell fate specification via control of asymmetric cell division in the chick inner ear, it is not clear that overexpression of a single Frizzled receptor will disrupt the process.
4.3.3.2 Practical and theoretical reasons governed our choice of c-Frizzled-1 and c-Frizzled-7 for misexpression experiments

The in situ and antibody data in chapter three suggested that four frizzled genes and one flamingo gene would all be interesting candidates for roles in either cell fate and/or PCP determination. I have only analysed two of these, c-frizzled-1 and c-frizzled-7 by RCAS-mediated misexpression.

The availability of full-length clones initially dictated this choice: both c-frizzled-1 and c-frizzled-7 were available, already cloned into RCAS vectors. This was not the case for c-frizzled-5 and c-frizzled-10 (the probes used in chapter 3 were generated from partial cDNA's). Another reason for selecting c-frizzled-1 and c-frizzled-7 was the sequence analysis, which revealed that both were closely related to other frizzled-7 genes, which have been implicated in convergent extension control in frog and fish (Medina et al. 2000). Therefore, my initial experiments concentrated on the use of c-frizzled-1 and c-frizzled-7 misexpression.

Although I did embark on the construction of a full-length c-frizzled-10 construct, I discontinued this in the light of the negative results with c-frizzled-1 and c-frizzled-7. Rather than continue with efforts to clone full-length c-frizzled-10 (and c-frizzled-5), I instead chose to pursue the option of making Dishevelled constructs (see chapter five) which, I hoped, would circumvent the need to misexpress the correct frizzled gene, and would be less subject to problems of redundancy. In vertebrates, as in Drosophila, there are many frizzled genes, but only a few dishevelled genes (one in Drosophila), on which Frizzled signalling depends. Moreover, the modular nature of the Dishevelled protein
makes it possible to target specific intracellular signalling pathways downstream from Frizzled receptors in a dominant-negative fashion. These experiments are described in chapter 5.
5 Chapter 5: Disruption of Frizzled signalling by modulating Dishevelled function

5.1 Introduction

In chapter 4, I described misexpression of c-Frizzled-1 and c-Frizzled-7 using RCAS viruses. The results did not reveal a function for these receptors in the chick inner ear. Yet these genes are expressed there in a highly suggestive pattern, and there are strong reasons to expect that the Frizzled signalling pathway should be important in ear development. The failure to detect a phenotype following overexpression of c-Frizzled-1 and c-Frizzled-7 does not rule out the existence of a function for Frizzled signalling in the ear. I therefore sought to assess Frizzled pathway function in the inner ear by a different means. In this chapter, I describe the use of Dishevelled-1 deletion constructs. These were designed to impinge specifically on canonical or PCP pathway functions of Frizzled signalling, in a dominant negative fashion. The results from these experiments, as from those in chapter 4, proved negative. Although Dishevelled deletion constructs were successfully tagged and shown to be expressed in the inner ear from E5 through E16, I was unable to detect any disruption of either cell fate or PCP determination in the inner. Though still not conclusive, when taken in conjunction with the data from chapter 4 this suggests that our original hypothesis about the role of Frizzled signalling is incorrect, or at least simplistic.
5.1.1 Misexpression of full-length Frizzled receptors is a limited approach to disrupting wild type Frizzled signalling

While exploring the consequences of misexpressing full-length frizzled genes in the chick inner ear, it became clear that a more incisive set of molecular tools would be useful. Even before I came to the conclusion that c-Frizzled-1 and c-Frizzled-7 overexpression gave no phenotype in the inner ear, there were theoretical reasons for wanting alternative viral constructs. Whatever results were obtained using the viruses driving overexpression of full-length receptors, it would be desirable to do an inverse experiment to test the effect of blocking the signalling pathway. With the full-length receptors as our only molecular tool, we had no way of doing this. One might contemplate using a dominant-negative Frizzled construct, but there is no well-established way to generate a dominant-negative allele. Another approach would be through gene knock-out in the mouse, but we do not currently know enough about frizzled expression patterns in the mouse inner ear to know the best candidates. In any case, it is always possible that genetic redundancy would mask effects at the receptor level, and could only be overcome by impinging on downstream signal transduction.

Another problem arises from the branched nature of the Frizzled signalling pathway, which is thought to trifurcate downstream from the receptor into Canonical, PCP and Ca\textsuperscript{2+} intracellular branches. Any phenotype observed through misexpression of a Frizzled receptor could be due to effects on any of these three downstream pathways. How to distinguish them?
To address these problems, I decided to make use of published data on the downstream effector, Dishevelled, to design a series of deletion constructs. These would serve as tools to block Frizzled signalling, so as to do the reciprocal experiment to overexpression of full-length Frizzled receptors and would also make it possible to interfere specifically with either the canonical or the PCP signalling pathway.

5.1.2 Dishevelled constructs can specifically inhibit canonical or PCP signalling

As briefly covered in the introduction, Dishevelled has three domains, differentially employed in the canonical and PCP branches of the Frizzled signalling pathway (the Ca\(^{2+}\) branch of the pathway may, in some cases, work independently of Dishevelled (Winklbauer et al. 2001), and I therefore did not consider in depth how one might impinge on it selectively). The DIX domain of Dishevelled appears to function specifically in the canonical signalling pathway; the PDZ domain appears to function in both canonical and PCP signalling; and the DEP domain is required specifically for PCP signalling.

My main interest was in finding a Dishevelled deletion construct which would interfere specifically with PCP and act in a dominant negative fashion.

In *Drosophila*, a Dishevelled construct which lacks the DEP domain is unable to translocate to the membrane and is PCP defective (Axelrod et al. 1998). Surprisingly, it has a dominant negative effect, blocking normal PCP determination even in the presence of a wildtype *dishevelled* allele. Since it does not locate to the presumed site of PCP signalling, and yet impedes wildtype function, the presumption is that it sequesters other signalling components, preventing them from binding wildtype Dishevelled in a Frizzled-Dishevelled
signalling complex. Conversely, a construct containing only the DEP domain is able to translocate to the membrane, but also blocks wildtype Dishevelled function in a dominant negative fashion. The effect is specific to PCP signalling; expressing the DEP domain alone has no effect on canonical Wnt signalling. Both removal and sole expression of the DEP domain can yield dominant negative PCP-specific effects in Drosophila, therefore.

While Drosophila studies indicated that two constructs could fulfill our criteria, vertebrate studies have tended to make use of the overexpressed DEP domain alone. A number of papers have found that overexpression of the DEP domain of Xenopus Dishevelled inhibits non-canonical Frizzled signalling and convergent extension movements, in both frog (Wallingford et al. 2000) and fish (Heisenberg et al. 2000). This form of Frizzled signalling has been shown to involve many of the components that are peculiar to the PCP pathway in Drosophila. Taken together, the literature indicated that the approach most likely to impinge on PCP signalling in the inner ear was overexpression of the DEP domain.

While a PCP-specific construct was a priority, I also wanted to test the effect of each of the three Dishevelled domains expressed separately. In this way I hoped to uncover other potential roles of Dishevelled in both canonical and PCP signalling. As controls, I also made full-length Dishevelled and a Dishevelled construct lacking all three conserved domains (in case there are other important regions within the gene which have not been identified to date). Figure 5.1 shows the design of these constructs. As a set, I hoped they would allow us to tackle Frizzled signalling in the inner ear in a more coherent and comprehensive manner than just overexpression of the Frizzled receptors.
5.1.3 How Dishevelled constructs elicit their effects depends on interacting partners

Apart from the in vivo effects of deletion constructs, much work has been done to identify the specific proteins that interact with the various domains of Dishevelled. The findings are summarised in table 5.1. A number of points deserve special consideration here. First, all components for which an interacting domain has been identified are consistent in their functions. For example, DIX interactors have known functions in the canonical branch of the Frizzled signalling pathway. Similarly, the only protein known to bind the DEP domain, Prickle, functions in PCP determination. This validates the approach of using individual domains to elicit pathway-specific effects, and also tallies well with the in vivo data generated (Tree et al. 2002). Second, the data hint at how Dishevelled might interact with the cytoskeleton, which is presumed to be the readout for inner ear PCP determination. For example, Dishevelled has been shown to interact with Daam I, which bridges it in a complex with Rho A (Habas et al. 2001).

An intriguing interaction with Notch has been reported (Axelrod et al. 1996). Given the role Notch is thought to play in cell fate determination in the inner ear, it is tempting to speculate on pathway crosstalk between Frizzled and Notch signalling, mediated directly by Dishevelled. But, at present, no evidence exists in favour of such an interaction in vertebrates, although recent evidence from the Drosophila eye is, at least, consistent with the idea of Dishevelled interacting directly with Notch (Strutt et al. 2002).
Table 5.1 Known Dishevelled interactors

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Protein type</th>
<th>Dishevelled interaction site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axin</td>
<td>Negative regulator of canonical signalling</td>
<td>DIX domain</td>
<td>(Li et al. 1999)</td>
</tr>
<tr>
<td>GSK-3β (shaggy)</td>
<td>Kinase. Targets β-catenin for degradation.</td>
<td>DIX domain</td>
<td>(Li et al. 1999)</td>
</tr>
<tr>
<td>Naked cuticle</td>
<td>Antagonises canonical signalling. May upregulate PCP signalling.</td>
<td>PDZ domain</td>
<td>(Rousset et al. 2001), (Yan et al. 2001)</td>
</tr>
<tr>
<td>Prickle</td>
<td>Antagonises dishevelled PCP function.</td>
<td>DEP domain</td>
<td>(Tree et al. 2002)</td>
</tr>
<tr>
<td>Dishevelled</td>
<td>Dishevelled interacts with itself</td>
<td>N-terminal region</td>
<td>(Rothbacher et al. 2000)</td>
</tr>
<tr>
<td>Strabismus</td>
<td>Antagonises canonical signalling. Activates PCP signalling.</td>
<td>PDZ domain</td>
<td>(Park and Moon 2002), (Darken et al. 2002)</td>
</tr>
<tr>
<td>Daam I and RhoA*</td>
<td>Daam I is a diaphanous homologue.</td>
<td>Not determined</td>
<td>(Habas et al. 2001)</td>
</tr>
<tr>
<td>Frodo</td>
<td>Positive regulator of Wnt signalling</td>
<td>Not determined</td>
<td>(Gloy et al. 2002)</td>
</tr>
<tr>
<td>Dapper</td>
<td>Dishevelled antagonist</td>
<td>Not determined</td>
<td>(Cheyette et al. 2002)</td>
</tr>
<tr>
<td>Par-1</td>
<td>Kinase. Positive for canonical, negative for PCP signalling.</td>
<td>Numerous potential phosphorylation sites</td>
<td>(Sun et al. 2001)</td>
</tr>
<tr>
<td>Notch</td>
<td>Cell fate determinant</td>
<td>Not determined</td>
<td>(Axelrod et al. 1996)</td>
</tr>
<tr>
<td>β-arrestin</td>
<td>Scaffolding protein</td>
<td>DIX domain</td>
<td>(Chen et al. 2001)</td>
</tr>
</tbody>
</table>

* DaamI binds Dishevelled directly, and also forms a ternary complex containing Dishevelled and Rho A.

