Identification and Characterisation of New Mouse Models for Hearing Loss

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A thesis presented for the degree of Doctor of Philosophy (PhD)

October 2016

Andrew Parker

Life and Biomolecular Science

The Open University, Milton Keynes, United Kingdom

Mammalian Genetics Unit, MRC Harwell Institute, United Kingdom

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Abstract

Mouse N-ethyl-N-Nitrosourea (ENU) mutagenesis programmes have been successfully employed for the identification of models of human disease, and allowed the discovery of novel gene associations. This thesis describes the characterization of two new ENU-induced mouse models of hearing loss, goya and Boycie, identified at the MRC Harwell Institute.

The goya mice carry a splice site mutation in the mitogen-activated protein kinase Map3k1 gene. In addition to an eyes-open-at-birth phenotype, Map3k1<sup>goya/goya</sup> mice initially develop supernumerary cochlear outer hair cells (OHCs) that subsequently degenerate, and a progressive profound hearing loss is observed by 9-weeks of age. Interestingly, heterozygote mice also develop supernumerary OHCs, but no cellular degeneration or hearing loss is observed. The extra OHCs were found not to arise from aberrant control of proliferation via p27KIP1. Expression of MAP3K1 is present in a number of inner ear cell types (inner and outer hair cells, spiral ganglion and stria vascularis), and investigation of targets downstream of MAP3K1 identified increased p38 phosphorylation in these cells. The goya mutant reveals a signalling molecule involved with hair cell development and survival.

Boycie mice were identified as exhibiting a high frequency progressive hearing loss with a semi-dominant mode of inheritance. Ultrastructural and morphological assessment of these mice identified interesting pathological findings, including: a consistent pattern of OHC loss in the basal and mid cochlear turns of both heterozygote and homozygote mice; thinning of the stria vascularis in similar cochlear regions; and, spiral ganglion neuron degeneration in the apical turn. The Boycie mutation was mapped to a ~3 Mb candidate interval on Chromosome 5 containing only one ENU-induced coding lesion, which is in the Orai1 gene that codes for a component of the calcium activated calcium entry (CRAC) channel.

These two novel mouse models highlight the important role for ENU mutagenesis in disease gene discovery.
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I have worked at the MRC Harwell Institute in three different decades and consider myself very lucky to have worked with some great people who have taught me a great deal. I would particularly like to thank Pat Nolan for giving me the opportunity to venture from the animal house to the lab, and Rachel Hardisty-Hughes, for initially teaching me about the ear, many valuable techniques and for believing in my abilities (and being a great boss!). All of the PI’s past and present have helped me in some way over the years and for that I am very grateful.

Massive thanks to Sara Wells, Lucie Vizor and all animal house staff from the Mary Lyon Centre, in particular Ward 4, for looking after my many mouse lines. I would also like to thank the Bioimaging, Histology, GEMS, Proteomics and Clinical Pathology core facilities at the MRC Harwell Institute for all their help and hard work.

I have to give a shout to my other work based support network, most of whom have now moved to pastures new: Kev and Steve from the old Imaging department for laughter on tap when required, Jeremy who not only taught me all I know about light microscopy but is a general all round good chap! Anne and Deb who taught me much - I miss you guys!

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<tbody>
<tr>
<td>ABC</td>
<td>Avidin/biotin complex</td>
</tr>
<tr>
<td>ABR</td>
<td>Auditory-evoked brainstem response</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AMPA</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ARC</td>
<td>Arachidonic acid receptor channels</td>
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<td>ARHL</td>
<td>Age-related hearing loss</td>
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<td>ARM</td>
<td>Armadillo</td>
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<td>AST</td>
<td>Aspartate aminotransferase</td>
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<td>Atf3</td>
<td>Activating transcription factor 3</td>
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<td>(also known as Math1) Atonal basic helix-loop-helix transcription factor 1</td>
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<td>Adenosine triphosphate</td>
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<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>Atp2a2</td>
<td>ATPase sarcoplasmic/endoplasmic reticulum (Ca^{2+}) transporting 2</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<td>BB-CK</td>
<td>Homodimer of creatinine kinase using two subunits from brain isoform</td>
</tr>
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<td>Abbreviation</td>
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<tr>
<td>B-CK</td>
<td>Creatinine kinase isoform most abundant in the brain</td>
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<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BGHR</td>
<td>Bovine growth hormone (reverse primer)</td>
</tr>
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<td>BK</td>
<td>‘Big K⁺’ channel</td>
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<tr>
<td>Bmp/BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine (5-bromo-2’-deoxyuridine)</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium (ionic)</td>
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<td>CAD</td>
<td>CRAC activation domain (see CRAC)</td>
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<tr>
<td>Camkk2</td>
<td>Calcium/calmodulin dependent protein kinase kinase 2</td>
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<td>CAPD</td>
<td>Central auditory processing disorder</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9 (see CRISPR)</td>
</tr>
<tr>
<td>CASPASE</td>
<td>Cysteine-aspartic proteases</td>
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<td>Caᵥ</td>
<td>Voltage gated Ca²⁺ channel</td>
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<td>Ccna1</td>
<td>Cyclin A1</td>
</tr>
<tr>
<td>Ccnd1</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>CCP4MG</td>
<td>CCP4 Molecular Graphics</td>
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CD  Cluster of differentiation

CDC42  Cell division control protein 42 homolog

CDH23  Cadherin23

Cdk4  Cyclin dependant kinase 4

Cdkn1b  (also known as p27\textsuperscript{kip1}) Cyclin-dependent kinase inhibitor 1B

Cdna  Complementary DNA

Chd7  Chromodomain-helicase-DNA-binding protein 7

Chr 5  Chromosome 5

CI  Confidence interval

CICR  Calcium Induced Calcium Release

CK  Creatine kinase

Cm\textsuperscript{R}  Chloromphenicol resistance

CN  Cranial nerve

CO\textsubscript{2}  Carbon dioxide

Col1a1  Collagen, type I, alpha 1

COME  Chronic otitis media with effusion

COOH  Carboxylic acid

Cos\textsuperscript{2}  Cosine squared

CRAC  Calcium Release Activated Calcium
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<tr>
<td>Cre</td>
<td>Cyclization recombination</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>crRNA</td>
<td>CRISPR targeting RNA</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle</td>
</tr>
<tr>
<td>Ctnbb1</td>
<td>Beta Catenin</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dB SPL</td>
<td>Decibel sound pressure level</td>
</tr>
<tr>
<td>DC</td>
<td>Deiters’ cels</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DDSA</td>
<td>Dodecenyl succinic anhydride</td>
</tr>
<tr>
<td>DFNA</td>
<td>Non-syndromic hearing loss, autosomal dominant</td>
</tr>
<tr>
<td>DFNB</td>
<td>Non-syndromic hearing loss, autosomal recessive</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>Diablo</td>
<td>Diablo IAP-binding mitochondrial protein</td>
</tr>
<tr>
<td>Dlk1</td>
<td>Delta-Like 1 Homolog</td>
</tr>
<tr>
<td>Dll1</td>
<td>Delta-like 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modification of Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
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<td>Abbreviation</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dOrai</td>
<td>Drosophila melanogaster Orai1</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s PBS</td>
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<tr>
<td>DPOAE</td>
<td>Distortion product optoacoustic emission</td>
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<tr>
<td>DSB</td>
<td>Double stranded break</td>
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<tr>
<td>DSD</td>
<td>Disorders of sexual development</td>
</tr>
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<td>dsDNA</td>
<td>Double stranded DNA</td>
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<td>dT</td>
<td>Deoxy-thymidine</td>
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<td>E2F transcription factor 1</td>
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<td>E</td>
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<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>Ethylenediaminetetraacetate</td>
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<td>EdU</td>
<td>5-ethynyl-2′-deoxyuridine</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td>Egfr</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid</td>
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<tr>
<td>EMPReSS</td>
<td>European Mouse Phenotyping Resource for Standardised ScreensEMRC</td>
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<td>Emx2</td>
<td>Empty Spiracles Homeobox 2</td>
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xxvii
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<tr>
<td>ENU</td>
<td>$N$-ethyl-$N$-Nitrosourea</td>
</tr>
<tr>
<td>EOB</td>
<td>Eyes open at birth</td>
</tr>
<tr>
<td>EP</td>
<td>Endocochlear potential</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal–regulated kinases</td>
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<tr>
<td>ES cell</td>
<td>Embryonic stem cell</td>
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<td>ESCM</td>
<td>Embryonic stem cell derived cardiomyocytes</td>
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<tr>
<td>ETOH</td>
<td>Ethanol</td>
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<td>EUCOMM</td>
<td>European Conditional Mouse Mutagenesis Program</td>
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<td>EUMODIC</td>
<td>European Mouse Disease Clinic</td>
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<td>FBS</td>
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<td>Fbxo11</td>
<td>F-Box Protein 11</td>
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<td>FESA</td>
<td>Frozen Embryo and Sperm Archive</td>
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<td>Ffgr/FGFR</td>
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<td>Fibroblast growth-factor</td>
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<td>FLP</td>
<td>Flippase recombinase</td>
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<td>Fos</td>
<td>FBJ Murine Osteosarcoma Viral Oncogene Homolog</td>
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<td>GWAS</td>
<td>Genome Wide Association Studies</td>
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<td>HDR</td>
<td>Homologous directed repair</td>
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<td>Hes</td>
<td>Hairy and enhancer of split</td>
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<td>HGU</td>
<td>Human Genetics Unit</td>
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<td>H-RAS</td>
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<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KOMP</td>
<td>Knockout Mouse Project</td>
</tr>
<tr>
<td>K-RAS</td>
<td>V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LDS</td>
<td>Lithium dodecyl sulfate</td>
</tr>
<tr>
<td>LER</td>
<td>Lesser epithelial ridge</td>
</tr>
<tr>
<td>Lfng</td>
<td>Lunatic fringe</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat-containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>LOCS</td>
<td>Lateral olivocochlear system</td>
</tr>
<tr>
<td>LoxP</td>
<td>Locus of X (cross)-over in P1</td>
</tr>
<tr>
<td>LRC</td>
<td>Leukotriene C4 regulated channels</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>LSM</td>
<td>Laser scanning microscope</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAP2K</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MAP3K</td>
<td>Mitogen activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MARVEL</td>
<td>MAL and related proteins for vesicle trafficking and membrane link</td>
</tr>
<tr>
<td>M-CK</td>
<td>Creatinine kinase isoform most abundant in skeletal muscle</td>
</tr>
<tr>
<td>Mef2c</td>
<td>Myocyte-specific enhancer factor 2C</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mechanoelectrical transduction</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MGD</td>
<td>Mouse Genome Database</td>
</tr>
<tr>
<td>MGI</td>
<td>Mouse Genome Informatics</td>
</tr>
<tr>
<td>MGU</td>
<td>Mouse Genetics Unit</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MM-CK</td>
<td>Homodimer of creatinine kinase using 2 skeletal muscle isoforms</td>
</tr>
<tr>
<td>MOCS</td>
<td>Medial olivocochlear system</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propane sulfonic acid</td>
</tr>
<tr>
<td>Mos</td>
<td>V-Mos Moloney Murine Sarcoma Viral Oncogene Homolog</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolts</td>
</tr>
<tr>
<td>Myo6</td>
<td>Myosin VI</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NH₂</td>
<td>Amine group</td>
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<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKCC</td>
<td>Potassium-chloride co-transporter</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMA</td>
<td>Nadic methyl anhydride</td>
</tr>
<tr>
<td>NorCOMM</td>
<td>North American Conditional Mouse Mutagenesis</td>
</tr>
<tr>
<td>Nr2f1</td>
<td>Nuclear Receptor Subfamily 2 Group F Member 1</td>
</tr>
<tr>
<td>OHC(s)</td>
<td>Outer hair cell(s)</td>
</tr>
<tr>
<td>OM</td>
<td>Otitis media</td>
</tr>
<tr>
<td>O₃O₄</td>
<td>Osmium tetroxide</td>
</tr>
<tr>
<td>OTO</td>
<td>Osmium tetroxide, thiocarbohydrazide processing</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>P2rx</td>
<td>P2X purinoreceptor</td>
</tr>
<tr>
<td>PAM</td>
<td>Protospacer adjacent motif</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS with Tween® 20</td>
</tr>
<tr>
<td>PCDH15</td>
<td>Protocadherin 15</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PHD</td>
<td>Plant homeodomain</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Ptk7</td>
<td>Protein tyrosine kinase 7</td>
</tr>
<tr>
<td>Ptpn11</td>
<td>Tyrosine-protein phosphatase non-receptor type 11</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription PCR</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RHOA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative quantification</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse-transcription PCR</td>
</tr>
<tr>
<td>RyR(s)</td>
<td>Ryanodine receptor(s)</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEK1</td>
<td>SAPK/Erk kinase</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum Ca$^{2+}$-ATPase</td>
</tr>
<tr>
<td>SGN(s)</td>
<td>Spiral ganglion neuron(s)</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SHIRPA</td>
<td>Smithkline-Beecham Pharmaceuticals, Harwell Mouse Genetics Centre and Mammalian Genetics Unit, Imperial College School of Medicine and St...</td>
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</table>
Mary’s, Royal London Hospital, St. Bartholomew’s and the Royal London School of Medicine Phenotype Assessment

SK  Small conductance calcium activated calcium channel

SNHL  Sensorineural hearing loss

SNP  Single nucleotide polymorphism

SOAE  Spontaneous optoacoustic emissions

SOC  Super Optimal broth with Catabolite repression

SOCE  Store operated calcium entry

Sox/SOX  SRY (sex determining region Y)-box

SPF  Specific pathogen free

SPL  Sound pressure level

Spry2  Sprouty 2

SR  Sarcoplasmic reticulum

STIM  Stromal interacting protein

SV  Stria-vascularis

SWIM  SWI2/SNF2 and MuDR

TAB1  TGF-β Activated Kinase 1/MAP3K7 Binding Protein 1

TAE  Tris-acetate-EDTA

TALENS  Transcription activator-like effector nucleases

xxxvi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween® 20</td>
</tr>
<tr>
<td>TCH</td>
<td>Thiocarbonyldrazide</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TGIM</td>
<td>Texas A&amp;M Institute for Genetic Medicine</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF Receptor Associated Factor</td>
</tr>
<tr>
<td>TRPA</td>
<td>Transient receptor potential ion channel</td>
</tr>
<tr>
<td>TRPC</td>
<td>Transient receptor potential cation channels</td>
</tr>
<tr>
<td>UCSF</td>
<td>University of California, San Francisco</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
</tr>
<tr>
<td>ZFNs</td>
<td>Zinc finger nucleases</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Zinc (ionic)</td>
</tr>
<tr>
<td>ZnF</td>
<td>Zinc finger</td>
</tr>
<tr>
<td>ZNPC</td>
<td>Zone of non-proliferating cells</td>
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</table>
Chapter 1

Introduction
1.1. Hearing Impairment

In Europe alone there are 22.5 million hearing impaired people, with hearing impairment affecting prelingual (1 in 800-1000) and postlingual children (1 in 400-500), which can hinder a child’s educational performance and cognitive development. Environmental effects such as trauma, noise exposure and ototoxic drugs can all result in hearing deficits, however over one third of all hearing defects are thought to have a genetic aetiology.

To date, 300 inherited syndromic and over 130 non-syndromic human loci predisposing people to hearing impairment have been identified. However, at the time the work contained in this thesis began, less than a third of the underlying genes and associated molecular defects had been characterized. At the time of writing, with the ever reducing cost of technologies such as next generation sequencing enabling improved mutation detection, the ratio of known/unknown genes has improved greatly; in the case of autosomal dominant non-syndromic hearing loss (termed DFNA) sixty out of eighty four are known, whereas for autosomal recessive non-syndromic hearing loss (termed DFNB), the responsible gene has been discovered in thirty three of fifty four reported loci.

1.2. The Ear

The organ required for both hearing and balance is the ear. The ear is a highly complex organ and can be split into three distinct areas, the outer ear, middle ear and inner ear (Figure 1.1). The correct development, anatomy and function of all three parts of the ear are required for accurate sensation of sound.
1.2.1. The Outer Ear

The outer ear consists of the auricle, or pinna, and auditory canal which collect and channel sound waves toward the tympanic membrane, (also known as the eardrum) (Fig. 1.1). The tympanic membrane is a thin layer of cells that separates the outer and middle ear, and plays a vital role in sound transmission. A sound wave is a fluctuation in air pressure caused by a noise source, when the sound waves hit the tympanic membrane, it moves in response to the altered pressure.

1.2.2. The Middle Ear

The middle ear is essentially an air filled cavity which houses the three smallest bones in the human body, the ossicles. The ossicular chain, made up of the malleus (hammer), incus (anvil) and stapes (stirrup), serves to transfer and amplify movements of the tympanic membrane to the inner ear, via a connection between the footplate of the stapes and the oval window. Attached to the bony capsule of the middle ear are the stapedius muscle and the tensor tympani, which connect to the stapes and malleus respectively. These two muscles can contract either together or independently when dangerously high intensity sound waves stimulate the tympanic membrane. This process, known as the tympanic reflex, reduces the amplification of the ossicular chain by restricting the movement of the joints and protects the inner ear from damage due to over stimulation. The middle ear is connected to the nasopharynx through the Eustachian tube which ventilates the middle ear and equalise pressure between the middle ear cavity and the throat (Figure 1.1).
The inner ear consists of the cochlea, which enables us to hear sound, and the vestibular system which senses balance and motion. (Figures 1.1 and 1.2). The shell of the inner ear is called the bony labyrinth, housed within is the membranous labyrinth. Located inside the membranous labyrinth are the sensory cells that enable the cochlea to transduce mechanical stimuli arriving at the oval window into electrical signals which travel along
the vestibulocochlear nerve (CN VIII) to the brain, and allow us to perceive sound, or hear.

The membranous labyrinth extends throughout both the cochlea and the vestibular system and contains a fluid called endolymph which is high in K\(^+\) and low in Na\(^+\) and Ca\(^{2+}\).

The space between the membranous labyrinth and the bony labyrinth is filled with a fluid called perilymph, which is very high in Na\(^+\) and low in K\(^+\), the high K\(^+\) concentration in the endolymph allows an electrical potential of around +80 mV when compared with perilymph, it is this potential that allows transduction from the sensory cells. In a cross section of the cochlea, the membranous labyrinth splits internal space into three sections or scala, the scala tympani, the scala media and the scala vestibule (Figure 1.2B). Although separated along the length of the cochlea, the scala tympani and scala vestibuli meet at the helicotrema, forming a continuous duct that surrounds the scala media, in which the sensory cells are found.

### 1.3. The Cochlea

The cochlea is a coiled structure that resembles a snail shell; it is named after the Latin term for snail. The organ of Corti is the structure in the cochlea that contains the outer hair cells (OHCs) and inner hair cells (IHCs). These sensory cells are normally arranged in one row of IHCs and three rows of OHCs, and sit on the basilar membrane which divides the scala media from the scala tympani. The IHC and OHC are separated by alternating inner and outer pillar cells, which join to form an arch surrounding the tunnel of Corti, a cross sectional illustration of the organ of Corti can be seen in Figure 1.2 and Figure 1.3.
Figure 1.2 Inner Ear Anatomy. A 3-D representation of the inner ear modelled from a micro-CT scan of a mouse skull. (A) The anatomy of the cochlea (C) and vestibular system (V) of the inner ear. (B) A cartoon of a cross section of the cochlea duct. The scala vestibuli, scala media and scala tympani are labelled as well as a number of important cochlea structures. Basilar membrane - BM, organ of Corti - OoC, Reissner’s membrane - RM, spiral ganglion neurons - SGN, spiral ligament - SL, stria vascularis - SV, tectorial membrane - TM.

Figure 1.3 The Organ of Corti. A cartoon of the organ of Corti and surrounding cells in more detail, highlighting the cellular architecture of the organ of Corti. (Claudius cells (CC), Deiters’ cells (DC), Hensen’s cells (HC), inner hair cells (IHC), inner sulcus cells (IS) nerve fibres (NF), outer hair cells (OHC), pillar cells (PC), tectorial membrane (TM)).
1.3.1. Cochlea Structure and Transduction of Sound

Actin rich organelles known as stereocilia protrude from the cuticular plate of both inner and outer hair cells. Although the scala media is wider at the base (closest to the oval window) the basilar membrane is narrowest (and stiffest) at this point, it gets wider and less stiff toward the apical end at the helicotrema. This gradient in basilar membrane physical properties along the length of the cochlea, in part influences the ability of different areas of the cochlea to be stimulated in a frequency specific, or tonotopic, manner (Békésy and Wever, 1960). Another factor that enables frequency discrimination is the tonotopic arrangement of OHC. At the base of the cochlea, the OHC are short, as are their stereocilia bundles, and at the apex both OHC bodies and stereocilia are longer (See Introduction 1.2.8). Underneath the OHC are Deiter’s cells, which support the OHC, and like the majority of supporting cells in the cochlea, research suggests they play a role in ion transport, essential for maintaining the unusual electrical potential of the endolymph (Kikuchi et al., 2000; Spicer and Schulte, 1993; Spicer and Schulte, 1994).

An acellular matrix, the tectorial membrane lies across the organ of Corti; the tallest stereocilia row of each OHC bundle make contact with the tectorial membrane (Kimura, 1966), although it is yet unknown if the same is true of IHC stereocilia (Matsumura, 2001). The stereocilia are mainly composed of actin filaments and form a bundle arranged in a ‘staircase’ formation. They initially grow towards a kinocilium on the apical side of the hair cell, in most mammals this kinocilium disappears when the bundle is fully mature. OHC contain three rows of stereocilia and the bundles take on a V or W shape, whereas IHC bundles contain two main rows arranged in a more subtle curve (Figure 1.4).

The apical end of stereocilia from neighbouring rows are joined by tip links (Pickles et al., 1984), which have been shown to consist of homodimers of protocadherin 15 (PCDH15)
at the bottom and cadherin 23 (CDH23) at the top (Kazmierczak et al., 2007). These tip links, as well as maintaining the structure of the stereocilia bundle, are involved with the opening of mechanoelectric transduction (MET) channels in the hair cell stereocilia, required for the transduction of sound (Assad et al., 1991; Wright, 1981). More recently it has been shown that the MET channels are located at the lower end of the tip link, after researchers observed very low calcium uptake by the tallest rows of stereocilia upon bundle deflection (Beurg et al., 2009).

Lateral to the OHCs in the organ of Corti are the Hensen cells, as with Deiters’ cells are thought to both play a structural role and be involved with ionic regulation in the cochlea (Fechner et al., 1998). Lateral to the Hensen cells are Claudius cells which again provide support for the organ of Corti and provide a barrier between endolymph and perilymph as a result of tight junctions holding the cells together.

The lateral wall of the cochlear duct consists of the spiral ligament and the stria vascularis. The spiral ligament is made up mainly of type I, type II, type III and type IV fibrocytes (Spicer and Schulte, 1991), it runs underneath the stria vascularis and in part serves to support this, and also anchor the lateral edge of the basilar membrane. The stria vascularis consists of three distinct cellular layers, basal, intermediate and marginal cells, the latter of which are exposed to the endolymph filled scala media. The basal cells act as

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![Figure 1.4 Scanning Electron Micrographs highlighting stereocilia arrangement.](image)

Panel A shows the single row of IHCs and three rows of OHCs. Panel B and C are higher magnification images of IHC (B) and OHC (C) stereocilia bundles. The ‘staircase’ row arrangement and W shaped bundle of the OHC are apparent in panel C. (Parker unpublished data)
a barrier between the spiral ligament and the stria vascularis. Intermediate cells of the stria vascularis are melanocytes, research using mice with pigmentation defects indicates that these are essential for the generation of the EP (Steel and Barkway, 1989). The marginal cells of the stria vascularis are polarised epithelial cells. They are covered in microvilli which secrete K⁺ into the endolymph (Wangemann et al., 1995). Separating the scala media from the scala vestibuli is Reissner’s membrane, which comprises two layers of epithelial cells.

1.3.2. Mechanoelectric Transduction in the Cochlea

Despite much research the identity of the mechanoelectric transduction (MET) channel is yet to be discovered; early research suggested that the transient receptor potential ion channel (TRPA1) was a likely candidate (Corey et al., 2004). A follow up study from the same group however indicated otherwise, after TRPA1 knockout mice exhibited no signs of hearing loss (Kwan et al., 2006). More recent studies using reporter tagged and knockout mice point toward transmembrane channel like proteins 1 and 2 (TMC1 and TMC2) being the MET channel (Kawashima et al., 2011; Kurima et al., 2015; Pan et al., 2013). Both localise to the MET channel site at the tips of the two shorter rows of stereocilia, and double knockouts have no MET current at all, however although likely involved in the MET channel complex, it has been suggested that neither form the pore (Fettiplace, 2016).

Whatever the identity of the MET channel, hair cell function and the mechanoelectric transduction process has been well described (reviewed in (Hudspeth, 2014)). Following the vibrations of the tympanic membrane being directed through the ossicles to the inner ear via the oval window, the basilar membrane vibrates up and down due to the fluid wave that propagates through the perilymph in the scala tympani and scala vestibule. The
upward movement of the basilar membrane lifts the organ of Corti towards the tectorial membrane creating a shearing force acting on the stereocilia. This causes deflection of the bundle towards the tallest row, which stretches the tip links allowing the MET channels (non-selective cation channels) in the stereocilia tips to open (Corey and Hudspeth, 1979; Shotwell et al., 1981) (Figure 1.5). As mentioned, the ~+80 mV potential of the endolymph, known as the endocochlear potential (EP) (Assad et al.), relies in part on the the unique ionic constitution of endolymph: high in K⁺, low in Na⁺ and extremely low in Ca²⁺, compared with the perilymph, which is low in K⁺, high in Na⁺ with more abundant Ca²⁺. The hair cells themselves have a resting membrane potential of around -60 mV, this creates a potential difference of 140 mV between the endolymph that bathes the apical surface and stereocilia of the hair cell are in contact with, and the hair cell body. This potential, driven by the EP allows a passive movement of K⁺ from endolymph into the hair cell eliminating the requirement for adenosine triphosphate (ATP). As the channel opens, K⁺ and Ca²⁺ ions flood into the body of the hair cell depolarising the membrane (Figure 1.5Bii). The channels are very slightly open when resting (Figure 1.5Bi), and when shearing force results in movement of the bundle away from the tallest stereocilia they close hyperpolarising the membrane (Figure 1.5.Biii). More recently it has been shown that the MET channels are located at the lower end of the tip link, after researchers observed very low calcium uptake by the tallest rows of stereocilia upon bundle deflection (Beurg et al., 2009).
In OHCs this change in ionic potential causes the cell body to lengthen or contract due to the voltage sensitive motor protein PRESTIN (Zheng et al., 2000a). This is thought to have the effect of amplifying the movements of the basilar membrane in a non-linear fashion,
allowing the detection of a wide dynamic range of sound intensity by altering the biomechanical properties of the organ of Corti (Ruggero and Rich, 1991) (Figure 1.6A-C).

**Figure 1.6 Electromotility and Neurotransmission resulting from MET Channel K⁺ influx.** Panels A – C show the effects of MET channel activity on the OHC. In a resting state (A) the OHC MET channels are slightly open, allowing a small amount of K⁺ to enter the OHC and maintaining a resting conformation. Upon deflection of the stereocilia towards the tallest row (B), K⁺ ions flood the cell, depolarise the membrane and cause the voltage sensitive motor protein PRESTIN to contract the cell membrane. When the bundle is deflected in the other direction (C), the MET channel closes, hyperpolarising the cell and causing PRESTIN to elongate the cell membrane. Panels D and E illustrate the result of MET channel K⁺ influx on IHC neurotransmission at the ribbon synapse, as described in the text. Mechanoelectric transduction channel – MET, outer hair cell – OHC, inner hair cell – IHC, potassium - K⁺, calcium - Ca²⁺, voltage gated calcium channel – VGCC, ribbon – R, glutamate containing vesicle – GV, spiral ganglion neuron – SGN, glutamate – G.
In IHCs the increase in membrane potential results in the activation of the voltage gated calcium channel $\text{Ca}_{v}1.3$ in the in cell membrane. This in turn allows $\text{Ca}^{2+}$ to enter the cell and results in exocytosis of vesicles carrying the neurotransmitter glutamate, from specialised ‘ribbon’ synapses, which consist of presynaptic active zones near the base of the hair cell, to the postsynaptic afferent spiral ganglion neurons (SGN) (Brandt et al., 2005; Brandt et al., 2003; Platzer et al., 2000) (Figure 1.6D).

**1.3.3. Innervation of the Cochlea**

Spiral ganglion neurons are afferent neurons which relay the signal transduced by the hair cell; they connect the ear to the cochlear nucleus and ultimately the brain. There are two distinct populations, type I SGN and type II SGN. IHCs connect with type I SGNs through a ribbon synapse in the basolateral membrane of the cell. Ribbon synapses are found in the eye and the ear and are able to produce a graded output of neurotransmitter, corresponding to the stimulus the sensor cell receives. This allows for very rapid encoding of the input signal, and also a constant variable output, essential for both hearing and vision (Khimich et al., 2005; tom Dieck and Brandstatter, 2006). The IHC synaptic ribbon tethers vesicles containing glutamate near a presynaptic cleft. Upon depolarisation of the membrane from $\text{K}^+$ influx, vesicles already at the active zone release their cargo of glutamate in response to $\text{Ca}^{2+}$ entering from the $\text{Ca}_{v}1.3$ channels into the synaptic cleft between the IHC and SGN (Figure 1.6E). The glutamate then binds to $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the SGN post synaptic density (Matsubara et al., 1996) and a graded potential is sent through the auditory nerve to the auditory cortex where it is decoded to allow the sense of hearing.

Afferent type I SGN contact only one IHC, however each IHC has contact with numerous type I SGNs (Figure 1.7A). The nerve fibres from type 1 SGN are also in contact with
efferent fibres originating in the lateral olivocochlear system (LOCS) (Figure 1.7A); little is known as to the true function of LOCS but they are thought to directly modulate the output from the IHC, partly due to their location (Groff and Liberman, 2003).

The OHC on the other hand are afferently innervated by type II SGN which account for less than 10% of the total number of SGN (Spoendlin, 1985). As a result, in contrast to the IHC which are innervated by multiple type I SGN, each type II SGN contacts several OHC, (Berglund and Ryugo, 1987) (Figure 1.7A). This observation reinforces the fact that the IHC are largely responsible for the transduction of sound. In addition, each OHC is efferently innervated by neurons originating in the medial olivocochlear system (MOCS) (Spoendlin, 1985). Although each OHC forms efferent synapses, the neurons themselves are much fewer in number than OHC (i.e. each neuron contacts numerous OHC), considerable dendritic branching occurs from each MOC neuron (Liberman and Brown, 1986) (Figure 1.7A). The efferent MOC neurons that contact the OHC are cholinergic neurons, meaning they use acetylcholine ACh as neurotransmitter (Fuchs and Murrow, 1992; Housley and Ashmore, 1991; Schuknecht et al., 1959). In the OHC the nicotinic acetylcholine receptors nAChRα9 and nAChRα10 mediate the incoming efferent signal (Elgoyhen et al., 1994; Elgoyhen et al., 2001). Once bound to ACh, they facilitate a short influx of extracellular Ca$^{2+}$ for which they are permeable; consequently this opens up small conductance calcium activated potassium (SK) channels in the OHC membrane allowing rapid efflux of K$^+$. (Fuchs and Murrow, 1992; Housley and Ashmore, 1991; Oliver et al., 2000) (Figure 1.7B). Findings suggest that these efferent signals act to regulate the electromotility of OHC in response to sound, as they can rapidly act on the membrane potential, regulating PRESTIN activity and thus the OHC length, affecting tuning, mechanics and ‘power output’ of the OHC (Dallos et al., 1997; Rabbitt et al., 2009). There is evidence to suggest that this control can protect OHC against damage from acoustic
trauma (Canlon and Fransson, 1995; Liberman, 1991). However it has been pointed out that the sound pressure levels often used to elicit this protective effect would not be likely in the real world, suggesting that although the effect is real, it is probably not the main role of efferent innervation in OHC (reviewed in (Christopher Kirk and Smith, 2003)).

![Diagram of Organ of Corti Innervation and the OHC Efferent Synapse](image)

**Figure 1.7** Organ of Corti Innervation and the OHC Efferent Synapse. Panel A is a cartoon of the organ of Corti indicating the afferent and efferent innervation. A single IHC is contacted by several type I SGN (green) and one synapse branching from an LOC efferent neuron (red) contacts the afferent fibre near the synapse. One type II SGN contacts a number of OHC (yellow). Dendrites branching from a single MOC efferent neuron contact numerous OHC (blue). Panel B illustrates activity of the OHC efferent cholinergic MOC synapse as described in the text. Inner hair cell – IHC, outer hair cell – OHC, potassium $K^+$, calcium - $Ca^{2+}$, acetylcholine – Ach, nicotinic acetylcholine receptors, small conductance calcium activated potassium channel – SK, medial olivocochlear – MOC, lateral olivocochlear – LOC.
1.3.4. Potassium Recycling in the Cochlea

As cochlear mechanoelectric transduction relies on the EP and K⁺ rich endolymph, it is important that the K⁺ concentration in the endolymph is maintained, for this reason, it must be recycled. It is well known that the EP is generated by the stria vascularis located on the lateral wall of the scala media, (Salt et al., 1987; Steel and Barkway, 1989; Tasaki and Spyropoulos, 1959). However, the exact physical and molecular path(s) that K⁺ ions taken up by the hair cells are recycled and returned to the endolymph are not conclusively understood. There is evidence for at least two separate routes; the lateral K⁺ recycling pathway thought to be responsible for recycling K⁺ from OHCs, and a medial K⁺ recycling pathway has been suggested for recycling K⁺ taken up by the IHCs (Figure 1.8A). In the case of the lateral pathway, following stimulation, influx of K⁺ from the endolymph opens potassium voltage-gated channel subfamily KQT member 4 (KCNQ4) channels in the base of the OHC (Kubisch et al., 1999) (Figure 1.8B).

Some believe that K⁺ ions move from the OHC freely into the perilymph in the scala tympani before being recovered by the spiral ligament (Zidanic and Brownell, 1990). There is, however, growing evidence that many of the supporting cells play a role in the trafficking of K⁺ back to the stria vascularis via the spiral ligament. Deiters’ cells express inwardly rectifying K⁺ channels (Kir4.1) and also two potassium-chloride co-transporters KCC3 and KCC4. All of these have been proposed to be involved with uptake of K⁺ released by the KCNQ4 channels in the OHC (Boettger et al., 2002; Boettger et al., 2003; Hibino et al., 1997; Rozengurt et al., 2003). The K⁺ is thought to be transported through the supporting cells and into the spiral ligament via a network of gap junctions comprising mainly CONNEXIN 26 and CONNEXIN 31 (Ahmad et al., 2003; Forge et al., 2003; Kikuchi et al., 1995; Lautermann et al., 1998). Mutations in the gap junction beta 2 (GJBZ) gene which encodes CONNEXIN 26 are by far the most common form of non-syndromic
autosomal recessive deafness (DFNB) in the developed world, accounting for roughly half of all cases (Denoyelle et al., 1997; Estivill et al., 1998; Kelsell et al., 1997; Mustapha et al., 2001; Zelante et al., 1997).

The type II and IV fibrocytes of the spiral ligament have been shown to express both the sodium-potassium-ATPase pump (Na,K–ATPase) (Ichimiya et al., 1994), and the sodium-potassium-chloride co-transporter NKCC1 (Crouch et al., 1997), which are thought to take up the recycled K⁺. The K⁺ then passes through another network of gap junctions connecting the spiral ligament to the basal and then intermediate cells of the stria vascularis (Kikuchi et al., 1995; Lautermann et al., 1998) (Figure 1.8C).

Between intermediate and marginal layers of the stria vascularis, is a tiny (~15 nm) extracellular space, the intrastrial space (Salt et al., 1987) (Figure 1.8C). This space is filled with intrastrial fluid, which is both positively charged, but has a very low K⁺ concentration and is separated from the perilymph surrounding the spiral ligament and basal/intermediate layers of the stria vascularis, and the endolymph in the scala media by tight junctions (Jahnke, 1975). Once K⁺ builds up in the intermediate cells after being pumped through the gap junction network, it passively diffuses through Kir4.1 channels in the apical membrane into the intrastrial space. It is thought that this K⁺ diffusion potential across the apical membrane of the K⁺ rich intermediate cells and the electrically isolated K⁺ poor intrastrial space is what generates the potential voltage jump which results ultimately responsible for the EP (Hibino et al., 1997; Salt et al., 1987; Takeuchi et al., 2000) (Figure 1.8C). Although Kir4.1 channels are known as inwardly rectifying they are also permeable in the opposite direction (Ishii et al., 1997).
Figure 1.8 Potassium Recycling in the Cochlea. Panel A shows an overview of both the lateral (black arrows) and medial (red arrows) routes that K\(^+\) is thought to be transported from the base of the hair cells back into the endolymph. (B) Shows K\(^+\) passively entering through a MET channel in an OHC stereocilia due to the diffusion gradient between endolymph and perilymph (+80 mV), before diffusing through KCNQ4 channels in the base of the cell. Panel C is a proposed model for movement of K\(^+\) from the spiral ligament, through the stria vascularis and into the scala media as described in the text. Stria vascularis – SV, spiral ligament – SL, supporting cells – SC, outer hair cell – OHC, spiral limbus – L, internal spiral sulcus – IS, mechanoelectric trasduction channel – MET, gap junctions – GJ, tight junctions – TJ, scala media – SM, marginal cells – MC, intrastrial space – IS, intermediate cells – IC, basal cells – BC, spiral ligament fibrocytes – SL(F), blood vessel – BV, potassium – K\(^+\), sodium – Na\(^+\), chloride - Cl\(^-\). Intrastrial space is exaggerated for illustrative purposes.
The basolateral membrane of the marginal cells of the stria vascularis also express NKCC1 and Na,K–ATPase. Once $K^+$ has diffused into the intrastrial space it is actively taken up via these pumps by the marginal cells (Figure 1.8C). The $Cl^-$ ions resulting from NKCC1 activity are removed from the marginal cells by CIC-K/Barttin chloride channels (Estevez et al., 2001) (Figure 1.8C) The marginal cells therefore have an extremely high concentration of potassium with a potential of ~+90 mV. The apical surface of the marginal cells can then secrete $K^+$ into the endolymph maintaining the majority of this electrical potential, as both marginal cells and the endolymph contain similar concentrations of $K^+$ ions. This is achieved via channels comprising potassium voltage-gated channel subfamily E member 1 (KCNQ1) and Potassium Voltage-Gated Channel Subfamily Q Member 1 (KCNE1), completing the recycling process (Neyroud et al., 1997; Vetter et al., 1996) (Figure 1.8C).

Much less attention has been given to the medial recycling pathway, although there is evidence for $K^+$ leaving the IHC and being recycled through gap junctions in the internal spiral sulcus and spiral limbus before returning to the endolymph (Spicer and Schulte, 1998) (Figure 1.8A). The likelihood is that both of these pathways, and possibly others yet to be identified, combine to contribute to this essential $K^+$ recycling process that maintains the EP in endolymph.

1.4. Retrocochlear Sound Transmission

The central auditory pathway is responsible for neurotransmission arising in the cochlea. Nerve impulses propagated from the cochlear hair cells travel through the SGN to the cochlear nucleus, from here they travel to the superior olivary complex. From here they are passed to the lateral lemniscus which relays the signal to the brainstem, and on to the inferior colliculus. From the inferior colliculus the neural information is passed through the medial geniculate nucleus to the auditory cortex (Fig. 1.9). As with the architecture of
the cochlea, a tonotopic organisation of the nerve fibre termini is maintained in the auditory cortex (Talavage et al., 2004).