A caveat to table 5.1 is that it is an amalgamation of data gleaned from a number of different organisms, and from different cellular and developmental contexts. It would be

158
wrong to assume that an interaction identified in one context will necessarily occur in another.

The table is a useful guide, but not a secure means for predicting function in unexplored tissues. Thus, it is not known what roles, if any, dishevelled may have in the inner ear. Some, or even all, of the interacting partners listed in the table may be expressed in the inner ear. Conversely, none of them may be present, and Dishevelled may interact with factors not listed above. We have been mindful of the biochemical data when designing the constructs and experiments described, but are also aware of the limitations of the data.

5.1.4 Vertebrate dishevelled homologues operate in the Frizzled signalling pathway

There are three dishevelled homologues in all completed vertebrate genomes to date, including Homo sapiens and Mus musculus. In the mouse, dishevelled-1, dishevelled-2 and dishevelled-3 are expressed in all tissues during embryonic development (Sussman et al. 1994), (Klingensmith et al. 1996), (Tsang et al. 1996). Further, biochemical investigation of murine Dishevelled proteins indicates that they operate in a broadly similar manner to their invertebrate counterparts within the Frizzled signalling pathway (Lee et al. 1999). It is difficult, from the data presented, to state what the expression patterns are in the inner ear. The overall impression given by the published data, however, is that dishevelled homologues are ubiquitously expressed during embryonic development, and that Dishevelled operates in the vertebrate Frizzled signalling pathway similarly to its role in invertebrate Frizzled signalling.
5.1.5 **Subcellular localisation, a key factor in PCP signalling, can be visualised using a tagged transgene**

Another unresolved issue arising from the Frizzled misexpression experiments is subcellular localisation. As discussed in the introduction, the asymmetric localisation of PCP determinants, including Frizzled, Dishevelled, Prickle and Flamingo, prefigures PCP emergence in the insect wing (although it is worth noting that, in insect sensory organ development, similar molecular asymmetries have not been described in relation to PCP). In *Drosophila*, overexpressed versions of Frizzled and Dishevelled, tagged with GFP, are thought to localise in the same manner as the wildtype proteins (Strutt 2001), (Axelrod 2001). It appears that the machinery for normal localisation of Frizzled and Dishevelled has spare capacity, enabling it to localise far higher levels of these proteins than occur normally in wildtype cells. By extension, one might hope that overexpressed versions of Frizzled and Dishevelled would show similar asymmetric localisation in the vertebrate inner ear and that this would mimic localisation of wildtype protein and prefigure the polarity of the hair bundle.

Neither the c-Frizzled-1 nor the c-Frizzled-7 RCAS constructs were tagged, and no antibodies are available against either protein. Attempts to tag them proved unsuccessful: the cloning strategy used to make them proved an insurmountable obstacle when trying to introduce either a FLAG or HA tag (restriction sites necessary for insertion of tags were destroyed during creation of the Frizzled RCAS constructs). It was also unclear where best to place the tag, since the c-Frizzled-1 and c-Frizzled-7 receptors have ligand-binding N-termini and putative PDZ-interacting C-termini, either of which might be disrupted by
introducing a tag. Therefore, I decided to focus my efforts on the Dishevelled constructs, tagging them with a FLAG epitope or, in later experiments, with an HA epitope. It was hoped that the different Dishevelled constructs might show differential ability to associate with the membrane, depending on the presence or absence of the DEP domain (which is believed to contain sequences required for membrane association). Also, I hoped to see asymmetries in cell membrane accumulation in hair cells, similar to both the subcellular localisation of Dishevelled in the *Drosophila* wing and to the subcellular localisation of c-Celsr-1 in the vestibular patches of the chick inner ear.
5.2 Results

5.2.1 RCAS viruses carrying tagged versions of Dishevelled were successfully produced

The RCAS Dishevelled viruses were constructed as described in chapter 2. After the virus was harvested, aliquots of the concentrated stocks were tested for infectivity on chick embryonic fibroblasts in culture. This was both to assay the viral titre and to check transgene staining.

Viruses carrying FLAG-tagged transgenes were successfully produced for all five Dishevelled constructs (figure 5.1 shows the structure of the different constructs). HA-tagged versions were made for all constructs except the full-length Dishevelled-1 (where addition of the HA tag would have yielded a 2.3kb insert, making it too big to be successfully expressed using the RCAS system).

Table 5.2 shows the titres of the different viral constructs. As a rough guide, a titre of $10^6$ viral units per ml is considered the minimum needed for successful generation of infected patches. $10^7$ units or above is preferable.
**Table 5.2** Dishevelled viral titres

<table>
<thead>
<tr>
<th>Inserted transgene</th>
<th>Tag</th>
<th>Approximate Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIX</td>
<td>HA</td>
<td>$10^9$</td>
</tr>
<tr>
<td>PDZ</td>
<td>HA</td>
<td>$10^8$</td>
</tr>
<tr>
<td>DEP</td>
<td>HA</td>
<td>$10^6$</td>
</tr>
<tr>
<td>DEP</td>
<td>FLAG</td>
<td>$10^7$</td>
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<tr>
<td>Full length</td>
<td>FLAG</td>
<td>$10^7$</td>
</tr>
<tr>
<td>Negative control (-)</td>
<td>HA</td>
<td>$10^8$</td>
</tr>
</tbody>
</table>

Figure 5.2 shows the relative staining intensities of FLAG-tagged and HA-tagged transgenes. It is clear that the HA tag system gives stronger expression (assuming the transgenes are being expressed at similar levels, which should be the case). Therefore, all misexpression experiments were initially carried out with HA-tagged versions of the Dishevelled constructs. Two FLAG-tagged Dishevelled viruses were also used: the full-length construct (for which there is no HA-tagged version) and the DEP-containing construct, because the HA-tagged DEP construct virus gives only low levels of infection.
Figure 5.1 Dishevelled deletion constructs and protein sequence alignments

Shown opposite are schematic representations of the five Dishevelled constructs. At the top is FL, the full-length Dishevelled protein. The only difference between this construct and endogenous Dishevelled is the addition of a C-terminal tag, which is either HA or FLAG.

The full length protein has three conserved domains, each expressed on its own in the next three constructs. The three constructs are termed DIX, PDZ and DEP. The final construct, designated (-), lacks all three conserved domains. It includes only the very start of the protein, the unconserved 3' end and a tag.

Shown overleaf is an alignment of the protein sequences of Dishevelled-1 from, respectively, Drosophila melanogaster, Homo sapiens and Mus musculus. The three conserved domains span the following amino acids (of the consensus sequence):

DIX 1-93
PDZ 265-353
DEP 439-514

Amino acids conserved between all three proteins are shown in green. Amino acids conserved between two of the three proteins are shown in red. Note the strong sequence conservation within the DIX, PDZ and DEP domains.
dishevelled alignment

**Section 1**

<table>
<thead>
<tr>
<th>Dishevelled-1 (fly)</th>
<th>Dishevelled-1 (human)</th>
<th>Dishevelled-1 (mouse)</th>
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</tr>
<tr>
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### Section 12

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<td>(507)</td>
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<td>CYYVFGELCSNLA LNLNSGSS SDSLTLALFELHF FVPLGOGY</td>
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### Section 14

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### dishevelled alignment

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<td>GGG</td>
<td>C</td>
<td>NN</td>
</tr>
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<td>S</td>
<td>A</td>
<td>ATV</td>
</tr>
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<td>OKAMGC</td>
<td>FEFV</td>
<td>DIM</td>
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<p>| | | | | | |</p>
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<thead>
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<td>-----</td>
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<tr>
<td>Dishevelled-1 (human)</td>
<td>G</td>
<td>SROSF</td>
<td>OKAMGC</td>
<td>FEFV</td>
<td>DIM</td>
</tr>
<tr>
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<td>G</td>
<td>SROSF</td>
<td>OKAMGC</td>
<td>FEFV</td>
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5.2.2 Dishevelled viruses successfully infect the inner ear

Having made the viruses, it was important to ascertain whether they would successfully infect the otic epithelium and express the transgene. Table 5.3 summarises the infections generated with the Dishevelled constructs.

Table 5.3 Dishevelled construct infections

<table>
<thead>
<tr>
<th>Inserted transgene</th>
<th>Stage analysed</th>
<th>Number of embryos surviving/injected</th>
<th>Infections</th>
<th>Markers used</th>
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<tr>
<td>DSH(DIX)</td>
<td>E5</td>
<td>6/12</td>
<td>5 of 6 embryos infected.</td>
<td>GAG</td>
</tr>
<tr>
<td></td>
<td>E10</td>
<td>13/36</td>
<td>10 embryos infected.</td>
<td>GAG</td>
</tr>
<tr>
<td></td>
<td>E14</td>
<td>17/30</td>
<td>4 infections in the basilar papilla.</td>
<td>GAG</td>
</tr>
<tr>
<td>DSH(PDZ)</td>
<td>E5</td>
<td>3/3</td>
<td>3 of 3 embryos infected.</td>
<td>GAG</td>
</tr>
<tr>
<td></td>
<td>E10</td>
<td>10/18</td>
<td>9 embryos infected.</td>
<td>GAG</td>
</tr>
<tr>
<td></td>
<td>E14</td>
<td>11/18</td>
<td>6 infections in the basilar papilla.</td>
<td>GAG</td>
</tr>
<tr>
<td>DSH(DEP)</td>
<td>E5</td>
<td>6/6</td>
<td>3 of 6 embryos infected.</td>
<td>GAG</td>
</tr>
<tr>
<td></td>
<td>E10</td>
<td>18/29</td>
<td>11 embryos infected.</td>
<td>GAG</td>
</tr>
<tr>
<td></td>
<td>E14</td>
<td>32/38</td>
<td>3 infections in the basilar papilla.</td>
<td>GAG</td>
</tr>
<tr>
<td></td>
<td>E16</td>
<td>5/8</td>
<td>2 infections in the basilar papilla.</td>
<td>GAG</td>
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<tr>
<td>DSH(-)</td>
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<td>3/3</td>
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<td>GAG</td>
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<td>8/10</td>
<td>7 embryos infected</td>
<td>GAG</td>
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<td></td>
<td>E14</td>
<td>9/16</td>
<td>3 infections in the basilar papilla.</td>
<td>GAG</td>
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<tr>
<td>DSH(FL)</td>
<td>E10</td>
<td>4/7</td>
<td>3 embryos infected.</td>
<td>GAG</td>
</tr>
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</table>
NB this table has the same format as tables 4.1 and 4.2 in chapter 4. Embryos at E10 were sectioned, and only sections including the inner ear were analysed. At E14 and E16, cochleae were dissected and stained as whole-mount specimens.

Infections were scored as positive if, at E10, GAG expression was seen in the inner ear. At E14 and E16, only infections within the basilar papilla were scored.

* The majority of E14 and E16 cochleae were assessed for infection with GAG immunostain only. A small number of cochleae (5 for DSH(DIX), DSH(PDZ) and DSH(DEP) each) were also stained for the transgene, using the HA tag. However, the HA immunostain protocol gives a relatively high level of background staining. Although this does not affect sectioned specimens, it makes visualisation of whole-mount cochlea PCP (using HCA immunostain) difficult. Having validated the use of GAG immunostaining (see figure 5.3), I decided to discontinue use of the HA immunostain at E14 or E16.

Figure 5.3 shows a successful viral infection, using the DSH(DIX) virus. Taken at E10, it shows robust infection of the sensory epithelium of the inner ear (in this case, of the lateral crista). There is also a strong infection of neurons and connective tissue below the crista itself. This is not unusual or unexpected; the founder cells for the neuronal population are present within the otic cup at the time of viral injection, and are often infected. The neurons, having delaminated from the epithelium, infect neighbouring connective tissue cells.

Of equal importance to demonstrating an infection is to demonstrate transgene expression. In chapter 4, I presented in situ data showing that *c-frizzled-1* and *c-frizzled-7*
Figure 5.2: Haemagglutinin (HA) tag versus FLAG tag staining. Both panels show chick embryonic fibroblasts infected with RCAS virus carrying the DSH(DEP) transgene. In (A), the transgene is HA-tagged, while (B) shows FLAG-tagged transgene expression. The HA tag gives more robust transgene detection than FLAG.
Figure 5.3 Detection of a Dishevelled deletion construct at E10

The figure shows a lateral crista of a DSH(DIX) infected embryo at E10. A-C are different channels of the same image, shown superimposed in D.

A is a combination of phalloidin and ToPro nuclear stain, B is GAG immunostain and C is a combination of HCA immunostain (showing the hair bundles) and HA immunostain (showing the DSH(DIX) transgene).

(A) Phalloidin stains the actin cytoskeleton. In hair cells, phalloidin highlights both the cell periphery and the hair bundle. The nuclear stain can differentiate between hair cells and supporting cells on the basis of the shape and location of the nucleus. Round, apical nuclei are hair cells (arrow), while supporting cell nuclei are more elongated and basal (arrowhead).

(B) GAG immunostain shows RCAS infected cells. In this case, both the epithelium and the neurons and mesenchymal cells basal to it are infected.

(C) HA immunostain shows the location of the expressed transgene (in this case, DSH(DIX)), while HCA immunostain marks the hair bundles.

(D) Superimposition of all three channels. Purple nuclei express the HA transgene (asterisk), while yellow cytoplasmic staining shows colocalisation of HA and GAG staining (+). Note that there is a good correlation between GAG staining and HA staining, although it appears that GAG-positive cells are more numerous (so that one sees some cells which are exclusively green, and contain no purple or yellow staining).
message could be detected in GAG expressing cells, indicating that the RCAS virus was successfully mediating gene misexpression. With my Dishevelled viruses, however, it is possible to go one step further: the addition of an HA tag allows detection of the protein product, confirming that the transgene has not only been transcribed, but also successfully translated. Using a combination of immunostains for GAG and the HA tag, it was possible to detect virus and transgene in the same cells. Not all GAG infected cells expressed the transgene; nor did HA-positive cells all show the same level of HA expression. This variation may reflect the stage of viral life cycle captured in the image, or may reflect a heterogeneous viral population, with inherently variable levels of transgene expression. Nevertheless, the image shows that GAG is still a good indicator that the transgene is also being expressed, and this is useful when interpreting images which do not, for practical reasons, include the HA tag immunostain. It is also worth noting that there are no cells which stain positive for HA but not for GAG. This indicates that the HA immunostain is specific for the product of the Dishevelled transgene.

As illustrated in figure 5.3, I combined the staining for GAG and HA with several additional markers: HCA staining for the hair bundle, phalloidin for the actin cytoskeleton and the DNA dye ToPro for the cell nucleus. In combination, these stains make it possible to both detect transgene misexpression and assay its effects on several features of sensory patch development. Hair cells can be counted, either by the number of hair bundles or by counting apically-located nuclei. Ratios of hair cells to supporting cells can also be assessed, using the nuclear marker. Finally, HCA and phalloidin stains allow effects on hair bundle morphology and polarity to be assessed.
5.2.3 Dishevelled constructs do not alter cell fate in the chick inner ear

One of the potential roles for Frizzled signalling in the inner ear is determination of cell fate. As work in *Drosophila* has shown, sensory organ cell fates are, in part, controlled by a Frizzled gradient. Further, the in situ data presented in Chapter 3 show certain frizzled genes to be expressed in one cell type only, which is consistent with a determining role in either hair cells or supporting cells. Therefore, I sought to explore the distribution of hair cells and supporting cells in sensory patches containing cells misexpressing Dishevelled constructs. Any effect on the final absolute numbers or ratio of these cell types would be indicative of a perturbation of wildtype cell fate determination.

Figures 5.4 through 5.8 show Dishevelled constructs overexpressed in the inner ear at E5, E10 and E14. Although both virus and transgene can be detected from E5 through E14, there is no obvious change in the distribution of cell types in the infected sensory patches. Counts confirm that the ratio of hair cells to supporting cells has not been altered to a statistically significant extent by any of the 3 Dishevelled constructs tested (DIX, PDZ or DEP), indicating that these Dishevelled transgenes do not affect cell fate determination. Table 5.4 summarises these findings. This suggests that neither the canonical nor the PCP signalling branches of the Wnt signalling pathway controls cell fate in the inner ear.
Table 5.4 Cell counts in Dishevelled construct-infected E10 inner ears

<table>
<thead>
<tr>
<th>Inserted transgene</th>
<th>Sensory patch (area analysed)</th>
<th>Uninfected hair cells/supporting cells per 10um</th>
<th>Ratio</th>
<th>Infected hair cells/supporting cells per 10um</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSH(DIX) Basilar papilla (50um)</td>
<td>2.8/7.4</td>
<td>0.38</td>
<td>2.2/6.8</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Anterior crista (50um)</td>
<td>1.6/7.2</td>
<td>0.22</td>
<td>2/8.8</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>DSH(PDZ) Utricle (50um)</td>
<td>1.6/7</td>
<td>0.23</td>
<td>1.4/6</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Lateral crista (50um)</td>
<td>2/4.4</td>
<td>0.46</td>
<td>2.2/4.4</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>DSH(DEP) Basilar papilla (20um)</td>
<td>1.5/7.5</td>
<td>0.20</td>
<td>1.5/7.5</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Utricle (50um)</td>
<td>1.8/6</td>
<td>0.30</td>
<td>1.6/5.8</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

NB counts were done on 1um thick optical slices, with the epithelium seen in transverse section, to ensure all counts were equivalent. Sections were stained with GAG and HCA immunostains. Hair cells were counted using both hair bundles and apical nuclei (in the event of a cell possessing one or the other, presence of a hair bundle was considered.
as defining a hair cell). Supporting cells were counted using basal, oblique spheroidal nuclei.

Wildtype counts were done on tissue adjacent to infected patches.

To confirm the accuracy of this approach, some of the counts were generated using additional hair cell markers. The basilar papilla count for DSH(DEP), for example, was generated using calbindin as a cytoplasmic hair cell marker (see figure 5.6 D). Also, the utricle count for DSH(PDZ) was generated using calretinin as a cytoplasmic hair cell marker. Note that both these counts are similar to the counts obtained using hair cell antigen and nuclear stains, confirming the validity of the approach.

5.2.4 Dishevelled constructs do not alter PCP determination in the basilar papilla

At E14 and E16, PCP can be readily assayed in the basilar papilla, using either HCA, or phalloidin or a combination of both. By superimposing the GAG pattern, it is possible to construct a PCP map of infected and uninfected cells. By this method, one can determine whether transgene expression causes any PCP defects (either cell autonomous or non-autonomous). Figure 5.9 shows an example of how such a pattern is plotted, using the HCA pattern from wildtype E14 and E16 cochleae. Although there is a clear PCP pattern, with all hair cells pointing in the same direction, there is a certain amount of variability. Individual hair cells deviate from the mean by around 20°. This places a limit on the detection threshold for any PCP phenotype: it is possible, with this degree of natural fluctuation of individual PCP vectors, that subtle PCP effects are missed. Nevertheless, the assay method should be sufficiently robust to detect gross PCP defects.
Figure 5.10 shows infected cochleae at E14. This time, an added complexity is determining which cells are GAG positive, as well as their PCP vectors. Not all cells have a vector defined, but those that do indicate that PCP is unaltered by the presence of either the DSH(DIX), DSH(PDZ) or DSH(DEP) transgene (either in infected cells or adjacent, uninfected ones). Table 5.5 summarises the data obtained from infected cochleae.