Figure 1.9 The central auditory pathway. Simplified diagram of the major structures along the auditory pathway involved with retro-cochlear sound transmission, as described in the text. Adapted from (Purves et al., 2001)
1.5. Organ of Corti Development

The inner ear develops from a pocket of epithelial cells called the otic placode. In mice, the transformation from a featureless ball of cells to the formation of the membranous labyrinth containing the identifiable inner ear structures, happens over a surprisingly short period of time between embryonic day (E) 11 and E17; by this stage features such as semi-circular canals and the extended, coiled cochlear duct containing a fully differentiated, albeit immature, organ of Corti are present. Although the exact interplay and molecular mechanisms governing these events are not yet fully understood, studies have identified many of the pathways and some of the critical genes that help regulate this highly complex and co-ordinated development of the cochlear and vestibular systems, in particular the highly specialised sensory epithelia (reviewed in (Liu et al., 2014)).

The Bone Morphogenetic Protein 4 encoding gene (*Bmp4*) is expressed at an early stage during inner ear development and is thought to play an important part in specification of the prosensory patch that gives rise to the organ of Corti (Morsli et al., 1998; Ohyama et al., 2010). As the cochlea duct starts to extend, at around E12, the expression pattern of *Bmp4* in the developing mouse inner ear is restricted to prosensory precursor cells in the semi-circular canal regions and a band of cells lateral to those that will become the organ of Corti (Morsli et al., 1998) (Figure 1.10A). By E13 the cells within this prosensory precursor domain express genes including the NOTCH ligand Jagged 1 (*Jag1*), SRY (sex determining region Y)-box (*Sox2*) and lunatic fringe (*Lfng*) (Figure 1.10B). Between E13 and E14, sonic hedgehog (*Shh*) expression is observed in the developing SGN (Driver et al., 2008; Liu et al., 2010) (Figure 1.10C). Both *Bmp4* and *Shh* have been shown to be essential for proper formation of the organ of Corti; however, instead of being ultimately
responsible for signalling resulting in the differentiation into specific sensory or supporting cells, it has been suggested that Bmp4 and Shh provide boundaries for the developing prosensory domain. In contrast to Bmp4, however, Shh is not expressed in the cochlear duct per se, rather it is thought that it may diffuse through from the SGN (Driver et al., 2008; Liu et al., 2010; Morsli et al., 1998; Ohyama et al., 2010). At the same time as the onset of Shh expression, a wave of expression of the cell cycle regulator cyclin-depantant kinase inhibitor 1B (p27Kip1) travels from the apex to the base of the cochlea restricted to the band of cells between those expressing Bmp4 and Shh (Figure 1.10B); this event is coincidental with these prosensory cells exiting the cell cycle forming a zone of non-proliferating cells (ZNPC) which extends throughout the whole cochlear spiral by E14.5 (Lee et al., 2006). As the wave of p27Kip1 expression is extending from apex to base, some cells already positive for p27Kip1 begin to express the atonal basic helix-loop-helix transcription factor 1 (Atoh1), also referred to as (Math1). As p27Kip1 expression is initiated at the apex of the cochlea, it is these apical precursor cells which exit the cell cycle first; conversely Atoh1 expression begins in the mid basal region of the cochlea and travels as a bi-directional wave throughout the cochlear spiral, (Figure 1.10C) (Woods et al., 2004). The onset of Atoh1 expression between E13.5 and E14.5 correlates with the first signs of differentiation of the prosensory precursor cells. In these Atoh1 expressing progenitor cells which will develop into the sensory hair cells, p27Kip1 is downregulated and NOTCH ligands including Jag2 and Delta1 are upregulated. This leads to NOTCH mediated lateral inhibition, with neighbouring cells expressing Notch1 destined for a supporting cell fate, due to upregulation of NOTCH effectors Hes1 and Hes5, which inhibit Atoh1 expression ((Li et al., 2008), reviewed in (Cai and Groves, 2015; Liu et al., 2014)). By E16 only immature hair cells express Atoh1 with p27Kip1 expression restricted to supporting cells, Fibroblast growth-factor receptor 3 (Fgfr3) expression is also seen
starting at E16, in developing supporting cells and pillar cells (Figure 1.10D) (Jacques et al., 2007). By birth (P0), the cellular architecture of the organ of Corti is complete, although cells are still immature, and the tunnel of Corti formed by the inner and outer pillar cells is not yet formed; \( p27^{kip1} \) expression is maintained in supporting cells (Kil et al., 2011; Lee et al., 2006; Lowenheim et al., 1999), \( Ffgr3 \) expression is restricted to pillar cells and Deiters’/Hensen cells (Jacques et al., 2007), and \( Atoh1 \) expression is confined to IHC and OHC (Woods et al., 2004). No expression of \( Shh \) is observed in the SGN (Liu et al., 2010), however expression of \( Bmp4 \) remains (Ohyama et al., 2010) (Figure 1.10E).

**Figure 1.10 Overview of gene expression during organ of Corti development.** (A) At E12, a band of cells expressing \( Jag1, Sox2 \) and \( Lfng \) is observed in the bottom of the developing cochlear duct. At the lateral edge of these cells, a band of \( Bmp4 \) expression is seen. (B) At E13, \( Shh \) which is strongly expressed in the SGN is thought to diffuse into a population of cells medial to those expressing \( Jag1, Sox2 \) and \( Lfng \). In the cells located between the areas of \( Bmp4 \) and \( Shh \) expression, an apical to basal wave of \( p27^{kip1} \) expression is observed, which is coincident with these cells exiting the cell cycle. (C) By E14 the wave of \( p27^{kip1} \) has extended throughout the cochlear spiral and a wave of \( Atoh1 \) starts travelling in the opposing direction. (D) By E16 \( p27^{kip1} \) is downregulated in developing hair cells and restricted to those that will become supporting cells, whereas \( Atoh1 \) is only expressed in developing hair cells. In addition \( Fgfr3 \) expression can be seen in developing supporting cells. (E) By P0 \( Shh \) expression is absent from SGN but \( Bmp4 \) expression remains at the lateral edge of the developing organ of Corti, \( p27^{kip1} \) remains in the supporting cells and \( Atoh1 \) in the hair cells, and \( Fgfr3 \) expression is seen in the developing inner and outer pillar cells and Deiters’/Hensen cells. Spiral ganglion neuron – SGN, Köllikers organ – KO, Inner hair cell – IHC, Outer hair cell – OHC, Inner pillar cell – IP, Outer pillar cell – OP. Figure adapted from (Wu and Kelley, 2012)
1.6. Frequency Response of the Cochlea

The ability of the ear to process complex sounds, made up of many frequencies allows us to perceive speech and other important sounds from the world around us. There is still debate as to how the ear manages to rapidly transduce such a wide range of frequency specific information, but two main theories have existed and remain for well over a century. ‘Place theory’ was introduced by Ohm and Helmholtz back in the mid-19th century, and hypothesized that different portions of the basilar membrane would respond to different frequencies of sound, a tonotopic response. Ohm suggested the cochlea worked as a frequency analyser, breaking complex sounds down into component sine waves, Helmholtz believed that the physical properties of the basilar membrane at different points along its length allowed different frequency sounds to cause it to resonate in sympathy. Research undertaken by Hungarian Nobel prize winner Georg von Békésy provided some evidence for place theory. Using dissected cochleae from a wide range of species, Békésy showed that a travelling wave propagated through the cochlear duct and caused the basilar membrane to move in sympathy. The portion of the cochlea where the wave reached its maximum amplitude related to the frequency of the sound being played; low frequency sounds stimulated the apical regions of the basilar membrane and higher frequency sounds stimulated the basal regions (Békésy and Wever, 1960). The fact that this research was performed on cadavers meant the travelling wave observed was a passive mechanism; this alone would probably be incapable of resolving complex sounds as accurately as they need to be. A challenge to the place theory was made by Seebeck, again in the mid-19th century, and proposes that frequency, or pitch, perception is related to the rate at which neurons fire in response to a sound. As previously mentioned the transduction channels in the stereocilia are partially open when resting and can either increase or decrease the membrane potential dependant on the
direction the stereocilia bundle is stimulated, this allows for a sinusoidal response of the hair cell. This has been termed ‘temporal theory’, again results from experiments investigating this revealed that at low frequencies there was indeed correlation between stimuli frequency and firing rate, however it would be impossible to resolve higher frequencies (reviewed in (De Cheveigné, 2004). It is probable that the ability of the cochlea to transduce such complex sounds is due to a combination of the two mechanisms, and the widely accepted theory is that rate of neuronal firing is largely responsible for the perception of low frequency sound, the place principle is dominant for high frequencies, and a combination of the two enables very accurate frequency resolution in the mid frequency range.

1.7. The Cochlear Amplifier

The idea of a cochlear amplifier was introduced by Thomas Gold in 1948. It was Gold who suggested that Békésy’s passive mechanism of the travelling wave would have encountered too much damping by the viscous nature of the endolymphatic fluid, and hypothesized that there must be some active positive feedback mechanism (Gold, 1948). Over three decades later David Kemp recorded the first sounds produced by the cochlea by placing a microphone in the ear canal, these were termed otoacoustic emissions (Kemp, 1978). The emissions can either be spontaneous (SOAE), or evoked by a stimulus, the most commonly investigated of these is the distortion product otoacoustic emission (DPOAE) (Kemp, 2002).

There are many proposed models for how they are exactly produced, but in mammals they are mostly based around movements of the OHC. As previously mentioned, the OHC are able to contract and elongate in response to membrane potential due to the presence of the voltage responsive motor protein PRESTIN (Brownell et al., 1985; Evans and Dallos,
1993; Liberman et al., 2002; Zheng et al., 2000b). The debate centres on whether it is this somatic motility of OHCs or complex active movement of the stereocilia bundle that allows for the fine tuning and amplification of vibrations in the basilar membrane (Chan and Hudspeth, 2005; Kennedy et al., 2005; Martin and Hudspeth, 1999; Ricci et al., 2002). This stereocilia motility has been shown to be responsible for driving the cochlear amplifier in other species such as the chick, where PRESTIN-like proteins are not present (He et al., 2003). The growing consensus is that in mammals both somatic and stereocilia bundle motility contribute to active cochlear amplification (Kennedy et al., 2006) (reviewed in (Ashmore et al., 2010; Hudspeth, 2014)).

1.8. The Vestibular System

The vestibular system deals with sensing motion and spatial orientation to enable the response of balance. There are five main sensory areas in the vestibular system, the sacculus, the utriculus and the three semi-circular canals. The semi-circular canals sense balance, whilst the sacculus and utriculus sense vertical movement and linear acceleration respectively, and similar to the cochlea all contain two types of hair cells which are bathed in endolymph. Vestibular hair cells differ slightly from those in the cochlea, they form on spots called maculae, the stereocila bundles retain the kinocilium, and are much taller than cochlear stereocilia. Another difference is that although the endolymph in the vestibular system is very similar in ionic composition, it does not have the driving force of the EP. Cells known as vestibular dark cells serve to recycle K⁺ similar to the stria vascularis, but they have no structure resembling the intermediate layer and consequently no positive potential (Zdebik et al., 2009). However, as the hair cells have a negative resting potential, K⁺ is still able to enter through MET channels in the stereocilia to depolarise the cell.
Figure 1.11 Overview of Otolithic Organs and Crista Ampularis Structure. Panel A shows a simplified general structure of the otolithic organs, as described in the text. When the otolothic membrane is displaced through acceleration, MET channels are opened in the embedded stereocilia of hair cells which are deflected towards the kinocilia. This increases the influx of $K^+$ from the normal resting levels and depolarises the membrane. Panel B indicates the orientation of stereocilia bundles on the utricular and saccular maculae. Panel C is a simplified representation of the crista ampularis from an ampulla of one of the semi-circular canals. When there is no movement the cupula is central and MET channels output a resting signal, upon deflection towards the kinocilia, the MET channel opens allowing a large influx of $K^+$, depolarising the membrane and increasing the output of neurotransmitter. When deflected away from the kinocilia the MET channel closes and hyperpolarises the membrane, decreasing the output of neurotransmitter. Kinocilia are indicated in red in panels A and C and by the point of the V in panel B. Potassium - $K^+$. 
The sacculus and utriculus are named the otolithic organs due to the presence of otoconia. Otoconia are calcium carbonate particles attached to a gelatinous matrix; these lay on top of the hair cells, and the MET channels in the stereocilia are opened due to deflection of the bundles toward the kinocilium caused by inertia due to the movement of the otoconia (Figure 1.11A). Stereocilia on the utricular macula are orientated with their kinocilia facing towards the striola, a separating ridge in the centre of the macula. Conversely on the saccular macula, hair cells are arranged with the kinocilia facing away from a midline of the striola (Figure 1.11B).

The horizontal, superior and posterior semicircular canals all work to sense rotation of the head, in different directions. In the semicircular canals, hair cells are located on the sensory epithelium named the crista, contained in bulbous structures at the base of each canal called ampulla. In contrast to the stereocilia in the otolithic organs, hair cells on the crista are all arranged with the kinocilia facing the same direction. Attached to the top of the hair cells is the cupula, a gelatinous structure which provides a barrier to restrict endolymph flow. Inertia from rotational movements pushes the cupula and depolarises or hyperpolarises the hair cells depending on the direction of movement (Figure 1.11C). Vestibular ganglion cells send the neural signals from all five sensory areas through the CN VIII enabling the sensation of motion and rotation, a good review of vestibular system structure and function can be found in (Purves D, 2001.).

1.9. Hearing Loss

Broadly speaking there are two types of hearing loss, conductive and sensorineural, although some cases of hearing loss are caused by a combination of the two. In addition to problems with the ear, deficits with the processing of auditory information by nuclei
along the CNVIII or in the auditory cortex can lead to problems with discriminating sound, termed central hearing loss.

1.9.1. Conductive Hearing Loss

Hearing impairments caused by factors affecting the outer or middle ear, the parts that conduct sound waves to the inner ear, are named conductive hearing loss. This can be caused by deformity of the outer ear, in particular the pinna, blockage of the external auditory canal, or damage/malformation of the tympanic membrane or ossicles. The majority of these can be treated, either with or without surgery. The most common cause of conductive hearing loss is due to a condition called Otitis Media (OM). OM is characterised by an effusion in the middle ear cavity, accompanied by inflammation of the middle ear epithelial lining. The effusion can increase the pressure on the tympanic membrane causing pain, and restricts movements of the ossicular chain resulting in reduced amplification of the vibrations of the tympanic membrane when they arrive at the oval window. The condition is common amongst young children; it is thought that around a quarter of all children will develop acute otitis media at least once by the age of 10. Occasionally some patients suffer recurring instances of OM with effusion (termed Chronic Otits Media with Effusion (COME)) and are usually treated by the surgical insertion of grommets, small plastic ventilation tubes, through the tympanic membrane. Although much debate exists about the exact cause of OM, it is widely accepted that infection and environmental factors play a large part in the condition. There is, however, a genetic component as well; a number of mouse models have been identified that develop COME, including the Jeff and Junbo mouse models discovered through N-ethyl-N-Nitrosourea (ENU) mutagenesis projects conducted at the MRC Harwell Institute. The Jeff mice harbour a semi-dominant mutation in F-Box Protein 11 (Fbxo11) resulting in OM (Hardisty et al., 2003), Junbo mice develop OM as a result of a mutation in the Ecotropic
viral integration site 1 (Evi1) gene (Parkinson et al., 2006). In addition to ENU mutants, recent studies at the MRC Harwell Institute involving homozygote Tgif knockout mice found that they also develop OM (Tateossian et al., 2013); All three of these genes are implicated in the regulation of the transforming growth factor beta (TGFβ) pathway.

1.9.2. Sensorineural Hearing Loss

Sensorineural hearing loss (SNHL) describes any form of hearing loss originating from problems with the inner ear, or the CN VIII. As mentioned this is a broad term and covers a wide range of distinct pathologies, although hearing loss can often be caused by a combination of factors. The term SNHL can be split further into sensory hearing loss and neural hearing loss. Sensory hearing loss refers to hearing impairment resulting from damage to or loss of cochlear hair cells and/or hair cell function, which can be caused by a number of factors both genetic and environmental. Environmental factors include damage due to noise, ototoxic drugs or infection. Unlike other vertebrates such as birds or amphibians, mammalian cochlear hair cells do not have the ability to regenerate; sensorineural hearing loss resulting from hair cell loss is therefore permanent. Neural hearing loss, as the name suggests, manifests when lesions occur in the neural pathway that the impulses released from the hair cells travel. This starts with the spiral ganglion neurons and continues along the route of the CN VIII to the auditory cortex. Around half the cases of SNHL are thought to have a genetic element. Although cochlear implants or hearing aids can help increase the perceived loudness of sound in some forms of SNHL, at present restoration of the accurate discrimination of sound to the level of an undamaged ear is not possible.
1.9.2.1. Presbycusis

As mammalian cochlear hair cells are non-regenerative, and in part due to the considerable stresses that they undergo, as people age they gradually lose these cells, and with them the ability to distinguish the same range of frequencies. This inevitable process is known as age-related hearing loss (ARHL) or presbycusis and is the most common form of SNHL. In addition to loss of hair cells, this can be caused by other age-related cellular pathologies or indeed a combination of different factors. Metabolic factors also play a part; atrophy of the stria vascularis results in a failure to maintain the EP, subsequently reducing the potential of the hair cells to respond normally. Neuronal degeneration also inevitably occurs with ageing, a loss of SGN will cause a hearing loss although the ear can tolerate loss of around 90% of SGN before serious effect on auditory thresholds occurs (Arvin et al., 2013).

1.9.3. Central Hearing Loss

Central hearing loss, perhaps more appropriately sometimes termed central auditory processing disorder (CAPD), refers to problems with the brain interpreting sound. Lesions in the brainstem or auditory cortex can lead to difficulties in ‘hearing’ even though the peripheral machinery of the ear is working correctly. It can be hard to diagnose central hearing loss from audiograms; ABR thresholds and latencies are often completely normal, however problems with speech recognition or noise discrimination can be a warning sign meriting further investigation.

1.10. The Mouse as a Model for Hearing Loss

The mouse is a valuable model for human hearing loss due to the similarities in the auditory system (Brown et al., 2008). The murine inner ear is a good representation of the
human cochlea with the obvious exception of size and frequency response, 20Hz – 20kHz in humans, and roughly 1-91kHz in mice. In addition to comparative size, the mouse cochlea only has one and three quarter turns as opposed to two and a half in the human.

The advantages of using mice to model human hearing loss in order to identify new genes and pathways involved are numerous. Firstly the lifespan and gestational period of mice are short compared to many other animal models. Secondly the environmental conditions can be controlled, also researchers are able to investigate the progression of pathological changes using various imaging techniques, which has led to a greater understanding of auditory function. In addition the mouse genome has been sequenced and is searchable online through genome browsers such as Ensembl (http://www.ensembl.org/index.html) and Vega (http://vega.sanger.ac.uk/index.html).

There are many techniques available to researchers which enable manipulation of the mouse genome, some of which will be outlined below. Reverse genetic approaches can be used to investigate function of orthologous genes suspected of causing hearing impairment identified though human GWAS studies, or those which are related to or function in the same pathways as known deafness genes. Forward genetics approaches can also be undertaken utilising inbred strains of laboratory mice. By inducing mutations in one strain and crossing to another, inherited phenotypes can be mapped to chromosomal regions with good accuracy by using known genetic markers that identify the parental strains. Both forward mutagenesis and reverse genetic approaches in laboratory mice have enabled the identification of many causative genes underlying hearing impairment (Avraham, 2003; Bowl and Dawson, 2015; Brown et al., 2008).

Many early models of hearing loss were derived from spontaneous mutations arising in inbred strains; the majority were known known as ‘waltzing’ mice as they tended to exhibit circling behaviour. These were caused by inner ear defects affecting both the
cochlea and vestibular apparatus. Examples of this are Ames-waltzer (av) found to be a mutation in Protocadherin 15 (Pcdh15) (Alagramam et al., 1999), Snell's waltzer (sv) for which Myosin VI (Myo6) was identified as the cause (Avraham et al., 1997), and waltzer (w), one of a number of known mutations in Cadherin 23 (Cdh23) (Di Palma et al., 2001). All of these mutations affect the stereocilia in some way, and are the cause of both hearing loss and vestibular dysfunction. 

For a number of years the vast majority of mouse models of hearing loss were discovered through the observation of this ‘waltzing’ head tilting phenotype and subsequently found to be deaf. In the present day, auditory function in mice can readily be assessed, and efficient mutagenesis of the mouse is easily achievable; phenotyping pipelines including a sensory assessment can be employed to assess basic vestibular and auditory function in mice (Hardisty-Hughes et al., 2010). This can then be combined with the various imaging techniques that can be utilised to examine anatomical features and inner ear pathologies to characterise the effects of mutations in affected mice (Parker et al., 2016). This approach employed in our laboratory has led to the discovery of a number of new mouse models of hearing loss that are either in completely novel genes with relation to hearing loss (Carrott et al., 2016; Hardisty et al., 2003; Mackenzie et al., 2009; Parker et al., 2015; Parkinson et al., 2006; Potter et al., 2016), or alleles of known hearing loss genes which are also useful in furthering functional understanding of the genes/proteins (Bortolozzi et al., 2010; Geng et al., 2013; Parker et al., 2010).
1.11. Assessment of Auditory Function in Mice

There are a number of phenotyping platforms available for auditory assessment in mice:

1.11.1. Clickbox

For a number of years the primary screening tool for assessing hearing in mice has been the clickbox. A clickbox is a small handheld device that emits a tone with a frequency around 20kHz at 90dBSPL (when operated at a distance of 30cm). The mouse will usually respond with a flick of the ear pinna known as the ‘Preyer’ reflex, occasionally accompanied by a slight startle response; the reduction or lack of a preyer reflex is indicative of possible auditory dysfunction (Nolan et al., 2000a). The clickbox is an efficient screening tool as it is a very quick test, but it produces a supra-threshold stimulus, therefore subtle phenotypes may not be identified. Another limitation is that it provides no frequency specific information.

1.11.2. Auditory-Evoked Brainstem Response

Techniques that are routinely used to diagnose hearing loss in humans can be modified to investigate auditory function in mice. The ‘gold standard’ of these is Auditory-Evoked Brainstem Response (ABR) (Zheng et al., 1999). An ABR is an auditory evoked potential and is obtained by playing a specified stimulus repeatedly into the ear of the mouse at a very high repetition rate (usually between 21 and 42.6/second) and measuring the electrical activity from the neuronal centres of the auditory pathway. In the mouse this is achieved by inserting sub-dermal electrodes, a ground electrode that can be placed anywhere, a reference electrode behind the ear that is being stimulated and the active recording electrode along the midline of the skull (Figure 1.12C). Hardware and computer software are then used to amplify and average the potential difference between the
reference and active electrodes in response to the stimuli (Figure 1.12A). The response is usually recorded for 10ms post stimulus and averaged up to 1000 times, any peak occurring at a consistent time in the recording is added to the overall average trace and those occurring at random times are subtracted. If the subject has normal auditory function, this results in an ABR trace that usually consists of five main peaks, corresponding to the different neuronal populations along the CN VIII and brainstem (Figure 1.12B).

![Figure 1.12 Auditory-Evoked Brainstem Response (ABR) Testing.](image)

(A) The ABR apparatus. The computer (1) is connected to the Tucker Davis Technology (TDT) system III hardware (2), this controls the stimulus speaker in the sound attenuated booth (4), where the mouse is placed on a heated mat for testing. The resulting trace is visualized on the display (3). (B) Typical response traces to a 12 kHz tone stimulus at 70 dB sound pressure level (SPL); the light blue trace is from a hearing wild-type mouse, the dark blue trace is from a mouse with a moderate hearing loss and the red trace is from a mouse with a severe hearing loss (i.e., no response at 70 dB SPL) (Parker, unpublished data). (C) The placement of the sub-cutaneous electrodes, (i) active, (ii) reference and (iii) ground, the stimulus is delivered free field to the right ear of the subject. The data from the ABR test are presented as an audiogram (D), which summarizes the mean hearing thresholds determined at each frequency, with error bars showing the standard error of the mean (in this case n = 5) (Parker, unpublished data). No response at the highest stimulus level (90 dB SPL) is recorded as 100 dB SPL for graphical representation. Figure taken from (Hardisty-Hughes et al., 2010).

The origins of these peaks appear to vary between species, however in mice, Henry in 1979 carried out research that suggested P1 originated in the cochlea, P2 in the cochlear nucleus, P3 the contralateral superior olivary complex, P4 the lateral lemniscus and P5 in the contralateral lateral inferior colliculus (Henry, 1979). The stimulus is reduced in
amplitude until no replicable peaks can be observed and the stimulus level above this is determined as the threshold for the stimulus; in mice the threshold is usually reported in decibels sound pressure level (dB SPL). ABR testing allows testing of different frequency stimuli, giving a good estimation of auditory function across the audible range, the data are reported in an audiogram, with ABR threshold plotted against stimulus frequency (Figure 1.12D).

### 1.11.3. Otoacoustic Emissions

As previously mentioned the cochlea produces low level sounds itself due to some form of OHC motility; as such these emissions can be measured and serve as an indicator of OHC function. The most commonly used is the DPOAE in which 2 distinct stimuli ($f_1$ and $f_2$) are played simultaneously directly into the ear canal and a distinct distortion product originating from the cochlea is recorded by a microphone included in the speaker probe. For assessment of auditory function in humans, the levels of $f_1$ and $f_2$ (L1 and L2) are normally slightly different where $L_1 = 65\text{dB SPL}$ and $L_2 = 55 \text{ dB SPL}$, although some protocols use both L1 and L2 at 65 dB SPL. The most reliable distortion product measured in mice is the cubic difference distortion product ($2f_1-f_2$) (Horner et al., 1985). Although it is generally accepted that the distortion product is an indicator of OHC function, it can also be affected by other pathologies; as the response is recorded in the ear canal it must be transmitted via the basilar membrane back through the middle and outer ear. Therefore, subjects with a conductive hearing loss or a reduction in mechanical movement of the basilar membrane will also display reduced DPOAE, however used in conjunction with ABR testing, a good assessment of auditory function can be obtained.
1.11.4. Observation and Assessment of Vestibular Function

As previously mentioned, the cochlea and vestibular system are closely linked, both through connection of the membranous and bony labyrinths and cellular similarities, vestibular dysfunction is often accompanied by hearing loss (and vice versa). There are a number of tests available to researchers to assess vestibular function in mice, beginning with simple observation. Mice with mutations causing severe vestibular defects will often exhibit circling or waltzing behaviour; they run repetitively in circles, this is sometimes accompanied by head bobbing, tilting or shaking. In mice with slightly less severe vestibular abnormalities, the phenotype may not manifest itself quite so obviously. A swim test may be performed; placing a mouse in water has the result of reducing proprioceptive stimuli from the ground, exaggerating mild phenotypes that may be hard to see in normal observation conditions. Another test, contact righting reflex, can also be useful in diagnosing a vestibular deficit. Mice are placed in a clear Perspex tube, and the tube inverted so the mouse is essentially walking on the ceiling of the tube. Mice will usually immediately attempt to return to walking along the floor of the tube using their righting reflex.

1.12. Genetic Manipulation in Mice

As mentioned, forward genetics as a hypothesis generating approach, and reverse genetics as a hypothesis testing approach, both have their advantages and disadvantages. In the case of forward genetics screens, they can be costly in terms of cage space and man hours to thoroughly investigate the phenotype of large numbers of mice, the vast majority of which will not display a phenotype for the particular screen. This is why large scale ENU screens are developed with pipelines investigating a wide range of phenotypes, partly for financial cost, but most importantly from a welfare point of view; as a
responsibility to the three Rs: replacement, reduction, refinement, progeny produced in these screens will all harbour many ENU induced mutations, and it is important that as many phenotypes as possible are discovered. This approach is ideal for discovering novel gene associations as no a priori assumptions about the gene involved are made. In addition until the mutation has been mapped and cloned, the gene causative of the phenotype is not known, this reduces experimental bias when screening (Nolan et al., 2000a).

Reverse genetics approaches such as targeting genes directly to ablate expression (knockout) enables the observation of the phenotypic effects of loss of function for the gene of interest. With the introduction of conditional knockout mice, aspects of a gene's expression can be controlled spatially or temporally. This is a very useful tool for studying genes in important developmental systems, and also for essential genes which ordinarily result in embryonic lethality when targeted. In addition to simply abolishing genetic function, genes and proteins can also be tagged to investigate localisation, or overexpressed. Whilst techniques to enable these manipulations have been available for many years, historically, producing mice that carry the desired alteration was error prone, expensive and time consuming. However, with the advent of new technologies such as CRISPR/Cas9 (outlined below), the efficiency of genetic manipulation is improving rapidly.

1.12.1. Transgenic mice

There are a number of strategies that can be employed to manipulate the mouse genome. Transgenic animals can be engineered by inserting fragments of deoxyribonucleic acid (DNA) or whole genes of interest into the target organism's genome with the use of vectors such as plasmids. This can be useful for many reasons including observation of in vivo overexpression of proteins, or incorporating reporter
constructs such as the bacterial LacZ gene to tag genes or proteins of interest so their expression patterns can be investigated. One common method of producing transgenic mice is by pronuclear injection of the desired DNA sequence into the freshly fertilised zygote before the first cell division, this ensures that if successfully incorporated, all cells in the embryo will contain the transgene, although integration into the genome is random and may occur more than once (Chandler et al., 2007); for this reason pronuclear injection is ideal for examining overexpression or copy number phenotypes for the target gene. One drawback with random integration is that multiple copies of the transgene may not be functional dependant on where they have integrated into the host genome; therefore a pure measure of copy number using techniques as quantitative real-time polymerase chain reaction (qRT-PCR) to look at mRNA levels may give misleading results on the actual level that the transgene is being functionally expressed. Other problems with random integration of the transgene can occur when the transgene incorporates within other genes, which can cause undesired phenotypic or potentially lethal effects which are not associated with the transgene itself.

One method of overexpressing genes is by using a bacterial artificial chromosome (BAC) as a vector. The advantage of using engineered BACs is that they can hold large fragments of DNA (normally 150 – 350 Kb), making it possible to insert whole genes and surrounding sequence meaning that the normal promotors and regulatory motifs can be incorporated. Another advantage is that BAC technology was used to sequence the human genome, huge libraries of BACs covering the vast majority of the genome are available, it is likely that a BAC is already available for purchase for a given a gene of interest.
1.12.2. Knockout Mice

Transgenic techniques can also be used to stop or reduce expression of a target gene, to investigate functionality by phenotypic observation. Traditionally this usually involves the use of plasmids as a vector, and is targeted to the gene of interest utilising the natural process of homologous recombination to incorporate engineered DNA sequences to replace wild-type copies of the target gene. This is done by creating a construct containing a selection cassette, such as a neomycin resistance gene, surrounded by sequence homologous to the gene/exon to be ‘knocked out’ and using transfection techniques to introduce the DNA sequence into totipotent cultured embryonic stem (ES) cells collected from donor blastocysts (Figure 1.13A and B). In vitro selecting techniques using antibiotics are then used to select only ES cells which contain the selection cassette and therefore have the targeted DNA removed (Figure 1.13C); these ES cells are then injected back into host blastocysts which are then implanted into a pseudo-pregnant female mouse (Figure 1.13D and E). The embryo develops and produces a chimeric mouse containing both the host animals and the donor ES cells carrying the transgene (Figure 1.13F). Subsequent breeding of chimeric mice, if the transgene has contributed to the production of gametes, can produce a mouse line that is heterozygous for the transgene in every cell via germline transmission (Figure 1.13G and H). If donor and host blastocyst have different coat colours, chimeric mice and heterozygotes can be identified by coat colour, alternatively mice can be genotyped. This technique allows for more precise manipulation of the genome as the constructs can be targeted to areas of the genome in the ES cells and selected for successful integration before the blastocyst injection stage. Mario Cappechi first used homologous recombination to target DNA constructs to specific genomic sites within mouse embryonic stem (ES) cells (Capcechi, 1989; Thomas and
Capecchi, 1987); he would later share the 2007 Nobel prize for Physiology along with Martin Evans and Oliver Smithies for their roles in the development of the technique.

**Figure 1.13 Creation of a knockout mouse.** A method of producing mice carrying a transgenic null allele of a targeted gene using the coat colour selection method as described in text. Embryonic Stem (cell) - ES cell. Figure based on (Adams and van der Weyden, 2008)
1.12.3. Conditional Knockout Mice

Although traditional mouse knockouts have helped researchers identify functional roles for many genes, the technique originally resulted in a mouse with the deletion of the functional gene in every cell in the body. Unsurprisingly as many genes are essential for the development of the embryo, some knockout mouse lines resulted in embryonic lethality. Although this confirms the target is an essential gene, some genes/proteins have multiple roles, many of which are tissue and time or stage specific. In addition some have distinct roles throughout the life cycle of the animal. A technique was developed that enabled genes to be knocked out in tissue(s) of choice at controlled times either during development or throughout the life of the animal. The Cre/LoxP system takes advantage of a protein found in a bacteriophage which causes recombination (hence the name Cre) between two small repetitive DNA sequences (LoxP) (Sauer and Henderson, 1988; Sternberg et al., 1981) (Figure 1.14A). Transgenic mice carrying the Cre gene under the control of either a tissue/developmental stage specific or inducible suitable mouse promoter sequence can be mated with transgenic mice which contain two LoxP sequences flanking the region to be deleted (Floxed); subsequent matings are performed to produce mice that are homozygous for the Floxed allele and hetero/hemizygous for Cre. Activation of the promotor will drive Cre expression resulting in knockout of the targeted region in a temporal, spatial or inducible manner according to the promotor (Gu et al., 1994; Sauer, 1998) (Figure 1.14B and C). A wide range of mouse lines have been produced and made available by the Jackson Laboratory with Cre under the control of different tissue specific and or temporally expressed gene promoters in addition to inducible lines, so that Cre is expressed only in certain tissues or at a particular time-point coincidental to that of a known gene or can either be induced by means of drugs such as Tamoxifen (https://www.jax.org/research-and-faculty/tools/cre-repository).
In addition to the use of the Cre/loxP system for knocking out single genes, integrating loxP sites at different loci can induce large chromosomal changes, although successful recombination can be less efficient. The orientation of the loxP sites will affect the
outcome of the recombination, with two LoxP sites in the same orientation Cre expression will excise the sequence in between, large deletions can be achieved if the LoxP sites are far apart. If the sites are orientated in opposing directions an inversion of the DNA in between will occur and if the LoxP sites are situated on different strands, chromosomal translocation will result (Nagy, 2000).

An alternative analogous system to Cre/LoxP is Flp/FRT. Flp/FRT is also now commonly used either instead of or in conjunction with the Cre/LoxP system to give even more control over the genome (Buchholz et al., 1998; Rodriguez et al., 2000; Yamamoto et al., 2009).

1.12.4. CRISPR/Cas9

In recent years, other systems for genome editing have become available to researchers including Transcription Activator-Like Effector Nucleases (TALENS) and Zinc Finger Nucleases (ZFNs) which are both based around artificially engineered restriction enzymes that can create double stranded breaks in DNA (reviewed in (Carlson et al., 2012)); however, the most promising breakthrough in genome editing in recent years is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system. This system makes use of a bacterial repeat sequence first observed in Escherichia coli (Ishino et al., 1987), and later discovered to be involved with bacterial acquired immunity (Barrangou et al., 2007). In prokaryotes the CRISPR locus contains short segments of pathogenic DNA (protospacers) in between the repeat sequences and subsequently transcribes a corresponding long RNA molecule (pre-CRISPR RNA). In the well described CRISPR system in Streptococcus pyogenes, short trans-activating RNAs (tracrRNA) bind with complimentary repeat sequences and promote cleavage into the shorter CRISPR RNA (crRNA) units, mediated by RNase III and the endonuclease CRISPR associated protein 9
(Cas9) (Deltcheva et al., 2011; Jinek et al., 2012); the crRNAs comprise the protospacer sequence preceded by one of the tandem repeats. The Cas9 protein forms an association complex with a crRNA and an activating tracrRNA to target the invading pathogenic DNA using the crRNA as a guide. The crRNA/Cas9/tracrRNA complex will only bind to the pathogenic DNA when a certain motif, the protospacer adjacent motif (PAM), is situated immediately downstream of the crRNA sequence on the non-complimentary strand. In the case of Cas9 in *Streptococcus pyogenes*, this PAM sequence is 5’-NGG-3’; when the crRNA/Cas9/tracrRNA associated crRNA binds its target DNA, a double stranded break (DSB) is introduced 3 bp upstream of the PAM by two active endonuclease domains contained within Cas9, termed HNH and RuvC (Jinek et al., 2012).

Researchers discovered that the system could be utilised to enable genome editing of model organisms. Commonly the CRISPR/Cas9 system already mentioned from *Streptococcus pyogenes* is the most commonly used for this purpose. A guide RNA (gRNA) construct is made containing the desired sequence to be targeted which resembles crRNA and is either attached to tracrRNA via a small linker (chimeric gRNA) (Jinek et al., 2012), or used in conjunction with tracrRNA. By taking advantage of the non-homologous end joining (NHEJ) DNA repair pathway which is prone to error and often results in insertions or deletions, creating knockouts of targeted genes is easily achievable. In addition through the addition of DNA donor templates, more precise changes including point mutations and insertion of reporters can be introduced by means of the homologous directed repair (HDR) pathway (Iliakis et al., 2004; Lin et al., 2014; Ran et al., 2013) (Figure 1.15).

In addition to using Cas9 to introduce DSBs, Cas9 proteins engineered to contain mutations in either or both of the endonuclease domains are available; these modified Cas9 proteins can act as a nickase to cut either strand of DNA individually or in the case of
the doubly mutated dCas9 (dead Cas9) to use the protein as a vector either by itself or associated with different factors. These factors include activators, repressors, fluorophores or chromatin modifiers which enable researchers to activate, “knockdown”, visualise or regulate the chromatin environment of specific genes (reviewed in (Xu et al., 2014)). Another advantage of the CRISPR/Cas9 system is that by introducing a number of different gRNAs, the system can also be used to promote many different genetic alterations at the same time, enabling complex pathway analysis without time consuming breeding schemes, reducing the economic and welfare costs. The 5′-NGG-3′ PAM motif occurs with high frequency in the human and mouse genomes, and other Cas9 proteins recognises other PAM motifs; therefore a huge portion of the genome is targetable and subsequently available for manipulation; indeed, premade gRNA vectors are readily commercially available for the vast majority of genes. As the CRISPR/Cas9 system is so versatile, and also because it can be used to introduce changes in the genome of living cells the potential benefits for gene discovery, basic science and gene therapy that this new technology promises are vast.

**Figure 1.15 The CRISPR/Cas9 system.** Overview of the CRISPR/Cas9 system – the Cas9 endonuclease domains introduce DSBs at the sequence of interest (as determined by the PAM sequence). This will start the NHEJ pathway (red arrows) which is prone to error and can introduce insertions or deletions potentially causing a frameshift and premature stop codon, creating a null allele of the targeted gene. The homologous directed repair pathway can be ‘programmed’ to perform accurate precision editing of the genome by using gRNA to repair the DSBs with a sequence of choice, allowing for introduction of point mutations and reporters. Double stranded break – DSB, non-homologous end joining – NHEJ, homologous directed repair – HDR, deoxyribonucleic acid – DNA, guide ribonucleic acid – gRNA. Figure adapted from (Ran et al., 2013)
1.12.5. ENU Mutagenesis

There are a number of ways of introducing random mutations into the genome including radiation, viruses or mutagenic chemicals. One of the more popular and successful methods of mutagenesis is the use of the alkylating agent ENU. ENU has the ability to transfer its ethyl group to nucleic acids which results in point mutations, it preferentially targets adenine or thymine (Popp et al., 1983). One of the advantages of using ENU as a mutagen is the fact it induces mainly point mutations resulting in either missense, nonsense or alternate splicing events. This can result in amorphic, hypomorphic, hypermorphic, antimorphic or neomorphic gene function, and can therefore be more useful than gene deletion alone in ascertaining the functionality of a gene product. In recent years a number of research establishments have set up large scale screens using comprehensive phenotyping pipelines to investigate the effects of ENU induced mutations, and have successfully identified many novel mouse models for a wide range of hereditary diseases (Hrabe de Angelis et al., 2000; Nolan et al., 2000b). ENU screens for both dominant and recessive mutations can be investigated, the former is relatively simple to perform; males from an inbred strain of choice are treated with ENU, they are then mated to females from another inbred strain that is used to map the mutation. In this breeding scheme progeny of this mating, generation 1 (G1) mice, are screened using a battery of phenotyping tests to investigate a wide range of disease phenotypes of interest. All G1 mice will be heterozygous for a large number of ENU induced mutations; the efficiency of ENU varies with dose but research has shown that with optimal dosing, a new mutation in a particular locus will occur in around 1 in 700 gametes (reviewed in (Balling, 2001)). If a phenotype is observed, the G1 mice are mated again to the mapping strain and the G2 offspring tested for the phenotype, according to Mendelian inheritance half of the G2 offspring should be affected if the phenotype is inherited. In a recessive
screen G2 females are mated back to the founder G1 male to homozygose recessive mutations, if inherited around one in eight animals of the G3 pedigrees produced will display the phenotype. Mutations can then be mapped using known single nucleotide polymorphisms (SNPs) between the mutagenised strain and the mapping strain. At the MRC Harwell Institute, sperm from all G1 males is collected and stored cryogenically in an ENU DNA/Sperm archive, along with large amounts of tissue for DNA extraction. Plates of pooled DNA are made available to researchers to screen for ENU induced mutations in their gene of choice. If ENU mutant alleles are discovered and confirmed, a request can be made to the MRC Harwell Institute’s Frozen Embryo and Sperm Archive (FESA) core facility for the re-derivation of the mouse line through in vitro fertilisation (IVF). This has the advantage of allowing both forward and reverse genetic screens to be performed on mice produced in ENU mutagenesis programs (Coghill et al., 2002).