**Table 5.5** PCP of wildtype and infected cells in E14 cochleae

<table>
<thead>
<tr>
<th>Construct</th>
<th>Number of cells</th>
<th>Average angle of wildtype cells (± standard deviation)</th>
<th>Average angle of infected cells (± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>analysed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wildtype/infected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSH(DIX)</td>
<td>44/19</td>
<td>84 ± 7.65</td>
<td>78 ± 11.71</td>
</tr>
<tr>
<td>DSH(PDZ)</td>
<td>28/16</td>
<td>89 ± 11.91</td>
<td>97 ± 14.28</td>
</tr>
<tr>
<td>DSH(DEP)</td>
<td>30/15</td>
<td>83 ± 13.85</td>
<td>85 ± 16.92</td>
</tr>
</tbody>
</table>

NB Angles are calculated as described in (Stone and Cotanche 1991). The basilar papilla is viewed en face, as shown in figure 5.9, with a 90° angle defined relative to the Y axis, which corresponds to the proximal-distal axis of the basilar papilla.

5.2.5 *DSH(DEP) infected hair cells and c-Celsr-1 expression*

As previously explained, vestibular patch PCP is not easily visualised by HCA immunostaining. In whole-mount specimens at E14, only the basilar papilla can be
accurately scored. This means that, of a possible nine sensory patches, only one can be analysed for our primary phenotype, a disturbance in PCP.

I sought to explore planar polarity of vestibular hair cells by an alternative means. In chapter 3, c-Celsr-1 was shown to have a biased subcellular distribution. We currently lack the markers necessary to prove that this biased distribution reflects the PCP vector of hair cells. Nevertheless, it is a strong possibility. I therefore looked at c-Celsr-1 expression in hair cells infected with the DSH(DEP) transgene, and sought to determine if the biased subcellular distribution is maintained. Also, since it has been reported that a Dishevelled construct containing the DEP domain can interact with the cell membrane, I sought to determine the subcellular localisation of the DSH(DEP) transgene. The preliminary data, shown in figure 5.11, are hard to interpret, and no firm conclusions could be drawn. However, it appears likely that hair cells expressing the DSH(DEP) transgene have a similar c-Celsr-1 expression pattern to adjacent, uninfected hair cells. Further, there may be a faint enrichment of DSH(DEP) at the cell membrane, consistent with reports from other systems. However, the staining pattern is weak and it should be stressed that these results represent too small a data set to present as strong evidence.

5.2.6 Dishevelled constructs and other Frizzled signalling functions

Embryos infected with Dishevelled viruses were analysed primarily to detect a PCP or cell fate phenotype. However, neither has emerged from the data set presented. Therefore, embryos were also analysed to determine if the viruses were responsible for any other
scorable phenotype which might provide evidence for Frizzled signalling pathway function in the inner ear.

No consistent phenotype emerged supporting a role for Frizzled signalling in the inner ear. In a small number of cases (n=2), DSH(DIX) infected cochleae showed an overgrowth of the otoliths which overly the lagenar macula, while a similarly small number of DSH(DEP) cochleae (n=4) showed reduced otoliths. Also, a small number of DSH(DIX) cochleae (n = 5) showed abnormal vasculature, with increased size blood vessels. However, none of these phenotypes presented consistently, nor were they strongly correlated with transgene expression, as judged by GAG staining.
**Figure 5.4** DSH(DIX) misexpression in the chick inner ear at E10 and E14

A through C are images taken at E10, while D is an E14 basilar papilla. In all the images, HCA immunostain is shown in red and GAG immunostain is green. In addition, A and C have phalloidin and ToPro nuclear stain in blue, while D has phalloidin only in blue.

(A) Low power image of the basilar papilla at E10. Towards the distal end, there is a large infected patch, which is shown in higher power in C. Both hair cells and supporting cells are infected (arrow and arrowhead, respectively).

(B) Projection of a z-series, in which a number of images have been superimposed upon each other to give a 3D rendering. In this case, the posterior crista is shown.

(C) Hair and supporting cells are infected, without any apparent associated defects.

(D) Optical slice through a whole-mount E14 cochlea, again showing hair and supporting cell infection.
Figure 5.5: DSH(PDZ) misexpression in the inner ear at E5, E10 and E14.

A-D show DSH(PDZ) RCAS virus infection from E5 through E14.

(A) Three days after injection of viral particles GAG expression (green, anti-GAG immunostaining) can be observed at E5, in the epithelium of the inner ear. Anti-HA immunostaining shows where the transgene is expressed (red). Cell bodies that appear yellow express both GAG and the DSH(PDZ) transgene. Note that nuclear expression of the transgene is also visible.

(B) Section of an E10 embryo, showing the lateral crista. GAG staining (green) again co-localises with HA-staining (red), which shows transgene expression. Anti-HCA immunostaining labels hair bundles (also in red), ToPro labels nuclei and phalloidin labels actin filaments (both blue). The ratio of hair cells to supporting cells is the same in the infected patch as in neighbouring, unlabelled epithelium.

(C) Shown is the utricle at E10, stained as in (B). Note that both hair cells (arrow) and supporting cells (arrowhead) are infected. Once again, the ratio of hair cells to supporting cells is the same in infected as in uninfected regions.

(D) An E14 whole-mount cochlea, stained as in (B) and (C), except that the HA staining was omitted. The image shows the surface of the basilar papilla; individual hair bundles can be seen, staining purple (co-localisation of HCA and actin stains). Two patches of infected cells can be seen by GAG staining. The patch on the left is within the basilar papilla, while the patch on the right is outside the basilar papilla, and the type of cells infected is unknown. Again, the distribution of hair cells within the infected part of the basilar papilla appears normal.
Figure 5.6 DSH(DEP) misexpression in the chick inner ear at E10

A through D are images of E10 sectioned embryos, showing the inner ear epithelium. All the images have HCA immunostain in red, GAG immunostain in green and ToPro and phalloidin and blue. In addition, D has anti-calbindin immunostain in red (along with HCA). Calbindin is present in hair cells of the basilar papilla only.

(A) Low power image, showing the utricle (u) and lateral crista (lc). There are several patches of infection, in both sensory patches and the non-sensory region between them.

(B) Infection in the utricle at higher power.

(C) High power image of the lagena (la). Both hair cells and supporting cells are infected (arrow and arrowhead, respectively). No obvious defects can be seen in this, or any other infected patch.

(D) Basilar papilla (bp). In this instance, the hair cells are stained red, with calbindin immunostain. Infected hair cells therefore show up yellow (asterisks). Infected hair cells appear to have normal hair bundles. Supporting cells are also infected (arrowhead).
**Figure 5.7** DSH(-) misexpression in the chick inner ear from E5 to E14

(A) and (B) Sections of the otic vesicle at E5; GAG immunostain is shown in green, and HA immunostain is in red, showing transgene expression. Transgene expression can be seen from early stages. Embryos were injected at E2, which is 72 hours before these images were taken. Yellow colour indicates that transgene stain correlates with the viral marker. Note in (B) that some transgene expression can be seen in the nucleus, and therefore shows up red (this does not indicate that cells expressing the transgene are not positive for viral markers. Rather, the two proteins have different subcellular localisations).

(C) Section of utricular macula at E10; GAG immunostain is green, HCA immunostain is red, ToPro and phalloidin are blue. DSH(-) virus has infected both hair cells and supporting cells (arrow and arrowhead, respectively), without appearing to alter normal patch morphology.

(D) Optical section showing a side-on view of a dissected cochlea at E14, showing a large portion of the basilar papilla, stained as in (C). Proximal is to the right. There are two large patches of infection within the basilar papilla. Viral expression does not appear to disturb basilar papilla development, however.

(E) Higher power image of one of the infected patches shown in (D), confirming that both hair cells and supporting cells are infected.
Figure 5.8 DSH(FL) misexpression in the chick inner ear at E10

(A) Low power image of a sectioned embryo at E10; GAG immunostain is in green and HCA immunostain is in red. The lateral crista (lc) is heavily infected, as are many non sensory regions of the epithelium. By contrast, the utricle (u) is largely uninfected.

(B) Low power image of a sectioned embryo at E10, stained as in (A). Shown is the basilar papilla. There is a small patch of infected cells at the proximal end, and a larger infected patch at the distal end of the basilar papilla.

(C) Shown is the utricle at higher power. Staining is as in (A), except for the addition of ToPro nuclear dye and phalloidin in blue.

(D) High power image of the anterior crista (ac). Staining as in (A). Both hair cells (arrow) and supporting cells (arrowhead) are infected, as judged by GAG immunostain.

In all four images, infection with the DSH(FL) RCAS virus has no discernible effect on wildtype sensory patch development.
Figure 5.9: Creating PCP diagrams for the basilar papilla

(A), (C) Optical sections tangential to the surface of wildtype basilar papillae, at E14 and E16 respectively. In both cases, HCA immunostain is in red. The hair bundle is clearly visible as a bar of intense staining, positioned to one side of the cell. In both (A) and (C), one cell is marked with a white arrow, demonstrating how a vector is assigned to each cell based on the shape and position of its bundle.

(B), (D) Patterns of PCP vectors in (A) and (C). Each arrow represents a single cell. The overall polarity of the patch is clearly demonstrated in both cases.
Figure 5.10: PCP determination in Dishevelled virus infected basilar papillae at E14

Images shown are tangential optical sections of whole mount basilar papillae at E14.

(A-D) DSH(PDZ) infected cochlea.

(E-H) DSH(DIX) infected cochlea.

(I-L) DSH(DEP) infected cochlea.

All three image series were prepared in the same fashion (i.e. the explanation given for A through D, the DSH(PDZ) infected papilla, applies to the other two virus constructs also).

A-C are different channels of the same confocal image.

(A) Phalloidin stain, showing hair bundles and the outline of cell apices.

(B) GAG immunostain, marking infected cells. One infected hair cell (+) and one infected supporting cell (*) are marked; note that the shape of the cell distinguishes the two types: hair cells are hexagonal in shape, and larger than the thin apical projections of the supporting cells.

(C) Immunostain for HCA.

(D) The pattern of PCP vectors. Arrows indicate the vector for each individual hair cell. Green arrows indicate infected cells, while black arrows are uninfected. Dots indicate cells whose PCP is not clear.
**Figure 5.11** DSH(DEP) misexpression and c-Celsr-1 subcellular distribution

(A) Section of anterior crista (ac) at E10; c-Celsr-1 immunostain is shown in green, while HCA and HA immunostain are shown in red. There is a patch of DSH(DEP) transgene expression, encompassing both sensory and non-sensory cells. C-celsr-1 expression can be seen below the hair cell bundle, at the apical surface of hair cells.

(B) Section of utricle (u) at E10, stained as in (A). Similar to (A), there are a number of cells infected with the DSH(DEP) transgene.

(C) and (D) are, respectively, the green and red channels of (E), which is a blow-up of a portion of (B). This is a high power view of the most apical part of the utricular macula epithelium. In C, c-Celsr-1 expression is shown. Two cells are marked, (*) and (+) both of which show a biased subcellular distribution of c-Celsr-1. These same cells are marked in D, where one can see transgene expression that appears similar to the c-Celsr-1 distribution (HCA stain is also visible, but is distinguishable from the transgene stain by its intensity and shape). The merge image, E, shows that there is a degree of overlap between c-Celsr-1 and DSH(DEP) transgene, which appears yellow.
5.3 Discussion

The overexpression of Dishevelled constructs had no demonstrable effect on the normal development of the chick inner ear. All five were successfully tagged and produced. Robust transgene expression was shown from E5: certainly early enough to disrupt cell fate or PCP determination. This transgene expression was maintained through E10, E14 and E16, again indicating that RCAS mediated misexpression had worked. Nevertheless, no clear defects were observed, either on general sensory patch morphology at E10 or on PCP determination in the basilar papilla at E14 or E16.

There is a clear similarity between the results presented in chapters 4 and 5. Both chart attempts to disrupt Frizzled signalling in the inner ear. Neither present positive data pinpointing a role for the pathway in sensory patch development, be it cell fate, PCP determination or, indeed, any other scorable developmental process. I will therefore save the more detailed analysis of the reasons for the failure to obtain a phenotype for chapter 6, rather than duplicate discussions at the end of chapters 4 and 5.

Below I consider issues which more specifically relate to the use of Dishevelled itself, since it is a distinct experimental approach from misexpression of Frizzled receptors.

5.3.1 Is dishevelled expressed in the inner ear?

The experimental approach described in this chapter is based on the use of dominant-negative deletion constructs of the Frizzled signalling effector, Dishevelled. The ability of these constructs to elicit an effect on Frizzled signalling rests on the assumption that
Dishevelled is expressed in the inner ear, and functions downstream of the Frizzled receptors whose expression patterns were characterised in chapter 3.

Ideally one would want to establish, either by in situ hybridisation or immunohistochemistry, that a chick Dishevelled homologue (or homologues) is expressed in the inner ear during sensory patch development, co-localised with expression of Frizzled receptors. However, this has not been possible. No chick *dishevelled* gene has been cloned, which means that in situ hybridisation cannot be performed. There are a number of anti-Dishevelled antibodies, raised against human, mouse and rat Dishevelled homologues that might cross-react with a chick Dishevelled. However, none of the antibodies I tested showed any expression of Dishevelled on chick sections through the hindbrain region at E4, E7 or E10 (data not shown), either in the inner ear or any other tissue (for example, one would expect to find Dishevelled expressed in the neural tube). Without an antibody or a chick Dishevelled clone, therefore, we assume on indirect grounds that Dishevelled is expressed in the inner ear. Is it possible that this assumption is flawed, and that Dishevelled is not present in the inner ear?

I would argue that the available evidence strongly points to Dishevelled being expressed in the inner ear. To my mind, the most convincing data is the presence of Frizzled receptors. It has been shown in numerous studies that Frizzled signalling requires Dishevelled (Shulman et al. 1998). If Dishevelled were not expressed in the chick inner ear, it would mean that four Frizzled receptors, c-Frizzled-1, c-Frizzled-5, c-Frizzled-7 and c-Frizzled-10, signal by a Dishevelled-independent mechanism. This would go against the weight of evidence from other systems. Frizzled receptor expression alone, therefore, argues that Dishevelled is likely to be present in the inner ear. Expression studies in mouse
also support the notion that Dishevelled is expressed in the inner ear, showing that Dishevelled homologues are virtually ubiquitously expressed during embryonic development (Sussman et al. 1994).

5.3.2 Did we chose the correct Dishevelled deletion constructs?

Another reason why the Dishevelled deletion constructs might have failed to disrupt Frizzled signalling is that the design was incorrect. However, I feel that is unlikely. The design was based on a wealth of published data; a number of groups have successfully employed this approach in the past (Moriguchi et al. 1999), (Rothbacher et al. 2000), (Axelrod et al. 1998), (Boutros et al. 1998), (Heisenberg et al. 2000), (Wallingford et al. 2000). This is not to say that I have used all the constructs described in these publications. Rather, I concentrated on those constructs which disrupted PCP signalling, and decided that overexpression of the DEP domain alone had given the most consistent results in this regard. It is likely that there are deletion constructs which would have proved useful for assaying canonical signalling, for example one that contains both the DIX and PDZ domains, that I did not employ. Overall, however, I feel the constructs are not obviously flawed, and represented the best chance of disrupting Frizzled signalling in the inner ear.

5.3.3 Can dishevelled work in a heterologous system?

201
As stated above, chick *dishevelled* homologues have not been cloned. Time constraints meant that I was unable to pursue the strategy of first cloning a full-length chick *dishevelled*, from which we could create Dishevelled deletion constructs. Instead I used human Dishevelled-1 as the basis for the deletion constructs. Is it possible that this contributed to the failure of the experiments, and that the human Dishevelled domains are too divergent from those of the chick to disrupt wildtype signalling?

There are a number of reasons for believing that the constructs based on human Dishevelled-1 should work in the chick. First, the three Dishevelled domains, DIX, PDZ and DEP are highly conserved. The main differences between Dishevelled protein sequences lies outside these domains (see figure 5.1). Since our constructs relied on these well-conserved domains, there was good reason to believe that they would be functional between species.

In addition, Dishevelled has been shown to maintain its function in heterologous systems in two ways. First, *Drosophila* Dishevelled constructs expressed in *Xenopus* animal cap cells are active and behave as expected, based on their domain structure (Axelrod et al. 1998); i.e. constructs containing the DEP domain associated with the membrane, while those lacking the DEP domain did not. Second, and more pertinent for our purposes, *Xenopus* Dishevelled deletion constructs can disrupt convergent extension in zebrafish embryos (Heisenberg et al. 2000). Expressing the DEP domain of x-Dishevelled-1 in the zebrafish disrupts a PCP–like process. This finding strengthens the logic behind our approach, and we felt it appropriate to proceed with the experiments using human Dishevelled-1 deletion constructs.
Nevertheless, my own evidence points to the contrary conclusion; that the Dishevelled-1 deletion constructs are not biologically active. The DIX, PDZ and DEP RCAS viruses were all tested in the chick limb bud, for effects on formation of the vasculature, a process known to involve Frizzled signalling in some developmental contexts (Ishikawa et al. 2001). No defects were observed for any of the three constructs (this work was done with the kind assistance of Neil Vargesson). Nor were any defects observed in the neural tube, which was often infected during the inner ear misexpression experiments. Frizzled signalling is known to have a mitogenic role in the neural tube, and it might be expected that some of the Dishevelled constructs would cause abnormal growth (or lack of it) in this tissue (Megason and McMahon 2002). But, again, no such phenotype was ever observed.

As the most important of our constructs, it would have been preferable to at least demonstrate that the DEP domain construct was biologically active in the chick. The obvious way to test this would be to misexpress it during chick gastrulation, since convergent extension movements represent a PCP-like process that the DEP domain alone ought to disrupt. However, this was not possible. The RCAS virus cannot be injected early enough to produce transgene expression in time to disrupt gastrulation. If we placed the DEP domain in another expression system, it would no longer be a valid control, since our experiments rely on Dishevelled construct expression under the control of the RCAS viral promoter. Demonstrating their efficacy outside this expression system would not mean that the Dishevelled constructs are active within it.

Therefore it seems that the approach we undertook, while valid in theory, may have failed practically. Without clear evidence that our deletion constructs are active in the chick, it must be considered a strong possibility that the Dishevelled experiments failed
because human Dishevelled constructs do not interact with chick Frizzled signalling pathway components.
6 Chapter 6: Discussion

"If you dissemble sometimes your knowledge of that you are thought to know, you shall 
be thought, another time, to know that you know not."

(Bacon 1625)

6.1 Summary

In this study, I have considered the role of Frizzled signalling, a known regulator of 
both asymmetric cell divisions and PCP in invertebrates, in cell fate specification and 
planar cell polarity of hair cells of the chick inner ear.

I have shown the several frizzled genes and one flamingo gene are expressed in the 
chick inner ear during sensory patch development. Further, I have shown that the Flamingo 
homologue c-Celsr-1 has a biased subcellular distribution in hair cells, strikingly 
reminiscent of Flamingo localisation in cells of the Drosophila wing. Also, I have shown 
that frizzled genes expressed in sensory patches tend to shift expression from all cells to 
cell-type specific expression at late stages of sensory patch development. Therefore, the 
descriptive data is consistent with a role for Frizzled signalling in PCP determination and/or 
cell fate specification.