1.13. International Consortia Investigating Gene Function in Mice

The publication of the mouse genome has led to a large number of multi-national consortia which are working toward the ultimate goal of creating a functional annotation of the entire mouse genome (~20000 genes). This is obviously a huge undertaking, but the international mouse genetics community have been working closely together pooling resources, sharing expertise and finding solutions to the task of recording and handling the enormous amounts of data produced; an overview of a selection of these consortia follows.

1.13.1. Eumorphia

(European Union Mouse Research for Public Health and Industrial Applications)

Eumorphia was a large scale European funded project that succeeded in developing and standardising phenotyping platforms across major research laboratories throughout
Europe. The main aim was to try and make experimental data more comparable. As a result of the efforts of the Eumorphia consortium a phenotyping platform was created named EMPReSS (European Mouse Phenotyping Resource for Standardised Screens), which contained more than 100 standard operating procedures that had been validated across several different European research centres. This resource enables scientists to perform reliable phenotyping on mice and is invaluable in assessment of gene function in mutant mice.

1.13.2. IKMC

(International Knockout Mouse Consortium)

The IKMC encompasses a number of separately funded projects including KOMP (Knockout Mouse Project), EUCOMM (European Conditional Mouse Mutagenesis Program), NorCOMM (North America Conditional Mouse Mutagenesis Project) and TGIM (Texas A&M Institute for Genetic Medicine). The aim of this consortium is to create an ES cell resource containing null and conditional null alleles of every protein coding gene in the mouse genome (C57BL/6).

1.13.3. EUMODIC

(European Mouse Disease Clinic)

EUMODIC used a phenotyping pipeline derived from the EMPReSS resource to perform high throughput screening on 500 mutant mouse lines created by the EUCOMM project. The phenotyping was performed at centres involved in the EUMORPHIA project, and data made publically available on the EuroPhenome website.
1.13.4. IMPC

(The International Mouse Phenotyping Consortium)

The IMPC follows on from work carried out on the EUMODIC project. It is a consortium of research centres from all over the world including Europe, America, Japan and Australia. The IMPC will attempt to perform high throughput phenotypic analysis of the remainder of the 20000 mutant lines that will be created by the IKMC.

1.14. Thesis Aims

The aims of this thesis, as the title suggests, are to identify new mouse models of hearing loss, and characterise the effects of the causative mutations. In order to achieve this I am investigating two new mouse models that have been identified during ENU mutagenesis screens.

At the time my PhD registration began, the underlying mutation had already been discovered for the first model discussed, goya, and preliminary auditory phenotyping had been performed to confirm hearing loss. My specific aims for this project are:

- Confirm (or otherwise) that the mutation in Map3k1 is causative of the hearing loss phenotype.
- Investigate the hearing loss phenotype over a time-course to study progression.
- Investigate the pathological basis of the hearing loss.
- Investigate localisation of MAP3K1 in the inner ear.
- Perform studies relating to possible function of MAP3K1 in the inner ear, with a view to gain insight into the mechanisms involved.
For the second model, Boycie, the specific aims are:

- Identify the causative mutation underlying the hearing loss
- Investigate the hearing loss phenotype over a time-course to study progression
- Investigate the pathological basis of the hearing loss.
- Perform studies relating to possible function of the identified gene in the inner ear with a view to gain insight into the mechanisms involved.
Chapter 2

Materials and Methods
2.1. Mice

2.1.1. Animal Husbandry

All animals were housed and maintained under specific pathogen-free (SPF) conditions in individually ventilated cages in the Mary Lyon Centre, MRC Harwell Institute, in adherence to environmental conditions as outlined in the Home Office Code of Practice. Mice were fed and watered ad-libitum and kept in a 12 hour light/dark cycle. All procedures were licenced by the Home Office and complied with the Animals (Scientific Procedures) Act 1986, under the project licences 30/2540, 30/2567 and 30/3015 with the approval of a local institutional ethical review committee. Mice were euthanized by Home Office Schedule 1 methods.

2.1.2. Mice Used and Breeding Schemes

2.1.2.1. goya

The goya mutant line was identified from the collaborative ENU mutagenesis vision screen undertaken by the Mammalian Genetics Unit (MRC Harwell Institute) and MRC Human Genetics Unit (HGU) Edinburgh. G0 C57BL/6J male mice treated with the potent chemical mutagen ENU were mated to C3H.Pde6b+ female mice to produce G1 progeny. G1 males were then mated to C3H.Pde6b+ female mice to produce G2 progeny. Female G2 mice were backcrossed to the G1 fathers to produce G3 mice that were screened for recessively inherited phenotypes (Figure 2.1). The goya line was then maintained on a C3H/HeH genetic background by outcrossing and intercrossing successive generations. The majority of the animal data presented in this study were generated from G7 mice, nearly congenic on the C3H/HeH background.
Figure 2.1 Breeding scheme for recessive ENU screen (A) G0 C57BL/6J mice are treated with ENU, after fertility returns they are mated to C3H females (in the case of the vision screen C3H.Pde6b+). (B) The G1 male mice are then crossed again to C3H females. (C) G2 female progeny (either wild-type or heterozygote for mutation of interest) are mated with the founder G1 male (who would be obligate heterozygote). (D) The resulting G3 progeny (pedigree) are screened for phenotypes of interest. According to Mendelian inheritance, roughly 1 in 8 G3 mice should be homozygous for a given recessive mutation using this breeding scheme. Wild-type - wt, heterozygote – het, homozygote – hom. Figure adapted from (Acevedo-Arozena et al., 2008).
2.1.2.2. Map3k1<sup>tm1Yxia</sup>

Map3k1 kinase deficient mice (Map3k1<sup>tm1Yxia/tm1Yxia</sup>) were imported from Ying Xia’s group at the University of Cincinnati medical centre (Cincinnati, OH 45267, USA) and re-derived by in vitro fertilisation by the FESA core into the Mary Lyon Centre to maintain SPF status. The Map3k1<sup>tm1Yxia</sup> allele has a LacZ reporter cassette in the place of the kinase domain and encodes a MAP3K1-ß-Galactosidase fusion protein which has the first 1188 amino acids of MAP3K1 but lacks the kinase domain (Xia et al., 2000). The imported Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice were originally on a mixed genetic background including C57BL/6J, upon arrival at our facility they were crossed to C3H/Heh for re-derivation and maintained by inter-crossing of heterozygous offspring.

2.1.2.3. Map3k4<sup>tm1Flv</sup>

The Map3k4<sup>tm1Flv</sup> null mice were obtained from Dr Andy Greenfield (Sexual Development Group, MRC Harwell Institute). These mice were generated by Cre/LoxP mediated deletion of exon 3, and shown to be null by absence of protein detection by western blot using antibodies to both the N and C termini of MAP3K4 (Chi et al., 2004). The mice were maintained on a C57BL/6J background.

2.1.2.4. Map3k4<sup>BAC</sup>

Mice overexpressing Map3k4 were also obtained from Dr Andy Greenfield (Sexual Development Group, MRC Harwell Institute). These mice were created using BAC transgenesis using a BAC clone containing the transcriptional unit of Map3k4 and surrounding upstream and downstream sequence (NOD/MrkTac BAC clone bQ279c06 (c06) (Centre for Applied Genomics, Toronto, Canada); mice carrying this transgene have
been previously shown to overexpress Map3k4 in vivo (Warr et al., 2012; Warr et al., 2014). The mice were maintained on a C57BL/6J background.

2.1.2.5. Boycie

The Boycie line was identified from a recessive ENU screen performed at the MRC Harwell Institute (separate to the vision screen), originally showing a hearing loss phenotype with a dominant mode of inheritance. ENU-treated G0 C57BL/6J male mice were mated to C3H/HeH female mice to produce G1 progeny. G1 males were mated to C3H/HeH female mice to produce G2 progeny. Female G2 mice were backcrossed to the G1 fathers to produce G3 mice that were screened for recessively inherited phenotypes. The Boycie line was maintained on a C3H/HeH genetic background by out-crossing initially and subsequently inter-crossing to produce homozygote mice.

2.1.2.6. Orai1\textsuperscript{L187Q}, Orai1\textsuperscript{V135E}, Orai1\textsuperscript{I123N}

Mice carrying additional ENU-induced mutations in Orai1 identified from screening of the MRC Harwell Institute ENU DNA archive, were re-derived using cryopreserved sperm collected from the G1 founder male. This was achieved by in vitro fertilisation performed by the FESA core in the Mary Lyon Centre. The G1 founder males resulted from crossing C3H/HeH females with either ENU-treated BALB/c (EMRC/323.9h - Orai1\textsuperscript{L187}), or C57BL/6J (CBMLC/583.10b – Orai1\textsuperscript{V135E}, CBMLC/392.7e – Orai1\textsuperscript{I123N} males. All lines were re-derived using a C3H/HeH background, before obligatory heterozygote progeny were inter-crossed in an attempt to produce wild-type, heterozygote and homozygote offspring for phenotyping.
2.2. Deoxyribonucleic Acid (DNA) Extraction

2.2.1. For Linkage Analysis

To prepare DNA to be used for linkage analysis or fine mapping, extraction of DNA from ear or tail biopsies was performed using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer’s instructions. Briefly biopsies were lysed with proteinase K overnight at 55° C, the DNA was precipitated with ethanol (ETOH) and centrifuged at 10000 x g through a silica membraned column. The DNA remains bound in the column and subsequent wash steps were performed to remove contamination. The DNA was eluted into Qiagen AE buffer, 60 µl for ear biopsies or 200 µl for tail biopsies.

2.2.2. For Genotyping by Enzymatic Digestion or Pyrosequencing

To prepare DNA to be used for genotyping, samples were either extracted as described above or with the DNA releasy™ kit (Anachem). For samples which DNA releasy™ was used, ear biopsies were placed in a 96 well plate, 10 µl of DNA releasy™ reagent was added and samples were heated using a thermocycler. The lysed solution was then diluted 1 in 20 before subsequent use as a DNA template for Polymerase chain reaction (PCR).

**DNA releasy™ Thermocycler Conditions:**

- 75° C for 5 minutes
- 96° C for 2 minutes
- 20° C hold
2.2.3. For Genotyping using TaqMan® Probes

To prepare DNA to be used for TaqMan® genotyping assays (both copy number variants and allelic discrimination), DNA extraction was performed using the DNA Extract All Reagents Kit contained within the TaqMan® Sample-to-SNP Kit (Applied Biosystems®) as per the manufacturer’s instructions. Briefly, ear biopsies were lysed in 50 µl of Lysis Solution for 3 minutes at 95°C, cooled for 30 seconds before 50 µl of DNA Stabilizing Solution was added. The extracted DNA was used at a 1 in 10 dilution for subsequent TaqMan® assays.

2.2.4. For Whole Genome Sequencing

To prepare DNA to be used for whole genome sequencing, extraction of DNA from a tail biopsy was performed using the Illustra™ Nucleon DNA Extraction Kit (GE Healthcare) as per the manufacturer’s instructions. Briefly biopsies were lysed with proteinase K before sodium perchlorate was added to deproteinase the DNA. Chloroform was then added to solubilise protein and lipids in the sample, before the addition of Nucleon Resin. Centrifugation was performed to separate into the lower organic phase containing protein and lipids, a middle phase of resin and the DNA-containing phase at the top. The top phase containing the DNA was aspirated and precipitated using EtOH, before resuspension in 100 µl of 10 mM Tris hydrochloride (Tris-HCl) (pH 8.5).

2.3. Linkage Analysis of the Boycie Mutation

DNA from ear or tail biopsies was prepared as described in 2.2.1., DNA samples from the parental strains (C57BL/6J and C3H/HeH) as well as a total of 17 G3 progeny with an absent click-box response, were sent for whole genome linkage analysis using KBioscience SNP panel (KBioscience Ltd.).
2.4. Fine Mapping of the Boycie Mutation

The candidate region identified by whole genome linkage analysis was narrowed by fine mapping using additional SNP markers contained within the interval, and DNA prepared from mice from subsequent generations. SNPs that are informative between C57BL/6J and C3H were identified using the dbSNP database through the Mouse Genome Informatics (MGI) website (http://www.informatics.jax.org/snp). Primers were designed to amplify the sequence surrounding the SNP and genotypes of mice were determined either by restriction enzyme digest of the resulting PCR product, or by high resolution DNA melting analysis (HRM) using the Lightscanner system (Idaho Technology Ltd.). For restriction enzyme digest, NEBcutter 2.0 (http://nc2.neb.com/NEBcutter2) was used to identify restriction enzymes that would differentially cut the amplicon according to parental strain. All digests were carried out using 10 µl of PCR product, 0.5 µl restriction enzyme, 2 µl 10 x appropriate enzyme buffer, 2 µl BSA (all New England BioLabs) and 5.5 µl ddH₂O. If no restriction enzyme could be found to discriminate between the two parental strains, a Lightscanner assay was performed. Briefly a PCR was performed with the inclusion of an intercalating fluorescent dye, LCGreen, which readily fluoresces when bound to double-stranded DNA (dsDNA). Samples were heated in the light scanner to denature the dsDNA, at which point the fluorescent signal became weaker. The curve at which the fluorescence diminishes was used to identify mutations. If a sample has contains a heterozygous SNP it will denature at a lower temperature than a wild-type sample due to hetroduplexes caused by mismatch annealing during PCR amplification. Comparison of the melting curves can therefore be used to identify wild-type and heterozygote genotypes. A summary of the SNPs and methods used for fine mapping can be seen in Table 2.1.
2.5. Genotyping

2.5.1. goya

For genotyping of the goya mice, a pyrosequencing assay was used. PCR was performed in a thermocycler using Taq PCR Master Mix (Qiagen). Following PCR amplification the Genotyping and Mutation detection core facility at the MRC Harwell Institute ran the assay on a PSQ HS 96A pyrosequencer (Biotage (Qiagen)) using a biotinylated sequencing primer from the reverse strand (5’-TTCTGATTTCACGTAGGATT-3’). Analysis of pyrogram peaks was performed to identify the genotype of each sample (Figure 2.2).

**Per 10 µl reaction:**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl</td>
<td>Qiagen PCR Master Mix</td>
</tr>
<tr>
<td>2.6 µl</td>
<td>ddH₂O</td>
</tr>
<tr>
<td>0.2 µl</td>
<td>Forward primer 5’-TAGTCTACCACCTGCGGGATA-3’ 10 µM</td>
</tr>
<tr>
<td>0.2 µl</td>
<td>Reverse primer 5’-CAAGCTGAGCCTGTGAA-3’ 10 µM</td>
</tr>
<tr>
<td>2 µl</td>
<td>DNA</td>
</tr>
</tbody>
</table>
Thermocycler program:

Step 1  95°C  5 minutes
Step 2  95°C  15 seconds
Step 3  60°C  30 seconds
Step 4  72°C  15 seconds
Step 5  repeat steps 2-4, 44 times
Step 6  72°C  5 minutes
Step 7  16°C  hold

Figure 2.2 Example pyrograms used to genotype *goya* mice. Panel A shows a single wild-type peak (A/A), panel B shows two peaks demarking a heterozygote *goya* sample (A/G) and panel C shows the single peak of a homozygote *goya* sample (G/G). N.B. The *goya* mutation is a T>C substitution, however sequencing primer is on the reverse strand, accounting for the complimentary nucleotides.
2.5.2. Map3k1^{tm1Yxia}

The Map3k1^{tm1Yxia} allele contains a LacZ reporter gene in place of the kinase domain. The presence or absence of LacZ was identified by assessing copy number using a TaqMan™ Copy Number Assay (Applied Biosystems®). A probe was designed to be complimentary to a region of the target amplicon within the LacZ gene containing a fluorescent reporter (FAM™) at the 5’ end and a quencher located at the 3’ end. When the reporter and quencher are in close proximity, as they are when the probe is intact, the quencher supresses the fluorescent signal. Upon extension of the forward primer during amplification cycling, the reporter is cleaved from the probe and a fluorescent signal can be detected. Further extension of the primer releases the quencher and blocks the signal. An internal reference (Dot1) was run in duplex with a different reporter label (VIC™). As with TaqMan® gene expression the amount of fluorescence is directly proportional to the amount of the target sequence present; therefore with correct threshold settings when compared to the Dot1 reference, one, two or no copies may be detected indicating the genotype. A second probe designed to recognise a region of the wild-type kinase domain, was used in a second qRT-PCR copy number assay for increased confidence of genotype detection. Assays were run and analysed on the 7500 Fast Real-Time PCR system (Applied Biosystems®) by the genotyping and mutation detection screens (GEMS) core facility at the MRC Harwell Institute.
Primers:

*LacZ* forward 5’-CTGCCACTTCAACATCAAC-3’

*LacZ* reverse 5’-TTATCAGCCGAAAAACCTACC-3’

*LacZ* probe (FAM™ labelled) 5’-TCGCCATTTGCCATCATCAATCC-3’

Wild-type forward 5’-AGCTGCAAGGTAATGGAGAAG-3’

Wild-type reverse 5’-CACGTCCGTCTTCCCAGTAG-3’

Wild-type probe (FAM™ labelled) 5’-CATCATTCAGCAGGAGTATATAAACC-3’

*Dot1l* forward 5’-GCCCATCGCCATTATCCTTACTAC-3’

*Dot1l* reverse 5’-TAGTTGGCATCCTTATGCTTCATC-3’

*Dot1l* probe (VIC™ labelled) 5’-CCAGCTTCAAGTCGTACGTAGTATAAACC-3’

qRT-PCR mix per 10 μl reaction:

5 μl ABI GTX TaqMan master mix

0.225 μl Forward primer (20 μM)

0.225 μl Reverse primer (20 μM)

0.2 μl Probe (5 μM)

0.3 μl FAM™ Assay (probe 5 μM & primers 15 μM each)

1.55 μl ddH₂O

2.5 μl DNA (1 in 10 dilution of Sample-to-SNP prep)

Real-time cycling conditions:

Step 1 95°C 20 seconds

Step 2 95°C 3 seconds

Step 3 60°C 30 seconds

Step 4 repeat steps 2-3, 40 times
2.5.3. *Map3k4<sup>tm1Flv</sup>*

The *Map4k4<sup>tm1Flv</sup>* was genotyped using a mixture of 3 primers, a forward primer and two reverse primers, one distinct for the wild-type allele, and one distinct for the mutant allele. PCR amplification of the different alleles produced different size fragments used to determine genotype.

**Per 20 µl reaction:**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.5 µl</td>
<td>ReddyMix PCR Master mix (ThermoFisher)</td>
<td></td>
</tr>
<tr>
<td>0.5 µl</td>
<td>Forward primer 5’-TAAGACAGAGGCAGGGATTGGC-3’</td>
<td>(10 µM)</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>Reverse primer (Wild-type) 5’-CCGTTGTTCAGAGTTGCTCG-3’</td>
<td>(10 µM)</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>Reverse primer (Mutant) 5’-GAGCAGTATTGTGGATTTCCGA-3’</td>
<td>(10 µM)</td>
</tr>
<tr>
<td>1 µl</td>
<td>DNA</td>
<td></td>
</tr>
</tbody>
</table>

**Thermocycler program:**

- **Step 1:** 95°C 5 min
- **Step 2:** 94°C 30 sec
- **Step 3:** 61.5°C 30 sec
- **Step 4:** 72°C 30 sec
- **Step 5:** repeat steps 2-4, 36 times
- **Step 6:** 72°C 5 min

As these mice were crossed to mice carrying the *Map3k1<sup>tm1Yxia</sup>* allele, separate genotyping assays were run for each sample.
2.5.4. Map3k4\textsuperscript{BAC}

The Map3k4 overexpressing mice were identified by the presence of a chloramphenicol resistance (\textit{Cm}\textsuperscript{R}) gene in the backbone of the BAC plasmid by PCR amplification using the following conditions:

**Per 20 µl reaction:**

- 18 µl ReddyMix PCR Master mix (ThermoFisher)
- 0.5 µl Forward primer (GC\textcolor{red}{G}TG\textcolor{red}{G}TTAC\textcolor{red}{G}G\textcolor{red}{G}TG\textcolor{red}{A}AAAC\textcolor{red}{C}T) (10 uM)
- 0.5 µl Reverse primer (GGGCAC\textcolor{red}{C}CAATAACTG\textcolor{red}{C}T\textcolor{red}{T}A) (10 uM)
- 1 µl DNA

**Thermocycler program:**

- Step 1: 94°C 4 min
- Step 2: 94°C 20 sec
- Step 3: 57°C 20 sec
- Step 4: 72°C 20 sec
- Step 5: repeat steps 2-4, 36 times
- Step 6: 72°C 5 min

As these mice were crossed to the Map3k1\textsuperscript{tm1Yxia} allele, separate genotyping assays were performed for each sample.
2.5.5. Orai\textsuperscript{1L187Q}

The Orai\textsuperscript{1L187Q} mice were genotyped using a restriction digest of PCR product:

Per 10 µl reaction

5 µl Qiagen PCR Master Mix
3.6 µl ddH\textsubscript{2}O
0.2 µl Forward primer 5’- GCTGGCCTGGGCCTTCT -3’ 10 µM
0.2 µl Reverse primer 5’- AGGCCACAGGGAACCAT -3’ 10 µM
1 µl DNA

Thermocycler program:

Step 1 95°C 5 min
Step 2 95°C 15 sec
Step 3 60°C 30 sec
Step 4 72°C 15 sec
Step 5 repeat steps 2-4, 44 times
Step 6 72°C 5 min

The resulting PCR product was digested using the restriction enzyme AluI (as described in 2.4.). The amplicon was 228 bp originally, digest with AluI produced bands of 190 and 38 for the wild-type allele but failed to cut the mutant allele.
**2.5.6. Orai\(^{V135E}\) and Orai\(^{I123N}\)**

The *Orai\(^{V135E}\) and Orai\(^{I123N}\)* mice were both genotyped using TaqMan® Allelic Discrimination Assays (Applied Biosytems). This assay works in a similar way to the TaqMan® Copy Number Variant Assay described in 2.5.2., although two probes are used; one that detects the wild-type allele (labelled with FAM™) and one to recognise the mutant allele (labelled with TET™). As the qRT-PCR is performed, the level of each fluorophore is measured and compared to elucidate the number of each allele present in the sample, therefore the genotype of the mouse.

**Orai\(^{V135E}\)**

Forward Primer (Antisense Strand) - AGGTGCTGATCATGAGGGCAAAC

Reverse Primer (Sense Strand) - AGGTAGCGATGGTGGAAGTC

Mutant Probe (Antisense) (FAM™-Labelled) - AGGTGCACGGCCACT

Wild-type Probe (Antisense) (TET™-Labelled) – AGGTGCTCGGCCACT

**Orai\(^{I123N}\)**

Forward (Sense) - GCTGGACACAGACCATGAC

Reverse (Antisense) - GCAGGATGCAGGTGCTGAT

Mutant Probe (sense) (FAM™-Labelled) - AGGGTTGCTCAACGTCTT

Wild-type Probe (Sense) (TET™-Labelled) - AGGGTTGCTCATCGTCTT

**qRT-PCR master mix**

- ABI GTX Taqman master mix 5µl
- Assay (Probes 5µM each & Primers 15µM each) 20uM 2µl
- Water 0.5µl
- DNA 2.5µl
Real-time cycling conditions:

Step 1 60°C 1 minute
Step 2 95°C 20 seconds
Step 3 95°C 3 seconds
Step 4 60°C 30 seconds
Step 5 repeat steps 3-4, 40 times
Step 6 60°C 1 minute

2.6. Agarose Gel Electrophoresis

Agarose gels for DNA analysis consisted of the desired concentration (0.7 – 2.5 %) of agarose (Sigma) dissolved in 1 x TAE buffer (40 mM tris acetate; 1 mM ethylenediaminetetraacetate (EDTA)) by heating in a microwave oven. On cooling, 0.2 µg/ml ethidium bromide was added (Alfar Aesar). Gels were poured and left to set, and loaded with sample pre-mixed with a loading dye (2 µl Orange G (Sigma)). HyperLadder™ 100 base pair (bp) ladder was also loaded to indicate size of DNA fragments. The gels were electrophoresed in 1 x TAE buffer until sufficient separation had occurred (usually 20 – 30 minutes) at 150 volts (V). Visualisation of DNA bands was achieved using ultraviolet (UV) light in a Gel Doc (Bio-Rad).

2.7. Screening of the MRC Harwell Institute ENU DNA Archive

The ENU archive contains ~10000 parallel DNA and sperm samples from ENU mutagenised G1 male mice. This enables a reverse genetics approach whereby DNA from the archive can be screened for mutations in a gene of interest; sperm from the complimentary sperm archive can then be used to re-derive mouse lines carrying alleles of interest using IVF. The archive was screened by HRM analysis using the Lightscanner
system (Idaho Technology Ltd.) to identify heterozygote SNPs in 96 well plates containing pooled (4 samples per well) of archived DNA. The individual DNA samples from the corresponding pool are then recovered for SNP identification and confirmation by Sanger sequencing. Exon 2 of Orai1 which harbours the c392T>C mutation in Boycie mice was PCR amplified as three separate amplicons which were used to identify alleles of Orai1 in the ENU DNA archive.

**Primers:**

Exon 2.1 Forward primer: 5’-GCGAGCGGCTCAGTTTC-3’
Exon 2.1 Reverse primer: 5’-TCGTGGGGTGACTCTTTG-3’
Exon 2.2 Forward primer: 5’-GATCAGCAGCTGCATCC-3’
Exon 2.2 Reverse primer: 5’-GTGCCGACAGTGACTG-3’
Exon 2.3 Forward primer: 5’-CTGCTGGGTCAAGTTCTT-3’
Exon 2.3 Reverse primer: 5’-GGTCATGGAGAAGGGCATAA-3’

**Per 10 µl reaction:**

5 µl Hotshot PCR Mastermix (Clent Life Science)
1 µl LCGreen Plus (Clent Life Science)
0.1 µl Primer forward (20 mM)
0.1 µl Primer reverse (20 mM)
1.8 µl ddH₂O
2 µl DNA

Reactions were loaded into black white-well plates (4Titude), with 20 µl mineral oil (Sigma Aldrich) per well, sealed with transparent film (ABgene) and centrifuged for 1 minute.
Thermocycler program:

Step 1  95°C  2 minutes
Step 2  95°C  30 seconds
Step 3  60°C*  30 seconds
Step 4  72°C  30 seconds
Step 5  repeat steps 2-4, 44 times
Step 6  95°C  30 seconds
Step 7  25°C  30 seconds
Step 8  16°C  30 seconds

* (Step 3 = 60°C for amplicons 2.1 and 2.3, but 58.7°C for amplicon 2.2)

2.8. Auditory Assessment

2.8.1. Click-box Testing

Mice were placed in the palm of hand and stimulated with a click-box. The click-box is a handheld unit which emits a high frequency (approximately 20 kHz) tone stimulus at a level of 90 decibels sound pressure level (dB SPL) when positioned 30 cm above the animal (obtained from the MRC Institute of Hearing Research, Nottingham, UK). The usual response of a hearing mouse is the Preyer’s reflex, a rapid flick back of the ear pinnae, and is often accompanied by a jump or startle response. The response to the click-box is scored as 2 – present, 1 – reduced or 0 – absent.
2.8.2. Auditory-evoked Brainstem Response (ABR)

Mice were anaesthetised using a mixture of ketamine hydrochloride (100 mg/kg) and xylasine (10 mg/kg), administered by interperitoneal injection, and placed in a heat-box (Datesand Ltd.) until immobilised. Animals were then placed on a heated mat to maintain body temperature at 37°C, inside an sound attenuating chamber (ETS-Lindgren SD enclosure). Recording electrodes (Grass Telefactor F-E2-12) were placed sub-dermally over the vertex (active), right mastoid (reference), and left mastoid (ground). As mice lose the ability to blink under anaesthetic, to prevent eye irritation from occurring ‘artificial tears’ were administered to the eyes of the mouse (Viscotears®, Novartis). Acoustic stimuli were delivered monaurally to the right ear at a distance of 1.5 cm via a free field transducer (ES1 Tucker Davis Technology (TDT), Alachua, FL), controlled by TDT SigGen/BioSig software, using TDT system III hardware. The transducer output was calibrated using a ¼” measuring microphone (7016 ACO-Pacific, Belmont, CA) and SigCal software (TDT). The normalised output was subsequently checked using a Brüel and Kjær PULSE system with a type 4939 ¼” measuring microphone attached to a high frequency 3110 processing module. For calibration the microphone capsule was placed inside a pistonphone calibrator (Aco-Pacific type 511e), which produced a 1 kHz tone at 94 dB SPL again using the Brüel and Kjær PULSE hardware and software. ABR responses were collected, amplified and averaged using TDT system III hardware and BioSig software (Tucker Davis Technology (TDT), Alachua, FL). Tone-burst stimuli totalled 7 ms duration including rise/fall gating using a 1 ms Cos² filter. Stimuli were presented at 90 dB SPL followed by decreasing steps of 5-10 dB SPL, until a threshold was determined visually by the absence of replicable response peaks. For the goya project stimuli consisted of tone-bursts at 8 kHz, 12 kHz, 20 kHz and 26 kHz presented at a rate of 21/sec for a total of 312 averages for all frequencies. For the Boycie project stimuli consisted of 0.1 ms broadband
clicks of alternating polarity presented at a rate of 21/sec for a total of 300 averages and
tone-burst stimuli at 8 kHz, 16 kHz and 32 kHz, presented at a rate of 42.5/sec for a
maximum of 1000 repetitions. The difference in chosen stimuli between the two projects
owed to equipment limitations at the beginning of the goya project. Where repeat testing
was performed, recovery of mice was accelerated by administration of atipamezole
(Antisedan™, 5mg/ml) at the rate of 1mg/kg of body weight.

2.9. Morphological Assessment of Inner ears

2.9.1. Scanning Electron Microscopy

Mice were euthanized by cervical dislocation before heads were removed, skinned,
bisected and inner ears rapidly excised. The oval window was pierced and a small hole
made at the apex of the cochlear shell. The cochleae were gently perfused with 2.5 %
glutaraldehyde (GTA) in 0.1M phosphate buffer (PB) (4.44 g monobasic sodium
phosphate, 17.04 g dibasic sodium phosphate both Sigma-Aldrich, 789 ml ddH₂O),
through the oval window, using a syringe with a 30G needle. Inner ears were
subsequently submerged in the same fixative and placed on a rocking platform at 4° C
overnight, and rinsed three times for 15 minutes in 0.1M PB. To ease removal of the bony
otic capsule, inner ears were then decalcified for 24-48 hours in 4.3 % EDTA (Sigma-
Aldrich) dissolved in 0.1M PB at room temperature. Fine dissection was performed to
reveal the organ of Corti (Figure 2.3. A-D), before osmium tetroxide - thiocarbohydrazide
(OTO) processing (adapted from (Hunter-Duvar, 1978)) was carried out either by hand or
using a Leica EM-TP (Leica Microsystems). The OTO processing involved the samples being
immersed in 1 % v/v osmium tetroxide (OsO₄) (Electron Microscopy Supplies) in 0.1M
sodium cacodylate buffer (Sigma-Aldrich) for 1 hour, washed six times for 5 minutes each
in ddH₂O, immersed in 1 % w/v thiocarbohydrazide (TCH) (Fluka) in ddH₂O for 30 minutes,
washed six times for 5 minutes each in ddH₂O, immersed in 1 % v/v OsO₄ in 0.1M sodium cacodylate buffer for 1 hour and finally washed six times for 5 minutes each in ddH₂O. Samples were then dehydrated through increasing strength ETOH solutions, 25 %, 40 %, 65 %, 80 %, 95 % and 100 % (Fisher Scientific) for 45 minutes each, before being transferred to 100 % acetone (Fisher Scientific) and critical point dried using either an Emitech K850 (EM Technologies LTD) or, more recently, a Leica EM CPD300 (Leica Microsystems). Inner ears were then mounted on stubs using silver paint (Agar Scientific) and sputter coated with platinum in an argon (BOC) atmosphere using a Quorum Q150T sputter coater (Quorum Technologies). Prepared cochleae were visualised with a JEOL LSM-6010 (Jeol Ltd.) scanning electron microscope.

**Figure 2.3 Fine dissection of the inner ear for SEM.** (A) Removal of the decalcified shell surrounding the cochlea. (B) When the cochlear shell is removed, the pigmented cells in the stria vascularis (SV) become less opaque. (C) Removal of the stria vascularis and spiral ligament/lateral wall of the cochlear duct. (D) The cochlea with the shell, stria vascularis and lateral wall removed to reveal the sensory epithelia. Figure adapted from (Parker et al., 2016).
2.9.2. Histological Examination

Mice were euthanized by cervical dislocation, heads removed, skinned and bisected. Either half heads were fixed by submersion in 10 % neutral buffered formalin (NBF), or inner ears were removed and fixed as described in 2.9.1. using 10 % NBF as a fixative in place of glutaraldehyde. In both cases fixation was performed for 1-3 days on a rocking platform at 4°C. Samples were then washed in 1x phosphate buffered saline (PBS) three times for 15 minutes before decalcification for a week in 4.3 % EDTA in 1 x PBS. Paraffin embedding and microtome sectioning was performed by the Histology Core Facility at the MRC Harwell Institute using standard methods. Sections were either all stained with Haematoxylin and Eosin (H&E), or alternate slides were H&E stained or left as charged slides for subsequent immunohistochemistry.

2.9.3. Resin Embedded Semi-thin Sections

Mice were euthanized by cervical dislocation, heads removed, skinned and bisected. Inner ears were then removed and fixed as described in 2.9.1., samples were then washed by submersion in 0.1 M PB three times for 15 minutes. Inner ears were then post-fixed with 1% OsO4 in 0.1 M sodium cacodylate buffer for 1 hour or until the cochlea was visibly stained through the shell, and the rinsed six times for 3 minutes each in ddH2O. Decalcification was performed in 4.3% EDTA dissolved in 0.1 M PB on a shaker at room temperature for one week, the EDTA was replaced daily. When decalcified, the inner ears were rinsed in 0.1 M PB and dehydrated through graded ETOH series for 30 minutes each (25 %, 40 %, 60 %, 85 %, 95 % and twice in 100 %) at 4°C, before immersion in acetone twice for 30 minutes at 4°C.

A 100 % resin mixture containing 13 g dodeceny succinic anhydride (DDSA), 13 g nadic methyl anydride (NMA) and 24 g TAAB 812 resin (TAAB Laboratories equipment Ltd.) was
combined in a falcon tube. For infiltration, inner ears were the immersed in 50 % resin in acetone, 70 % resin in acetone and twice in 100 % resin for 6 hours each at room temperature with gentle agitation. For embedding, inner ears were transferred to coffin moulds containing 100 % with 2 % w/v benzyl di-methyl amine accelerator added (TAAB Laboratories equipment Ltd.) and orientated for sectioning (Figure 2.4). The moulds were placed in the oven to polymerise for 2-3 days at 65° C. Polymerised blocks were trimmed and semi-thin (0.5 µm) sections cut using glass knives on an Ultracut E ultra-microtome (Reichert-Jung), before staining was achieved with 1% toluidine blue in sodium borate.

![TOP VIEW](image)

**Figure 2.4** Resin embedding for semi-thin sectioned histology. A representation of the desired orientation of the dissected inner ear in a coffin mould. The inner ear is positioned on its side with the cochlea (C) pointing toward the cutting (angled) end of the mould and the vestibular system (V) positioned at around a 45° angle to the cochlea, with the oval window (OW) facing toward the non-angled end. Figure adapted from (Parker et al., 2016)

### 2.10. X-Gal Staining

Mice were euthanized and inner ears removed and fixed for 2 hours at 4°C in 0.1M PB containing 1 % paraformaldehyde (PFA) (Sigma-Aldrich) 2 mM MgCl₂ (Sigma-Aldrich), 0.25 % GTA (Sigma-Aldrich) and 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Merck Millipore). Inner ears were then washed in 0.1 M PB containing 2 mM MgCl₂ (Sigma-Aldrich) and 0.02 % NP-40 (Fluka). Staining was performed overnight at room temperature in a solution of 0.1 M PB containing 2 mM MgCl₂ (Sigma-Aldrich), 5 mM potassium ferrocyanide (Sigma-Aldrich), 5 mM potassium ferricyanide
(Sigma-Aldrich), 0.02 % NP-40 and 1mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) (Sigma-Aldrich). Post-staining, ears were decalcified in 4.3 % EDTA in 0.1M PB (Sigma-Aldrich) for 48 hours at 4°C, and either prepared for whole-mount imaging by dissection of the cochlear duct, or processed using standard paraffin embedding histological techniques sectioning at 10 µm; sections were counterstained using Nuclear Fast Red (Sigma). Sections and whole-mount dissected cochlea were both imaged on a Zeiss Axio Observer Z-1 microscope using extended focus image capture.

2.11. Cloning

To enable in vitro functional investigation of the Boycie mutation in Orai1, constructs were generated to express enhanced green fluorescent protein (EGFP) and c-Myc tagged wild-type ORAI1 and also EGFP and c-Myc tagged ORAI(V131A), the peptide substitution resulting from the Boycie mutation.

2.11.1. TA Cloning

A RIKEN complementary DNA (cDNA) clone (D530032D3) was purchased from Source BioScience containing the full sequence of wild-type Orai1. Primers were designed to amplify Orai1 with the addition of restriction enzyme specific motifs (EcoRI and SacII) to enable subsequent directional sub-cloning of the construct into the EGFP and c-Myc expression vectors:

EcoRI-Orai1 Forward primer: 5’-TCGAATTCCatccgagcctgccgccg-3’
SacII-Orai1 Reverse primer: 5’-TCCCGGgcagcataggtggggccccggt-3’

GeneClean II was used to purify the resulting PCR product prior to TA cloning using the pGEM®-T Vector system I kit (Promega). TA cloning enables insertion of DNA into a vector without the need for restriction enzymes. During PCR the Taq polymerase adds an
adenosine (A) residue to each 3’ end of the PCR product, the linearised pGEM®-T vector contains a thymine (T) residue at both 3’ ends. Simple ligation of the linearised vector and PCR product mediated by T4 DNA ligase results in a circular plasmid containing the PCR product. Ligation reactions were prepared as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl</td>
<td>2x Rapid ligation buffer (Promega)</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>pGEM-T® vector (25 ng) (Promega)</td>
</tr>
<tr>
<td>3.28 µl</td>
<td>Orai1 PCR Product</td>
</tr>
<tr>
<td>1 µl</td>
<td>T4 DNA ligase (3 Weiss units) (Promega)</td>
</tr>
<tr>
<td>0.22 µl</td>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

Ligation reactions were incubated at RT for 1 hour, and then used for subsequent transformation of JM109 cells.

2.11.2. Transformation of Competent Cells

High efficiency competent JM109 cells (Promega) were transformed with the ligation reaction prepared in 2.11.1. This was achieved by placing 20 µl JM109 cells on ice with 2 µl of the ligation reaction for 30 minutes before heat-shocking at 42°C for 45 seconds in a water bath. The tubes were then returned to the ice for 2 minutes before 200 µl of pre heated (37°C) SOC media (Sigma) was added. The transformations were then placed in a shaking incubator (150 rpm) for 1.5 hours at 37°C. After incubation, 150 µl of the transformations was streaked onto lysogeny broth (LB) agar plates containing ampicillin (100 µg/ml) (Sigma), isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) (Promega) and X-Gal (80 µg/ml) (Sigma) to enable blue/white selection. The plates were incubated overnight at 37°C. Blue/white selection is possible due to the presence of the β-Galactosidase enzyme gene in the pGEM-T® vector. IPTG induces transcription of β-Galactosidase which reacts with the X-gal and results in blue staining of the colony; this
occurs if no recombination of the insert is present in the vector. If DNA has successfully integrated into the vector, expression of β-Galactosidase is disrupted resulting in a lack of staining or ‘white’ colonies. Isolated white colonies were selected and transferred into 100 µl LB broth and then used as templates for a colony PCR using the insert specific primers. Following colony PCR, 10 ml of LB broth containing 100 µg/ml ampicillin, was inoculated with the LB containing the isolated colony and incubated overnight at 37°C shaking at 200 rpm, in order to amplify the plasmid.

2.11.3. Isolation of Plasmid DNA

DNA was isolated from the overnight cultures using the Wizard® Plus SV Miniprep Purification System (Promega), which uses a spin column with a silica membrane to isolate the plasmid DNA. Briefly, cells in the bacterial cultures were pelleted by centrifugation at 2000 rpm for 10 minutes and the media removed. Cells were then resuspended and lysed before centrifugation through the spin column. Wash steps were then performed to remove contaminants whilst the plasmid DNA remains bound on the silica membrane inside the column. Finally DNA was eluted in 100 µl ddH₂O, and sent for Sanger sequencing using T7 and SP6 insert primers (Source Bioscience) to confirm the presence of the EcoRI-Orai1-SacII insert.

2.11.4. Site Directed Mutagenesis

To introduce the c.392T>C Boycie mutation into the wild-type Orai1 construct the QuikChange® Lightning (Agilent Technologies) site directed mutagenesis kit was used. This involves a pair of complimentary primers both containing the desired mutation and utilises a rapid acting high fidelity DNA polymerase. Following thermal cycling and incorporation of the primers, the enzyme DpnI which digests methylated DNA was used
to remove the plasmid DNA, leaving the freshly synthesised, unmethylated Orai1 DNA containing the mutation. The following primers were used for mutagenesis:

Orai1 T392C sense: 5'-GCCTGCACCACAGCGCTAGTGCGCCGTG-3'

Orai1 T392C antisense: 5'-CACGGCCACTAGCGCTGTGGTGTCAGGC-3'

Following mutagenesis, JM109 cells were transformed again using the mutagenised DpnI treated DNA and sent for sequencing as previously described in 2.11.2. and 2.11.3.

2.11.5. Construct Generation

Mammalian expression vectors, pEGFP-N1 (Clontech) and pcDNA3.1/Myc-His (B) (Invitrogen) were selected in order keep the Orai1 sequence in frame with the C-terminal EGFP and c-Myc tags. Plasmid DNA prepared and checked by Sanger sequencing as described in 2.11.3., was digested with both EcoRI and SacII (both New England Biolabs) in order to release the Orai1 insert sequence. Electrophoresis was then used to isolate the insert from the plasmid backbone. The Orai1 DNA product was subsequently excised from the agarose gel and purified using the GeneCLean II kit (MP Biomedicals). An identical digest was also performed using DNA prepared from the EGFP and c-Myc expression vectors in order to linearise them ready for ligation of the insert.

Digest reactions:

5 µl DNA (Miniprep of pGEM-T containing insert or expression vector)

5 µl Restriction enzyme buffer

1 µl EcoRI (New England Biolabs)

1 µl SacII (New England Biolabs)

38 µl ddH2O

Enzyme digest reactions were incubated for 2 hours at 37°C.
Ligation reactions were then prepared as follows:

0.5 µl Digested expression vector
1 µl Digested and isolated insert
1 µl T4 DNA ligase (Promega)
5 µl 2x Ligase buffer (Promega)
2.5 µl ddH₂O

The ligation reactions were incubated for 1 hour at RT, and JM109 cells transformed again as described in 2.11.2, and plated on LB agar plates. For pcDNA3.1/Myc-His(B) plasmids, the LB agar plates contained 100 µg/ml ampicillin, for pEGFP-N1 plasmids, plates contained 100 µg/ml kanamycin. As the plasmid vectors contain corresponding antibiotic resistance cassettes, only successfully transformed colonies should survive. After incubation of plates overnight at 37°C isolated colonies were selected and used to inoculate 10 ml of LB containing appropriate antibiotics which were cultured overnight at 37°C at 200 rpm. Plasmid DNA was isolated from overnight cultures and sent for Sanger sequencing as described in 2.11.3. using vector specific sequencing primers to confirm the correct Orai1 sequence and tag was present in each sample:

**Vector specific sequencing primers:**

pEGFP-N1 pEGFP-N1 (Reverse): 5’-CGTCGCCGTCCAGCTCGACCAG-3’
pDNA3.1/Myc-His(B) T7 (Forward): 5’-TAATACGACTCTATAGGG-3’
pDNA3.1/Myc-His(B) BGHR (Reverse): 5’-TAGAAGGCACAGTCGAGG-3’

**2.11.6. Cell Culture**

Cell culture was carried out using sterile technique in a BioMAT2 Class 2 Microbiological Safety Cabinet (Contained Air Solutions). Cos-7 cells (derived from the kidney of an
African green monkey (Gluzman, 1981)) were kindly provided by Dr Chris Esapa (MRC Harwell Institute). Frozen aliquots of Cos-7 cells were defrosted in a water bath at 37°C and transferred into 75 cm² flasks (Greiner Bio-One) containing 14 ml high-glucose Dulbecco’s Modified Eagle Medium with 10 % foetal bovine serum (FBS) and 1 x penicillin/streptomycin (all Invitrogen), pre warmed to 37°C. The flasks were placed in an incubator (Sanyo) at 37°C with CO₂ at 5 % to culture until confluent.

2.11.7. Splitting Cells

Upon reaching 80-90 % confluence, cells were washed in pre-warmed (37°C) Dulbecco’s PBS (DPBS) (Gibco) before the addition of 2 ml 0.05% Trypsin-EDTA (Gibco). Flasks were placed in the incubator for at 37°C until cells detached from the surface (2 - 5 minutes), 8 ml of Dulbecco's modification of Eagle's medium (DMEM) was then added to inactivate the trypsin. The cell suspension was then centrifuged at 1000 rpm for 2 minutes at RT to pellet cells, the medium was aspirated and cells were resuspended in 14 mls fresh medium. The new suspension was mixed gently by pipette, divided between 2 new flasks and returned to the incubator.

2.11.8. Freezing Cells

For freezing of Cos-7 cells, washing and trypsinisation was performed as in 2.11.7.. Cells were then pelleted in a centrifuge at 1000 rpm for 5 minutes and resuspended in 10 ml freezing media (DMEM containing 50 % FBS and 10 % dimethyl sulfoxide (DMSO)). The suspension was then aliquoted to 1 ml cryotubes for storage at -80°C.

2.11.9. Seeding of Cells for Subcellular Localisation

Confluent Cos-7 cells were washed and trypsinised as described in 2.11.7.. Cell density was then calculated using a haemocytometer (Hawksley) in combination with an Olympus
IMT-2 microscope. Cells were seeded on to 22 x 22 mm² sterile coverslips placed in 6-well tissue culture plates at a volume of 1 x 10⁵ and incubated at 37°C with 5 % CO₂ for 24 hours.

2.11.10. Transient Transfection of Cos-7 Cells

Transient transfections of Cos-7 cells were achieved using the JetPEI® transfection reagent (PolyPlus Transfection) according to manufacturer’s guidelines. Briefly, 6 µl of JetPEI® was mixed with 100 µl 150 mM sodium chloride (NaCl) (PolyPlus Transfection) and added to 1 µg of plasmid DNA (containing the appropriate construct as described in 2.11.5.) also diluted in 100 µl 150 mM NaCl. The JetPEI®/DNA complex was then incubated at RT for 30 minutes and gently added dropwise to the seeded cells in the 6 well tissue culture plates (from 2.11.9.). The transfected cells were then incubated again for 24 hours at 37°C with 5 % CO₂ before immunocytochemistry was performed. A non-transfected control coverslip was also prepared for each construct.

2.11.11. Subcellular Localisation

After incubation for 24 hours after transfection, the coverslips seeded in 2.11.9. were rinsed twice with cold 1 x PBS, and immersed in 4 % PFA for 10 minutes at RT to fix the cells. For cells transfected with the Myc tagged constructs, blocking was carried out in 10 % donkey serum in 1 x PBS with 0.1 % TWEEN® 20 (PBST) for 1 hour at RT, before incubation with rabbit anti-Myc (Santa Cruz (SC789)) primary antibody in PBS with 1 % donkey serum overnight at 4°C. The primary antibody was then washed away with 2 rinses of cold PBS and the Alexa Fluor® 488 conjugated secondary antibody was added in PBS and incubated protected from light for 1 hour at RT. The secondary antibody was then removed and the cells rinsed again twice with cold PBS and 4',6-diamidino-2-phenylindole (DAPI) was added (1 µg/ml) for 1 minute. No antibodies were necessary for
cells transfected with the EGFP-tagged constructs, however DAPI was added as above. Cells were given a final rinse and coverslips were mounted onto charged microscope slides using SlowFade® Gold (Life Technologies) and protected from light. Non transfected controls were used to eliminate the possibility of unspecific background fluorescence. Cells were imaged Zeiss LSM 700 inverted fluorescence confocal microscope.

2.11.12. Calcium Release Activated Calcium (CRAC) Channel Function

The calcium imaging used to assess effects of the Boycie mutation on CRAC channel function were kindly performed by Prof Anant Parekh and Dr Dan Bakowski (University of Oxford). To assess Ca\(^{2+}\) entry, a re-addition assay was performed using the Ca\(^{2+}\) sensitive ratio-metric fluorophore Fura-2, excited at wavelengths of 356/380 nm. Briefly HEK293 cells were either mock transfected, or co-transfected with STIM1 and either wild-type or Boycie ORAI1 tagged with EGFP (as described in 2.11.5.) and cultured using standard methods. Cells were loaded with Fura-2/AM (acetoxymethyl) (Molecular Probes), to fluorescently label intracellular Ca\(^{2+}\). To measure Ca\(^{2+}\) entry through the CRAC channel, cells were placed in a Ca\(^{2+}\) free solution to remove extracellular Ca\(^{2+}\) and treated with 1 μM thapsigargin (Sigma) to deplete intracellular Ca\(^{2+}\) stores. Extracellular Ca\(^{2+}\) was then re-added to the solution and level of intracellular Ca\(^{2+}\) over time was calculated by ratiometric analysis. The rate of Ca\(^{2+}\) entry was deduced using these measurements. Mock-transfected HEK293 cells were used to identify the basal levels of CRAC channel activity.
2.12. RNA Techniques

2.12.1. RNA Extraction

Inner ears were excised and the organ of Corti micro-dissected from P1 mice and placed in RNAlater until extraction. For each sample RNA was pooled from four (two mice) organs of Corti, which were homogenised using a Polytron™ homogeniser (KINEMATICA). Isolation of RNA was achieved using the RNeasy micro kit (Qiagen) as per the manufacturer’s instructions; this kit uses guanidine-isothiocyanate to lyse the tissue and silica-membrane columns to isolate RNA by centrifugation. The column was treated with DNasel to remove DNA before elution in 20 µl of DNase/RNase-free water; extracted RNA was stored at -80°C until required.

2.12.2. Complementary DNA (cDNA) synthesis

2.12.2.1. For Analysis of Map3k1 Transcription in the Cochleae of goya Mice.

For Map3k1 transcriptional analysis, cDNA was synthesised using a high capacity cDNA reverse transcription kit (Life technologies) as per manufacturer’s instructions. For each sample 150 ng of RNA was used in a 10 µl reaction. Primers used were a combination of oligo-(dT), random hexamers and a Map3k1 specific primer spanning the exon 14 – exon 15 boundary. cDNA was stored at -20°C until use.

2.12.2.2. For Use in RT² Profiler™ PCR Array

For optimal results, the RT² Profiler PCR Array requires cDNA to be synthesised using the RT² First Strand Kit (both SABioscience). As such 500 ng of RNA was used in each 20 µl reaction and diluted using 91 µl RNase free water as per the manufacturer’s instructions.
2.12.2.3. For Gene Expression Analysis using TaqMan™ Hydrolysis Probes

For gene expression analysis cDNA was synthesised as in 2.12.2.1 except that only random hexamers were used to prime the cDNA synthesis reactions.

2.12.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The cDNA synthesised in 2.12.2.1 was used as a template for PCR amplification to investigate transcription of Map3k1 in the mouse cochlea using a forward primer spanning the end of exon 11 and the beginning of exon 12 and a reverse primer from exon 14 of Map3k1. This strategy was employed to investigate possible alternative splicing of Map3k1 resulting from the goya mutation in the intron 13 splice donor site.

2.12.3.1. Gel Extraction and Sequencing of RT-PCR Product

RT-PCR products obtained in 2.12.3. were separated by agarose gel electrophoresis in a 0.7 % low melting point agarose gel. Separated RT-PCR fragments were then excised from the gel and purified using the GeneClean II Kit (MP Biomedicals). Purified RT-PCR products were then TA cloned into pGEM-T vector, JM109 cells transformed and plasmid DNA isolated as described in 2.11.1, 2.11.2. and 2.11.3. Plasmid DNA was then sent for Sanger sequencing using SP6 and T7 primers (Source Bioscience) to investigate gene splicing events.

2.12.4. Gene Expression

2.12.4.1. RT² Profiler™ PCR Array Mouse MAP Kinase Signaling Pathway

The RT² Profiler™ PCR Array Mouse MAP Kinase Signaling Pathway (SABiosciences) was used to investigate differential gene expression in a large number of MAPK pathway members. The 96-well plate assay was prepared according to the manufacturer’s instructions; briefly 102 µl of the cDNA synthesis reaction detailed in 2.12.2.2. was
combined with 1350 µl 2 x RT² SYBR Green Mastermix and 1248 µl RNase-free water (both SABioscience). 25 µl of the PCR mix was added to each well, and the RT² Profiler PCR Array plate was centrifuged for 1 minute at 1000 x g at RT. The plate was then run on a 7500 Fast real time PCR machine (Applied Biosystems®) as per the manufacturer’s instructions.

2.12.4.2. TaqMan® Gene Expression Assays

Quantitative Reverse Transcription PCR (qRT-PCR) of selected target genes was used to assess gene expression by detection of mRNA using TaqMan® (Applied Biosystems) hydrolysis probes according to manufacturer’s instructions (Table 2.2). These kits use gene specific primers and a probe 5’ labelled with FAM™ dye with a quencher molecule at the 3’ end. In a similar manner to the TaqMan® Copy Number Assays described in 2.5.2., the intact probe anneals to sequence in between the primers but does not fluoresce due to the close proximity of the quencher and FAM™ dye reporter. As the Taq polymerase extends from the 5’ primer, the dye is cleaved and fluoresces. The number of PCR cycles taken to reach a certain threshold of accumulated fluorescence can then be compared between samples and used to determine the relative amount of mRNA present during cDNA synthesis. For each reaction 5 µl (10 ng) of cDNA was combined with 10 µl TaqMan® Fast Advanced PCR Master Mix, 1 µl TaqMan Gene Expression Assay (20 x) and 4 µl RNase free water (all Applied Biosystems®). Samples were run in triplicate on a 7500 Fast real time PCR machine (Applied Biosystems®) as per manufacturer’s recommended instructions. Gene expression was normalised to the geometric mean of the reference genes Gapdh and β-actin. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method.
Gene symbol | TaqMan Gene Expression Assay ID
--- | ---
Lgr5 | Mm00438890_m1
Axin2 | Mm00443616_m1
Ctnnb1 | Mm00483039_m1
Ccna1 | Mm00432337_m1
E2f1 | Mm00432936_m1
Col1a1 | Mm00801666_g1
Rb1 | Mm00485586_m1
Dhfr | Mm00515662_m1
Map3k1 | Mm00803707_m1
Ccnd1 | Mm00432359_m1
Jun | Mm00495062_s1
Fos | Mm00487426_g1
β-actin | Mm00607939_s1
Gapdh | Mm99999915_g1

Table 2.2. TaqMan Gene Expression Assays used for qRT-PCR.

2.13. Immunodetection

2.13.1. Total Protein Extraction

Mice were euthanised by cervical dislocation, and whole inner ears removed and immediately snap frozen on dry-ice before storage at -80°C. Inner ears from 3 P1 mice (1 ear per mouse) were pooled and lysed in 20mM Bicine with 0.6 % Chaps (both Sigma) supplemented with phosphSTOp™ and cOmplete mini™ inhibitor cocktails (both Roche), using a Precellys 24 homogeniser with a soft tissue kit (Precellys) for 3 x 20 seconds. Lysates were then chilled on ice for 30 minutes and centrifuged at 12000 rpm for 20 minutes at 4°C, the supernatant (total protein) was aspirated to a fresh tube on ice and either quantified immediately or frozen at -20°C.

2.13.2. Protein Quantification

Total protein lysate concentration was determined by a colorimetric detergent compatible (DC) method using the DC™ Protein Assay kit (BioRad). A dilution series of bovine serum albumin (BSA) was prepared ranging from 10 – 0.15 mg/ml protein and used as standards. Samples were diluted 1:3 with the lysis buffer detailed in 2.13.1., 5 μl of each sample (and standard) were pipetted in duplicate in a 96-well Corning® UV-
transparent plate (Sigma). 20 µl Protein Assay Reagent S (a surfactant) was combined per 1 ml of Protein Assay Reagent A (an alkaline copper tartrate solution) and 25 µl of this solution was added to each well. 200 µl Protein Assay Reagent B (a dilute Folin reagent) was then added to each well and the plate was covered and incubated with gentle agitation for 15 minutes at RT. Protein concentration was measured using an Epoch Spectrophotometer at 750 nm (BioTek).

2.13.3. Western Blotting

For immuno-detection of protein by western blotting, samples were first separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). For denaturing, 5 µg each total protein lysate was added to 2.5 µl 4 × NuPAGE® lithium dodecyl sulfate (LDS) Sample buffer and 1 µl NuPAGE® Reducing Agent (both Invitrogen) the solution was then made up to 10 µl with ddH$_2$O and incubated at 70 °C for 10 minutes. Pre-cast NuPAGE® Novex® 4-12% Bis-Tris gels were placed into an XCell SureLock™ Mini-Cell (Invitrogen), and loaded with 10 µl of each sample and 10 µl of a SeeBlue® Plus2 pre-stained protein standard (Invitrogen). The gel was run using 1 x NuPage® 3-(N-morpholino) propane sulfonic acid (MOPS) SDS buffer 200 V for 50 minutes. Proteins were transferred to a Novex® nitrocellulose membrane using the XCell II™ Blot Module in 1 x NuPage® Transfer buffer containing 0.1 % antioxidant (all Invitrogen) 10 % methanol, for 1 hour at 30 V. Membranes containing transferred protein were then incubated in 5 % non-fat milk (Sigma) in 1 x tris-buffered saline containing 0.1 % Tween-20 (TBST) (Bio-Rad) for 1 hr at RT with gentle agitation to prevent non-specific binding of antibodies. After this blocking step the solution was removed and the membranes were incubated with the primary anti-MAP3K1 antibody (either sc-252 (Santa Cruz) at 1:500 dilution, or LF-
PA41823 (AbFrontier) at 12 µg/ml) in 1.5 % non-fat milk overnight at 4°C with gentle agitation. The primary antibody was then discarded and membranes were washed three times with TBST for 10 minutes each. The membranes were incubated with a secondary horseradish peroxidase (HRP) conjugated goat-anti-rabbit antibody (#1706515 Bio-Rad) at a dilution of 1:3000 for one hour at RT with gentle agitation. The secondary antibody solution was discarded and membrane again washed three times with TBST for 10 minutes, and then placed in PBS. Chemiluminescent detection of the HRP-conjugated, primary-bound secondary antibody was achieved using Amersham ECL Western Blotting Detection Reagent before the membrane was exposed to Hyperfilm ECL autoradiography film inside a Hypercassette (all GE Healthcare). A Compact X4 automated X-ray film processor (Xograph Healthcare Ltd.) was used to develop the films in a dark room.

2.13.4. Simple Western Peggy System

Capillary based immuno-detection was performed using the automated Peggy™ system (Simple Western™). This system allows for numerous immuno-detection assays to be performed with a small amount of protein. Briefly, lysates were mixed with Simple Western™ sample dilution buffer (Protein Simple) containing reducing agent and fluorescent standards, and denatured at 95°C for 5 minutes. Samples were then loaded into a 384 well assay-plate and subsequently the Peggy™ system. Proteins migrate through a stacking matrix and then through a size resolving matrix for separation and are immobilized in the capillary upon stimulation with ultraviolet (UV) light. Proteins of interest are identified with primary antibodies (Table 2.3), and labelled with HRP-conjugated secondary antibodies before in-capillary detection using chemiluminescence (Figure 2.5).
Antibody | Supplier | Host species | Dilution
--- | --- | --- | ---
Anti-p38 MAPK | Cell Signalling Technology (CST 9212) | Rabbit Monoclonal | 1:50
Anti-phospho p38 MAPK (Thr180/Tyr182) | Cell Signalling Technology (CST 9215) | Rabbit Polyclonal | 1:50
Anti-p44/42 MAPK (ERK1/2) | Cell Signalling Technology (CST 9102) | Rabbit Polyclonal | 1:50
Anti-phospho p44/42 MAPK (ERK1/2) (Thr202/Tyr204) | Cell Signalling Technology (CST 4377) | Rabbit Monoclonal | 1:50
Anti-SAPK/JNK | Cell Signalling Technology (CST 9252) | Rabbit Polyclonal | 1:50
Anti-phospho SAPK/JNK (Thr183/Tyr185) | Cell Signalling Technology (CST 9251) | Rabbit Polyclonal | 1:50

Table 2.3. Primary antibodies used for Peggy™ Simple Western Immunodetection

Figure 2.5 Capillary based immunodetection. Illustration of a size based protein immuno-detection assay on the Simple Western Peggy™ capillary based system. After protein samples are automatically loaded from the assay plate to the capillary, they are separated by size due to migration through stacking and separation matrices. A proprietary method using UV light is used to attach the protein to the capillary wall before in-capillary immunoprobing using primary and HRP conjugated secondary antibodies is performed. Chemiluminescence is used to detect the amount of secondary antibody bound to the target protein, which is recorded as a quantifiable peak. Fluorescent protein standards are included in the sample mix to facilitate predicted molecular weight of a given peak. Figure adapted from Protein Simple® website (http://www.proteinsimple.com).
2.13.5. Immunohistochemistry

Mice were euthanized by decapitation and bisected heads fixed in 4 % PFA in PBS for 1 hour at 4°C. The bisected heads were then dehydrated and embedded in paraffin wax and 5µm sections collected onto charged slides. Sections were de-paraffinised by submersion in Sub-X (Surgipath®) followed by ETOH, before endogenous peroxidase activity was quenched by submersion in 3 % hydrogen peroxide (H₂O₂). Slides were then washed in 1 x TBST and blocked in 1 x TBST containing 10 % goat serum. Sections were then incubated overnight at 4°C with primary antibodies (see Table 2.4 for details).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Host species</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>Anti-phospho p38 MAPK (Thr180/Tyr182) XP®</td>
<td>Cell Signalling Technology (CST 4511)</td>
<td>Rabbit (M)</td>
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</tr>
<tr>
<td>Anti-phospho p44/42 MAPK (ERK1/2) (Thr202/Tyr204)</td>
<td>Cell Signalling Technology (CST 4377)</td>
<td>Rabbit (M)</td>
<td>1:1000</td>
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<tr>
<td>Anti-phospho SAPK/JNK (Thr183/Tyr185)</td>
<td>Cell Signalling Technology (CST 9251)</td>
<td>Rabbit (P)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-MAP3K1</td>
<td>AbFrontier (LF-PA41823)</td>
<td>Rabbit (P)</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Anti-MAP3K1</td>
<td>SantCruz (sc-252)</td>
<td>Rabbit (P)</td>
<td>1:50</td>
</tr>
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Table 2.4. Primary antibodies used for chromogenic immunohistochemistry

Immuno-detection was achieved using the VECTASTAIN® Elite ABC Rabbit IgG avidin biotin kit (Vector Laboratories) according to manufacturer’s instructions. This uses an avidin-biotin peroxidase complex (ABC) as a means of signal amplification. Briefly, a biotinylated secondary antibody is used to detect the primary antibody, avidin has a high affinity for biotin, and each avidin molecule can bind 4 molecules of biotin. Separately avidin is mixed with biotinylated HRP to form complexes but not in high enough quantities to saturate, when the sample is incubated with these complexes, the biotinylated secondary antibody is recognised by avidin and the complex binds. This results in each epitope recognised by the primary antibody being attached to a complex of multiple HRP conjugated biotin
molecules, therefore increased sensitivity. Chromogenic detection was performed using 3,3'-Diaminobenzidine DAB+ Chromogen (Dako), and slides were counterstained with haematoxylin. Controls were run alongside each experiment without the addition of a primary antibody to investigate possible background signal from the secondary antibody.

2.13.6. Whole-mount Immunofluorescence

Mice were euthanised by cervical dislocation, inner ears removed and fixed in 4 % PFA for 1 hour at RT. After 3 washes in 1 x PBS for 10 minutes each, the cochlear duct was micro-dissected and the lateral wall and stria vascularis removed using a SteREO Discovery.V20 stereo microscope (Zeiss) to aid visualisation. Samples were permeabilised for 20 minutes in 0.25 % Triton X-100 in 1 x PBS before blocking was performed at RT for 1 hour using 10 % goat serum (Sigma) in PBST. Whole-mount preparations were incubated overnight at 4°C with an anti-β-Galactosidase antibody (Chemicon® AB1211) diluted 1:1000 in blocking solution. The following day, samples were washed again in PBST (3 x 15 minutes) and incubated with a combination of Alexa Fluor® 568-conjugated phalloidin to label filamentous actin, and goat-anti-rabbit Alexa Fluor® 488 secondary antibody (both Invitrogen, both at 1:200 dilution) for 1 hour at RT. Another wash step (3 x 15 minutes PBST) was then performed to remove unbound secondary antibody before samples were mounted on microscope slides with SlowFade® Gold Antifade Reagent (Life Technologies). Confocal fluorescence imaging was performed using a Zeiss LSM 700 inverted microscope.


5-ethynyl-2'-deoxyuridine (EdU) is a small molecule that can replace thymidine in newly synthesised DNA; as such it is a marker of proliferation. It is similar to bromodeoxyuridine (BrdU) although it can be detected using a copper catalysed reaction
between an alkyne contained within EdU, and an azide labelled probe, a process termed a ‘click’ reaction or ‘click’ chemistry. For detection of BrdU harsh denaturing steps are needed to allow binding of the large antibody to the BrdU which can be detrimental to the sample (Figure 2.6). The Click-iT Plus™ EdU Alexa Fluor 594 Imaging Kit (Life Technologies) was used to identify proliferating cells in embryonic cochleae. Pregnant females were injected with 100µg 5-ethyl-2'-deoxyuridine (EdU) in 200 µl PBS twice, at 2 hour intervals, before embryos were harvested 2 hours after final injection at either E14.5 or E18.5. Embryonic heads were fixed in 4 % PFA in PBS for 1 hour at 4°C and tail collected for genotyping. Fixed heads were then dehydrated and embedded in paraffin wax and 5µm sections collected onto charged slides. The copper-azide ‘click’ Alexa Fluor 594 reaction for detection of EdU was performed as per the manufacturer’s instructions.

Figure 2.6. Illustration of Edu vs BrdU based detection. Panel A illustrates the accessibility of the Click-iT® Alexa Fluor® azide reagent to the EdU incorporated in freshly synthesised DNA. Panel B highlights the increased size of the anti-BrdU antibody to the Click-iT® reagent; because of this, denaturation of dsDNA must be performed before detection which can be detrimental to the sample. Figure adapted and colourised from (https://tools.thermofisher.com/content/sfs/manuals/mp10338.pdf).
For dual labelling with EdU and anti-p27KIP1, the EdU labelling and ‘click’ reaction was performed as above then processed slides were immediately washed in PBS containing 3 % BSA at RT, Slides were then blocked with 1 x PBS containing 5 % donkey serum and 0.5 % Triton X-100 (Sigma), before incubation with a rabbit polyclonal anti-p27KIP1 (Cell Signalling Technology CST 2552) at 1:200 dilution overnight at 4°C. Slides were washed in PBS before incubation with Alexa Fluor 488 conjugated donkey anti-rabbit secondary antibody at 1:200 dilution. Slides were either mounted with SlowFade® Gold Antifade Reagent (Life Technologies) or SlowFade® Gold Antifade Reagent with DAPI (Life Technologies). Confocal fluorescence imaging was performed using a Zeiss LSM 700 inverted microscope. Controls were run alongside each experiment without the addition of a primary antibody to investigate possible background fluorescence.

2.15. In silico Analysis

For in silico predicted effect of mutations on protein structure and function the following platforms were used:

- Protein Variation Effect Analyser (PROVEAN) ([http://provean.jcvi.org/index.php](http://provean.jcvi.org/index.php))
- Simple Modular Architecture Research Tool (SMART) ([http://smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/))
- MutationTaster ([http://www.mutationtaster.org](http://www.mutationtaster.org))
- Sorting Intolerant From Tolerant (SIFT) ([http://sift.jcvi.org/](http://sift.jcvi.org/))

The following software was used to further predict tertiary protein structure and visualise 3-Dimensional (3D) protein prediction models:

- RaptorX ([http://raptorx.uchicago.edu/](http://raptorx.uchicago.edu/))
• Iterative Threading ASSEMBly Refinement (I-TASSER)  
(http://zhanglab.ccmb.med.umich.edu/I-TASSER/)
• CCP4 Molecular Graphics (CCP4MG) (http://www CCP4.ac.uk/MG/)
• UCSF Chimera (https://www.cgl.ucsf.edu/chimera/)

2.16. Clinical Pathology

I would like to thank the IMPC group at the Mary Lyon Centre for collection of samples for clinical pathology, and the Clinical Pathology core facility at the MRC Harwell Institute for running the experiments, and summarising the data.

2.16.1. Sample Collection

Samples for haemotology, clinical chemistry and fluorescence activated cell sorting (FACS) analysis were collected from the same mouse. Age and sex matched mice were euthanised by inhalation of isofluorane. Blood was then collected for haemotology by insertion of a fine glass capillary tube into the retro-orbital sinus, 200 µl of blood was collected into an EDTA-coated tube. Enough whole blood to isolate 160-200 µl of plasma was then collected into a lithium Heparin coated gel tube (Sarstedt) for clinical blood chemistry analysis. Following collection of blood, spleens were harvested and placed immediately on ice in 1 ml Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma).

2.16.2. Haematology

Immediately following blood collection as described in 2.16.1. blood samples were placed on a rotary agitator for 30 minutes at RT to prevent clotting. Samples were then analysed on an AE® 2120 Haematology System (Siemens) for complete blood, platelet and differential white blood cell counts.
2.16.3. Clinical Blood Chemistry

Following collection of blood as described in 2.16.1., samples were centrifuged at 5000x g for 10 minutes at 8°C and plasma harvested. Plasma was analysed on AU680 clinical chemistry analyser (Beckman Coulter) for the parameters listed in Table 2.5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Parameter</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>(mmol/l)</td>
<td>Albumin</td>
<td>(g/l)</td>
</tr>
<tr>
<td>Potassium</td>
<td>(mmol/l)</td>
<td>Total Cholesterol</td>
<td>(mmol/l)</td>
</tr>
<tr>
<td>Chloride</td>
<td>(mmol/l)</td>
<td>HDL</td>
<td>(mmol/l)</td>
</tr>
<tr>
<td>Urea</td>
<td>(mmol/l)</td>
<td>Glucose</td>
<td>(mmol/l)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>(µmol/l)</td>
<td>Triglycerides</td>
<td>(mmol/l)</td>
</tr>
<tr>
<td>Calcium</td>
<td>(mmol/l)</td>
<td>Total Bilirubin</td>
<td>(µmol/l)</td>
</tr>
<tr>
<td>Inorganic Phosphate</td>
<td>(mmol/l)</td>
<td>Iron</td>
<td>(µmol/l)</td>
</tr>
<tr>
<td>ALP</td>
<td>(U/l)</td>
<td>Amylase</td>
<td>(U/l)</td>
</tr>
<tr>
<td>ALT</td>
<td>(U/l)</td>
<td>CK</td>
<td>(U/l)</td>
</tr>
<tr>
<td>AST</td>
<td>(U/l)</td>
<td>Fructosamine</td>
<td>(µmol/l)</td>
</tr>
<tr>
<td>Total Protein</td>
<td>(g/l)</td>
<td>Haemolysis</td>
<td>Severity</td>
</tr>
</tbody>
</table>

Table 2.5 Clinical Blood Chemistry parameters and units.

2.16.4. Fluorescence Activated Cell Sorting FACS Analysis

After spleens were collected as described in 2.16.1., they were homogenised in 2.6 ml RPMI-1640 containing 2% FBS and 400 µl of 7 x enzyme cocktail (Table 2.7) using a gentleMACS™ Octo Dissociator (Miltenyi Biotec Ltd.).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACS buffer</td>
<td>1 x PBS; 5mM EDTA; 0.5% FBS (v/v)</td>
</tr>
<tr>
<td>RBC Lysis buffer</td>
<td>1 x solution (eBioscience)</td>
</tr>
<tr>
<td>Stopping buffer</td>
<td>1 x PBS; 0.1 M EDTA</td>
</tr>
<tr>
<td>7 x Enzyme cocktail</td>
<td>500 µL of DNase I (10 mg/ml); 500 µL Collagenase II (70 mg/ml); 4 ml RPMI with 2 % FBS (v/v)</td>
</tr>
</tbody>
</table>

Table 2.6 Solutions used for FACS sample preparation

Following homogenisation, the enzymatic digestion was halted by combining samples with 300 µl stopping buffer (Table 2.6). Homogenates were then filtered through a nylon mesh (70 µm) and cells were pelleted by centrifugation at 4°C for 5 minutes at 1300 rpm.
Pellets of cells were then resuspended in 1 ml FACS buffer (Table 2.6) and diluted 1:300 to a working solution. Cells were counted using a Moxi™ Z Mini Automated Cell Counter (ORFLO Technologies) and plated at 2 million cells per well in a 96 well plate. A wash step was performed by adding 100 µl of FACS buffer to each well before centrifugation for 5 minutes, 290 x g at 4°C, the supernatant was then discarded. Red blood cells were lysed by adding 100 µl of red blood cell (RBC) lysis buffer (eBioscience) to each well and mixing. Plates were then incubated on ice for 1 minute before centrifugation, removal of supernatant and another wash step. A blocking step to prevent non-specific antibody binding was performed by resuspension of cells in 50 µl Fc Block (1:100, BD Pharmingen) and incubation on ice for 15 minutes. Wells were washed again with FACS buffer, cells pelleted by centrifugation, resuspended in 50 µl of antibody cocktail and incubated on ice protected from light for 20 minutes. Two antibody cocktails (Panel 1 and Panel 2) were used in separate runs (Table 2.7). After incubation with antibodies another wash step was performed as before and cells were resuspended in FACS buffer containing 1:10000 SYTOX® Blue (Life Technologies) to identify live or dead cells. A BD FACSCanto™ II system (BD Pharmingen) was used to analyse cells, the system had 405 nM, 488 nM and 633 nM wavelength lasers and cell types were gated and determined according to the parameters outlined in Table 2.8.
<table>
<thead>
<tr>
<th>Panel 1:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marker / Antigen</strong></td>
<td><strong>Fluorochrome</strong></td>
</tr>
<tr>
<td>Live/dead</td>
<td>SytoxBlue</td>
</tr>
<tr>
<td>CD5</td>
<td>BV421</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC</td>
</tr>
<tr>
<td>CD8</td>
<td>PE-CF594</td>
</tr>
<tr>
<td>CD25</td>
<td>APC</td>
</tr>
<tr>
<td>CD62L</td>
<td>APC-Cy7</td>
</tr>
<tr>
<td>CD44</td>
<td>PE</td>
</tr>
<tr>
<td>CD161</td>
<td>PECy7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel 2:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Marker / Antigen</strong></td>
<td><strong>Fluorochrome</strong></td>
</tr>
<tr>
<td>Live/dead</td>
<td>SytoxBlue</td>
</tr>
<tr>
<td>F4/80</td>
<td>PE</td>
</tr>
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<td>CD19</td>
<td>BV510</td>
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<tr>
<td>IgD</td>
<td>APC</td>
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<td>Ly6C</td>
<td>FITC</td>
</tr>
<tr>
<td>Ly6G</td>
<td>BV421</td>
</tr>
<tr>
<td>CD5</td>
<td>BV421</td>
</tr>
<tr>
<td>CD11b</td>
<td>PE-CF594</td>
</tr>
<tr>
<td>CD11c</td>
<td>PECy7</td>
</tr>
<tr>
<td>MHCII</td>
<td>APC-Cy7</td>
</tr>
</tbody>
</table>

Table 2.7 Antibodies used for detection of cell types by FACS analysis
2.17. Statistical Analysis

Many thanks are extended to Dr George Nicholson (University of Oxford) for his excellent statistical advice, and help with analysis of hair cell loss in the goya mice. All data are presented as mean +/- SEM, unless indicated otherwise, in all cases significance levels on
graphs are represented by, n.s. = not significant, * = p≤0.05; ** = p≤0.005; *** = p≤0.0005; **** = p≤0.00005.

2.17.1. ABR Thresholds

All ABR data were analysed using either one-way analysis of variance (ANOVA) with TUKEY post-hoc test for instances where three or more genotypes were included, or unpaired students t-test for two genotypes.

2.17.2. Within-genotype Rates of Hair Cell Loss Across Weeks 2, 4, and 9

Counts were split into six distinct data sets for model fitting - according to hair cell type (inner or outer) and genotypic group. A Poisson generalized linear model was fitted to each data set separately. The model was specified as:

$$\Pr(y_{twi} = k) = \frac{\exp(-l_{twi}) l_{twi}^k}{k!}, \quad k = 0, 1, 2,...$$

$$\log l_{twi} = a_i + b_i \times (w - 2)$$

(i.e. with a turn-specific, log-linear relationship between mean hair-cell count and week), where

- $t \in \{1, 2, 3, 4\}$ indexes turn (1 = apical, 2 = mid-apical, 3 = mid, 4 = mid-basal),
- $w \in \{2, 4, 9\}$ denotes week,
- $i \in \{1, 2,..., n_{tw}\}$ indexes mouse within (turn, week) group,
- $y_{twi}$ is the observed hair cell count in week $w$, at turn $t$, in mouse $i$, and
- $l_{twi}$ is the mean hair cell count in week $w$, at turn $t$, in mouse $i$.

For estimates and confidence intervals for the weekly percentage change in mean hair cell count, at each turn $t$ (i.e. $100 \times \left[ \exp(b_t) - 1 \right]$) see supplementary materials Table S1.
Supplementary materials Table S2 displays p-values from testing the null hypothesis that the weekly percentage change is zero (i.e. $H_0: b_1 = 0$). Application of a variety of diagnostic tools suggested that the model provided a reasonably good fit to the data.