Using the RCAS retrovirus system, I have misexpressed two full-length chick Frizzled 
receptors, c-Frizzled-1 and c-Frizzled-7. Also, I have misexpressed five Dishevelled
deletion constructs. I have demonstrated that viruses containing these constructs are capable of infecting the otic epithelium from as early as E3 to as late as E16, covering the period of hair cell birth and PCP determination. Using tagged Dishevelled transgenes, I have shown that viral infection is accompanied by transgene protein expression in the majority of infected cells. However, the results suggest that none of these transgenes affects normal sensory patch development. More specifically, I have shown that viral infection does not alter hair cell to supporting cell ratios at E10 or wildtype PCP patterns in the basilar papilla at E14. Therefore, the virus data does not indicate a role for Frizzled signalling in PCP or cell fate determination.

In effect, my data set is split between descriptive experiments, which support a role for Frizzled signalling in sensory patch development, and gene misexpression experiments which suggest that interfering with Frizzled signalling has no effect on sensory patches. Experiments designed to complement one another have yielded apparently contradictory results. How best to reconcile these disparate findings?

The paradox can be explained if one of the experimental approaches fails for practical reasons or because my constructs were wrongly designed. I will consider below if it is possible that the viral misexpression results, which failed to produce a phenotype, could be explained in these terms.

Alternatively, the original hypothesis could be incorrect. It may be that Frizzled signalling has no role in the chick inner ear.

Finally, it is possible that the complete data set can be reconciled, if one takes the view that Frizzled signalling may operate in a different fashion to that seen in other PCP contexts, or may have a different importance in comparison with other mechanisms that
might operate in parallel. It is possible that the Frizzled signalling cassette in chick is such as to thwart disruption by the viral misexpression of Frizzled receptors and Dishevelled deletion constructs. An alternative approach, interfering with c-Celsr-1, is suggested by the expression data, and may yet prove an effective means of disrupting PCP determination in the chick inner ear.

Below I review the data presented in this thesis, and attempt to draw appropriate conclusions. I will focus on the question of Frizzled signalling involvement in PCP determination, and will only touch briefly on the question of asymmetric cell divisions. Although this latter process might be controlled by Frizzled signalling, we have not garnered sufficient evidence to warrant detailed discussion.

6.2 What has been accomplished

6.2.1 A description of wildtype PCP patterns in the basilar papilla

Using a combination of confocal microscopy, SEM and TEM, I have described wildtype PCP patterns. In particular, I have focussed on the basilar papilla, and shown that it has a single PCP vector running across the patch, perpendicular to the proximal-distal axis, that becomes overt between E8 and E12. My findings are in good agreement with previously published work, on which I have also relied heavily, particularly for the earliest manifestations of PCP.
6.2.1.1 Electron microscopy work on the basilar papilla

SEM is ideally suited for analysis of the structure of the hair cell bundle, and I have looked at the apical surface of E9 through E12 basilar papillae. I did not attempt to duplicate the rigorous and comprehensive previously published SEM studies of PCP in this system (Stone and Cotanche 1991), (Cotanche and Corwin 1991). Rather, as an adjunct to my main project, I wished to check that these existing findings were applicable to my own experimental material. I confirmed that they were. Working backwards, PCP is clearly defined by E12. The kinocilium is displaced to one side of the cell, and there is a well ordered stereociliary staircase. Hair cells across the patch are well aligned with one another, in both proximal and distal regions. At E11, the PCP vectors of individual hair cells are clearly defined; each cell has a kinocilium to one side and a number of stereocilia ordered in height away from the kinocilium. However, the PCP vectors of neighbours are less well coordinated than at E12. It appears that a refinement of the overall PCP field is taking place at this stage, by reorientation of hair cells that are already individually well polarised. Indeed, the SEM studies of (Stone and Cotanche 1991) and (Cotanche and Corwin 1991) indicate that, as early as E8-E9, individual hair cells in the basilar papilla have a PCP vector, with an asymmetrically placed kinocilium, and that this vector, although not totally random in its orientation, is only very roughly aligned with the PCP vectors of neighbouring cells.

While SEM is suited to analysis of the apical surface of sensory patches, TEM can be used to look at sections of the patch, revealing both the hair bundle and internal cellular architecture. It thus has the potential to identify internal asymmetries, not visible by other
means, which might prefigure PCP emergence on the cell surface and therefore allow one to further refine the time window for when PCP determination occurs. One such feature has been described by (Denman-Johnson and Forge 1999). The striated rootlet, which extends from the base of the kinocilium to one side of the cell apex, is seen to be polarised coincident with displacement of the kinocilium. However, it has not been established that striated rootlet asymmetry precedes kinocilium asymmetry. I did not see this structure, or any intracellular asymmetry foreshadowing asymmetric localisation of the kinocilium, in my more limited sets of TEM sections of the basilar papilla.

6.2.1.2 Using confocal microscopy to assess PCP

From the outset, our aim was to describe wildtype PCP in the major sensory patches (the basilar papilla, cristae, saccular macula and utriclar macula) so that disturbances of PCP could be recognised and scored. This undertaking turned out to be quite difficult, because of the need to use a technique for visualising PCP that would allow me at the same time to identify cells expressing transgenes (i.e. those we hoped would possess a PCP phenotype). Confocal microscopy seemed to be the only practicable means of combining these two requirements. SEM and TEM are powerful tools for analysing wildtype PCP, but are not suited to viral misexpression experiments, where mutant cells need to be identified amongst (the majority) of wildtype cells. The need to rely on confocal microscopy has, in turn, forced me to concentrate on the basilar papilla, since this is the only sensory patch where I found it possible to accurately assay PCP markers clearly visible by confocal imaging.
For visualisation of hair cell PCP by confocal microscopy I relied on the anti-HCA (hair cell antigen) mouse monoclonal antibody. Raised against a tyrosine phosphatase cell-surface receptor, the antibody is highly specific for the stereociliary array (Goodyear and Richardson 1997). In both sections and whole-mounts, staining is robust and reproducible. Once the bar-shaped bundle characteristic of basilar papilla hair cells emerges, around E12, HCA on its own can identify a PCP vector. In vestibular patches, however, the thinner, rounder bundle makes the HCA pattern altogether less informative. Even in combination with phalloidin, which has the added advantage that it marks the cell periphery as well as the hair bundle, no PCP information can be reliably obtained in vestibular patches.

A potential solution to this problem lies in pinpointing the location of the kinocilium. Positioned to one side of the hair bundle, it identifies the hair cell vector. In zebrafish, the anti-acetylated tubulin antibody has proven extremely effective at marking the position of the kinocilium and, as a result, the PCP vector of hair cells. Even in patches with complicated PCP patterns (such as are found in the zebrafish maculae) this approach has been very successful (Haddon et al. 1999).

I have attempted, without success, to use tubulin staining for PCP analysis in vestibular patches of the chick inner ear. A number of anti-tubulin antibodies have been tried, including anti-acetylated tubulin, anti-beta-tubulin and TuJ1. Of these, only anti-beta-tubulin marks the kinocilium, but the staining pattern is weak, heterogeneous (only some hair cell kinocilia can be identified) and not reproducible. Until this problem is resolved, scoring PCP phenotypes of infected vestibular patches will not be possible.

Thus, while I have established a system for analysing PCP determination at the same time as viral infection in the basilar papilla, I have not been able to do so for the vestibular
patches. This has necessarily limited the scope of my investigation into PCP and leaves the possibility that my experimental interference with Frizzled signalling may actually have affected vestibular patches, but I failed to visualise it. On the positive side, however, we have a robust assay system for the basilar papilla which, with its precise, uniform vector, bears the closest resemblance to the *Drosophila* paradigms and, potentially, offers the best opportunity to detect even subtle effects of Frizzled signalling on hair cell PCP.

### 6.2.2 Expression patterns of Frizzled signalling pathway genes

Using in situ and antibody stains, I have looked at the expression patterns of Frizzled signalling pathway genes in the inner ear. The aim of this body of work was, firstly, to ascertain if these genes were expressed in the inner ear at all and, secondly, to identify the best potential PCP candidate genes.

I have shown that four frizzled genes and one flamingo gene are expressed in the inner ear between E4 and E10 (the period of hair cell fate and PCP determination). Two iroquois genes, *c-iroquois-1* and *c-iroquois-2* (homologues of the *Drosophila* PCP gene *mirror*) are expressed in the inner ear before E3. This expression is too early to influence the relevant steps of hair cell development, and I will not consider them further.

*C-frizzled-1* and *c-frizzled-7* share similar expression patterns at early stages. At E4, both are expressed in all the future sensory patches, in areas encompassing expression of *c-Serrate-1* (a marker for sensory patches). Both also show complicated temporal changes, becoming restricted to distinct sensory and non-sensory regions. Notably, both show cell type specific expression within sensory patches, with *c-frizzled-1* expressed in hair cells
and c-frizzled-7 in supporting cells of the maculae by E9. C-frizzled-5 is also expressed exclusively in hair cells of the maculae, but is not visible prior to E8.

C-frizzled-10 is expressed only in the basilar papilla. At E7, all cells of the basilar papilla express c-frizzled-10, but by E9 only supporting cells maintain that expression.

C-celsr-1, a chick flamingo homologue, is expressed in hair cells of all patches from E9 onwards. An antibody against c-Celsr-1 protein reveals that c-Celsr-1 has an apical distribution in hair cells. Further, in cristae and maculae, and possibly also in the basilar papilla, c-Celsr-1 appears to have an anisotropic subcellular distribution similar to that seen for Flamingo in the Drosophila wing, where the protein is concentrated at the proximal and distal faces of the cell and depleted at the anterior and posterior faces. It is tempting to speculate that this biased c-Celsr-1 expression correlates with the emergence of PCP in hair cells.

6.2.2.1 C-celsr-1 has the most promising expression pattern

What conclusions can we draw from these expression patterns? In the context of a search for PCP candidates, the c-Celsr-1 protein pattern stands out as potentially the most informative finding. To find any protein with a biased distribution orthogonal to the apical-basal axis would be promising. But c-Celsr-1 is more than a random gene. Given that it is a homologue of a known core PCP gene, flamingo, and that its expression pattern in cristae and maculae is strikingly reminiscent of Flamingo protein distribution in the Drosophila wing, might the similar subcellular distribution reflect a conserved function? And if so, what further deductions can we draw?
If the c-Celsr-1 expression pattern is a marker of PCP, visible before the emergence of permanent, physical asymmetries, just as Flamingo is in the *Drosophila* wing, it tells us something important about the timing of PCP determination. *C-celsr-1* expression is not detectable before E8, suggesting that there is a critical time period during which PCP genes act, somewhere around E8-E10. This would fit with the SEM data for the basilar papilla, where the kinocilium becomes located to the cell periphery, and neighbouring cells align their PCP vectors, in the E8-E11 time window.