Model fitting, diagnostic plots, and hypothesis tests were performed using the glm()-based functionality of the package "stats" in R (R Development Core Team, 2013)

2.17.3. Inter-genotype Comparison of Hair Cell Counts at Week 2

For each cell type (inner and outer) and for each turn, cell counts were compared pair-wise between genotypic groups, p-values result from a Welch’s t-test applied to log-transformed hair cell counts.

2.17.4. Comparison of Qualitative Phenotypes Across Genotypic Groups

The proportion of mice carrying each particular qualitative phenotype (Normal, Extra row or Extra OHC) was estimated in each genotypic group ($Map3k1^{+/+}$, $Map3k1^{goya/+}$ and $Map3k1^{tm1Yxia/+}$). Phenotype proportions were compared pair-wise across genotypes to determine whether mice with a particular phenotype were over-represented in some genotypic groups relative to others. Specifically, estimates and exact binomial confidence intervals were obtained for the proportion of mice of a particular genotype carrying a particular phenotype (Brown et al., 2001). Fisher’s exact test was used to test the null hypothesis of equality of phenotypic proportion across a pair of genotypic groups.

2.17.5. TaqMan® Gene Expression Data

Significance between genotypes was assessed by unpaired student’s t-test using the $\Delta$CT values of each biological replicate.
2.17.6. Simple Western Peggy™ Protein Phosphorylation

Significance was assessed using one-way ANOVA with TUKEY post-hoc analysis.

2.17.7. Immuno-ratio Quantification of Positively Stained Nuclear Area

Significance between genotypes was assessed by unpaired students t-test

2.17.8. Clinical Pathology

All clinical pathology data were analysed either using one-way ANOVA with TUKEY post-hoc analysis (for Boycie and Orai1^{L187Q}) or by unpaired students t-test (Orai1^{V135E}, Orai1^{I123N}).

2.18. Image Manipulation

All fluorescent images were treated equally and taken using the same microscope settings (per experiment) in relation to laser power gain and were applicable zoom.

Identical crop tool settings (Adobe Photoshop) were used to maintain dimensions and resolution for figures that required comparable cropped images.

The highlighting of OHC on some scanning electron micrographs, and the stria vascularis on Boycie histological sections was performed using adjustment in Adobe Photoshop to aid visualisation. A colour balance adjustment layer was added with the required area highlighted and all colours were adjusted equally (per experiment).

All data are presented as mean +/- SEM, unless indicated otherwise, in all cases significance levels on graphs are represented by, n.s. = not significant, * = p≤0.05; ** = p≤0.005; *** = p≤0.0005; **** = p≤0.00005.
Chapter 3

Phenotypic Characterisation

of the *goya* Mutation
3.1. Introduction

3.1.1. The Collaborative ENU Recessive Vision Screen

To maximise the efficiency of the MRC Mammalian Genetics Unit ENU recessive screen, a collaboration was set up between ourselves and the MRC Human Genetics Unit (HGU) in Edinburgh to additionally screen pedigrees for eye defects and visual dysfunction. A number of pedigrees from the ongoing ENU recessive screen were bred using the sighted C3H line (C3H.Pde6b+) as the mapping strain (Hart et al., 2005). This enabled assessment of eye phenotypes without the confounding factor of the retinal degeneration known to occur in C3H inbred strains. The retinal degeneration (rd1) mutation responsible is found in a number of inbred mouse strains, and is the combination of an insertion of a murine provirus near the first exon of the Pde6b gene, and a point mutation which introduces a premature stop codon in exon 7 of the gene (Pittler and Baehr, 1991). The C3H.Pde6b+ strain have been bred to include the Pde6b gene from the BALB/c strain which do not carry the allele. Pedigrees that had undergone assessment for eyes and vision were subsequently made available for screening for other phenotypes.

3.1.2. Identification of the goya Mutant

As part of the ENU recessive vision screen a mouse mutant, goya, was identified with an eyes-open at birth (EOB) phenotype (Figure 3.1). Mouse eyelids normally begin to fuse around E15.5, with opposing epithelial sheets migrating towards one another until the fusion of the sheets covers the cornea at around E17.5. The eyelids remain fused until the second post-natal week, with separation usually complete by P12 (Findlater et al., 1993). Upon further phenotypic characterisation it became apparent that the EOB mice also lacked a response to a click-box test, indicative of hearing loss. Initial auditory brainstem response testing at 12 weeks of age confirmed hearing loss in the EOB mice.
Prior to my involvement in the goya project the underlying lesion was cloned by Dr Sally Cross at the MRC HGU. Briefly, a genome scan was performed on DNA extracted from five affected mice (with both EOB and lack of click-box response) using pyrosequencing assays covering a panel of 271 informative SNP markers spread across the mouse genome. Linkage was shown to a 24.7Mb region on chromosome 13, between the SNP markers rs13481942 and rs6316705. Within this chromosomal region was located the mitogen-activated protein kinase kinase kinase Map3k1 gene (also known as Mekk1). This was identified as being a strong candidate gene for the site of the goya mutation because mice deficient for Map3k1 are known to have open eyes at birth (Juriloff et al., 2004; Yujiri et al., 2000; Zhang et al., 2003). Indeed the encoded protein MAP3K1 is highly expressed in the migrating leading edge of the eyelid epithelium and it is thought that in its absence the migration of these cells is impaired leading to the failure of eyelid closure during embryogenesis (Sheth et al., 2006; Xia and Kao, 2004; Zhang et al., 2005; Zhang et al., 2003). Although previous studies have shown that mice lacking functional MAP3K1 have eye defects, no auditory phenotype has been reported in these mice. Sanger sequencing of the Map3k1 gene in goya mutant mice revealed a single nucleotide mutation, which was not present in the wild-type parental strain mice, in the intron 13 splice donor site (IVS13+2T>C) (Figure 3.2.).
Human mitogen-activated protein kinase kinase kinase 1 (MAP3K1) or MEK kinase 1 (MEKK1) is a 196 kDa serine-threonine kinase. MAP3K1 is among the signal transduction proteins which are cleaved by caspases early in the apoptotic response and data suggest that MAP3K1 cleavage is involved in a MAP3K1/CASPASE amplification loop (Widmann et al., 1998). CASPASE-3 cleaves MAP3K1 at Asp874 releasing a COOH-terminal 91 kDa fragment (p91) that encodes for the MAP3K1 kinase domain and a 105 kDa N-terminal fragment (p105) (Widmann et al., 1998). Only the p91 fragment and not the full length 196 kDa MAP3K1 is pro-apoptotic (Schlesinger et al., 2002; Widmann et al., 1998).

The 196 kDa form of MAP3K1 is membrane bound and following CASPASE activation, the p91 cleavage product localizes to the cytoplasm where the kinase activity of p91 leads to further activation of caspases (Schlesinger et al., 2002; Widmann et al., 1998; Zhang et al., 2003). Overexpression of full length MAP3K1 induces activation of extracellular signal-
regulated kinases (ERK), a signal responsible for cell survival and proliferation, but activation was not observed with p91 (Schlesinger et al., 1998). This suggests that MAP3K1 plays a pivotal role in regulating cell survival as well as cell death and that cleavage by caspases acts as a molecular switch. In addition it shows that full length MAP3K1 has distinct functionality from that of the cleaved p91 fragment.

Another unique role for full length MAP3K1 is that it can act as an E3 ubiquitin ligase, as yet it is the only MAPK protein known to have this function in addition to kinase activity. One of the established roles for MAP3K1 is the Lys48 poly-ubiquitination of ERK1/2 to mark it for proteasomal degradation (Lu et al., 2002). It has also been shown to do the same for c-Jun (Xia et al., 2000). In addition to turnover of proteins, the role of ubiquitination is important in MAPK signalling as a regulatory mechanism; the creation of different ubiquitin chains can affect protein-protein interactions, which could play a role in stimulus to response specificity. Indeed it was recently shown that MAP3K1 transfers a Lys-63 linked poly-ubiquitin chain to TGF-β Activated Kinase 1/MAP3K7 Binding Protein 1 (TAB1) in response to epidermal growth factor (EGF) and TGF-β stimulation, which amongst other things is critical for ES cell differentiation (Charlaftis et al., 2014).

3.1.5. MAPK Signalling Pathway

Classical MAPK signalling involves a cascade of phosphorylation events. Extracellular signals including growth factors, stress stimuli or cytokines are picked up by a variety of receptors at the cell membrane. The receptors activate proteins such as the small GTPases RAS or RAC1, which initiate the MAPK cascade by interactions with MAP3Ks, of which MAP3K1 is one. The MAP3Ks phosphorylate target MAP2K proteins; in the case of MAP3K1 the targets are usually MAP2K4 and MAP2K7 (Hirai et al., 1998; Xu and Cobb, 1997; Yan et al., 1994). The MAP2Ks then in turn phosphorylate MAPK proteins; MAP3K1
preferentially activates the JNK axis of the MAPK signalling pathway, although it can also lead to activation of both of the other two main branches, p38MAPK and ERK1/2 (Lin et al., 1995; Minden et al., 1994). The MAPK proteins go on to effect a vast array of target proteins and genes, many of which are transcription factors that regulate a diverse range of cellular processes and responses (Figure 3.3). In certain circumstances MAP4K proteins such as germinal centre kinase (GCK) can initiate the cascade by activating MAP3Ks (Chadee et al., 2002).

In addition to extracellular stimuli including growth factors and extracellular stress, intracellular stress stimuli such as reactive oxygen species (ROS) can also activate MAPK pathways (Nagai et al., 2007).

MAPK signalling is often portrayed as a series of distinct phosphorylation events, however much of the signal specificity is thought to be derived from the use of scaffolding proteins. This was first identified in budding yeast S. Cerevisiae, where protein-protein interactions of STE5 were investigated by multiple groups (Choi et al., 1994; Marcus et al., 1994); it was shown to form complexes with STE11, STE7 and FUS3 and be essential for the function of the cascade, thus it was hypothesised it acted as a scaffold protein. Experimentation with engineered complexes comprised of these proteins with the addition of another scaffold protein, PBS2, showed that it was possible to alter the standard response of the complex to a given stimuli (Park et al., 2003). In mammalian MAPK signalling, a number of scaffold proteins have been identified, however it has been harder to fully dissect and model scaffold complex systems (reviewed in (Witzel et al., 2012). The human orthologue of STE 11 is MAP3K1; studies indicate it may also have a function as part of a ‘scaffold’ signalling module in both the JNK and ERK cascades. It can bind multiple signalling proteins to regulate specific responses, although it remains to be
shown if this is sequential or in complex (Karandikar et al., 2000; Russell et al., 1995; Xia et al., 1998; Xu and Cobb, 1997)
3.2. Results

3.2.1. Complementation Test

One of the challenges for researchers using ENU-mutagenesis as a means of generating disease models is confirming which ENU induced lesion is in fact responsible for the observed phenotype. A method that can be utilised for recessively inherited phenotypes is to perform a complementation test. This is performed by crossing mice carrying the ENU lesion in question to mice carrying a null allele of the same gene in which the mutation is suspected. The progeny resulting from this cross that are heterozygous for both alleles, known as compound heterozygotes, will not carry a wild-type allele of the gene and therefore should recapitulate the observed phenotype in the homozygote ENU mutants.

In an attempt to confirm the goya mutation (IVS13+2T>C) in Map3k1 as being causative of both eye and auditory phenotypes, we obtained a MAP3K1 kinase deficient mouse line (Map3k1\textsuperscript{tm1Yxia}) from Ying Xia at the University of Cincinnati Medical Center. The allele Map3k1\textsuperscript{tm1Yxia} encodes a MAP3K1-β-Galactosidase fusion protein consisting of the first 1188 amino acids of MAP3K1 fused to the β-Galactosidase protein. The fusion protein does not contain any of the MAP3K1 kinase domain (Xia et al., 2000). Mice heterozygous for the goya mutation were mated to mice carrying the Map3k1\textsuperscript{tm1Yxia} allele to produce compound heterozygous mutant offspring. The progeny carrying both Map3k1 alleles (Map3k1\textsuperscript{goya/tm1Yxia}) were born with their eyes open as with mice homozygous for either allele, confirming that the goya mutation was indeed responsible for this previously reported phenotype.

To confirm that mutation of Map3k1 also caused the hearing loss phenotype observed in goya homozygote mice, and to investigate if any hearing loss was present in
Map3k1\textsuperscript{tm1Yxia/tm1Yxia} mice, auditory brainstem response (ABR) analysis was performed on both Map3k1\textsuperscript{goya} and Map3k1\textsuperscript{tm1Yxia} heterozygote and homozygote mice as well as compound heterozygote and wild-type mice (Figure 3.4.).

![9 week ABR](image)

**Figure 3.4. ABR and complementation test.** At 9-weeks of age Map3k1\textsuperscript{goya/goya} (n=6), Map3k1\textsuperscript{tm1Yxia/tm1Yxia} (n=6) and Map3k1\textsuperscript{goya/tm1Yxia} (n=6) mice exhibit severe hearing loss demonstrated by ABR thresholds 50 – 60dB above Map3k1\textsuperscript{+/+} mice. Mice heterozygous for both the goya (n=13) and tm1Yxia (n=6) alleles have thresholds not significantly different from wild-type at 9-weeks of age. This shows that the Map3k1\textsuperscript{tm1Yxia} allele fails to complement the Map3k1\textsuperscript{goya} allele and confirms the mutation in Map3k1 as causative of the hearing loss in goya homozygote mice. Data shown are mean ± standard error of the mean, p values calculated using one-way ANOVA with TUKEY post hoc analysis - *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001.

At 9-weeks of age both Map3k1\textsuperscript{goya/goya} and Map3k1\textsuperscript{tm1Yxia/tm1Yxia} homozygotes show a very significant hearing loss with average ABR thresholds around 80 dB SPL across all frequencies tested (p=<0.0001) (Figure. 3.4). Whereas, Map3k1\textsuperscript{goya/+} and Map3k1\textsuperscript{tm1Yxia/+} heterozygous mice have auditory thresholds similar to wild-type (Map3k1\textsuperscript{+/+}) mice. Importantly, the compound heterozygous mutant mice (Map3k1\textsuperscript{goya/tm1Yxia}) also demonstrate a severe hearing loss at 9-weeks of age with ABR thresholds in the region of 80 dB SPL, similar to the thresholds obtained for the homozygote mice (Map3k1\textsuperscript{goya/goya} and Map3k1\textsuperscript{tm1Yxia/tm1Yxia}). These observations demonstrate the Map3k1\textsuperscript{goya} allele fails to
complement the Map3k1<sup>tm1Yxia</sup> allele, and that the goya hearing loss phenotype is caused by the ENU-induced IVS13+2T>C lesion in Map3k1.

The 9-weeks of age ABR thresholds of the Map3k1<sup>goya/goya</sup> male and female mice were compared to investigate possible gender bias. No significant differences in ABR thresholds between genders were observed at any of the frequencies tested (see appendix) for this reason gender was not considered a variable in subsequent experiments.

3.2.2. The goya Mutant Displays an Early Onset Progressive Hearing Loss

To investigate the onset of hearing loss, ABR was performed on Map3k1<sup>goya/goya</sup>, Map3k1<sup>tm1Yxia/tm1Yxia</sup> and wild-type mice at 2- and 4-weeks of age. At 2-weeks of age, Map3k1<sup>goya/goya</sup> mice have ABR thresholds similar to those of wild-type mice at all but the highest frequency tested (26kHz) (Figure 3.5A). However, Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice show elevated thresholds compared to wild-type and Map3k1<sup>goya/goya</sup> mice at all frequencies tested. At 4-weeks of age, both homozygous mutants (Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup>) exhibit significantly elevated ABR thresholds (30 - 40dB SPL higher) at all frequencies tested when compared to wild-type controls (Figure 3.5B). As shown in the non-complementation test data, 9 week-old mice homozygous for either mutation have significantly elevated ABR thresholds, 50 – 60 dB SPL higher compared with wild-type controls (Figure 3.5C). These data show that although Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice already have a moderate to severe loss of auditory function at the onset of hearing, both mutants display an early onset, rapid hearing loss with a progressive element.

To determine the longitudinal effects of the goya mutation on auditory thresholds, ABR was performed on cohort of Map3k1<sup>+/+</sup>, Map3k1<sup>goya/+</sup> and Map3k1<sup>goya/goya</sup> mice at 1-year of age. At 1-year of age, Map3k1<sup>goya/+</sup> mice have ABR thresholds similar to those of wild-type mice, demonstrating that goya heterozygotes do not develop late-onset hearing loss
Moreover, there is no further decline in the auditory function of homozygote animals between 9-weeks and 1-year of age (Figure 3.5C, D).

**Figure 3.5. Time-course ABR Analysis** (A) Average ABR thresholds of *Map3k1*+/+ (n=4), *Map3k1*gyoa/gyoa (n=3) and *Map3k1*tm1Yxia/tm1Yxia (n=4) mice at 2-weeks of age (P16). *Map3k1*tm1Yxia/tm1Yxia show significantly elevated thresholds at all frequencies when compared to wild-type or *Map3k1*gyoa/gyoa mice. *Map3k1*gyoa/gyoa mice only show a significant difference from wild-type at 26kzh. (B) At 4-weeks of age *Map3k1*gyoa/gyoa (n=3) and *Map3k1*tm1Yxia/tm1Yxia (n=3) mice have significantly elevated average ABR thresholds (+30-40dB) when compared to *Map3k1*+/+ (n=3) mice across all frequencies tested. (C) As shown in the complementation test, by 9-weeks of age *Map3k1*gyoa/gyoa (n=6) (Figure 3.4), *Map3k1*tm1Yxia/tm1Yxia (n=6) mice exhibit severe hearing loss demonstrated by ABR thresholds 50 – 60dB above *Map3k1*+/+ mice. (D) ABR performed at 1-year of age show that *Map3k1*gyoa/ (n=6) mice have similar thresholds compared to *Map3k1*+/+ (n=5) mice. Also, *Map3k1*gyoa/ (n=7) mice have thresholds similar to those measured at 9-weeks of age suggesting no further decline in hearing function. Data shown are mean ± standard error of the mean, p values calculated using one-way ANOVA with TUKEY post hoc analysis - *p=<0.05, **p=<0.01, ***p=<0.001, ****p=<0.0001. (Figure adapted from (Parker et al., 2015))
3.2.3. Inner Ear Morphology

Given the elevated auditory thresholds observed in *Map3k1<sup>goya/goya</sup>* mice, the morphology of their inner ears were next examined in detail. Initially 9-week old inner ears were dissected and stained with haematoxylin and eosin (H&E). Inspection of cochlear sections suggest cellular degeneration within the organ of Corti of 9-week old *Map3k1<sup>goya/goya</sup>* mice in all cochlear turns, most notably the OHC, however, other inner ear structures such as Reissner’s membrane, the stria vascularis and spiral ganglion neurons appear normal (Figure 3.6.).

![Histological analysis of goya inner ears](image)

Figure 3.6. Histological analysis of goya inner ears. H&E stained cochlear sections from 9-week old *Map3k1<sup>+/+</sup>* (A, C, D, G, H, K, L) and *Map3k1<sup>goya/goya</sup>* (B, E, F, I, J, M, N) mice. Panels A and B show an overview of the apical turn of *Map3k1<sup>+/+</sup>* (A) and *Map3k1<sup>goya/goya</sup>* (B), indicating that Reissner’s membrane and the stria vascularis show no signs of degeneration at this time-point. Spiral ganglion neurons of *Map3k1<sup>goya/goya</sup>* mice also appear unaffected (F, J, N) when compared with *Map3k1<sup>+/+</sup>* (D, H, L). Higher magnification of the organ of Corti shows normal arrangement of sensory epithelial cells in all cochlear turns (C, G, K, apical, mid and basal turn respectively) in *Map3k1<sup>+/+</sup>* mice, outer hair cells are indicated for orientation. In contrast, cellular loss is observed in all regions (E, I, J, apical, mid and basal turn respectively) of the *Map3k1<sup>goya/goya</sup>* cochlea, most notably OHC and/or Deiters’ cells (E, I, J arrows). RM - Reissner’s membrane, SV - stria vascularis, OHC - outer hair cells, SGN – spiral ganglion neurons. Scale bars represent 50 µm.
3.2.4. Middle Ear Examination

The EOB phenotype in Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice has been attributed to dysfunction of TGF-β- and ACTIVIN-induced JNK activity involved with epithelial sheet movement in the migrating eyelid (Xia et al., 2000; Zhang et al., 2003). Previous studies have shown that mutations in regulators of this pathway can cause otitis media (OM) in mice (Hardisty et al., 2003; Parkinson et al., 2006). Upon removal of the middle ear bulla to expose the inner ear capsule, it is usually evident if a mouse is affected by OM by the presence of puss or exudate. During removal of inner ears of Map3k1<sup>goya/goya</sup> mice over the course of this project no such evidence of OM was observed. In addition, imaging of the tympanic membrane using a stereo microscope shows the malleus clearly within the middle ear cavity, and importantly, no clouding of the tympanic membrane which is an indication of effusion related to OM (Figure 3.7.A, B). Furthermore removal and imaging of ossicles revealed no morphological difference between Map3k1<sup>goya/goya</sup> and Map3k1<sup>+/+</sup> control mice (Figure 3.7.C, D). Taken together these suggest there is no conductive element to the hearing loss in goya homozygote mice.
3.2.5. Hair Cell Abnormalities in Map3k1 Mutants

To further assess the organ of Corti of Map3k1 mutant mice, scanning electron microscopy (SEM) was used to examine the ultrastructure of the cochlear sensory epithelium at ages matching the ABR time-points (2-, 4- and 9-weeks of age). Scanning electron microscopy allows for investigation of cochlear hair cells at high magnification, and is particularly useful for assessing the loss of sensory hair cells or abnormalities involving the stereocilia bundles. Regions of the cochlear spiral were defined as: apical (<90° from apex), mid apical (90-180° from apex), mid (180-360° from apex) and mid basal (360-540° from apex) (Figure 3.8 A). Assessment of hair cell number was performed by
counting the number of IHC or OHC in contact with 20 pillar cells (for OHCs, the second and third row were counted in a diagonal line from the first row OHC as per their cellular organisation.) (Figure 3.8. B). Cells were considered present if any stereocilia remained.

![Figure 3.8. Ultrastructural Analysis using Scanning Electron Microscopy. A) An overview of the cochlear spiral divided into the regions to be analysed for morphology and hair cell number; A, red -apical (<90° from apex), MA, green - mid apical (90-180° from apex), M, blue - mid (180-360° from apex) and MB, yellow - mid basal (360-540° from apex). B) Assessment of cochlear hair cell numbers was performed by counting the number of IHCs and OHCs cells in contact with 20 pillar cells, with OHC rows 2 and 3 counted in a diagonal line to the right from the row 1 OHC that contacts the pillar cell. Cells were classed as present if any stereocilia remained. A – apical, MA – mid apical, M – mid, MB – mid basal, IHC - inner hair cells, OHC1,2,3 – outer hair cell rows 1,2,3, PC – pillar cells. Scale bar in A = 100 μm, in B = 10 μm.]

As described in the Introduction (1.3.1), mice usually have one row of IHCs and three rows of OHCs. At 2-weeks of age, both Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia IHCs and OHCs display normal morphology of the stereocilia bundles (Figure 3.9).
In addition both showed normal cellular arrangement of the sensory epithelium with the exception that both mutants have more OHCs than wild-type (Figure 3.10.A). The additional OHCs were organised as an extra row extending throughout the majority of the cochlear regions examined (Figure 3.10.A). However, by 4-weeks of age both mutants showed degeneration of OHCs with an increasing severity from apex-to-base (Figure 3.10.B). By 9-weeks of age both mutants showed degeneration spreading further along the cochlea with very few OHCs remaining in the apical regions. As expected no OHC loss is observed in wild-type mice by 9-weeks of age (Figure 3.10.C).

At 2-, 4- and 9-weeks of age IHCs appeared normal in morphology in all except \textit{Map3k1}^{tm1Yxia/tm1Yxia} cochlea at 9-weeks, which showed a slight reduction in number again.
with an apical-to-basal gradient (Figure 3.10.C). Although the majority of IHCs were unaffected, at 9-weeks of age the extent of degeneration in small patches of the $Map3k1^{tm1Yxia/tm1Yxia}$ organ of Corti was very severe. IHCs, OHCs and pillar cells had disappeared and rosette-like formations of what appear to be Claudius and Hensen cells had formed in their place (Figure 3.10.D). A similar pattern of cellular remodelling has been previously reported in post aminoglycoside damaged cochleae (Taylor et al., 2012).

Figure 3.10. Time-course analysis of hair cell morphology in $Map3k1$ mutant mice. (A-C) Representative scanning electron micrographs from the apical (A), mid-apical (MA), mid (M) and mid-basal (MB) turns of organ of Corti from $Map3k1^{+/+}$, $Map3k1^{goya/goya}$ and $Map3k1^{tm1Yxia/tm1Yxia}$ mice, at 2-, 4- and 9-weeks of age. Both $Map3k1^{goya/goya}$ and $Map3k1^{tm1Yxia/tm1Yxia}$ mice have an extra row of OHCs at 2-weeks of age. A progressive loss of OHCs is seen between 2- and 9-weeks of age in both homozygous mutants, but not $Map3k1^{+/+}$ cochleae. An apical-to-basal increase in severity of degeneration was also observed. (D) Scanning electron micrograph demonstrating the rosette like cellular formation in a 9-week old $Map3k1^{tm1Yxia/tm1Yxia}$ mouse. The remains of some IHC stereocilia bundles can be seen, all OHC are missing (*) and pillar cells have been replaced in the rosette formation with Hensen and Claudius like cells. IHC – inner hair cell, OHC – outer hair cell, PC – pillar cells, HC – Hensen like, CC – Claudius like. Scale bars represent 10µm. (Figure adapted from (Parker et al., 2015))
3.2.6. Quantification of Hair Cell Number and Degeneration

(Statistical analysis performed by George Nicholson)

Sensory cell counts were performed as described to assess the progressive nature of the cell loss in different regions of the cochlear spiral, and to allow comparison between genotypes. IHC numbers were similar between Map3k1<sup>+/+</sup>, Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice at all four cochlear regions at 2 and 4-weeks of age. At 9-weeks of age IHC numbers are similar between Map3k1<sup>+/+</sup> and Map3k1<sup>goya/goya</sup> mice, whereas there is a trend for a reduced number of IHCs in the basal region of Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochlea (Figure 3.11.A, B, C). In Map3k1<sup>+/+</sup> mice OHC numbers are consistent across the different regions of the cochlea up to 9-weeks of age (Figure 3.11.D). At 2-weeks of age, Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice have an increased number of OHCs compared to wild-type, which is significant for most of the regions assessed (Figure 3.11.D, E, F). At 4-weeks of age, degeneration of OHCs progressed in Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice. In the apical region Map3k1<sup>goya/goya</sup> mutants show an average reduction of OHC numbers of 26%, and Map3k1<sup>tm1Yxia/tm1Yxia</sup> of 20%. In the mid-apical region Map3k1<sup>goya/goya</sup> mice show an OHC loss of 24% whereas Map3k1<sup>tm1Yxia/tm1Yxia</sup> lost 43% (Figure 3.11.E, F). In the mid-cochlear region Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice show an OHC loss of >67% and in the mid-basal region >95% loss (Figure 3.11.E, F). A similar apical-to-basal increase in severity is observed in 9-week old mutants (Figure 3.11.E, F). To determine statistical significance, the rate of hair cell loss was estimated under a Poisson generalized linear model (see Materials and Methods 2.17.). There was no statistical support for IHC loss in any genotypic group (Table 3.1. and 3.2.). There was strong statistical support for OHC loss in the Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> genotypic groups but no evidence in the wild-type group (Table 3.2.). The rate of OHC loss in the mutant groups increased consistently from apical-to-basal turns (Table 3.1.). The
rate of OHC loss did not differ significantly between the two mutant groups. These statistical analyses quantify and corroborate the obvious effects visible in Figure 3.10.

Figure 3.11. Quantification of hair cell loss in the organ of Corti of Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice. (A-C) Average number of IHC’s in contact with 20 pillar cells at 2, 4 and 9-weeks of age. No significant differences in IHC number were observed in Map3k1<sup>+/+</sup> (A), Map3k1<sup>goya/goya</sup> (B), and Map3k1<sup>tm1Yxia/tm1Yxia</sup> (C) mice, although by 9-weeks of age reduced numbers of IHCs were observed in some of the Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochleae. (D-F) Average number of Map3k1<sup>+/+</sup> (D), Map3k1<sup>goya/goya</sup> (E) and Map3k1<sup>tm1Yxia/tm1Yxia</sup> (F) OHCs in contact with 20 pillar cells at 2, 4 and 9-weeks of age. t-tests were performed to compare the mean numbers of OHCs between genotypic groups at 2-weeks of age (see Methods). Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice have more OHCs than Map3k1<sup>+/+</sup>. This difference was significant in the apical (***, p<0.001), mid-apical (*, p<0.05) and mid-basal turns (**, p<0.01) in Map3k1<sup>goya/goya</sup>. In Map3k1<sup>tm1Yxia/tm1Yxia</sup> the extra number of OHCs was significantly higher than Map3k1<sup>+/+</sup> in the mid-apical (*, p<0.05) and mid (**, p<0.01) turns. In the mid turn of Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochleae, significantly more OHCs were observed than in Map3k1<sup>goya/goya</sup> (**, p<0.005), however there were no other obvious differences between the mutant alleles. By 4-weeks of age, nearly all Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> OHCs are missing in the mid-basal turn, and in the mid turn we observed variable levels of OHC loss in Map3k1<sup>goya/goya</sup> cochleae, and substantial loss in Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochleae. In the mid-apical and apical turns OHC loss was evident but not to the extent of the mid and mid-basal turns. By 9-weeks of age the majority of OHCs are missing in the mid-basal and mid turns of both Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochleae, and severe loss is seen in the mid-turn and mid-apical turns. No significant difference in OHC numbers were seen across the time-points in Map3k1<sup>+/+</sup> cochleae. The rate of decrease in hair cell number over time was also analysed and found to be highly significant in both homozygous mutants (see Table 3.1 and 3.2 for 95% confidence intervals and p values). Data shown are mean ± standard error of the mean, n ≥3 for genotype at each cochlear turn - †,*p<0.05, **p<0.01, ***p<0.001. (Figure from (Parker et al., 2015)).
Table 3.1. Estimates and confidence intervals for the weekly percentage change in mean hair cell count at different cochlear regions.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Genotype</th>
<th>Apical</th>
<th>Mid-apical</th>
<th>Mid</th>
<th>Mid-basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>Map3k1+/+</td>
<td>0 [-4.5]</td>
<td>0 [-4.4]</td>
<td>1 [-4.6]</td>
<td>1 [-4.6]</td>
</tr>
<tr>
<td>IHC</td>
<td>Map3k1goya/goya</td>
<td>3 [-3.8]</td>
<td>1 [-4.6]</td>
<td>1 [-4.6]</td>
<td>1 [-5.6]</td>
</tr>
<tr>
<td>IHC</td>
<td>Map3k1tm1Yxia/tm1Yxia</td>
<td>0 [-5.5]</td>
<td>0 [-5.5]</td>
<td>-3 [-9.2]</td>
<td>-4 [-11.3]</td>
</tr>
<tr>
<td>OHC</td>
<td>Map3k1+/+</td>
<td>-1 [-3.2]</td>
<td>0 [-3.2]</td>
<td>0 [-3.3]</td>
<td>0 [-3.3]</td>
</tr>
<tr>
<td>OHC</td>
<td>Map3k1tm1Yxia/tm1Yxia</td>
<td>-13 [-16,-10]</td>
<td>-21 [-24,-18]</td>
<td>-38 [-43,-33]</td>
<td>-78 [-86,-69]</td>
</tr>
</tbody>
</table>

Table 3.2. p-values from testing the null hypothesis that the weekly percentage change in hair cell number is zero.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Genotype</th>
<th>Apical</th>
<th>Mid-apical</th>
<th>Mid</th>
<th>Mid-basal</th>
</tr>
</thead>
<tbody>
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<td>IHC</td>
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<td>0.95</td>
<td>0.72</td>
<td>0.79</td>
</tr>
<tr>
<td>IHC</td>
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<td>0.71</td>
<td>0.76</td>
<td>0.82</td>
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<tr>
<td>IHC</td>
<td>Map3k1tm1Yxia/tm1Yxia</td>
<td>0.92</td>
<td>0.98</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>OHC</td>
<td>Map3k1+/+</td>
<td>0.65</td>
<td>0.81</td>
<td>0.98</td>
<td>0.95</td>
</tr>
<tr>
<td>OHC</td>
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<td>3.70E-30</td>
<td>6.70E-28</td>
<td>8.80E-18</td>
</tr>
<tr>
<td>OHC</td>
<td>Map3k1tm1Yxia/tm1Yxia</td>
<td>8.70E-19</td>
<td>2.00E-30</td>
<td>3.80E-27</td>
<td>4.00E-15</td>
</tr>
</tbody>
</table>

3.2.7. Heterozygote Map3k1 Mutant Mice also Exhibit Hair Cell Abnormalities

Similar to homozygous mutants, Map3k1goya/+ and Map3k1tm1Yxia/+ mice display extra OHCs compared to wild-type mice, but unlike homozygous mutants no OHC degeneration was observed at any time-point investigated (Figure 3.12.A). A total of 54 Map3k1+/+, 11 Map3k1goya/+ and 30 Map3k1tm1Yxia/+ images were analysed (not all from the same mice, maximum of 4 per mouse). The number of images containing normal OHCs (3 rows), extra OHCs (1-4 OHCs in addition to the 3 normal rows), or extra rows of OHCs (≥ 5 OHCs in a continuous line, in addition to the 3 normal rows) was calculated. Images obtained from cochleae from both heterozygote mutants and wild-type mice had patches with isolated extra OHCs. However, the results clearly show a significant increase in OHC number in heterozygote mice, with 43% of the Map3k1tm1Yxia/+ and 27% of the Map3k1goya/+ images investigated showing extra rows of OHCs, compared to just 4% of wild-type mice (statistical summary in Table 3.3.) (Figure 3.12.B). These observations suggest distinct
roles for MAP3K1 in sensory hair cell development as well as maintenance and survival of the organ of Corti.

Figure 3.12. Assessment of heterozygote Map3k1 mutant hair cells. (A) Scanning electron micrograph showing an extra row of OHCs in the mid-basal region of a 9-week old Map3k1\(^{+/+}\) cochlea. (B) Clustered histogram representing the percentage of total images captured from Map3k1\(^{+/+}\) (n=54), Map3k1\(^{+/+}\) (n=11) and Map3k1\(^{tm1Yxia/+}\) mice (n=30), containing 3 rows of OHCs (normal), extra OHCs (1-4 OHCs in addition to the 3 normal rows), or extra rows of OHCs (≥ 5 OHCs in a continuous line, in addition to the 3 normal rows). Images of cochleae from both heterozygote alleles and Map3k1\(^{+/+}\) mice showed isolated extra OHCs, however 27% of Map3k1\(^{+/+}\) and 43% of Map3k1\(^{tm1Yxia/+}\) heterozygote images contained extra rows of OHCs, significantly higher than the 4% of Map3k1\(^{+/+}\) images. The percentage of Map3k1\(^{tm1Yxia/+}\) images containing the normal 3 rows of OHCs was also significantly lower when compared to Map3k1\(^{+/+}\). * p<0.05, *** p<0.0001, p values calculated using Fisher’s exact test, see Methods and Table 3.3 for estimates confidence intervals and p-values). Scale bar represents 10 µm. (Figure adapted from (Parker et al., 2015)).

3.2.8. Localisation of MAP3K1 to the Inner Ear

3.2.8.1. Immunohistochemistry with Commercially Available Anti-MAP3K1 Antibodies

The subcellular localisation of MAP3K1 has been the subject of much debate, studies using a range of antibodies show differing results including membrane bound, nuclear and cytosolic fragments (Geh, 2011; Schlesinger et al., 2002; Xu et al., 1996). In vitro studies investigating different roles for MAP3K1 have used a variety of cell lines for functional studies, however very few have described endogenous expression of MAP3K1 in vivo. A further confounding factor is that the protein is cleaved from a full length membrane bound protein into an enzymatically active fragment containing the kinase domain; the antigen used to create the antibody may detect one or more proteins, either the full length protein, the resulting cleavage products or both. In an attempt to
investigate the localisation of MAP3K1 within the inner ear immunohistochemistry using the Vectastain Elite ABC kit (Vector laboratories) with DAB chromogenic detection, was undertaken using new-born inner ear paraffin embedded sections prepared from control (either Map3k1^{+/+}, Map3k1^{goya/+}) and Map3k1^{goya/goya} mice (Figure 3.13.). Commercially available anti-MAP3K1 antibodies were obtained from SantaCruz Biotechnology (sc-252 (C22)) and AbFrontier (LF-PA41823). The SantaCruz antibody gave fairly weak cytoplasmic staining in epithelial cells lining the cochlear duct, and also in the fibrocytes of the spiral ligament lateral to the stria vascularis (Figure 3.13.A, B). The AbFrontier antibody also gave similar weak cytoplasmic staining but also revealed nuclear expression in both the epithelial cells and the supporting cells (Figure 3.13.C, D). The SantaCruz antibody was generated by using an immunising peptide mapping to the C-terminus of rat MAP3K1 that contains the kinase domain, whereas the AbFrontier antibody was raised to a recombinant protein from the N-terminus of human MAP3K1. Encouragingly both antibodies labelled similar cell types within the cochlea, but differences in sub-cellular localisation makes interpreting the results difficult. It is possible that the different epitopes for the two antibodies is the cause of the difference; however the difference may also be a result of non-specific binding of the primary antibodies.
3.2.8.2. Western Blot Analysis using Commercially Available Anti-MAP3K1 Antibodies

Given the differential subcellular localisation of the two antibodies used for immunohistochemical detection of MAP3K1 in the inner ear, western blotting using protein extracted from the inner ear of Map3k1+/+; Map3k1goya/goya; Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia mice was performed to investigate specificity of the antibodies (Figure 3.14.). Bands were detected in all samples, including Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia. Both antibodies detected proteins that migrated with similar molecular weights (~50 kDa and ~130 kDa); however the migration of the bands did not mirror the expected molecular weights of MAP3K1 as reported in the literature. The sc-
252 antibody, which is raised to an immunogen comprising part of the kinase domain, detected proteins in the \textit{Map3k1}\textsuperscript{tm1Yxia/tm1Yxia} lysate. This is confusing as the kinase domain of this mutant is replaced with a \textit{lacZ} reporter cassette (Xia et al., 2000; Zhang et al., 2003). The strongest band detected in all samples with this antibody was \textasciitilde 50 kDa, there is also a weaker band that migrated at \textasciitilde 130 kDa. The same bands were observed in the western blot using the LF-PA41823 antibody, although the \textasciitilde 130 kDa band was much stronger compared with that seen with the sc-252 antibody. Interestingly the LF-PA41823 antibody also detected a larger band (\textasciitilde 230 kDa) in the \textit{Map3k1}\textsuperscript{tm1Yxia/tm1Yxia} lysate (Figure 3.14.).

As previously mentioned, the reported molecular weight of full length human MAP3K1 is 196 kDa, the pro-apoptotic cleaved fragment is 91 kDa leaving a 105 kDa N-terminus fragment of which little is known (Deak and Templeton, 1997; Schlesinger et al., 2002; Widmann et al., 1998). Murine MAP3K1 has a predicted molecular weight \textasciitilde 3 kDa less than human, due to a 19 amino acid difference in the peptide sequence. Molecular weight prediction software such as Swiss-Prot predict human full length MAP3K1 to be 164 kDa, with murine at 161 kDa, with the murine cleaved fragment predicted to be 67 kDa; neither the reported or in silico predicted weights match that of the bands observed with the inner ear lysates. The MAP3K1-\textit{ß}-Galactosidase fusion protein was reported to migrate at 242 kDa (Xia et al., 2000); it is possible that the larger band seen in the \textit{Map3k1}\textsuperscript{tm1Yxia/tm1Yxia} lysate corresponds to this fusion protein; however this lysate should contain no wild-type full length protein or kinase domain, so it is contradictory that the other bands would also be present, albeit slightly weaker.

It is interesting that both bands were present using both antibodies, which would suggest specificity, however the size is still confusing. A possible explanation is that they are proteolytic products of the full length protein, however, combined with the presence of
identical protein bands in the kinase deficient mice, it would be unwise to trust immunohistochemical labelling performed with either of these antibodies.

![Western blot analysis](image.png)

**Figure 3.14. Immunodetection of MAP3K1 using commercially available anti-MAP3K1 antibodies.** Western blot analysis of Map3k1+/+, Map3k1gy/gy, Map3k1gy/gya and Map3k1tm1Yxia/tm1Yxia inner ear protein lysates, using two commercially available anti-MAP3K1 antibodies, AbFrontier LF-PA41823 and SantaCruz sc-252. Both antibodies detected protein bands that migrated at ~50 kDa and ~130 kDa in all samples including Map3k1gy/gy, Map3k1gy/gya and Map3k1tm1Yxia/tm1Yxia, although the 130 kDa band was weaker when the sc-252 antibody was used. The LF-PA41823 antibody also detected a larger product at ~230 kDa in the Map3k1tm1Yxia/tm1Yxia lysate which may correspond to the larger predicted weight of the MAP3K1-ß-Galactosidase fusion protein (242 kDa), however the presence of the other bands is contradictory.

### 3.2.8.3. Whole-mount Cochlear Immunofluorescence using Anti-ß-Galactosidase Antibody

Given the inconclusive immunolabelling data using commercially available anti-MAP3K1 antibodies, Map3k1tm1Yxia mice which express a MAP3K1-ß-Galactosidase fusion protein were exploited (Xia et al., 2000; Zhang et al., 2003). Whole-mount immunolabelling of 2-week old Map3k1tm1Yxia/tm1Yxia cochlea, using a commercially available anti-ß-Galactosidase antibody, was performed to investigate the localisation of MAP3K1-ß-Galactosidase. Widespread punctate expression of MAP3K1-ß-Galactosidase was observed throughout the cochlear duct (Figure 3.15.C, D, E). From the punctate labelling a pattern emerged consistently that looked similar to the localisation of basal bodies of the kinocilium on Deiter’s cells and pillar cells. It also appears the tips of stereocilia are labelled in a small
number of the homozygote cochleae, although this was not observed in all samples (Figure 3.15.E). A small amount of background labelling was observed in C3H control samples at all dilutions that were used (Figure 3.15.A, B), but not in the non-primary negative control (data not shown).

3.2.8.4. X-Gal Detection of MAP3K1-β-Galactosidase

A second strategy employed to assess localisation of MAP3K1 in the inner ear was 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) staining. Whole-mount X-Gal staining of inner ears from P12 Map3k1^{tm1Yxia/tm1Yxia} and wild-type mice was performed, prior to decalcification and dissection of the cochlear duct. Cochleae from
\( \text{Map3k1}^{\text{tm1Yxia/tm1Yxia}} \) mice showed widespread expression of MAP3K1-β-Galactosidase (Figure 3.16.A). Higher magnification examination of the cochlear duct using extended focus image capture showed X-gal staining in IHCs and OHCs, border cells of the internal spiral sulcus, Claudius and Hensen cells as well as SGN (Figure 3.16.C). To investigate further cellular localisation across the cochlea, whole-mount X-Gal stained ears were decalcified, embedded in paraffin wax and sectioned sagittally at 10 µm. This confirmed the localisation in the cell types observed in the whole-mount images and also revealed expression in cell types such as Deiters’ cells, pillar cells, Reissner’s membrane, marginal cells in the stria vascularis and the tympanic border cells of the basilar membrane (Figure 3.16.E, G, O, P). A transverse section of the organ of Corti also highlighted the expression of the fusion protein in IHCs and OHCs (Figure 3.16.Q). In addition to the expression in the cochlea, X-gal staining was observed in the vestibular system. The apical surface of supporting cells and hair cells in both otolithic organs and the cristae of the semi-circular canals all showed expression of MAP3K1-β-Galactosidase (Figure 3.16.K, L, M). Positive staining in all \( \text{Map3k1}^{\text{tm1Yxia/+}} \) samples mirrored that of homozygotes, whereas no staining was seen in either the vestibular system or cochlea of the \( \text{Map3k1}^{+/+} \) mice (Figure 3.16.B, D, F, H, J, L, N).

3.2.9. Vestibular Function in goya Homozygote Mice.

No obvious signs of vestibular dysfunction, such as head tilting or circling behaviour were observed in either \( \text{Map3k1}^{\text{goya/goya}} \) or \( \text{Map3k1}^{\text{tm1Yxia/tm1Yxia}} \) mice. Given the expression of MAP3K1 observed in the apical surfaces of hair cells in the vestibular system, possible vestibular effects of the mutation were investigated further. Swim testing of 1-year old \( \text{Map3k1}^{\text{goya/goya}} \) mice was undertaken (Mrs S. Morse). All mutant mice swam normally, performing as well as \( \text{Map3k1}^{+/+} \) control animals, suggesting vestibular function is not affected by MAP3K1 deficiency.
Figure 3.16. MAP3K1-ß-Galactosidase expression in the inner ear of P12 Map3k1<sup>tm1Yxia/tm1Yxia</sup> and Map3k1<sup>tm1Yxia/+</sup> Mice. Panels A and B show X-gal stained cochlea from Map3k1<sup>tm1Yxia/tm1Yxia</sup> and Map3k1<sup>+/+</sup> mice respectively. The Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochlea (A) shows widespread staining due to the presence of a MAP3K1-ß-Galactosidase fusion protein in these mice. Panels C and D are extended focus images showing staining in the cochlear duct. In Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochlea (C) strong staining can be observed in Claudius cells, Hensen cells, outer hair cells, inner hair cells, border cells of the internal spiral sulcus and spiral ganglion neurons. This staining is not observed in the Map3k1<sup>+/+</sup> cochlea (D). Panels E-N show X-gal stained sagittal sections of the cochlea and vestibular systems from Map3k1<sup>tm1Yxia/tm1Yxia</sup> and Map3k1<sup>+/+</sup> mice: E, F – stria vascularis, G, H – spiral ganglion neurons, I, J – sacular macula, K, L – utricular macula, M, N – crista ampullaris. X-gal positive staining is present in the marginal cells of the stria vascularis (E), the spiral ganglion neurons (G) and the apical surfaces of supporting cells and hair cells in all of the otolithic organs in the vestibular system of Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice. Panel O shows the distribution of MAP3K1-ß-Galactosidase in the cochlear duct of Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice. Staining is observed in the stria vascularis (SV), Reissner’s membrane (RM), Claudius cells (CC), Hensen cells (HC) and border cells of the internal spiral sulcus (BC). Panel P is an enlargement of the organ of Corti (dashed box in panel O) and shows MAP3K1-ß-Galactosidase expression in the apical surface of IHCs, OHCs, Deiters’ cells (DC), pillar cells (PC) and tympanic border cells of the basilar membrane (BM). Panel Q shows a transverse section of the organ of Corti from a Map3k1<sup>tm1Yxia/+</sup> mouse highlighting more clearly X-Gal positive staining in the IHCs, pillar cells and OHCs. CC - Claudius cells, HC - Hensen cells, OHC - outer hair cells, IHC - inner hair cells, BC - border cells of the internal spiral sulcus, SGN - spiral ganglion neurons, SV - stria vascularis, RM - Reissner’s membrane, DC - Deiters’ cells, PC - pillar cells (Figure from (Parker et al., 2015)).
3.3. Discussion

3.3.1. The ENU-induced Lesion in \textit{Map3k1} is Causative of the Hearing Loss in \textit{goya} Mice.

Mice homozygous for the \textit{goya} mutation are born with their eyes open, develop various adult eye phenotypes and also display a lack of response to a clickbox. Whilst investigating the \textit{goya} mutant for the vision based phenotypes, Dr Sally Cross from the HGU in Edinburgh identified an ENU-induced lesion in the \textit{Map3k1} gene as being the likely causative mutation. In order to confirm that the mutation was responsible both for the EOB phenotype and for the lack of clickbox response, I performed a complementation test by crossing heterozygote \textit{goya} to mice heterozygous for the previously reported kinase negative allele \textit{Map3k1}^{tm1Yxia} (Xia et al., 2000), and performed ABR analysis at 9-weeks of age. These data showed that \textit{goya} homozygote, \textit{Map3k1}^{tm1Yxia} homozygote and compound heterozygote mice all display a severe hearing loss at 9-weeks of age. This confirms that the hearing loss observed in the \textit{goya} mice is due to the mutation (IVS13+2T>C) in \textit{Map3k1}, and also provides a previously unreported phenotype for mice homozygote for the \textit{Map3k1}^{tm1Yxia} allele.

3.3.2. Mice Deficient for MAP3K1 Display an Early Onset Progressive Hearing Loss

Time course ABR analysis indicated that the hearing loss had a progressive element. The ABR thresholds in \textit{goya} homozygote mice at 2-weeks of age (the onset of hearing in mice) were not significantly different from wild-type at any of the frequencies tested except 26kHz; this suggests that auditory function develops quite normally in \textit{goya} homozygote mice. The thresholds at 4-weeks of age are intermediate between the 2-week of age and 9-week of age thresholds suggesting a rapid decline of auditory function directly after the onset of hearing. Mice homozygous for the kinase deficient \textit{Map3k1}^{tm1Yxia/tm1Yxia} allele also showed a progressive hearing loss, however the ABR thresholds at 2-weeks of age were
significantly higher than both wild-type and $Map3k1^{goya/goya}$ across all the frequencies tested. It is possible that this is an effect of the different nature of the mutations, however another possibility is the different genetic backgrounds of the two mutants, with $goya$ maintained on C3H and $tm1Yxia$ containing C57BL/6J. It is well known that different inbred strains of mice can have an influence on phenotypic effects of mutations, due to the presence of alleles with the ability to modify a given phenotype arising from generations of inbreeding (Balmer et al., 2013; Kiernan et al., 2007; Marshall et al., 2013; Rubinstein et al., 2015; Silva et al.; Threadgill et al., 1995). In addition to general background effects, C57BL/6J mice are known to develop ARHL due to the age related hearing loss (ahl) mutation in cadherin 23 (Cdh23) gene (Erway et al., 1993; Johnson et al., 1997; Noben-Trauth et al., 2003). Apart from the early auditory thresholds, no other major differences in phenotypes were noted between the $goya$ and the $tm1Yxia$ alleles of $Map3k1$; this suggests that even if modifiers were involved, the eventual outcome of the mutation is unaffected.

### 3.3.3. Hair Cell Abnormalities in $Map3k1$ Mutants

Initial histological examination of $Map3k1^{goya/goya}$ inner ears indicated a loss of hair cells and supporting cells in the organ of Corti, although other cell types and cochlear structures showed no obvious abnormalities. Subsequent ultrastructural analysis of the sensory epithelial surface of the organ of Corti using scanning electron microscopy revealed two distinct hair cell phenotypes. Firstly, both $Map3k1^{goya/goya}$ and $Map3k1^{tm1Yxia/tm1Yxia}$ mice develop supernumerary hair cells in the form of an extra row of OHC. Secondly, temporal analysis over the same time course as the ABR analysis revealed a progressive cellular degeneration in the organ of Corti with an apical-to-basal increase in severity. Cellular loss was first seen in the OHCs, although by 9-weeks IHCs and pillar cells were also missing in some mid and mid-basal regions, although not statistically
significant. High magnification examination of IHC and OHC stereocilia bundles showed no overt abnormal morphology.

Mice heterozygous for either the Map3k1<sup>goya</sup> or the Map3k1<sup>tm1Yxia</sup> allele also develop extra OHCs, but interestingly they do not show progressive cellular degeneration as seen in the respective homozygotes. Indeed, at 1-year of age Map3k1<sup>goya/+</sup> and wild-type mice have similar auditory thresholds. The fact that haploinsufficiency of Map3k1 is enough to replicate the supernumerary OHC phenotype, but not the hearing loss and cellular degeneration, suggests that within the organ of Corti MAP3K1 plays multiple distinct roles in cellular development and survival.

**3.3.4. Localisation of MAP3K1 to the Inner Ear**

The commercially available anti-MAP3K1 antibodies tested were deemed unreliable for immunohistochemistry. Although both that were tested identified somewhat comparable protein bands by Western blotting, the size of the bands (~50 kDa and ~130 kDa) was contradictory to the reported (196 kDa and 91 kDa), or the bioinformatically predicted molecular weights (161 kDa and 67 kDa) of the full length and cleaved fragments of MAP3K1. In addition when immunostaining was performed, the antibodies showed differences in sub-cellular localisation, although many of the same cell types were labelled.

To overcome the reliability issues surrounding the anti-MAP3K1 antibodies, detection of the MAP3K1-β-Galactosidase fusion protein expressed by mice carrying the Map3k1<sup>tm1Yxia</sup> allele was preferred. Both whole-mount immunofluorescence and X-Gal staining approaches were used; the results show that MAP3K1 is widely expressed in the inner ear. The expression in OHCs and IHCs, along with Deiters’ cells, in the organ of Corti is consistent with the observed phenotype of additional rows of OHCs and OHC
degeneration in Map3k1 mutants. These findings of MAP3K1 expression in the cochlea are consistent with the observations of another study of mice carrying the Map3k1\textsuperscript{tm1Yxia} allele that was performed coincidentally with this investigation (Yousaf et al., 2015). They too report expression in Deiters’ cells, Reissner’s membrane and the stria vascularis, but did not mention additional sites of expression including Claudius cells, Hensen cells and Border cells, or the basilar membrane. The whole-mount immunofluorescence they report using an anti-β-Galactosidase antibody was in agreement to the results obtained in this investigation.

3.3.5. Conclusions from Phenotypic Characterisation of the goya Mutant

The goya mutant has revealed previously unreported distinct novel roles for MAP3K1 both in the development and maintenance of the auditory system. However, the widespread expression of MAP3K1 in the inner ear, combined with its broad range of reported roles and cellular functions, makes postulating the mechanism of hearing loss and cellular loss resulting from MAP3K1 deficiency somewhat challenging. The rate of decline in auditory function of goya homozygote mice seems to be consistent with the loss of OHC. It should be noted however that the kinase negative Map3k1\textsuperscript{tm1Yxia/tm1Yxia} mutant already displayed a moderate hearing loss at the 2-week of age time-point, which could suggest that OHC loss is not the primary cause of auditory decline. Irrespective of whether OHC loss is a secondary effect or not, their rapid degeneration suggests that MAP3K1 plays an essential role in OHC survival. The increased number of OHC in both heterozygote and homozygote goya and Map3k1\textsuperscript{tm1Yxia} mice also highlights that MAP3K1 regulates the correct development of the organ of Corti. The fact that neither heterozygote develops an auditory deficit or suffers OHC loss shows that these are distinct mechanisms; therefore MAP3K1 plays multiple roles in auditory function.
In summary, the characterisation of the *goya* phenotype has shown that the previously discovered IVS13+2T>C ENU-induced lesion in *Map3k1* is the causative mutation underlying both the EOB and auditory phenotypes in the *goya* mutant. These findings demonstrate a new involvement of MAP3K1 in auditory function.
Chapter 4

Functional Investigation of the *goya* Mutation and MAP3K1 Deficiency in the Inner Ear
4.1. Introduction

4.1.1. Functional Consequences of MAP3K1 Deficiency

In mice, loss-of-function mutations in the Map3k1 gene lead to defects in epithelial migration that manifest as an EOB phenotype (Xia and Kao, 2004; Zhang et al., 2003), due to defects in actin polymerisation and c-JUN phosphorylation. Studies in keratinocytes demonstrate that activation of c-Jun N-terminal kinase (JNK) by TGF-beta and Activin requires MAP3K1, leading to c-JUN phosphorylation, actin stress fibre formation and cell migration (Zhang et al., 2005; Zhang et al., 2003). Furthermore MAP3K1 has also been shown to act independently of JNK during the regulation of cell proliferation and apoptosis in the retina. It is reported that in the retinas of Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice there is an increase in proliferation of Müller glial cells, followed by apoptosis, which has been attributed to the p27<sup>Kip1</sup> axis; the study showed upregulation of the p27KIP1 targets Ccna1 (Cyclin A1), Ccnd1 (Cyclin D1) and Cdk4/6 (Cyclin dependant kinase 4/6) as well as E2F1 targets such as dihydrofolate reductase (Dhfr) in Map3k1<sup>tm1Yxia/tm1Yxia</sup> retinas, although expression of the key pathway members p27<sup>Kip1</sup>, E2f1 and Rb1 were unchanged from wild-type (Mongan et al., 2011).

While it is clear a MAP3K1-JNK cascade is critical for epithelial sheet movements during eye organogenesis, it may also be expected to have a role in the development of other epithelia. Indeed, MAP3K1 is required during wound healing where injury up-regulates MAP3K1 and leads to changes in the expression of genes associated with extracellular matrix homeostasis. Conversely, knock-down of MAP3K1 impairs wound healing (Deng et al., 2006).

In humans, MAP3K1 mutations have been shown to cause 46,XY disorders of sexual development (DSD) (Loke et al., 2013; Pearlman et al., 2010). A number of these
mutations have been studied, and they all result in the increased phosphorylation of
downstream MAPK proteins p38 MAPK and ERK1/2.

Large scale projects gathering and analysing datasets to identify common mutations in
various human cancers have indicated that MAP3K1 mutations may be causative of a
number of different cancers, with by far the most common association being luminal
breast cancer (Cerami et al., 2012; Gao et al., 2013; Kan et al., 2010; Pham et al., 2013).

It is also thought that MAP3K1 mediated JNK activity plays a role in cardiac disease. It has
been shown that Map3k1−/− mice were protected against cardiac hypertrophy and showed
reduced JNK activity following forced expression of the G protein Gαq, one of the most
established mediators of the pathology; this suggests that Map3k1 promotes hypertrophy
through JNK activation in response to stimulation by Gαq (Minamino et al., 2002).
Another study, however, showed that cardiac hypertrophy was more severe in Map3k1−/−
than in wild-type than wild-type mice when pressure overload was induced by aortic
restriction (Sadoshima et al., 2002). Taken together these contradictory studies highlight
the complexities of MAP3K1 signalling whereby it can both inhibit and promote the
development of the same pathological condition dependant on stimulus.

4.1.2. Stress Response Pathways in the Inner Ear

A number of studies indicate that MAPK pathways are heavily involved with cell death
and cell survival in the organ of Corti in the inner ear. For example, apoptotic death of
cochlear sensory hair cells damaged by acoustic trauma is mediated by the MAPK/JNK
signalling cascade (Dinh and Van De Water, 2009). Exposure to noise has also been shown
to result in an increase of p38 phosphorylation and Fas cell surface death receptor (FAS)
expression in the sensory epithelia, suggesting a role for the FAS-induced mitochondrial
death pathway, regulated by p38, in noise damage (Jamesdaniel et al., 2011).
Aminoglycoside antibiotics such as gentamicin are well known to have ototoxic effects, resulting in a loss of hair cells, particularly OHC, and sensorineural deafness. The exact cause of cellular loss as a result of aminoglycoside treatment is not yet fully understood, but a body of evidence suggest that mitochondrial dysfunction and a build-up of free radicals is responsible (Dehne et al., 2002; Fischel-Ghodsian, 2003; Hobbie et al., 2008; Huth et al., 2011).

The ERK1/2 and JNK and p38 MAPK signalling pathways are all reported to play roles in the apoptosis resulting from aminoglycoside induced damage. Studies have shown that inhibitors of JNK can protect against noise-induced and aminoglycoside-induced hearing loss (Eshraghi et al., 2007; Pirvola et al., 2000; Wang et al., 2003). Another investigation suggested that the K-RAS/RAK/JNK pathway is important for sensory hair cell death, while the H-RAS/RAF/MEK/MAPK (ERK1/2) pathway is important for their survival (Battaglia et al., 2003). However, a contrasting study in guinea pigs showed the opposite result; treatment with gentamicin resulted in the upregulation of the proapoptotic B-cell lymphoma 2 (BCL2) family member Harakiri in OHC, and activation of CASPASE-3. Inhibition of ERK1/2 in gentamicin treated cells blocked CASPASE-3 activation and apoptosis, conversely, JNK inhibition had the opposite effect, suggesting that for aminoglycoside ototoxicity JNK signalling plays a protective role whereas ERK1/2 promotes apoptosis. A role has also been suggested for p38 in aminoglycoside ototoxicity. Inhibition of p38 phosphorylation results in a partial (~50%) reduction in gentamicin induced hair cell loss in cochlear explants, although greater protection was achieved with inhibition of both p38 and CASPASE-3 (Wei et al., 2005).

Taken together these studies highlight that MAPK signalling plays a vital but highly complex mediatory role in sensory hair cell survival from different stress factors. Understanding the exact mechanisms behind the contrasting protective and proapoptotic
effects of different branches of the MAPK signalling pathway is complicated by crosstalk between the pathways, and remains elusive.

4.1.3. Mouse Models with Extra OHC

As previously mentioned the organ of Corti normally consists of one row of IHCs and three rows of OHCs. There are a number of mouse models which have previously been reported to exhibit increased numbers of hair cells. The Mouse Genome Database resource (MGD) (http://www.informatics.jax.org/) (Eppig et al., 2015) was used to search for known mouse mutants with the phenotype annotated term ‘increased cochlear outer hair cell number’; a list of 17 genes was returned (Table 4.1.).

<table>
<thead>
<tr>
<th>Gene symbol</th>
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<tr>
<td>Cdkn1b (p27&lt;sup&gt;Kip1&lt;/sup&gt;)</td>
<td>(Chen and Segil, 1999)</td>
<td>Hes5</td>
<td>(Zine et al., 2001)</td>
</tr>
<tr>
<td>Chd7</td>
<td>(Pau et al., 2004)</td>
<td>Jag2</td>
<td>(Lanford et al., 1999)</td>
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<td>Crsl</td>
<td>(Hawker et al., 2005)</td>
<td>Nog</td>
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<td>Dfna5</td>
<td>(Van Laer et al., 2005)</td>
<td>Notch1</td>
<td>(Lanford et al., 1999)</td>
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<td>Dll1</td>
<td>(Kiernan et al., 2005)</td>
<td>Nr2f1</td>
<td>(Tang et al., 2006)</td>
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<td>Emx2</td>
<td>(Rhodes et al., 2003)</td>
<td>Ptk7</td>
<td>(Lu et al., 2004)</td>
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<td>Eya1</td>
<td>(Zou et al., 2008)</td>
<td>Rb1</td>
<td>(Sage et al., 2006a)</td>
</tr>
<tr>
<td>Fgfr3</td>
<td>(Hayashi et al., 2007)</td>
<td>Spry2</td>
<td>(Shim et al., 2005)</td>
</tr>
<tr>
<td>Hes1</td>
<td>(Zine et al., 2001)</td>
<td>Map3k1</td>
<td>(Parker et al., 2015)</td>
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Table 4.1. Genes linked with supernumerary OHC phenotype. List of genes returned from the MGD database with the annotated phenotype search term ‘increased cochlear outer hair cell number’. Mice harbouring mutations in these genes have been reported to exhibit supernumerary OHC. The entry for Map3k1 in red corresponds to the work in this thesis and resulting publication (Parker et al., 2015).

A number of components of the Notch signalling pathway are reportedly involved with the production of supernumerary OHC (Figure 4.1.). The genes responsible are spread throughout all levels of the pathway from membrane bound ligands (delta-Like 1 (Dll1), Jag2) and receptor (Notch1) to subsequent downstream targets, hairy and enhancer of split 1 and 5 (Hes1, Hes5). This suggests a crucial role for Notch signalling in the regulation of OHC number. As with a large number of canonical signalling pathways there is evidence to suggest crosstalk between the Notch and MAPK pathways (Mittal et al., 2009; Yamashita et al., 2013).
In addition to Notch related genes, included are regulators of cell proliferation, which affect cell-cycle exit in the inner ear. The cyclin-dependent kinase inhibitor, \(p27^{kip1}\), is a key regulator that arrests the cell cycle at G1. Its expression in the developing cochlea results in the formation of the ZNPC, and these cells subsequently differentiate into sensory hair cells and supporting cells. It is known that these ZNPC cells undergo their final division by embryonic day 14.5 (E14.5). However, in \(p27^{kip1}\) homozygous null mice, there is an extended period of pro-sensory precursor cell proliferation leading to increased numbers of hair cells and supporting cells (Lowenheim et al., 1999).

The \(E2f1\) inhibitor \(Rb1\) is also associated with increased hair cell number. Mice lacking retinoblastoma protein develop extra inner and outer hair cells, and analysis of progenitor cell proliferation indicates that RB1 is involved in cell cycle exit of sensory progenitor cells (Sage et al., 2005). The additional hair cells in \(Rb1\) knockout mice can

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**Figure 4.1. NOTCH pathway members involved with increased OHC number.** The NOTCH signalling pathway with genes returned from the MGD database with the annotated phenotype search term ‘increased cochlear outer hair cell number’ circled in red. The list included NOTCH ligands Dll1 and Jag2, the receptor Notch1 and target transcription factors Hes1 and Hes5. The pathway shows that in certain circumstances the crosstalk with the MAPK signalling pathway by means of Ras activation at the receptor level (highlighted in orange). Pathway image adapted from KEGG pathways (Kanehisa et al., 2016) (http://www.genome.jp/kegg-bin/show_pathway?org_name=mmu&mapno=04330&mapscale=&show_description=hide)
transduce mechanical stimuli, but they undergo apoptosis and are completely missing by 3-months of age (Sage et al., 2006b).

4.2. Results

4.2.1. Analysis of Map3k1 Transcription in the Cochlea

Past experiments performed by Dr Sally Cross at the MRC HGU had identified abnormal splicing of the Map3k1 transcript in the brain, kidney and spleen of goya mice as a result of the intron 13 splice donor site mutation (IVS13+2T>C). In an attempt to ascertain the effect of the goya mutation on splicing in the cochlea, reverse transcription polymerase chain reaction (RT-PCR) analysis of RNA isolated from postnatal day 1 (P1) Map3k1<sup>goya/goya</sup>, Map3k1<sup>goya/+</sup> and Map3k1<sup>+/+</sup> organ of Corti was performed. Using the RNA extracted from the P1 organ of Corti as a template, cDNA was synthesised using a combination of oligo DT, random hexamers and Map3k1 specific primers. The RT-PCR was then performed using the cDNA as a template and a forward primer spanning the exon 11/12 boundary so as to not amplify any possible contamination from genomic DNA, and a reverse primer from exon 14. As expected a single PCR product of 339 bp was obtained from wild-type samples. However, in mutant samples the wild-type product was absent and two smaller products could be seen. In the heterozygous samples both the wild-type and aberrant RT-PCR products were found (Figure 4.2.)

![Figure 4.2 RT-PCR analysis of RNA extracted from the organ of Corti of P1 mice. In the Map3k1<sup>+/+</sup> RT-PCR product, a single amplicon corresponding to the expected 339 bp of wild-type Map3k1 sequence was observed. For Map3k1<sup>goya/goya</sup>, the wild-type product was absent, instead two smaller amplicons were identified the most abundant between 200 and 300 bp and a less abundant product between 100 and 200 bp. All three amplicons were present in the Map3k1<sup>goya/+</sup> RT-PCR product. (Figure adapted from (Parker et al., 2015)).](image-url)
4.2.2. Sanger Sequencing of Inner Ear Map3k1 RT-PCR products

To determine the effect of the goya mutation on splicing, the Map3k1+/+ and two Map3k1goya/goya RT-PCR products were excised from the gel, cloned and sent for Sanger sequencing (Figure 4.3.A).

As expected, the Map3k1+/+ product shows the consensus sequence of exons 12, 13 and 14 correctly spliced, whereas the two Map3k1goya/goya products show aberrant splicing. The more abundant product found in homozygotes (Map3k1goya/goya RT-PCR 1) demonstrates the use of a cryptic splice donor site within exon 13. In this case 81 nucleotides are skipped leaving the transcript in-frame, but producing a protein

![Figure 4.3. Sanger sequencing reveals aberrant splicing in Map3k1goya/goya inner ears.](Figure adapted from (Parker et al., 2015))
with an internal deletion of 27 amino acids (L760 to R786 of murine MAP3K1). The less abundant product (Map3k1\textsuperscript{goya/goya} RT-PCR 2) shows exon 12 spliced directly to exon 14, skipping exon 13 (Figure 4.3.). Skipping of the 190 nucleotide exon 13 creates a transcript with a frameshift. Translation of this transcript would lead to the production of a protein containing the first 723 amino acids of MAP3K1 followed by incorporation of seven novel amino acids before a stop codon is encountered. If translated this truncated protein would lack the C-terminal 770 amino acids of MAP3K1 including the kinase domain.

4.2.3. \textit{In silico} Analysis

4.2.3.1. PROVEAN Mutation Prediction

\textit{In silico} analysis was performed to investigate predicted effects of the most abundant splice variant of \textit{goya Map3k1} as shown by the RT-PCR experiment; which uses the cryptic splice donor site within exon 13 (henceforth referred to as MAP3K1\textsuperscript{CS}). Using the PROVEAN online prediction software the wild-type peptide sequence for murine MAP3K1, and the MAP3K1\textsuperscript{CS} variant containing the deletion of amino acids L760 to R786 were compared. Unsurprisingly the prediction was that this internal deletion was deleterious for the protein and resulted in a PROVEAN score of -24.642 (anything < -2.5 is considered deleterious).

Although the effect of a substantial internal deletion may be expected to be deleterious, the nature of MAP3K1 structure and function may mean that some kinase activity may be retained; as previously mentioned, full length MAP3K1 is reported to be a membrane bound 196 kDa protein, this protein can be cleaved by caspase-3 at the aspartate residue 874 (murine) to release the C-terminus including the kinase domain into the cytosol, where it becomes enzymatic and pro-apoptotic (Schlesinger et al., 2002). The location of the deletion in MAP3K1\textsuperscript{CS} is not in close proximity to the caspase cleavage site in the
primary structure, leaving the possibility that if translated and present the MAP3K1\textsuperscript{CS} C-terminus including the kinase domain may still be cleavable by caspase-3.

### 4.2.3.2. Predicted Domain Differences between MAP3K1 and MAP3K1\textsuperscript{CS}

Analysis of protein domains contained within wild-type MAP3K1 and MAP3K1\textsuperscript{CS} proteins was performed using two different prediction software platforms. Firstly the SMART protein server (Schultz et al., 1998) (http://smart.embl-heidelberg.de/) was used. This software predicted both proteins to contain a zinc-finger SWIM type domain and a plant homeobox (PHD) domain containing a zinc-finger RING (Really Interesting New Gene) motif in addition to a serine-threonine kinase domain (Figure 4.4.B) The main difference between the two full length proteins apart from the position of the kinase domain, reflecting the 27 amino acid internal deletion in the MAP3K1\textsuperscript{CS} sequence, was the omission of a low complexity region between L750 and L762 (Figure 4.4.B).

Low complexity regions have been shown to be important for protein binding and also folding (Seldeen et al., 2008). The importance of these regions is highlighted in protein phosphorylation cascades (Iakoucheva et al., 2004), in particular those low complexity regions centrally located in the protein (Coletta et al., 2010).

When the same analysis was performed using the InterPro platform (Mitchell et al., 2015) (https://www.ebi.ac.uk/interpro/) an additional domain was identified; in wild-type MAP3K1, a 221 amino acid armadillo-type fold sequence was predicted between Q558 and S770 (Figure 4.4.C). The last 25 amino acids of this domain are contained within the deletion in the MAP3K1\textsuperscript{CS} protein, and in the mutant the protein is predicted to truncate and slightly shift the armadillo-type fold domain to a 145 amino acids located between D551 and S695 (Figure 4.4.C).
Armadillo-type repeats are so called due to structural similarities with domains originally found in β-CATENIN for which armadillo is a drosophila homolog. They are super-helical structures consisting of multiple repeats of around 40 amino acids and act as protein

Figure 4.4 Prediction of protein domain motifs in murine MAP3K1 and MAP3K1\textsuperscript{CS}. A) scale representation of MAP3K1 showing the exons (odd blue, even red) and the CASPASE-3 cleavage site (D874). The purple section and corresponding peptide sequence demarks the deletion resulting from the use of the cryptic splice donor site in exon 13. Panels B and C show predicted protein domains derived from the peptide sequences of MAP3K1 and MAP3K1\textsuperscript{CS} using the SMART (B) and InterPro (C) software. Both predicted zinc finger SWIM and PHD/RING type domains in addition to the kinase domain. The pink areas on the SMART domain prediction correspond to low complexity regions, one of these is missing as a result of the deletion in MAP3K1\textsuperscript{CS} (red circle). The InterPro software also predicts an Armadillo-type fold domain in both proteins, which is slightly shifted and truncated in the MAP3K1\textsuperscript{CS} splice variant of the protein (red arrow).
binding domains, usually contained within proteins of diverse cellular functions (reviewed in (Tewari et al., 2010)). Although many proteins share similar structural repeats, the sequence varies greatly which may explain why InterPro was the only prediction software used to identify the feature within MAP3K1.

4.2.4. Secondary and Tertiary Structure Prediction

4.2.4.1. Full-length MAP3K1

To investigate possible alterations in predicted secondary and tertiary structure, two protein structure prediction platforms were used: I-Tasser (Roy et al., 2010; Yang et al., 2015; Zhang, 2008) (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and RaptorX (Kallberg et al., 2014; Ma et al., 2013; Peng and Xu, 2011a; Peng and Xu, 2011b) (http://raptorx.uchicago.edu/). The full length sequences of MAP3K1 and MAP3K1CS were entered for analysis to produce .pdb (protein database) files containing predicted secondary and tertiary 3-D structures. The crystal structure of MAP3K1 is as yet unresolved; however, structures that have been resolved which include kinase domains (e.g Serine/Threonine kinase 10 (STK10)) showed a high structural similarity with the kinase domain in the MAP3K1 RaptorX prediction model but not with the I-Tasser prediction. For this reason the RaptorX model was used for further analyses.

When the predicted 3-D structures of the full-length wild-type MAP3K1 and the cryptic-splice site MAP3K1 variant are aligned using a superimpose algorithm within the CCP4MG software, two main differences can be seen. Firstly the kinase domains differ in position, being opposed to one another (Figure 4.5). Secondly the MAP3K1CS sequence lacks a predicted structure at the N-terminus. It is unclear as to why this would be the case as the peptide sequence coding this structure is still present in MAP3K1CS, being situated well
upstream of the internal deletion (Figure 4.5); presumably this is either due to slight changes in protein folding inhibiting the formation of the domain, or a prediction error.

**Figure 4.5. RaptorX prediction of secondary and tertiary structure.** Graphical representation of predicted secondary and tertiary structure of wild-type MAP3K1 (blue) and the cryptic splice site variant, MAP3K1\textsuperscript{CS} (gold) created using the RaptorX structure prediction server and visualised using the CCP4MG 3-D molecular modelling software. The MAP3K1 model (A) shows an extra structural domain (arrow) at the N-terminus that was not predicted in the MAP3K1\textsuperscript{CS} model (B). (C) When the models are superimposed using an algorithm in the CCP4MG software in addition to the N-terminus domain (arrow) the kinase domains of the proteins appear to be in positional opposition to one another (red box).
4.2.4.2. Kinase Domain

Although when the full length proteins are aligned the kinase domains are in positional opposition to one another (Figure 4.6.A), if the two domains are isolated and superimposed using the CCP4MG software algorithm used to align the full length protein models, there is very little predicted structural difference (Figure 4.6.B, C, D).

Figure 4.6. Alignment of predicted MAP3K1 and MAP3K1\textsuperscript{CS} kinase domains. (A) Relative orientation of the kinase domains of MAP3K1 (blue/green) and MAP3K1\textsuperscript{CS} (gold/red) in the full length superimposed models. The domains are similar in position relative to their own tertiary structures, however the domains appear opposed in relation to one another when the two models are aligned. (B, C, D) When isolated from the rest of the model, the MAP3K1 (B, blue) and MAP3K1\textsuperscript{CS} (C, gold) kinase domains are well matched (D).
4.2.4.3. CASPASE-3 Cleavage Site

The relative size of the caspase cleavage site (DTVD, amino acids 871-874) in MAP3K1$^{CS}$ is predicted to be roughly half that of wild-type MAP3K1, due to subtle differences in the tertiary structure (Asp 871, Thr 872, Val 873, Asp 874) (Figure 4.7.A, B). However, it also appears to be accessible in both models. It is unclear if the predicted size difference would affect interactions with CASPASE-3, if not, this leaves the possibility that cleavage and activation of the pro-apoptotic C-terminus fragment of MAP3K1$^{CS}$ may still occur.

![MAP3K1 CASPASE-3 cleavage site](image)

![MAP3K1$^{CS}$ CASPASE-3 cleavage site](image)

Figure 4.7. Accessibility of predicted MAP3K1 and MAP3K1$^{CS}$ CASPASE-3 cleavage sites. The CASPASE-3 cleavage site is accessible in both MAP3K1 (A, blue) and MAP3K1$^{CS}$ (B, gold) prediction models, suggesting that cleavage and subsequent release of the proapoptotic kinase domain may still be possible in goya homozygote mice.
4.2.4.4. Residues Essential to Kinase Function

Other important motifs contained within the MAP3K1 kinase domain are the ATP binding site (amino acids 1230 – 1253), and the active site (amino acids 1346 - 1358) essential for kinase function. In addition research has shown that phosphorylation of residues Thr 1381 and Thr 1393 of MAP3K1 is necessary for its catalytic function (Chadee et al., 2002; Deak and Templeton, 1997; Siow et al., 1997). To investigate if the predicted tertiary structure of the two proteins had any effect on relative positions of these important residues, the motifs were highlighted within the aligned kinase domain models. The location of the activation loop site containing the important Thr 1381 and Thr 1393 residues is predicted to be similar in the both models, however there are slight alterations in orientation of Thr 1381 and the side chains of other residues (Figure 6.8.A, B, C). The ATP binding site is very well preserved in both predictions, however interestingly the phenyl ring of the Phe 1235 residue appears to be opposed between the two models (Figure 6.8.D). The active site including the important catalytic aspartate residue Asp 1350 was almost identically positioned in both the MAP3K1 CS and MAP3K1 prediction models.
4.2.4.5. The Zinc-Finger SWIM Domain

The zinc-finger binding SWI2/SNF2 and MuDR (ZnF SWIM) domain found in MAP3K1 has been shown to be required for the binding of MAP3K1 to Jun to promote c-Jun ubiquitination (Rieger et al., 2012). When the two full length protein models are
superimposed the ZnF SWIM domains are located in a well matched portion of the 3-D structure; position and accessibility appear similar in both proteins.

When the two ZnF SWIM domains are isolated and superimposed, it is apparent that the MAP3K1CS predicted structure contains a longer α-helical domain than the wild-type MAP3K1. The longer α-helix structure contains the Cys 350 and His 352 residues.

Figure 4.9. Alignment of predicted MAP3K1 and MAP3K1CS ZnF SWIM domains. Panels A and B show predicted ZnF SWIM domains in MAP3K1 (A, green/blue) and MAP3K1CS (B, red/gold). Panel C shows that when the full length models are superimposed, the domains are situated in a similar orientation and position within both prediction models.
important for zinc binding in the domain. It is possible that this increase in α-helical structure, and position of these important residues, could affect interactions between MAP3K1CS and c-Jun or have an effect on the function or specificity of the ZnF SWIM domain (Figure 4.10.).

![Figure 4.10. Predicted structures of isolated MAP3K1 and MAP3K1CS ZnF SWIM domains.](image)

4.2.4.6. The Zinc-Finger RING Domain

Contained within the plant homeobox domain (PHD) is the ZnF RING domain. This motif has been shown to be essential for the E3-Ubiquitin ligase activity that makes MAP3K1 unique amongst MAP3Ks (Lu et al., 2002). Similar to the ZnF SWIM domain, the location of the ZnF RING domain within the tertiary folding is maintained when the two full length protein models are superimposed (Figure 4.11.).