It is frustrating that the biased distribution of c-Celsr-1 was only clearly observed in vestibular patches, on which we have little data on PCP emergence. It has not been possible to visualise a biased subcellular distribution of c-Celsr-1 in the basilar papilla (its expression appears weaker than in the vestibular patches), on which we have most PCP information. Therefore, although it is an obvious suggestion that the bias in c-Celsr-1 reflects the PCP vector of each hair cell, we are currently unable to confirm this. With perseverance it might, however, be possible: the c-Celsr-1 antibody became available only at the very end of my series of experiments, and this left me very little time to optimise the immunostaining protocol.

I feel that the biased distribution of c-Celsr-1 around the apical surface of hair cells is the single most compelling piece of evidence that a mechanism homologous to the *Drosophila* PCP system is involved in PCP determination in the chick inner ear.
6.2.2.2 Interpreting the frizzled gene expression patterns depends on PCP timing

Because the frizzled genes show such temporally dynamic patterns, one must closely consider the timing of PCP determination before interpreting them. If we suppose that the E8-E11 period is the critical one for PCP determination, as SEM studies and c-Celsr-1 expression suggests, the frizzled data are at least consistent with a PCP function, provided we accept that different combinations of frizzled genes may be responsible for this in different sensory patches.

Might PCP be determined even before the total hair cell complement of a patch has been born? If PCP is determined early, even frizzled expression which is eventually restricted to supporting cells may have a PCP function. The basilar papilla-specific expression of *c-frizzled-10* perfectly illustrates this point. If E9 onwards is the critical time window for PCP, and the PCP of hair cells is presumed to depend on frizzled expression within the hair cells themselves, then *c-frizzled-10* is not a candidate gene, since it is only expressed in supporting cells at this stage. If, however, you hold out the possibility that PCP may be determined before this, *c-frizzled-10* does fulfil the criteria of a candidate gene since, at E7, it is expressed in all cells of the basilar papilla.

Another point to consider, and one not raised previously, is what role, if any, supporting cells may play in PCP development, given that supporting-cell specific expression is observed for *c-frizzled-7* and *c-frizzled-10*. Does PCP coordination between hair cells require the involvement of supporting cells, which surround them even on the apical surface of the sensory patch? Do supporting cells relay a signal, or become polarised themselves? Supporting cells have microvilli on their apical surfaces, but they are not
visibly polarised (SEM images at E12 demonstrate this, at a stage when hair cells clearly are polarised). The only evidence available on this issue argues against a requirement for supporting cells. In the zebrafish mind bomb mutant, sensory patches consisting entirely of hair cells have been analysed, and found to display essentially normal PCP (Haddon et al. 1999). This observation argues that hair cells by themselves have the apparatus for correct PCP determination, although it leaves open the possibility that, in normal development, where hair cells are separated from one another by supporting cells, the supporting cells may be important in transmitting the influences that coordinate the orientation of each hair cell with that of its neighbours. It could be that Frizzled in supporting cells has a role of this sort.

As discussed in chapter 3, however, there is a strong possibility that Frizzled signalling may have other functions in the inner ear. The cell type specific expression suggests a role for frizzled genes in defining the differentiated character of the expressing cell. Also, the expression adjacent to patches suggests that frizzled genes may mediate signalling that controls the dimensions or integrity of the sensory patches. In support of this final suggestion, (Stevens et al. 2002) report that misexpression of an activated β-catenin construct within the epithelium of the inner ear leads to overgrowth and even merger of sensory patches.

Overall, the expression patterns of Frizzled signalling pathway genes indicate that the pathway is active during inner ear development, and may have several distinct developmental functions.
A comparison of electroporation and virus injection

An unexpected offshoot of the project has been the work undertaken on methods for use of the RCAS virus as a gene vector in chick embryos, comparing the relative efficacy of electroporation of viral DNA and virus particle injection in achieving RCAS-mediated gene misexpression in the inner ear. Initially, we hoped and expected that electroporation of the RCAS DNA genome would give us efficient and spreading RCAS infections, without the need for laborious production of virus particles (which must be done to a very high titre to ensure good infections) (Momose et al. 1999). However, this turned out not to be the case. Although electroporation consistently gave us viral gene expression up to E8 (with associated high levels of transgene message), we were unable to detect infected patches after this. Given that the main assay for PCP determination was assaying E14 whole-mount cochleae, the lack of detectable late infection was the death-knell for my electroporation experiments. I was obliged to return to production of viral particles, if we were to score the effect of transgenes on PCP determination in the basilar papilla.

It is unclear why electroporation should fail to establish a long-lived infection. The RCAS DNA that I electroporated codes for all necessary components of the viral life cycle. I showed that both transgene message and GAG protein were being produced, and therefore felt safe in the assumption that other viral components needed for propagation of the infection should also be produced normally. There is no question of a mutation in the DNA used; the same DNA which failed to elicit long-lived infections in the embryo was used to successfully transfect chick embryonic fibroblasts, from which we eventually harvested viral particles. Nevertheless, my observation, backed by large numbers of electroporated
embryos (and in the absence of published material to the contrary), is that electroporation does not give long-lived viral expression.

By contrast, using viral particles does give long-lived infections (up to E16), along with strong sustained transgene expression. It therefore remains the method of choice for introducing genes into chick embryos, at least where infections beyond E8 are required.

6.2.4 Misexpression of Frizzled signalling pathway components

Using virus particle injections, I have successfully misexpressed c-Frizzled-1, c-Frizzled-7 and a number of Dishevelled deletion constructs in the chick inner ear. For each viral construct, I have generated a large number of infections within all eight sensory patches. Further, by tagging the Dishevelled constructs with an HA epitope, I have shown that the transgene can be directly visualised, and correlates well viral markers.

What I have failed to do, for any of the contracts, is demonstrate an effect on wildtype sensory patch development. At E10, all the sensory patches appear grossly normal, and cell counts indicate that none of the constructs perturb the ratio of hair cells to supporting cells. At E14, I have shown that the viral constructs do not interfere with normal PCP specification in the basilar papilla.

6.3 Wrong hypothesis, wrong approach or neither?

As stated above, the descriptive data are suggestive of Frizzled signalling pathway function in the inner ear. However, the functional experiments, misexpressing Frizzled
receptors and Dishevelled deletion constructs, failed to corroborate the hypothesised roles for Frizzled signalling. One can suppose that the hypothesis about Frizzled signalling controlling PCP and/or cell fate specification is wrong. Alternatively, the hypothesis may be correct and we somehow failed in our experimental approach. Finally, it is possible that the entire data set can be explained if one supposes that Frizzled signalling in the inner ear has certain novel characteristics. I will consider these possibilities in turn.

6.3.1 Frizzled signalling might not control hair cell PCP

We have no direct evidence, either from the data presented in this thesis, or in the wider literature, that Frizzled signalling controls hair cell PCP in the vertebrate inner ear. Rather, we have combined three types of suggestive observations to formulate the hypothesis. The first is that the systems in which the molecular control of PCP has been elucidated rely on Frizzled signalling. This is true for all the polarised tissues and organs of *Drosophila* (Shulman et al. 1998), as well as for the convergent extension movements of vertebrate gastrulation (Darken et al. 2002), which one may regard as a PCP-like process, but one where the cell polarity is manifest in polarised cell movement rather than polarised rigid cell structures. Second, there is evidence that vertebrate homologues of *Drosophila* PCP genes, when mutated, cause hair bundle defects. These include two cadherin genes and Myosin VIIa (Alagramam et al. 2001), (Di Palma et al. 2001), (Gibson et al. 1995). The third observation, though it appears to be a statement of the obvious, is nevertheless worth including at this juncture: hair cell PCP *must* be under developmental control. The co-ordinated polarity of hair cells within each sensory patch requires a molecular control
mechanism. The implication is that, if Frizzled signalling is not involved in hair cell PCP, another molecular pathway must be. It is, to my mind, difficult to contemplate that PCP determination in the inner ear is controlled by a molecular pathway hitherto unlinked to this process in any other system. We are also far from alone in believing that the same components that govern PCP in *Drosophila* are likely to be involved in hair cell PCP (Winter et al. 2001), (Tree et al. 2002).

Overall, I feel the hypothesis that Frizzled signalling is involved in PCP determination is more likely to be correct than incorrect. I will now consider the implications of that statement.

6.3.2 *Frizzled signalling might control hair cell PCP*

If one supposes that Frizzled signalling controls PCP determination in the chick inner ear, how are we to explain the lack of phenotypes observed with my viral experiments? There are a number of reasons why we may have failed to achieve a disruption of wildtype PCP signalling using our viral constructs, based on full-length Frizzled receptors and Dishevelled deletion constructs, which I will consider in turn.

6.3.2.1 *Wrong frizzled?*

In *Drosophila*, there is only one known PCP effector amongst the four frizzled genes in the genome (Janson et al. 2001). Is it possible, of the ten chick frizzled genes identified,
that we missed the crucial one, or that some other chick frizzled, as yet unidentified, is the key to PCP?

From the outset, we knew that there was no clearly defined unique chick orthologue of *d-frizzled-1*, which is the frizzled gene controlling PCP signalling in the fly. There is a chick homologue of zebrafish and *Xenopus frizzled-7*, which have been implicated in convergent extension (Darken et al. 2002), (Winklbauer et al. 2001), *c-frizzled-7*. But overexpression of this Frizzled receptor does not, in my hands, alter PCP determination in the basilar papilla.

There are two other chick frizzled genes which are closely related to *frizzled-7*: *c-frizzled-1* and *c-frizzled-2*. Of those, *c-frizzled-1* is expressed in hair cells of the maculae at E8, which is consistent with a PCP role. *C-frizzled-2* was not tested. In the worst case scenario, *c-frizzled-2* is the key frizzled gene, and we did not identify it. How likely this is, it is hard to judge. However, for completeness it will be desirable to check expression patterns in the inner ear for all frizzled genes.

Setting aside *c-frizzled-2*, I found no inner ear expression for *c-frizzled-4*, *c-frizzled-6*, *c-frizzled-8* and *c-frizzled-9*, while *c-frizzled-3* was not available. Failure to detect expression does not amount to proof that frizzled genes tested are definitely not expressed in the inner ear. However, I feel the chance that these frizzled genes have a PCP role is small, given that I did successfully visualise patterned expression of *c-frizzled-1*, *c-frizzled-5*, *c-frizzled-7* and *c-frizzled-10* (which shows that the in situ technique works).

Use of Dishevelled deletion constructs provided another test of the involvement of Frizzled signalling, and a more stringent one, since the many different Frizzled receptors are thought to all depend on a much smaller set of Dishevelled proteins for their action.
This of course assumes that the Dishevelled constructs were effective as dominant-negative inhibitors— an assumption that has been validated in other contexts (Rothbacher et al. 2000), (Axelrod et al. 1998), (Heisenberg et al. 2000). That the Dishevelled constructs, like the Frizzled constructs, failed to elicit a phenotype suggests that there may be a common reason behind the failure of both approaches, and argues against the hypothesis that we have merely failed to identify the correct PCP Frizzled receptor.

6.3.2.2 Wrong experimental approach?

Supposing that Frizzled signalling is the PCP determinant, is it possible that we took the wrong experimental approach? There were two main tests of the function that I undertook; overexpressing full-length Frizzled receptors and overexpressing Dishevelled deletion constructs. Are there reasons why both prongs of this twin approach might have failed even if the hypothesis behind their design was correct?

There are clear differences between the two types of experiment, notably in the assumptions one makes when designing them. The first type of experiment is based on the expectation that overexpressing full-length Frizzled receptors will suffice to elicit a phenotype. The presumption is that endogenous levels of Frizzled protein are well below the levels required to produce maximal activation of the pathway and that there is no limiting factor elsewhere in the pathway. If asymmetric localisation of Frizzled proteins is the critical aspect for PCP control, overexpression will only be expected to upset PCP if the localisation machinery is overwhelmed by the excess of Frizzled protein. And, of course, as already discussed, production of an effect depends on selection of the ‘correct’ Frizzled: it
is not certain that c-Frizzled-1 and c-Frizzled-7, the two Frizzled receptors tested, are
necessarily the PCP receptors, even though they have suggestive expression patterns.
Finally, there is the possibility that genetic redundancy will thwart experiments based on
single components. Genetic redundancy is especially a problem in experiments where one
seeks to test function by eliminating one of a set of functionally similar components, but it
can also cause difficulties in experiments like mine, where the selected component is
overexpressed (i.e. overexpression of one Frizzled receptor may have little effect, for
example, if the pathway can compensate by altering expression of another receptor).
Overlapping functions are an occupational hazard for the vertebrate developmental
biologist, and are always a possibility where genes of the same family are coexpressed. In
the inner ear, there is partial overlap in expression patterns, most notably at early stages.
For example, at E4, \textit{c-frizzled-1} and \textit{c-frizzled-7} have extremely similar expression patterns
and, if they also shared a similar function, it is possible that misexpression of just one of the
two would fail to disrupt the wildtype phenotype. At later stages, however, their expression
patterns are more complementary than similar.

Taking these factors into consideration, we felt that, while Frizzled overexpression had
a reasonable chance of giving a phenotypical effect if our general hypothesis was correct,
the most revealing experiments were likely to be those based on the Dishevelled constructs.
However, this approach also is not without pitfalls. There are a number of problematic
factors one might consider, given the negative results obtained, which I covered at the end
of chapter 5. They include Dishevelled construct design, the use of human Dishevelled-1
and uncertainties as to which dishevelled genes are likely to be critical for Frizzled
signalling in the chick inner ear.
Overall, therefore, there are a number of reasons why the misexpression of Frizzled receptors and Dishevelled constructs might have failed to give a phenotype, and my observations need not lead to the conclusion that Frizzled signalling has no function in the chick inner ear.

6.3.2.3 Wrong assay method?

Given that I only ever assessed PCP in the basilar papilla, and several days after it is determined, might I have missed a PCP phenotype? Certainly, PCP phenotypes in the vestibular patches would have escaped me, and remain a strong possibility.

In the basilar papilla, I could have missed a disruption of PCP at early stages, if this was corrected and then masked by subsequent realignment of hair cell vectors. As noted in the description of wildtype PCP, hair cells do normally go through a process of adjustment: they do not emerge with their vectors already precisely aligned, unlike the polarised cells in systems such as Drosophila wing, in which PCP is precise at the outset and not subsequently altered. In the basilar papilla, an initial roughly organised hair cell asymmetry is seen between E8 and E9, several days before we assay the patches. It is therefore conceivable that, if Frizzled signalling is responsible for this initial rough asymmetry, but is not required for the subsequent refinement step, I scored hair cell PCP too late to detect an effect that had actually occurred.
6.3.2.4 Foiled by low transgene expression?

While analysing the viral and transgene markers in the otic epithelium, I was struck by the relative intensity of staining in sensory versus non-sensory regions. Though it was by no means always the case, there were a number of instances where one could unambiguously pinpoint the site of a sensory patch, based solely on the lack or reduced expression of viral and transgene markers. Figure 6.1 illustrates this point. Whatever the cause of this relatively low expression of the genes carried in the viral genome in the region of greatest interest for my experiments, it is likely to have reduced the chance of seeing a phenotypic effect. Indeed, all my experiments require that the transgene be strongly expressed. Thus, a dominant-negative form of a protein (encoded by the transgene) will generally block a pathway only if its concentration is enough to ‘swamp’ the wildtype form and out-compete it in at least one of its essential interactions. In my attempts to block PCP signalling with the DSH(DEP) construct, for example, one can anticipate that the pathway will only be blocked if interactions between the Frizzled receptor and the construct, which lead to no productive signal, prevent interactions between the Frizzled receptor and the wildtype Dishevelled, which lead to normal signal transduction. If transgene expression of the dominant-negative transgene is too low, the reduction in wildtype signalling may be too slight to produce a scorable PCP phenotype.

My other approach, overexpressing full-length Frizzled receptors, may also be vulnerable to reduced transgene expression. If, to produce a PCP phenotype, one needs to overcome an existing Frizzled signalling gradient, since it is this that is hypothesised to
**Figure 6.1** Low GAG and transgene expression in sensory patches

A through D are images of sectioned, E10 embryos, showing the inner ear. A and B both show expression of the DSH(DEP) transgene, with HA immunostain in red. Also in red is HCA immunostain, which shows the position of the sensory patch (in this case, the anterior crista, ac).

C and D show GAG immunostain in green (the virus carries the c-Frizzled-7 transgene). HCA immunostain is in red. C shows the anterior crista, while D shows the anterior crista and utricle (u).

In all four cases marker expression is stronger in non-sensory regions than it is within the sensory patches, be it GAG or HA immunostain.
orient hair cells, the signal generated by misexpressed receptors must at least equal, and probably surpass endogenous Frizzled signalling. If transgene expression is too low, it may be that hair cells orient their hair bundles normally because the strongest ‘PCP signal’ they receive is from endogenous Frizzled signalling, and not from the misexpressed c-Frizzled-1 and c-Frizzled-7 forms.

Similar considerations apply if hair cell polarity is dictated by an asymmetric distribution of Frizzled protein inside the individual cell. In this case, a disturbance of PCP will be produced by artificial overexpression of Frizzled only if the level of overexpression is so high as to overwhelm the intracellular localisation machinery.

6.3.3 Could a different Frizzled signalling system explain our results?

I have considered two diametrically opposed conclusions from my results: that Frizzled signalling does or, alternatively, does not determine PCP of chick inner ear hair cells. I will now briefly consider a third possibility, that the PCP machinery in the chick inner ear, while involving some homologous components, does not use them in the same way as the fly. This might allow us to reconcile all the data presented in this thesis.

The c-Celsr-1 expression pattern provides a strong indication of the conservation of one key facet of the invertebrate PCP signalling mechanism- biased subcellular localisation of Flamingo; but we do not know if the function of Frizzled signalling in PCP is conserved. It is possible that, in the inner ear, c-Celsr-1 may signal in a PCP pathway which does not rely on Frizzled and Dishevelled. Frizzled signalling may have taken on some different
function, and the requirement for it in PCP may have been replaced, either by a transfer of function to the Celsr proteins, or by a new developmental function for other molecules, whose role we have not suspected. If this were the case, it would explain why the strongly suggestive expression data for c-Celsr-1 have not been matched by my results with Frizzled and Dishevelled construct misexpression experiments.

6.4 Future work

The question of the function of Frizzled signalling in the inner ear remains open. I have hypothesised that Frizzled signalling is involved in PCP determination, in cell fate specification and in defining the extent of the sensory patches. All those possibilities remain to be proven. Indeed, it is still possible that there is no function for the Frizzled signalling pathway in the chick inner ear, in spite of the expression data presented in chapter 3.

There are a number of experiments one might propose, based on the findings of this thesis, that would help decide among these possibilities. For example, conditional knockouts of selected Frizzled receptors in the mouse could allow their function in the inner ear to be investigated. A similar strategy could also be employed using morpholino (a modified anti-sense RNA) knockdown in zebrafish. Both these approaches have the advantage of knocking out Frizzled function, which may prove more informative than Frizzled overexpression.

However, the experiment most likely to clarify the basis of PCP in the inner ear, I feel, is to interfere with the function of c-Celsr-1. It is expressed in the most suggestive pattern
of all Frizzled signalling pathway components analysed, and is therefore the prime candidate for PCP involvement.

In summary, this thesis represents the beginning of an analysis of the roles of Frizzled signalling and of the mechanisms of PCP in sensory patch development in the ear. The data show that several Frizzled signalling components are expressed in sensory patches and, despite the failure of the functional experiments using the RCAS retrovirus system to reveal a phenotype, there remains the strong possibility that Frizzled signalling will, in future, be shown to play a role in PCP determination and, potentially, other aspects of sensory patch development.
7 References


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230


236