When the ZnF RING domains from the two models are isolated and superimposed the general α-helical domains are reasonably well aligned, however many of the important zinc binding residues, notably Cys 454 and Cys 483, show considerable orientation and positional differences between the two predicted models (Figure 4.12.). These residues in conjunction with neighbouring cysteine residues, Cys 458 and Cys 474, are pairs two and
four of the eight metal binding residues contained in the domain. ZnF RING domains are known to bind two zinc atoms by the formation of a ‘cross-brace’ system; these four residues are responsible for binding one of these. It is unclear whether the predicted relative position of these residues would alter specificity or functionality of the ZnF RING domain in MAP3K1CS. Although the positions in the tertiary structure appear altered, they are in regions with no secondary structure which are known to be flexible and so may still permit zinc binding; in the MAP3K1CS model however, the Cys 458 residue is located within an α-helix structure which may affect this.
**Figure 4.11. Alignment of predicted MAP3K1 and MAP3K1\textsuperscript{CS} ZnF RING finger domains.** The highlighted ZnF RING domain in wild-type MAP3K1 (A, blue/green) and MAP3K1\textsuperscript{CS} (B, gold/red). When the full length models are superimposed, the domains are situated in a similar orientation and position within both prediction models (C).
4.2.4.7. The Armadillo-type Fold Region

When the armadillo-type fold (ARM) region is examined on the full length models the difference in size is apparent, although the structure of both predicted domains agrees with the definition of the super-helical ARM repeat domain as described in the literature (Figure 4.13.). When the domains are isolated, alignment of the remaining helices is good.
(Figure 4.13.D), however, if the predicted size of the domain is correct it would be hard to see how functionality would not be perturbed in some way by the truncation.

Figure 4.13. Analysis of predicted MAP3K1 and MAP3K1<sup>CS</sup> ZnF ARM domains. Panel A shows the ARM domains of wild-type MAP3K1 (blue/green) and MAP3K1<sup>CS</sup> (gold/red) highlighted within the full length superimposed models. When the ARM domains are isolated it is clear the MAP3K1 (B) is larger and contains more α-helices than the MAP3K1<sup>CS</sup> (C) ARM domain. Aside from size differences, the remaining α-helices align well (D)
4.2.5. Protein Phosphorylation

4.2.5.1. Simple Western Peggy™ Size Assay

To investigate effects of the goya mutation downstream of MAP3K1, the Peggy™ (Simple Western) system was used. The Peggy™ system uses capillary based separation and immuno-detection of proteins, which can be identified either by size or charge. As previously described, canonical MAPK signalling consists of a cascade of phosphorylation events. Once activated by a stimulus, a MAP3K protein phosphorylates a MAP2K protein, in the case of MAP3K1 this is ordinarily MAP2K4 or MAP2K7, although in certain conditions may be other MAP2K proteins. The MAP2K’s in turn phosphorylate a MAPK protein, for MAP3K1 this cascade usually results in phosphorylation and activation of the MAPK JNK, although it can also signal through ERK1/2 and p38. In order to ascertain if phosphorylation of any of these MAPK’s was affected as a result of the goya mutation, antibodies for both total and phosphorylated forms of these main branches of the MAPK cascade were used.

Whole inner ears were dissected from litters of P1 mice, immediately snap frozen on dry ice, and a tail biopsy taken for genotyping purposes. A total of three ears from separate individual mice were pooled according to genotype and total protein was extracted. No differences in phosphorylation were detectable in ERK1/2 or JNK (data not shown). However, a trend towards increased p38 phosphorylation in Map3k1^{goya/goya} inner ear lysates was observed (Figure 4.14.).
4.2.5.2. Immunohistochemistry

To further investigate p38 MAPK phosphorylation in Map3k1\(^{goya/goya}\) inner ears, immunohistochemistry was performed on paraffin embedded cochlear sections of P1 littermates (Figure 4.15). Map3k1\(^{goya/goya}\) mice (Figure 4.15.Bi, Bii) show intense nuclear staining of phosphorylated-p38 in all cell types in the cochlear duct and SGN. The majority of nuclei in the surrounding structures such as the spiral ligament and spiral limbus were also intensely stained. Map3k1\(^{+/+}\) mice displayed a similar pattern of nuclear expression. However, for identical experimental conditions the staining was much weaker and fewer nuclei in the surrounding structures stained positive for phosphorylated-p38 MAPK (Figure. 4.15.Ai, Aii).
To quantify the increased phospho-p38 positive staining in the P1 mutant cochlea, the ImageJ (https://imagej.nih.gov/ij/) plugin ImmunoRatio was used (Figure 4.16A). Sections from the mid-turn of all *Map3k1<sup>goya/goya</sup>* and *Map3k1<sup>+/+</sup>* mice in the litter were analysed. ImmunoRatio has been designed to diagnostically assess the percentage area of positively DAB stained nuclei in a given sample; this is achieved by a combination of colour deconvolution and adaptive thresholding, however the analyses do not take intensity of stain into account (Tuominen et al., 2010). The results show an increase in positively stained nuclear area in *Map3k1<sup>goya/goya</sup>* mice (n=3) when compared with *Map3k1<sup>+/+</sup>* (n=2) (Figure 4.16B).

Figure 4.15. Phosphorylated p38 expression in the mid-turn of P1 cochleae. Immunohistochemistry of P1 *Map3k1<sup>+/+</sup>* (A) and *Map3k1<sup>goya/goya</sup>* (B) cochlear sections using an anti-phosphorylated p38 (pp38) antibody. Widespread nuclear expression of pp38 is seen in cells surrounding the cochlear duct in both *Map3k1<sup>+/+</sup>* (Ai) and *Map3k1<sup>goya/goya</sup>* (Bi) mice, however, the depth of staining is greatly increased in *Map3k1<sup>goya/goya</sup>* cochleae. In addition to the depth of staining, nearly all nuclei are stained in the *Map3k1<sup>goya/goya</sup>* cochlea, however some nuclei in spiral ligament, spiral limbus and basilar membrane in the *Map3k1<sup>+/+</sup>* cochlea remained unstained with this length of chromogenic exposure. The same increased positive staining for pp38 was observed in the spiral ganglion neurons (SGN) of *Map3k1<sup>goya/goya</sup>* (Bi) mice compared to *Map3k1<sup>+/+</sup>* (Aii). These results were consistent for littermate controls (*Map3k1<sup>+/+</sup>* n=2 *Map3k1<sup>goya/goya</sup> n=3). Cochlear duct – CD, spiral ganglion neurons – SGN. Scale bars represent 50 µm. (Figure adapted from (Parker et al., 2015)).
Immunohistochemistry was also performed using anti-phospho-JNK and anti-phospho-ERK1/2 antibodies (Figure 4.17). Consistent with the Peggy™ analysis, no obvious differences in intensity or expression pattern were observed between the genotypes for either antibody. It is worth noting that in contrast to the nuclear expression pattern of phospho-p38 MAPK, both antibodies show strong labelling beneath the basal surface of the IHCs and OHCs, consistent with the location of SGN neurite extensions at the P1 time point. In addition low-level anti-phospho JNK was detected in the cytoplasm and nuclei of cells throughout the organ of Corti of both Map3k1<sup>goya/goya</sup> and Map3k1<sup>+/+</sup> mice (Figure 4.17.A, B).

![Figure 4.16. Quantification of increased p38 phosphorylation.](image)

Parker et al., 2015
4.2.6. Gene Expression

4.2.6.1. MAPK Pathway Array

One outcome of MAPK phosphorylation cascades is transcriptional regulation of genes by activation or repression of transcription factors. To this end gene expression analysis was performed on RNA extracted from P1 cochlear ducts of Map3k1^{+/+} and Map3k1^{goya/goya} mice. Initially the RT^2 Profiler PCR Array Mouse MAP Kinase Signaling Pathway (SABiosciences) was trialled. This array contained qRT-PCR probes for 84 genes within the MAPK signalling pathway, and was used as a cheaper and faster alternative to RNAseq to obtain an overview of the effects of the goya mutation on downstream gene expression. Initially one plate for Map3k1^{goya/goya} and one for Map3k1^{+/+} was used with a view to repeating to confirm interesting results. Cochlear ducts were dissected from P1 Map3k1^{goya/goya} and Map3k1^{+/+} mice and the lateral wall and stria vascularis removed to leave the organ of Corti. Four samples collected from two animals per genotype were
pooled and total RNA isolated, before first strand cDNA synthesis was performed using random hexamer oligonucleotides. The MAPK pathway array assay was then run according to manufacturer’s instructions. Table 4.2. shows genes that were identified as differentially regulated between $\text{Map3k1}^{+/+}$ and $\text{Map3k1}^{goya/goya}$ organs of Corti, using Beta-actin ($\text{Actb}$) as a reference gene. Interestingly out of the 84 target genes only 10 were differentially regulated, nine upregulated and one downregulated (Table 4.2.). Furthermore, the relative fold changes were lower than we had expected given the integral part MAP3K1 is known to play in the MAPK signalling cascade; only six genes, Collagen, type I, alpha 1 ($\text{Col1a1}$), delta-like 1 homolog ($\text{Dlk1}$), V-Mos Moloney Murine Sarcoma Viral Oncogene Homolog (Mos), FBJ Murine Osteosarcoma Viral Oncogene Homolog (Fos), Epidermal growth factor receptor (Egfr) and Heat shock 70 kDa protein 5 ($\text{Hspa5}$) had a relative fold change >2 in $\text{Map3k1}^{goya/goya}$ organ of Corti when compared to $\text{Map3k1}^{+/+}$. The only gene downregulated in $\text{Map3k1}^{goya/goya}$ organ of Corti was $\text{Ccna1}$ ($\text{CyclinA1}$). Given the relatively low number of differentially expressed genes and the low fold changes observed, combined with the required financial outlay, instead of repeating the MAPK pathway array assay, individual hydrolysis probes were utilised to further study gene expression.

<table>
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<th>average Ct- hom</th>
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</table>

Table 4.2. Genes showing differential expression between $\text{Map3k1}^{+/+}$ and $\text{Map3k1}^{goya/goya}$ in P1 organs of Corti. Using the 84 gene MAPK pathway array, five genes $\text{Col1a1}$, $\text{Dlk1}$, $\text{Mos}$, $\text{Fos}$, $\text{Egfr}$ and $\text{Hspa5}$ had a relative fold change (RQ) >2 in $\text{Map3k1}^{goya/goya}$ organ of Corti when compared to $\text{Map3k1}^{+/+}$. Another four genes, heat shock 70 kDa protein 5 ($\text{Hspa5}$), $\text{Mef2c}$, $\text{E2f1}$ and $\text{Map3k1}$ itself were upregulated <2 fold. Only $\text{Ccna1}$ ($\text{CyclinA1}$) was downregulated in $\text{Map3k1}^{goya/goya}$ organ of Corti relative to $\text{Map3k1}^{+/+}$. 164
4.2.6.2. Gene Expression Using TaqMan® Hydrolysis Probes

To validate the results obtained in the MAPK pathway array assay, and to investigate other potential MAP3K1 targets, total RNA was isolated from the organs of Corti of additional Map3k1^goya/goya and Map3k1^+/− mice (n≥3 pools per genotype/assay) and analysed using gene specific hydrolysis probes. For each pool cDNA was synthesised and run in triplicate to assess mRNA levels for each specific assay. TaqMan® hydrolysis probes were obtained for several of the genes showing differential expression between genotypes in the MAPK profiler array. In order to try and elucidate the molecular mechanism underlying the extra OHC phenotype in Map3k1^goya/goya mice, expression levels of additional genes that previously reported to be involved with causing similar phenotypes in mice.

4.2.6.2.1. p27KIP1/RB Pathway

As mentioned earlier, in the retina of Map3k1^tm1Yxia/tm1Yxia mice a number of genes relating to activity of the p27KIP1/RB pathway were found to be upregulated. In order to assess if the same was true in the organ of Corti of Map3k1^goya/goya mice, expression of several of these genes were investigated. In the MAPK profiler array assay Ccna1 (Cyclin A1) was 2 fold downregulated in Map3k1^goya/goya organs of Corti, however, qRT-PCR analysis using TaqMan® hydrolysis probes revealed this was not the case; no difference was seen between wild-type or Map3k1^goya/goya samples. No large fold differences were observed in any of the genes analysed (Ccnd1 (Cyclin D1), Dhfr, E2f1 and Rb1), however E2f1 showed a small but significant upregulation (p=0.0379, relative fold change (RQ)=1.5) in Map3k1^goya/goya samples when compared with wild-type (Figure 4.18.).
4.2.6.2.2. WNT Signalling Pathway

In the study investigating the involvement of MAP3K1 in human patients with 46 X,Y sex reversal, it was concluded that mutations of MAP3K1 result in enhanced WNT signalling; in NT2/D1 cells transfected with MAP3K1 constructs containing the various mutations, all resulted in a large upregulation of Ctnbb1 (β-catenin) and increased binding of MAP3K1 to AXIN1 and Ras homolog gene family member A (RHOA) (Loke et al., 2014). In the cochlea, increased expression of Ctnbb1 (β-catenin) has been shown to upregulate Axin2, and Lgr5, two markers of WNT signalling important for hair cell development (Chai et al., 2011). At P1, the age that RNA was isolated from the organ of Corti samples used in this experiment, Lgr5 is expressed in the third row of Deiters’ cells on the lateral edge of the greater epithelial ridge (GER) (Chai et al., 2011). When Ctnbb1 (β-catenin) is
overexpressed in new-born cochlear explants, it has been shown that post-mitotic Lgr5 expressing supporting cells can trans-differentiate into hair cells and replace damaged cells (Bramhall et al., 2014; Shi et al., 2013; Shi et al., 2012). Expression of Ctnnb1 (β-catenin), Axin2, and Lgr5 in P1 Map3k1goya/goya and wild-type organ of Corti samples was investigated. Axin2 showed a significant relative fold change in the homozygote cochleae (RQ=1.3), but no significant fold changes were observed in Lgr5 or Ctnnb1 (Figure 4.19.).

4.2.6.2.3. JNK Target Genes

Although no differences in JNK phosphorylation were seen at the protein level, expression of the JNK targets, Jun and Fos were also analysed. Both JNK targets showed a trend of upregulation; a ~1.5 relative fold change was observed in relation to Jun expression in Map3k1goya/goya cochlea compared to wild-type, however neither Fos or Jun showed significant differences in expression levels (Figure 4.20.).
4.2.7. Investigation of Cellular Proliferation in the Developing Cochleae of Map3k1 Mutant Mice

4.2.7.1. Immunohistochemistry Using an Anti-Ki-67 Antibody

Initially to investigate if cellular proliferation was increased in developing Map3k1 mutant cochleae, an anti-Ki-67 antibody was used. Ki-67 is a protein expressed at active stages in the cell cycle, and as such is frequently used as a marker of proliferation (Scholzen and Gerdes, 2000). The prosensory cells in the mouse cochlea are known to undergo their final division by embryonic day 14.5 (E14.5). Inter-crossed pregnant females were plug checked to confirm successful mating, subsequently sacrificed at 16.5 days post coitum (d.p.c.) and embryos were harvested. Immunohistochemistry using the anti-Ki-67 antibody was performed on sections prepared from the embryonic heads. No positively stained nuclei were observed in the developing organ of Corti, or in any of the cells contained within the epithelial lining of the cochlear duct in either Map3k1<sup>+/+</sup> (n=3) or Map3k1<sup>goya/goya</sup> (n=3); whereas positively stained cells are seen in the surrounding tissue, confirming the efficacy of the antibody (Figure 4.21.). These data suggested that no cells...
were proliferating within the developing organ of Corti in *Map3k1<sup>goya/goya</sup>* mice at this time-point.

**Figure 4.21. Investigation of proliferation in E16.5 *Map3k1<sup>goya/goya</sup>* cochleae using anti-Ki-67 antibody.** Immunohistochemistry using anti-Ki-67 antibody to identify proliferating cells did not label any cells within the immature organ of Corti in either E16.5 *Map3k1<sup>+/+</sup>* (A,B,C) or *Map3k1<sup>goya/goya</sup>* (D,E,F) sections. Organ of Corti – OoC. All scale bars represent 50 µm.

### 4.2.7.2. EdU Click Chemistry

Although the immunostaining using the anti-Ki-67 antibody indicated that proliferating cells were not present in the *Map3k1<sup>goya/goya</sup>* at E16.5, a more sensitive approach was employed to investigate if there were subtle differences either spatially or temporally. The thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) can incorporate itself in place of thymidine in *de novo* synthesised DNA in cells undergoing the S phase of the cell cycle.
EdU has an advantage over the more traditionally used 5-bromo-2'-deoxyuridine (BrdU) in that it is detected using a copper catalysed reaction between an alkyne contained within EdU, and an azide labelled probe; a process termed a ‘click’ reaction. BrdU is detected with an antibody and requires DNA denaturation which can damage tissue and also affect antigenicity of the specimen. The small size of the azide labelled probe means that it can penetrate quickly into tissue and react with the EdU without the need for denaturing steps, resulting in faster and more sensitive labelling, with no damage to the specimen (Salic and Mitchison, 2008) (see materials and methods).

Inter-crossed pregnant \textit{Map3k1}\textsuperscript{tm1Yxia/+} females were injected at 14.5 d.p.c with EdU, twice at 2 hourly intervals, and sacrificed 2 hours after the second injection. Embryos were collected and heads removed and processed as for immunohistochemistry. The click reaction was performed on sections using Click-iT Plus™ EdU Alexa Fluor 594 Imaging Kit (Life Technologies) and DAPI (4',6-diamidino-2-phenylindole) was used as a nuclear stain (Figure 4.22.). Using EdU at E14.5, proliferating nuclei were observed around the cochlear duct of both \textit{Map3k1}\textsuperscript{+/+} and \textit{Map3k1}\textsuperscript{tm1Yxia/tmY1xia} mice, in particular in the GER and Köllikers organ (KO). Interestingly EdU positive nuclei were observed also in the region of the lesser epithelial ridge (LER) from which the OHCs and Deiters’ cells are derived.

\textbf{4.2.7.3. Spatial Expression and Localisation of p27KIP1}

To investigate if cochlear p27KIP1 expression or localisation was affected by MAP3K1 deficiency, and to see if the EdU positive cells in \textit{Map3k1}\textsuperscript{tm1Yxia/tmY1xia} cochlea were localised within the ZNPC, EdU was used in addition to an anti-p27KIP1 antibody. The click reaction was performed on sections from the same E14.5 cochleae used in the previous experiment, and immediately afterwards the sections were processed for immunofluorescence imaging using the anti-p27KIP1 antibody and an Alexa Fluor 488
secondary antibody. Figure 4.23. shows that p27KIP1 localisation is similar to $\text{Map3k1}^{+/+}$ in the $\text{Map3k1}^{tm1Yxia/tm1Yxia}$ cochlea at E14.5.

Figure 4.22. Investigation of proliferation in E14.5 $\text{Map3k1}^{tm1Yxia/tm1Yxia}$ cochleae using EdU. Click chemistry based detection of EdU (Red) in E14.5 developing $\text{Map3k1}^{+/+}$ and $\text{Map3k1}^{tm1Yxia/tm1Yxia}$ cochleae showed proliferating nuclei in the GER and KO of both wild-type and mutant. In the mid turn of the $\text{Map3k1}^{tm1Yxia/tm1Yxia}$ cochlea proliferating cells are also labelled in the LER (arrow) that gives rise to OHC and supporting cells. Greater epithelial ridge – GER, lesser epithelial ridge – LER, Köllikers organ – KO. Scale bars represent 20 µm.
In higher magnification images of Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochleae, EdU positive nuclei can be seen in the LER in the mid and apical turns, however, they do not overlap with the ZNPC as demarked by p27KIP1 expression, this was the case for all cochleae examined (Map3k1<sup>tm1Yxia/tm1Yxia</sup> n=3, Map3k1<sup>+/+</sup> n=3) (Figure 4.24.). This shows that by E14.5, the ZNPC is established in Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochleae as it is in wild-type cochleae, and that sensory precursor cells have exited the cell cycle.

4.2.7.4. Temporal Expression and Localisation of p27KIP1

To investigate if there is a temporal effect on p27KIP1 expression or localisation the experiment was repeated with pregnant females injected with EdU at 18.5 d.p.c. By this time-point expression of p27KIP1 is known to be downregulated in hair cells and upregulated in supporting cells (Chen and Segil, 1999). Again no difference was found in localisation of p27KIP1 in mutant cochleae. Moreover, no proliferating cells were seen anywhere in the cochlear duct in either Map3k1<sup>tm1Yxia/tm1Yxia</sup> or Map3k1<sup>+/+</sup> littermate controls in any of the cochlear turns (Figure 4.25)
Figure 4.24. Co-detection of p27KIP1 and EdU. Higher magnification (40X) immunofluorescent detection of EdU and anti-p27KIP1. Panels A,C,E,G,I and K show both EdU (red) and p27KIP1 (green) labelling of the basal, mid and apical cochlear turns from $Map3k1^{+/+}$ (A,E and I) and $Map3k1^{tm1Yxia/tm1Yxia}$ mice (C,G and K). The greater and lesser epithelial ridges are labelled, and the boundaries of p27KIP1 expression are highlighted (white lines). Panels B,D,F,H,J and L are identical images to the corresponding images on the left with the p27KIP1 channel removed to allow assessment of EdU labelling. Although the basal and mid cochlear turns of $Map3k1^{tm1Yxia/tm1Yxia}$ show EdU positive nuclei in close proximity to the lesser epithelial ridge (D and H), none overlap with the p27KIP1 expression as demarked by the white lines). Lesser epithelial ridge – LER, greater epithelial ridge – GER. Scale bars represent 20 µm.
Taken together these data indicate that the mechanism resulting in extra OHC in Map3k1 mutant mice appears not to involve increased or extended proliferation of pro-sensory precursor cells, and that MAP3K1 deficiency does not have any obvious effect on p27KIP1 expression or localisation.

4.2.8. Investigation of Possible Map3k1 and Map3k4 Interactions or Functional Redundancy

4.2.8.1. Generation of Map3k1 and Map3k4 Double Mutants

Both MAP3K1 and MAP3K4 can activate the JNK and p38 MAPK pathways. To investigate any potential functional redundancy between these two MAP3Ks, matings were set up to produce progeny heterozygous for both the kinase deficient allele of Map3k1 and Map3k4 null allele (Map3k1<sup>tm1Yxia/+</sup>:Map3k4<sup>tm1Fly/+</sup>). The double heterozygote mice

Figure 4.25. Co-detection of EdU and p27KIP1 at E18.5. Immunofluorescent detection of EdU (red) and p27KIP1 (green) EdU and anti-p27KIP1 at E18.5. The basal, mid and apical cochlear turns of Map3k1<sup>+/+</sup> (A,B and C) and Map3k1<sup>tm1Yxia/tm1Yxia</sup> (D,E and F) respectively, all show no EdU positive cells in the epithelial lining of the cochlear duct. By this time-point p27KIP1 expression is mainly restricted to the supporting cells around the developing organ of Corti. Scale bars represent 50 µm.
subsequently underwent analysis to see if any of the phenotypes observed in the \textit{Map3k1} mutants were replicated.

All progeny from this cross were born with their eyes closed including \( \text{Map3k1}^{\text{tm1Yxia}/+}:\text{Map3k4}^{\text{tm1Flv}/+} \) mice. ABR analysis at 9-weeks of age revealed no significant differences between the double heterozygote mice (n=4) and wild-type (n=2) or \( \text{Map3k4}^{\text{tm1Flv}/+} \) (n=3) mice (Figure 4.26).

![Figure 4.26. 9-week old ABR analysis of \( \text{Map3k1}^{\text{tm1Yxia}/+}:\text{Map3k4}^{\text{tm1Flv}/+} \). No differences were observed in ABR thresholds between \( \text{Map3k1}^{\text{tm1Yxia}/+}:\text{Map3k4}^{\text{tm1Flv}/+} \) and either \( \text{Map3k1}^{+/+}:\text{Map3k4}^{\text{tm1Flv}/+} \) or wild-type mice at 9-weeks of age. Data shown are mean ± standard error of the mean.](image)

As expected from the ABR result, analysis of the organs of Corti from \( \text{Map3k4}^{\text{tm1Flv}/+} \) and \( \text{Map3k1}^{\text{tm1Yxia}/+}:\text{Map3k4}^{\text{tm1Flv}/+} \) mice using SEM, revealed no degeneration of hair cells. Moreover, all \( \text{Map3k1}^{\text{tm1Yxia}/+}:\text{Map3k4}^{\text{tm1Flv}/+} \) organs of Corti displayed extra OHC with similar regularity seen in \( \text{Map3k1}^{\text{tm1Yxia}/+} \) mice, presumably due to the presence of this allele. No hair cell phenotype was observed in \( \text{Map3k4}^{\text{tm1Flv}/+} \) mice which displayed the normal arrangement of one row of IHCs and three rows of OHCs (Figure 4.27.).
These data seem to suggest there is no genetic interaction between Map3k1 and Map3k4 in the pathways determining EOB or sensory hair cell development and survival; however, it may be that haplo-insufficiency of both genes is permissible in relation to the degenerative aspects of MAP3K1 deficiency.

4.2.8.2. Generation of Map3k1 Deficient, Map3k4 Overexpressing mice

In in vitro studies, NT2/D1 cells transfected with a variety of MAP3K1 mutant constructs all resulted in differential gene expression effects; interestingly co-transfection of the same cells with both mutant MAP3K1 and wildtype MAP3K4 constructs rescued the dysregulation in all cases investigated (Loke et al., 2014). The sexual development group at the MRC Harwell Institute had previously generated BAC transgenic mice that overexpress Map3k4, and kindly made these mice available.

Analysis of Map3k1tm1Yxia/+:Map3k4tm1Flv/+ mice indicated there was no redundancy between these two genes in relation to the EOB or auditory phenotypes. However, the previously reported total phenotypic rescue in ES cells suggested that MAP3K4 is capable of ameliorating MAP3K1 deficiency. To investigate if this was the case in vivo and,
importantly, in the cochlea, mice were produced that were homozygous for the \( \text{Map3k1}^{\text{tm1Yxia}} \) allele, and also carried the \( \text{Map3k4} \) over-expressing BAC transgene.

In order to see if an auditory phenotype resulted from \( \text{Map3k4} \) over-expression alone, and to reduce any potential masking effects of modifiers within the mixed genetic background of the progeny, all genotype combinations were investigated.

The \( \text{Map3k1}^{\text{tm1Yxia}/\text{tm1Yxia}}:\text{Map3k4}^{\text{BAC/+}} \) mice displayed EOB the same as the \( \text{Map3k1}^{\text{tm1Yxia}/\text{tm1Yxia}} \) mice (data not shown). Subsequent ABR analysis of 9-week old mice showed no significant differences between \( \text{Map3k1}^{\text{tm1Yxia}+/+} \) or \( \text{Map3k1}^{+/+} \) mice carrying the \( \text{Map3k4} \) BAC transgene and those that were wild-type for \( \text{Map3k4} \) at any frequency tested. The \( \text{Map3k1}^{\text{tm1Yxia}/\text{tm1Yxia}}:\text{Map3k4}^{\text{BAC/+}} \) ABR thresholds were also not significantly different to \( \text{Map3k1}^{\text{tm1Yxia}/\text{tm1Yxia}}:\text{Map3k4}^{+/+} \) (Figure 4.28.).

![Figure 4.28. Investigation of the effect of \( \text{Map3k4} \) overexpression on auditory function. ABR analysis of all genotypic combinations of wild-type, \( \text{Map3k1}^{\text{tm1Yxia}} \) heterozygote or homozygote mice, either with or without the \( \text{Map3k4} \) overexpressing BAC transgene. The mice carrying the \( \text{Map3k4} \) BAC transgene showed no significant differences in ABR thresholds from those that did not for any of the genotypes tested. Data shown are mean ± standard error of the mean. Data were analysed using unpaired students t-test between each genotype with and without the BAC transgene.](image)

Overexpression of \( \text{Map3k4} \) also seemed not to have any effect on the extra OHC phenotype exhibited by both \( \text{Map3k1}^{\text{tm1Yxia}} \) heterozygote and homozygote mice (Figure
When Map3k1tm1Yxia/tm1Yxia SEM images were analysed, OHC degeneration with an apical to basal gradient of severity was observed in mice that carried the BAC transgene and also those that did not. However, it appeared that Map3k1tm1Yxia/tm1Yxia::Map3k4BAC/+ cochleae were not as severely affected as those that did not overexpress Map3k4 (Figure 4.29.Q – X). To quantify this observation, cell counts were performed to assess the total number of OHCs spanning the apical, mid apical and mid turns (≤360° from the apex). Two of the four Map3k1tm1Yxia/tm1Yxia::Map3k4BAC/+ mice examined had a far greater number of OHCs remaining at 9-weeks of age than any of the other animals, however the remaining two mice had numbers similar to those not carrying the Map3k4 BAC transgene; this variance resulted in no significant difference in OHC numbers between Map3k1 deficient mice overexpressing Map3k4 and those which did not (p=0.1864) (Figure 4.30.).

Taken together, these data suggest that there is no functional redundancy between Map3k1 and Map3k4 in the pathways responsible for regulating epithelial sheet movement during eyelid development, OHC number or maintaining auditory function in mice. The slower rate of OHC degeneration in two of the four Map3k1tm1Yxia/tm1Yxia::Map3k4BAC/+ cochleae may be a sign that MAP3K4 can, at least in part, compensate for a lack of functional MAP3K1 in the pathway(s) ultimately responsible for the OHC loss; however the lack of significance due to the remaining two mice would suggest this is more likely a result of individual variability. It is possible this could be introduced due a modifier in the genetic background, however the Map3k4BAC/+ mice are congenic on the C57BL/6J background which often exacerbates auditory phenotypes rather than having a protective effect.
Figure 4.29. Investigation of the effect of \textit{Map3k4} overexpression on cochlear hair cells. Scanning electron micrographs from the apical, mid-apical, mid and mid basal regions of 9-week old \textit{Map3k1}^{+/+}:\textit{Map3k4}^{+/+} (A, B, C, D), \textit{Map3k1}^{+/+}:\textit{Map3k4}^{BAC/+} (E, F, G, H), \textit{Map3k1}^{tm1Yxia/+}:\textit{Map3k4}^{+/+} (I, J, K, L), \textit{Map3k1}^{tm1Yxia/+}:\textit{Map3k4}^{BAC/+} (M, N, O, P), \textit{Map3k1}^{tm1Yxia/tm1Yxia}:\textit{Map3k4}^{+/+} (Q, R, S, T) and \textit{Map3k1}^{tm1Yxia/tm1Yxia}:\textit{Map3k4}^{BAC/+} (U, V, W, X). The BAC transgene appeared to have no effect on wild-type or \textit{Map3k1}^{tm1Yxia/+} hair cell numbers (A-P). The \textit{Map3k1}^{tm1Yxia/tm1Yxia} mice showed OHC degeneration with an apical to basal gradient of severity with or without the BAC transgene (Q-X), however the degeneration did not seem as severe in some of the \textit{Map3k1}^{tm1Yxia/tm1Yxia}:\textit{Map3k4}^{BAC/+} (U, V, W). Scale bars represent 10 µm.
Figure 4.30. Quantification of OHC number in 9-week old Map3k1tm1Yxia/tm1Yxia:Map3k4BAC/+ mice. Representative scanning electron micrographs showing an overview of the cochleae of 9-week old Map3k1tm1Yxia/tm1Yxia:Map3k4BAC/+ (A and C) and Map3k1tm1Yxia/tm1Yxia:Map3k4+/+ (B and D) mice. The areas still containing OHC have been highlighted in purple to aid visualisation. Two of the Map3k1tm1Yxia/tm1Yxia:Map3k4BAC/+ cochleae (shown here in A and C) had many more OHC remaining than any of the Map3k1tm1Yxia/tm1Yxia:Map3k4+/+ mice not carrying the BAC transgene (B and D). Quantification of OHC number in the entire first turn of four mice from each genotype highlighted this large increase, however the difference between the genotypes was not significant (p=0.1864). Data analysed by student’s t-test. Scale bars represent 100 µm.

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E. OHC counts
4.3. Discussion

4.3.1. Effects of the *goya* Mutation on MAP3K1 Structure

The IVS13+2T>C ‘goya’ mutation in the intron 13 splice donor site of *Map3k1* results in aberrant splicing of exons 12 and 13/14. Analysis of mRNA in the organ of Corti of P1 *goya* mice showed that two main splice events occur as a result of the mutation; firstly a low abundance splice variant which skips exon 13 completely and would produce a severely truncated protein lacking the kinase domain; and a greatly more abundant splice variant which utilises a cryptic splice donor site within exon 13, which if translated would produce an in-frame deletion of 27 aa.

Analysis of the predicted domains within *goya* MAP3K1<sup>CS</sup> indicated that an area of low complexity was missing, and also a predicted ARM-type fold was shifted and truncated from the wild-type MAP3K1. These could have both structural and functional effects on the protein; both motifs have been associated with protein binding, and they are located in close proximity to the ZnF RING domain of MAP3K1. This could be important as the ZnF RING containing PHD domain has been shown to be responsible for the wide ranging E3 ubiquitin ligase activity which makes the protein unique amongst the MAP3Ks (reviewed in (Suddason and Gallagher, 2015).

The analysis of secondary and tertiary protein structure differences caused by the *goya* mutation is complicated by the lack of a solved crystal structure for MAP3K1. This is most likely due to insolubility of the protein; as seen in the SMART protein analysis MAP3K1 contains a number of low complexity regions, it is reported that proteins containing these motifs are not readily crystallised (Le Gall et al., 2007). For this reason the 3D structure analysis of MAP3K1 and MAP3K1<sup>CS</sup> is entirely predicted and therefore may, or may not,
be representative of physiological structural differences resulting from the \textit{goya} mutation.

Notwithstanding this entirely computational method, the main differences in predicted structure were perhaps unsurprisingly observed in domains in close proximity to the site of the deletion in the primary structure. This would to an extent suggest that rather than a pure lack of kinase activity, alterations in protein binding and possibly ubiquitination, associated with normal function of full length MAP3K1 could be responsible for the observed phenotype in \textit{goya} mice. This is interesting as the observed phenotypes of Map3k1\textsuperscript{goya/goya} and Map3k1\textsuperscript{tm1Yxia/tm1Yxia} mice are almost identical. One explanation for this could be that auto-phosphorylation of residues outside the active site may be necessary for some functions of full length MAP3K1; it has previously been shown that Thr 1381 and Thr 1393 of the active site in the kinase domain undergo auto-phosphorylation (Deak and Templeton, 1997) which is required for activation. The extent of auto-phosphorylation that occurs in MAP3K1 is far greater than just these two residues, spanning the whole full length polypeptide (Chadee et al., 2002). However, it is unclear as to the exact mechanisms of auto phosphorylation, or for which MAP3K1 functions, if any, the additional auto-phosphorylation is required. Perhaps a more plausible explanation is the fact that oligomerisation with additional proteins can activate or prime MAP3K1 kinase activity (Baud et al., 1999; Chadee et al., 2002); the MAP3K1 regulated processes regulating the observed eye and inner ear phenotypes may require binding of certain proteins that the deletion interferes with. In a similar manner the previously described functionality of MAP3K1 as a scaffold protein may be perturbed in both Map3k1\textsuperscript{goya/goya} and Map3k1\textsuperscript{tm1Yxia/tm1Yxia} mice.
4.3.2. The Effects of the goya Mutation on Downstream Protein Phosphorylation

Both capillary based immunodetection using the Peggy™ system and subsequent immunostaining highlighted an increase in p38 phosphorylation in the cochlear duct of \textit{Map3k1}^{goya/goya} mice at P1. The increase was not significant in the case of the Peggy data, but this is probably due to an outlier in one of the wild-type pooled lysates; the levels of phosphorylation in this one lysate were even higher than the goya homozygote lysates. An explanation for this could be a compromised health status of one or more of the wild-type animals used in this pooled sample, however, if the pups were overtly unhealthy or ‘runty’ they were not included in collection. Analysis of the whole dataset using the non-linear regression based ROUT method for identifying outliers in the PRISM software (Motulsky and Brown, 2006), highlighted this lysate as the only outlier. It was still identified when the stringency of the algorithm (Q) was increased to 0.2%, meaning that 99.8% of outliers detected would be true outliers, however it was still included in the analysis to avoid bias. When one way ANOVA using TUKEY post hoc analysis is performed on the dataset with the outlier removed the differences between the genotypes becomes significant (p=0.0418). Future studies may benefit from utilising additional proteomics platforms such as mass spectrometry to investigate possible differential phosphorylation events resulting from the goya mutation in more detail.

The increase in p38 phosphorylation indicated by the Peggy™ data was in agreement with observations made in previously reported \textit{in vitro} studies using MAP3K1 constructs harbouring known human mutations (Loke et al., 2014; Pearlman et al., 2010). For this reason immunohistochemistry was used to investigate p38 phosphorylation in \textit{Map3k1}^{goya/goya} cochlear ducts. The anti-phosphorylated p38 antibody labelled more nuclei and to a greater depth of staining in all three \textit{Map3k1}^{goya/goya} animals when compared to two wild-type littermates. This suggests that the trend seen in the Peggy™
data was real, however nearly all nuclei in the epithelia lining the cochlear duct and surrounding structures was positively labelled. This makes it hard to posit as to the effect increased p38 phosphorylation in relation to the hair cell and auditory phenotypes observed in MAP3K1 deficient mice. It is well known that both the JNK and p38 pathways can be activated either individually or simultaneously by common upstream stimuli (reviewed in (Kyriakis and Avruch, 1996)). As MAP3K1 preferentially activates the JNK axis, it is possible that effectively inhibiting this side of the pathway by way of MAP3K1 deficiency results in increased p38 phosphorylation. It is worth noting, however, that no differences in JNK (or ERK1/2) phosphorylation were observed in either the P1 Peggy™ data or by immunohistochemistry. This suggests that either JNK (and ERK1/2) activity in the cochlea at this time-point is mediated by a MAP3K1 independent mechanism, or that goya MAP3K1 is still able to activate JNK (and ERK1/2). This is still a possibility inasmuch as the immunohistochemistry was performed on sections from Map3k1<sup>goya/goya</sup> mice; the kinase domain of the cryptic splice site variant appears structurally very similar to wild-type and CASPASE-3 cleavage site is still exposed in the predicted 3-D model. There is therefore a possibility that kinase function may be retained in goya MAP3K1.

4.3.3. Effects of the goya Mutation on Gene Expression

The analysis of gene expression in P1 organ of Corti showed that E2f1 and Axin2 are slightly but significantly upregulated in Map3k1<sup>goya/goya</sup> cochleae when compared to wild-type. Whilst significant the relative fold changes were all <1.5; as a single PCR cycle represents a two-fold change, this is often set as a minimum threshold for confidence in the validity of a qRT-PCR experiment. This is particularly true when using sample sizes similar to those used in this experiment (n=3 pools per genotype). The MAPK pathway array assay, although only performed on one sample per genotype, also indicated that differences in gene expression were either subtle or absent. Only the E2f1 upregulation
was consistent between the MAPK pathway array and TaqMan® assays. It was also somewhat surprising that the subtle changes observed were in fewer genes than may be expected, given the pivotal role of MAP3K1 in the signalling pathway.

It is possible that these small fold changes may have an effect at a physiological level, in particular for complex regulatory mechanisms in signalling pathways; however, although technical replicates were very consistent, there was some individual variability between biological replicates. This could suggest the observed differences may not be representative of the true effects of the goya mutation on gene expression, or slight differences (hours) in age at time of sample collection may have introduced variability. It does highlight however that certainly at the P1 time-point investigated, none of the genes studied were drastically dysregulated in the Map3k1^goya/goya organs of Corti.

The main mode of regulation performed by MAP3K1, as the name suggests is phosphorylation, so it may not be surprising that few differences in gene expression were noted in the MAPK profiler array. Ultimately, however, transcriptional changes often occur following the phosphorylating signalling cascade. As the potential downstream targets are so large in number, a more in depth transcriptomic approach such as RNA sequencing (RNA Seq) may be required, ideally at a number of developmental stages.

4.3.4. Effects of MAP3K1 Deficiency on Cellular Proliferation in the Developing Cochlea

Evidence from previous studies point toward a possible role for MAP3K1 in regulating proliferation within the developing pro-sensory domain in the cochlear duct. The observed sustained proliferation and subsequent apoptosis of Müller glial cells in the retina of Map3k1^tm1Yxia/tm1Yxia mice was attributed to the p27KIP1/RB axis which regulates the cell cycle (Mongan et al., 2011). The fact that both p27Kip1 and Rb1 null mice exhibit supernumerary hair cell phenotypes similar to Map3k1 mutant mice, suggested that
dysregulation of this pathway may be the mechanism underlying the extra row of OHC in Map3k1\textsuperscript{goya/goya} and Map3k1\textsuperscript{tm1Yxia/tm1Yxia} cochleae (Chen and Segil, 1999; Kil et al., 2011; Lowenheim et al., 1999; Sage et al., 2005; Sage et al., 2006a).

As previously described, between E12.5 and E14.5, expression of p27KIP1 extends from the apex to the base of the cochlear duct, coinciding with cell cycle exit of all precursor cells in this domain, and resulting in the ZNPC (Lee et al., 2006). Two methods were used to investigate proliferation in the developing cochlea of Map3k1 deficient mice. The first, immunohistochemistry with an anti-Ki-67 antibody on E16.5 cochlear sections, showed no proliferating nuclei in the immature organ of Corti of either Map3k1\textsuperscript{goya/goya} or Map3k1\textsuperscript{+/+} mice.

The second and more sensitive approach of Click-chemistry allowed labelling of proliferating cells after treatment with the thymidine analogue EdU. At E14.5, positively labelled nuclei were observed in the cochlear duct of both Map3k1\textsuperscript{tm1Yxia/tm1Yxia} and Map3k1\textsuperscript{+/+} mice, in particular within the GER region. In the Map3k1\textsuperscript{tm1Yxia/tm1Yxia} mice, some nuclei in the LER region were also labelled, suggesting possible extended proliferation of pro-sensory precursor cells. However, upon further investigation using a combination of both EdU Click-chemistry and anti-p27KIP1 immunohistochemistry, it was apparent that none of the EdU positive cells overlapped with the ZNPC as defined by expression of p27KIP1. The expression pattern of p27KIP1 was consistent with that previously reported (Lee et al., 2006). Moreover no positively labelled cells were seen in the cochlear duct epithelia of either Map3k1\textsuperscript{tm1Yxia/tm1Yxia} or Map3k1\textsuperscript{+/+} mice at the E18.5 time-point, indicating that proliferation is not extended in Map3k1\textsuperscript{tm1Yxia/tm1Yxia} cochleae. These data suggest that the increase in OHC number is not related to aberrant proliferation arising from dysregulation of the p27KIP1 axis.
4.3.5. Investigation of Map3k1 and Map3k4 Interactions or Functional Redundancy

It is well known that MAP3K4 and MAP3K1 can both activate the JNK and p38 pathways under certain conditions. Often haploinsufficiency of two genes in the same pathway is enough to replicate a phenotype caused by a homozygous mutation of either gene, indicating interaction at a genetic level. Map3k4 has recently been reported to also be involved with cochlear patterning and auditory function. In our hands the Map3k4<sup>tm1Flv</sup> allele was homozygote lethal (Bogani et al., 2009), however this recent study used the Map3k4<sup>K1361R/K1361R</sup> allele and found that around a third of the homozygote mice reached adulthood (Haque et al., 2016). In contrast to Map3k1 mutants, Map3k4<sup>K1361R/K1361R</sup> mice have fewer cochlear hair cells, and exhibit defects in differentiation and neuronal patterning (Haque et al., 2016). To see if any genetic interaction occurred between Map3k1 and Map3k4 in the pathways determining the EOB or the auditory phenotypes observed in goya mice, Map3k1<sup>tm1Yxia/+</sup>:Map3k4<sup>tm1Flv/+</sup> mice were produced, heterozygous for kinase deficient alleles of both genes.

The Map3k1<sup>tm1Yxia/+</sup>:Map3k4<sup>tm1Flv/+</sup> mice were born with their eyelids fused as normal, and no differences in ABR thresholds were observed at 9 weeks of age compared to wild-type or single heterozygote mice. Moreover the double heterozygote mice displayed a similar extra OHC phenotype to that observed in Map3k1<sup>tm1Yxia/+</sup> or Map3k1<sup>goya/+</sup> animals, whereas Map3k4<sup>tm1Flv/+</sup> cochleae showed no such phenotype with the OHC arrangement and number mirroring that of wild-type mice. The failure of the double heterozygote mice to replicate the EOB or degenerative auditory phenotypes would initially suggest there is no genetic interaction between Map3k1 and Map3k4 in the responsible pathways, however, the results could be interpreted in a number of different ways. It may be that one wild-type copy of each gene is sufficient to protect against these phenotypes; ideally the effects of Map3k1 haploinsufficiency in mice that were null for Map3k4 would be...
studied, however, as mentioned in our hands the \textit{Map3k4}^{tm1Flv} mice are homozygous lethal (Bogani et al., 2009). The study by Haque and colleagues saw reduced numbers of hair cells in \textit{Map3k4} kinase deficient mice (Haque et al., 2016), whereas \textit{Map3k1} mutant mice have an excess of OHC. This suggests that MAP3K1 and MAP3K4 play antagonistic roles in specification of hair cell number, with MAP3K4 normally promoting an increase in OHC number and MAP3K1 negatively regulating. A balance of these opposing signals is therefore necessary for the usual three rows of OHC to develop correctly.

Co-expression of wild-type MAP3K4 along with MAP3K1 constructs harbouring human mutations rescued the effects of the mutations (Loke et al., 2014). This finding, combined with the fact that MAP3K1 and MAP3K4 can activate both JNK and p38 suggested possible functional redundancy between the two proteins. To investigate if functional redundancy occurred in the MAPK pathways regulating eyelid morphogenesis or auditory function, a \textit{Map3k4} BAC transgenic mouse line was used to produce \textit{Map3k1} kinase deficient, \textit{Map3k4} overexpressing mice (\textit{Map3k1}^{tm1Yxia/tm1Yxia}:\textit{Map3k4}^{BAC/+}).

The \textit{Map3k1}^{tm1Yxia/tm1Yxia}:\textit{Map3k4}^{BAC/+} mice displayed EOB, immediately confirming that the overexpression of \textit{Map3k4} does not rescue this aspect of MAP3K1 deficiency. The 9-week old ABR data highlighted that the overexpression of \textit{Map3k4} itself does not protect MAP3K1 deficient mice from developing a severe hearing loss by 9-weeks of age, nor affect the auditory function of wild-type or \textit{Map3k1}^{tm1Yxia/+} either positively or negatively. It cannot be ruled out, however, that the BAC transgene may not be being expressed in the inner ear.

As previously discussed, the normal role of MAP3K4 in cochlear patterning appears to be promoting the generation of OHC. However the wild-type mice overexpressing \textit{Map3k4} still had the normal arrangement of three rows of OHC, suggesting that an increase in
MAP3K4 alone is not sufficient to promote additional rows. It is unclear as to why half of the $\text{Map3k1}^{\text{tm1Yxia/tm1Yxia}}:\text{Map3k4}^{\text{BAC/+}}$ mice still retain many more OHC than observed in $\text{Map3k1}^{\text{tm1Yxia/tm1Yxia}}$ mice. It may be that $\text{Map3k4}$ overexpression somehow slows the rate of OHC degeneration. However, the fact that the remaining $\text{Map3k1}^{\text{tm1Yxia/tm1Yxia}}:\text{Map3k4}^{\text{BAC/+}}$ cochleae had similar numbers of OHC to the $\text{Map3k1}^{\text{tm1Yxia/tm1Yxia}}$ mice points more toward either individual variability or perhaps underlying effects of genetic background. In conclusion it would appear that $\text{Map3k4}$ overexpression fails to significantly rescue any of the observed phenotypes in $\text{Map3k1}^{\text{tm1Yxia/tm1Yxia}}$ mice, suggesting there is no redundancy between the two MAP3Ks in the responsible mechanisms.

This could mean that the effects seen in the previous in vitro study (Loke et al., 2014) are not replicated under physiological conditions. Alternatively, the changes in gene expression rescued by wild-type MAP3K4 may not be involved with either the eye or inner ear phenotypes in $\text{Map3k1}^{\text{tm1Yxia/tm1Yxia}}$ mice. Another possibility is that these phenotypes in MAP3K1 mutants do indeed result from a kinase independent mechanism as previously hypothesised from the nature of the goya mutation.
Chapter 5

Identification and Characterisation

of the ‘Boycie’ Mouse Mutant Line
5.1. Introduction

5.1.1. Identification of Hearing Loss in the Muta-Ped-8 (Boycie) Pedigree

One of first pedigrees to be screened during a recessive ENU mutagenesis screen for bone, liver and innate immunity mutants performed at the Mary Lyon Centre at the MRC Harwell Institute was MutaPed-8. As part of the phenotyping pipeline a SHIRPA (Smithkline-Beecham Pharmaceuticals, Harwell Mouse Genetics Centre and Mammalian Genetics Unit, Imperial College School of Medicine and St Mary’s, Royal London Hospital, St. Bartholomew’s and the Royal London School of Medicine Phenotype Assessment) test was performed on all pedigrees. The SHIRPA is a battery of 40 different observable or measurable phenotypic assessments to identify phenodeviants that could be indicative of a wide range of defects or diseases. Included in the SHIRPA test was a sensory battery including the click-box test for assessment of auditory function. When the G3 pedigree of Muta-Ped-8 underwent SHIRPA testing a number of the mice were found to have a reduced or absent click-box response at 12 weeks of age. Further initial screening of G3 mice revealed that nearly half (56 of 115) displayed the click-box phenotype; this ratio is suggestive of a dominantly inherited trait. Subsequently a preliminary ABR assessment using a click stimulus was performed, which highlighted a significant (p=0.0009) ~20dB SPL elevation in click evoked ABR thresholds (Figure 5.1). Whilst this is not a severe elevation, the click stimulus can have a masking effect on frequency specific thresholds as a large area of the cochlea is being stimulated. The line was renamed Boycie after the late composer William Boyce who developed a sensorineural hearing loss.
5.1.2. Whole Genome SNP Mapping of the Boycie Mutation

Tail biopsies were taken from 17 affected G3 mice, DNA was extracted and sent for whole genome SNP mapping using the Kbiosciences SNP panel. As a result the hearing loss phenotype was mapped to a ~65 Mb region on Chromosome 5 by identification of a SNP marker at Chr5:117214009, for which all affected mice were either heterozygote or homozygote for the C57BL/6J variant (Figure 5.2).

![Figure 5.1. Initial click-evoked Boycie ABR. ABR using a click stimulus showed an increase of ~20 dB SPL in mice deemed affected (n=9, click-box = 0 or 1) and those deemed unaffected (n=3, click-box 2). Data shown are mean ± standard error of the mean. Data were analysed using unpaired students t-test (p=0.0009).](image1)

### Figure 5.1
Initial Boycie Click evoked ABR

- **Figure 5.1.** Initial click-evoked *Boycie* ABR. ABR using a click stimulus showed an increase of ~20 dB SPL in mice deemed affected (n=9, click-box = 0 or 1) and those deemed unaffected (n=3, click-box 2). Data shown are mean ± standard error of the mean. Data were analysed using unpaired students t-test (p=0.0009).

### Figure 5.2
Whole genome SNP mapping of the *Boycie* mutation. The Kbiosciences SNP panel using alleles informative between C57BL/6J and C3H showed linkage of the *Boycie* phenotype to chromosome 5, between markers positioned at Chr 5:085931742 and Chr 5:149044358 with 16 of the 17 affected mice being heterozygotic between C57BL/6J and C3H, and the remaining mouse homozygous for the C57BL/6J SNP variant. (? indicates a failed call)

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5.2. Results

5.2.1. Fine Mapping of the Boycie Mutation

To reduce the candidate interval, fine mapping was performed. Additional SNP markers polymorphic between C57BL6/J and C3H were selected within the region of linkage on chromosome 5. Mice from subsequent generations (G4-G12) were then genotyped for these SNPs using PCR and restriction enzyme digest (see Table 2.1) to attempt to narrow the region. DNA from any mice that narrowed the interval by restriction enzyme genotyping was then sent for Sanger sequencing for confirmation.

This method enabled the region to be narrowed to just over 3 Mb between the SNP markers rs29681199 (Chromosome 5:119708589) and rs50469018 (Chromosome 5:123039216). This narrowed candidate interval contains 64 known protein coding genes. A number of genes within this region were selected as candidate genes because of previously reported or potential involvement in auditory function. ATPase sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) transporting 2 (Atp2a2), tyrosine-protein phosphatase non-receptor type 11 (Ptpn11), P2X purinoreceptors 4 and 7 (P2rx4, P2rx7), calcium/calmodulin dependent protein kinase kinase 2 (Camkk2), and Orai1 were screened for ENU-induced mutations using Sanger sequencing in an attempt to find the causative mutation of the observed phenotype. In addition the gene Diablo located just downstream of the region was also screened as it was found to be the causative gene for DFNA64 (Cheng et al., 2011a). A summary of these genes and the outcome of Sanger sequencing can be seen in Table 5.1.
As shown in Table 5.1, the only coding mutation discovered in these seven prioritised candidate genes was a c.392T>C point mutation in the gene *Orai1*.

### 5.2.2. The c.392T>C Mutation in *Orai1*

The c.392T>C mutation in *Orai1* was found to be present in all 17 affected mice originally used for mapping and those displaying a click-box phenotype from subsequent generations. The mutation would result in a valine to alanine (V131A) substitution in the encoded ORAI1 protein product, also known as CRACM1 (*Orai Calcium release-activated calcium channel protein 1*). The bio-chemical properties of valine and alanine are similar; both are neutral, non-polar and hydrophobic amino acids, however the valine residue is highly conserved across species (Figure 5.3).
In silico analysis using Mutation Taster (http://www.mutationtaster.org) (human sequence) indicated the T392C substitution was predicted to be disease causing, returning a probability score of 0.9999999991756 (between 0 minimum and 1 maximum).

Although the basic characteristics of valine and alanine are similar, the amino acid change gave a score of 64 (from a range of 0 – 215), which is calculated using the Grantham matrix to compare physico-chemical properties of the two amino acids. Investigation using the PROVEAN prediction software also suggested the V131A change in the peptide sequence may be deleterious, although the confidence was not as high as Mutation Taster, returning a PROVEAN score of -2.7 (< -2.5 considered deleterious). A similar result was achieved by the SIFT prediction software, which also predicted the change to be deleterious with a score of 0.02 (threshold 0.05), although the prediction stated it was low confidence. The SMART protein prediction software highlighted no changes in domain number, type or location within the sequence between the wild-type and Boycie mutant proteins.

**Figure 5.3 Conservation of p.131V across species.** The valine residue that is changed to alanine (red arrow) as a result of the Boycie c.392T>C mutation in Orai1 is highly conserved across species. The substitution lies within the second transmembrane domain of the ORAI1 protein (TM2 – black box), in a region of 13 residues that remain conserved through all the species listed (stars).
5.2.3. Whole Genome Sequencing

As the candidate region still contained 57 genes that had not been investigated by Sanger sequencing, the strategy of whole genome sequencing (WGS) was employed to see if the mutation in *Orai1* was the only ENU-induced coding change in the interval. Mice with an established hearing loss that were heterozygous for the c.392T>C mutation in *Orai1* were inter-crossed to produce progeny homozygote for the mutation. In addition to WGS, homozygote mice were used for further fine mapping and characterisation of the phenotype. High quality DNA was extracted from an affected female homozygous for the c.392T>C mutation in *Orai1* and sent for WGS at The Wellcome Trust Centre for Human Genetics (High-Throughput Genomics group, University of Oxford, UK).

Briefly, the DNA was sheared and used to create libraries containing short paired end fragments of around 100 nucleotides which were examined using massively parallel sequencing on the Illumina HiSeq 2000 platform. Data were returned to the Bioinformatics group at the MRC Harwell Institute for analysis. The dataset was aligned to the Mm10 build of the C57BL/6J reference genome, and filtered for known SNPs between C57BL/6J and C3H, the two parental strains. Sequence variants that were not filtered out were than classified as low, mid or high confidence based on a quality score which was calculated by assessing read depth and frequency of the variant within those reads. The remaining variants within the candidate interval only contained one high confidence ENU induced non-synonymous coding lesion, the c.392T>C substitution in *Orai1*. In fact this was the only variant found within any coding gene in the region at any confidence level. (Table 5.2).
Within the candidate interval there were a total of 13 gaps that the WGS data did not cover. The gaps ranged between 1 and 155 nucleotides; all were checked using Sanger sequencing and no additional ENU-induced lesions were identified.

### 5.2.4. Orai1 and Store Operated Calcium Entry

#### 5.2.4.1. Orai1

The gene *Orai1* encodes the ubiquitously expressed ORAI1 protein, named after the Orai in Greek mythology who were the gatekeepers of heaven. ORAI1 forms the pore sub-unit of the Calcium Release Activated Calcium (CRAC) channel (Prakriya et al., 2006), essential for correct Store Operated Calcium Entry (SOCE). SOCE is a process that maintains intracellular Ca\(^{2+}\) levels and also can activate Ca\(^{2+}\) based signalling pathways, predominantly the nuclear factor of activated T cells (NFAT) pathway in non-excitable cells (Cheng et al., 2011b); Ca\(^{2+}\) entry through CRAC channels has been linked with regulation of a number of cellular processes including migration (Yang et al., 2009), proliferation (Abdullaev et al., 2008) and is known to play roles in cancer (Yang et al., 2009) as well as being essential for correct immune function (Feske et al., 2006). The two most important proteins involved with replenishing intracellular Ca\(^{2+}\) stores via the CRAC channel are ORAI1 itself, first identified by Feske and colleagues (Feske et al., 2006), and

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the sensor protein Stromal Interacting Protein 1 (STIM1) (Roos et al., 2005). ORAI1 is located in the plasma membrane of the cell and consists of 4 transmembrane domains, 2 extracellular and 1 intracellular linker regions, and cytoplasmic N- and C-termini (Figure 5.4A); STIM1 is a transmembrane protein primarily located in the ER membrane, the N-terminal contains EF hand domain of STIM1 inside the lumen of the ER which is bound to \( \text{Ca}^{2+} \) when it is present, promoting an inactive state. The C-terminal of STIM1 contains 3 coiled coil domains which are bound when the protein is inactive; two of which form the CRAC Activation Domain (CAD). Also at the C-terminus of STIM1 a lysine rich motif is present which promotes association between STIM1 and the plasma membrane when activated. Activation occurs through the depletion of ER \( \text{Ca}^{2+} \) stores, which strips the EF hand of previously bound \( \text{Ca}^{2+} \), resulting in a conformational change of STIM1 (Figure 5.4B).

Figure 5.4 ORAI1 and STIM1. Panel A shows a cartoon indicating the conformation of the ORAI1 protein within the plasma membrane in monomeric form. The N- and C- termini are both located within the cytoplasm, with a coiled coil domain at the C-terminal required for binding to STIM1 during channel formation. The 4 transmembrane domains span the plasma membrane, and are joined by 2 extracellular and one intracellular linker regions. Panel B shows the STIM1 protein located within the ER membrane, the left hand panel highlights the conformation of STIM1 when ER \( \text{Ca}^{2+} \) stores are full, the N-terminal EF hand in the ER lumen is bound to \( \text{Ca}^{2+} \) which promotes association of the EFh and sterile alpha motif domains and maintains a resting state. Cytoplasmic coiled coil domains, including those which form the CAD are bound in this resting state. Upon store depletion, shown in the right panel, the \( \text{Ca}^{2+} \) becomes disassociated from the EF hand which results in a conformational change including unfolding of the sterile alpha motif/EF hand in the ER lumen, and the cytoplasmic coiled coil complex and puts STIM1 into an active state. A lysine rich domain at the C-terminal of STIM1 aids association with the plasma membrane. \( \text{Ca}^{2+} \) ions are shown as red circles. Plasma Membrane – PM, Transmembrane domain 1-4 - TM1-4, amino terminal - NH\(_2\), carboxyl terminal – C, coiled coil domain – cc, lysine rich domain – K, channel activating domain - CAD, endoplasmic reticulum membrane – ERM, sterile alpha motif – SAM, EF hand - EFh. Figure adapted from (Cahalan, 2009; Shaw et al., 2013)
In order to form the pore of the CRAC channel, ORAI1 monomers must first form a homomeric structure, a subject of some controversy. Historically, research suggested that the active CRAC channel pore consisted of a tetramer of ORAI1 molecules (Ji et al., 2008; Madl et al., 2010; Mignen et al., 2008b; Penna et al., 2008); however the crystal structure of the drosophila homologue of ORAI1, dOrai, was published in 2012 and revealed a hexameric stoichiometry (Hou et al., 2012). This homologue is highly similar both structurally and in sequence identity to mammalian ORAI1, with the transmembrane domains being almost identical (Yeromin et al., 2004). The debate still remains, however it was shown that in mammalian cells, a hexameric ORAI1 pore does not display the correct selectivity of the CRAC channel, whereas in tetrameric form it does (Thompson and Shuttleworth, 2013). There are also contradictory reports about the resting state of ORAI1 in the plasma membrane with some studies suggesting it is dimeric (Demuro et al., 2011; Penna et al., 2008) and others that it is already tetrameric (Ji et al., 2008; Mignen et al., 2008b) when not functional in the CRAC channel.

There are two known protein coding isoforms of Orai1, the Orai1α isoform is less abundant due to a relatively weak kozak sequence and has a molecular mass of 32 kDa; another isoform arises due to translational initiation at an alternative methionine residue towards the end of the N terminal. This isoform produces the shorter, but more abundant 23 kDa Orai1β, which lacks a poly-arginine sequence associated with binding of phosphatidylinositol-4,5-bisphosphate (PIP2); the fact that Orai1β lacks this domain is thought to result in increased mobility compared to the Orai1α isoform, although both can successfully form CRAC channels (Fukushima et al., 2012). There are also two homologues of ORAI1, ORAI2 and ORAI3. In vitro, research suggests that there may be some functional redundancy between homologues and that ORAI2 and ORAI3 may able to compensate in facilitating CRAC channel activity to some extent in the absence of
functional ORAI1 (Mercer et al., 2006). Although the exact function of these additional ORAI proteins has not been investigated to the same extent as ORAI1, research undertaken recently is suggesting a role for them in SOCE of excitable cells. It appears that SOCE in numerous cell types in the mouse brain are regulated either by STIM1 or its homolog STIM2, using ORAI2 rather than ORAI1 to form the pore of the channel (Berna-Erro et al., 2009; Hartmann et al., 2014; Sun et al., 2014). Interestingly ORAI1 was shown in these studies not to be required for SOCE in neurons from the mouse cerebellum, hippocampus or cortex. Conversely another study showed that in the case of rat hippocampus and cortex neurons, STIM1 and STIM2 could both form channels with ORAI1 to facilitate SOCE; STIM1 in the CRAC channel to compensate ER Ca\(^{2+}\) depletion, but STIM2 to potentially regulate basal levels of intracellular Ca\(^{2+}\) (Gruszczynska-Biegala et al., 2011). A consensus on the role of SOCE in excitable cells is yet to be agreed.

5.2.4.2. SOCE and CRAC Channel Activation

Although the exact configuration of the pore is still a cause of controversy, the activation and properties of the CRAC channel have been well studied and explored by in vitro experiments. The channel is an inwardly rectifying cation channel with a high selectivity for Ca\(^{2+}\) above other divalent cations. The high affinity for Ca\(^{2+}\) has been shown to be a result of glutamate residues at p.E106 and p.E190 (human) located in transmembrane domains 1 and 3 respectively, which when mutated impair the entry of Ca\(^{2+}\) and allow movement of monovalent ions through the channel (Prakriya et al., 2006). One of the biggest intracellular stores of Ca\(^{2+}\) in mammalian cells is the endoplasmic reticulum (ER); influx of extracellular Ca\(^{2+}\) related to low levels in the ER was noted over 30 years ago (Putney, 1986); since then a large body of research has helped elucidate the proteins involved, the mechanisms by which they facilitate calcium entry, and also the functions of SOCE (reviewed in (Hogan and Rao, 2015)). SOCE is required for Ca\(^{2+}\) regulation in non-

200
excitable cells, and is essential for Ca\textsuperscript{2+} signalling in many cell types, the most studied of which are immune cells. In the case of T- cells the CRAC current I\textsubscript{CRAC} has been shown to be largely responsible for NFAT activation, and partially required for nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation, as the names suggest both are essential for transcriptional regulation related to immune function (Cheng et al., 2011b).

In immune cells, the SOCE pathway involves ligand binding of membrane bound receptors, such as T-cell/B-cell receptors (TCR/BCR), or G-Protein coupled receptors (GPCR) located in the plasma membrane; when receptors are not bound to ligands, ORAI1 and STIM1 are resting distributed throughout the plasma membrane or ER membrane respectively (Figure 5.5A). Upon receptor activation by ligand binding a signalling cascade is initiated involving the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}) by phospholipase C (PLC) which produces cytosolic inositol 1,4,5-triphosphate (InsP\textsubscript{3}). InsP\textsubscript{3} in turn binds to 1, 4, 5-inositol triphosphate receptors (InsP\textsubscript{3}R) in the ER membrane, priming them to become activated by subsequent cytosolic Ca\textsuperscript{2+} release from the ER. Upon store depletion and the resulting disassociation of the EF hand with bound Ca\textsuperscript{2+}, STIM1 dimerises, translocates and aggregates at ER/plasma membrane junctions. ORAI1 located in the plasma membrane also aggregates near the ER/plasma membrane junctions (Figure 5.5B). STIM1 dimers then bind to both the N and C termini of ORAI1 via the CAD domain, opening the channel and facilitating replenishment of intracellular Ca\textsuperscript{2+} from extracellular sources. Intracellular Ca\textsuperscript{2+} can then be actively returned into the ER by the sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) (Park et al., 2009; Yuan et al., 2009) (Figure 5.5C).
Diseases Resulting from ORAI1 Mutations

Given the reported role of CRAC in immune cells, it is not surprising that loss-of-function mutations in both Orai1 and Stim1 in humans lead to immunological deficiencies. First reported in the case of Orai1 was a dominant negative mutation leading to a substitution of tryptophan for arginine (p.R91W) in patients with a severe combined immune deficiency (SCID) like phenotype (Feske et al., 2006). It is proposed that this mutation results in the closure of the CRAC channel due to the p.R91W substitution in transmembrane domain 1, introducing an aromatic side-chain which potentially blocks the pore (Hou et al., 2012; Lacruz and Feske, 2015). Other reported loss of function mutations in ORAI1 in human patients with immune deficiencies are recessively inherited frameshift (p.A88SfsX25, p.H165PfsX1) or compound heterozygotic mutations (p.A103E/p.L194P) which all result in a loss of ORAI1 protein expression; in addition to the immune deficiency phenotypes, all patients also presented with congenital muscular

Figure 5.5 SOCE and CRAC channel activation. Panel A shows a resting state with Ca\(^{2+}\) stores in the ER high and the EFh of STIM1 bound to Ca\(^{2+}\) and inactive, ORAI1 tetramers are distributed throughout the plasma membrane. Panel B shows the process of receptor based store depletion, using GPCR as an example. When the receptor is activated by ligand binding, a cascade is initiated with PLC hydrolysing PIP2 at the plasma membrane to produce InsP \(_3\). InsP \(_3\) then can bind to InsP \(_3\)Rs in the ER membrane priming them to be activated by Ca\(^{2+}\) in the cytosol. Once InsP \(_3\)R are activated they allow efflux of Ca\(^{2+}\) from the ER; when stores are depleted, Ca\(^{2+}\) disassociates from the EFh of STIM1, changing the conformation of the protein and enabling dimerization and mobilisation to plasma/ER membrane junctions. Panel C illustrates the process of CRAC channel formation; ORAI1 is recruited to the plasma/ER membrane junction to form the channel pore to facilitate Ca\(^{2+}\) entry when activated by interactions between both the N- and C-terminal domains of ORAI1 and the CAD domains of STIM1. Influx of extracellular Ca\(^{2+}\) through the activated CRAC channels raises cytosolic Ca\(^{2+}\) levels, allowing SERCA pumps replenish the ER store. N.B. as there are discrepancies in the exact stoichiometry of the CRAC channel, ORAI1 is represented as a tetramer in both resting and active states, and the ratio of STIM1 to ORAI1 is simplified for illustrative purposes. Figure based on (Lacruz and Feske, 2015)
hypotonia (Chou et al., 2015; McCarl et al., 2009). More recently, however, a series of gain-of-function dominant mutations (p.G98S, p.P245L and p.L138F) have been reported that all result in increased or constitutive CRAC channel activity; phenotypes related to these gain of function include the skeletal muscle defect tubular aggregate myopathy and miosis (OMIM 160565, OMIM 615883) (Endo et al., 2015; Nesin et al., 2014) (Figure 5.6).

![Figure 5.6 Known loss-of-function and gain-of-function mutations in ORAI1.](image)

Figure 5.6 Known loss-of-function and gain-of-function mutations in ORAI1. Panel A highlights known loss-of-function mutations in ORAI1 and their location within the protein. The R91W mutation is the cause of a SCID like phenotype in human patients and results in a present but non-functional ORAI1 protein (Feske et al., 2006). The two frameshift mutations (A88SfsX25 and H165PfsX1) and the reported compound heterozygote mutation of A103E/L194P all result in a loss of ORAI1 protein and therefore function. Panel B shows the location of mutations known to result in a gain of function that leads to increased SOCE activity ((P245L) (Nesin et al., 2014)) or constitutive Ca$^{2+}$ entry ((G98S, L138F) (Endo et al., 2015)). The relative size of the blue arrows in both panels indicates reduced/absent (A) and increased (B) Ca$^{2+}$ entry resulting from the mutations. N-termini – N, C-termini – C, coiled coil – cc, transmembrane domain 1-4 – M1-4. Figure adapted from (Lacruz and Feske, 2015)

### 5.2.4.3. Additional Roles of ORAI1

Although the importance of ORAI1 in CRAC channel function is clear, research has suggested it has additional functions, both store dependent and independent. The transient receptor potential channels (TRPC) have also been implicated in SOCE. In the case of TRPC1 it is also known to form a channel with STIM1 in response to store Ca$^{2+}$ depletion however the channel is less selective than the CRAC channel; as a result the TRPC1/STIM1 channel is a much larger current (I$_{SOC}$), presumably due to the reduced selectivity for Ca$^{2+}$ compared to the pore comprised of ORAI1. Interestingly, although this
appears to be distinct from the CRAC channel, and not containing ORAI1, research suggests that ORAI1 is required to target TRPC1 to the ER/plasma membrane junctions where it can be activated by STIM1 (Cheng et al., 2011b).

Independently of store depletion ORAI1, ORAI3 and STIM1 are required components of arachidonic acid receptor channels (ARC) (Mignen et al., 2007; Mignen et al., 2008a) and also leukotriene C4 regulated channels (LRC) (Gonzalez-Cobos et al., 2013; Zhang et al., 2013). These are again highly selective Ca\textsuperscript{2+} channels but require accumulation of arachidonic acid/leukotriene C4 on the cell surface for activation, rather than responding to low levels of intracellular Ca\textsuperscript{2+}. These examples may well not be exhaustive and highlight a diverse number of ways that channels involving ORAI1 may be formed and activated.

5.2.5. Evaluation of Auditory Function

5.2.5.1. Click-box Testing

As previously mentioned, the original pedigree contained 56 out of 115 mice with a click-box deficit; these mice were tested between 8 and 12 weeks of age. Subsequent to this, affected mice with the heterozygous haplotype underwent backcrossing to the C3H inbred strain to commence the process of making the line congenic on the C3H genetic background. It quickly became apparent that there may a progressive element to the hearing loss; nearly all of the first litters of G4 mice exhibited a normal response to the click-box at 4 weeks of age, but then upon re-testing at later time-points many failed to respond. Examination of click-box data collected from a large number of G4 progeny at weekly intervals between 3 and 11 weeks of age shows a steady decline in the number of Boycie heterozygote mice with a normal click-box response (Figure 5.7). Whilst these data show a variability in the onset of severe hearing loss (the click-box is a supra-threshold
response), the progression from normal to reduced, to absent response was evident amongst mice that were repeatedly tested, indicating the mutation results in a progressive decline in auditory function.

Figure 5.7 Click-box evaluation of 3-11 week old G4 wild-type and Boycie heterozygote mice. Percentages of G4 wild-type and Boycie heterozygote mice, between 3 and 11 weeks of age, showing either the normal response (Preyer’s reflex), a reduced response or an absent response to a click-box. Panel A shows that the vast majority of wild-type mice exhibited the normal response at all time-points, only a small number showing a reduced response and none failed to respond. Panel B shows that the number of Boycie heterozygote mice exhibiting a normal response to the click-box gets progressively fewer each week, with all heterozygotes 3 weeks of age; in contrast by 11 weeks of age 80% showed no response and 20% only a reduced response.
5.2.5.2. ABR Analysis

Cohorts of wild-type, Boycie heterozygote and Boycie homozygote mice underwent ABR assessment at 4, 6, 8 and 12 months of age to investigate further the progressive element of the hearing loss in mice carrying the Boycie mutation, and also to examine the effects of the allele in homozygote form. In addition a cohort of wild-type and Boycie heterozygote mice was also tested at 19 months of age (Figure 5.8). It should be noted that the majority of mice making up these cohorts were congenic for the mutation (G10 +), although a small number including those used for the 19 month ABR were from G6 or G7; the onset of the hearing loss appeared to be somewhat delayed and more variable in the mice from subsequent generations than the original or G4 click-box analysis, suggesting that the genetic background has an effect on the hearing loss phenotype. Statistical analysis was performed using One-way ANOVA with Tukey’s post hoc analysis and multiple comparisons (for p values see Figure 5.8E). For each genotype and time-point n≥3, see appendix for individual thresholds indicating specific numbers.

At 4 months of age, no significant differences in ABR thresholds were found between Boycie heterozygote and wild-type mice, for either the click stimulus or any of the frequency specific stimuli. In the case of the 32 kHz stimulus, 3 of the 5 heterozygote animals tested had ABR thresholds 20-30 dB SPL higher than either the remaining 2 heterozygotes or the wild-type (see appendix for individual ABR values). The Boycie homozygote mice however, exhibit significantly elevated thresholds (+30-50 dB SPL) for all stimuli tested when compared to either wild-type or heterozygote mice; no homozygote mice showed a response to the 32 kHz stimulus at the highest level tested (90 dB SPL). (Figure 5.8A).
At 6 months of age, Boycie heterozygote mice showed a significant elevation in ABR thresholds (~+40 dB SPL) at 32 kHz when compared to wild-type by this time-point, despite 1 of the 3 heterozygote animals showing a similar response to wild-type. The Boycie homozygote mice again display significantly elevated thresholds when compared to wild-type for all stimuli, however, thresholds were only significantly higher than heterozygote mice for the 8 kHz and 16 kHz stimuli. There was no real change in ABR threshold levels between the homozygote mice at 4 and 6 months of age (Figure 5.8B).

By 8 months of age, significant differences are seen between all genotypic combinations for the click stimulus. For the 8 kHz and 32 kHz stimuli, both Boycie heterozygote and homozygote mice exhibit significant threshold increases over wild-type mice. At 16 kHz, homozygote mice show a significant elevation when compared to either wild-type or heterozygote mice. Again the average homozygote thresholds are similar to homozygote thresholds at 4 and 6 months of age (Figure 5.8C).

The pattern of significance in ABR thresholds at 12 months of age mirrors that of the 8 month time-point, however p values differ (Figure 5.8D and E). The average threshold for Boycie homozygote mice at 16 kHz is elevated when compared to the 8 month time-point, however, as the error bars suggest there was high variability between individuals (Figure 5.8D and appendix).

The data from the time-course ABR analysis suggest that by 4 months of age, Boycie homozygote mice display a moderate to severe hearing loss at low and mid frequencies (+30-50 dB SPL), and are essentially deaf at the higher 32 kHz frequency. On the other hand, Boycie heterozygote animals have similar thresholds to wild-type mice at 4 months of age, but develop a progressive, high frequency hearing loss by 6 months of age. By 8 months of age the heterozygote mice also display mild to moderate hearing loss (+15-20
dB SPL) at low frequencies, which also impacts on the thresholds for the click stimulus. Figure 5.8F shows the progression of decline in *Boycie* heterozygote average ABR thresholds over the time-course.

To investigate if *Boycie* heterozygote low and mid frequency ABR thresholds progressed to comparable levels to that of the homozygote with time, a cohort of heterozygote mice was aged to 19 months (Figure 5.8G). There were no significant differences between the thresholds at 12 and 19 months of age for any stimuli, suggesting that between 8 and 12 months the extent of auditory decline has already been reached.

Taken together the ABR data show a semi-dominant mode of inheritance of the hearing loss phenotype in mice carrying the *Boycie* mutation; the onset of hearing loss in heterozygote animals is delayed to that of homozygotes, but by 8 months of age, they display average ABR thresholds intermediate between those of wild-type and homozygote mice.
Figure 5.8 ABR assessment of Boycie mutant mice. Panels A-D show audiograms highlighting average ABR thresholds of wild-type (blue), Boycie heterozygote (red) and homozygote (yellow) at 4 months (A), 6 months (B), 8 months (C) and 12 months (D) of age, as described in the text. Panel E shows comparative inter-genotype p values for the 4 – 12 month ABR thresholds. The Boycie heterozygote mice show a steady progressive predominantly high frequency hearing loss highlighted in panel (F). Panel G shows that there is no further elevation in Boycie heterozygote ABR thresholds between 12 and 19 months of age.
5.2.6 Histological Assessment

5.2.6.1. Organ of Corti

To investigate the effect of the Boycie mutation on general cochlear morphology, histological examination of paraffin embedded head sections was undertaken. It should be noted that these mice were from G4 pedigrees and all heterozygote mice had lost the response to the click-box. Examination of the organ of Corti revealed degeneration in 5 month old heterozygote Boycie mice. The H&E stained sections, as is often the case with paraffin embedded inner ears, did not completely preserve organ of Corti morphology, however evaluation of nuclei remaining revealed that the OHCs and occasionally Deiters’ cells were missing in the basal, and in some cases, the mid cochlear turns of Boycie heterozygote mice, although IHCs appeared to be unaffected (Figure 5.9).

Figure 5.9 Histology of the organ of Corti. H&E stained histology sections showing sections of the organ of Corti from the apical (A), mid (B) and basal (C) cochlear turns of wild-type (Ai,Bi,Ci) and Boycie heterozygote (Aii,Bii,Cii) mice. Wild-type mice show the normal arrangement of nuclei for OHC (black arrows), Deiters’ cells (red arrows) and IHCs (blue arrows) in all cochlear turns (Ai,Bi,Ci). Boycie heterozygote mice show the expected cellular arrangement in the apical turn (Aii), however the mid and basal turns show degeneration of the organ of Corti including a complete loss of OHC nuclei (Bii and Cii) and in this case most of the Deiters’ cells in the mid turn (Bii). IHC nuclei are visible in all sections. Scale bars represent 50 µm, n=6 per genotype, images are representative.
5.2.6.2. Stria Vascularis

In addition, the stria vascularis of 5 month old Boycie heterozygote mice was visibly thinner in the basal turn when compared with wild-type mice (Figure 5.10).

Figure 5.10 Histology of the stria vascularis. H&E stained histology sections showing examples of the stria vascularis from the Basal (A), Mid (B) and Apical (C) cochlear turns of 5 month old wild-type (Ai-iii, Bi-iii and Ci-iii) and Boycie heterozygote (Aiv-vi, Biv-vi and Civ-vi) mice. The stria vascularis has been highlighted for easier identification using colour balance in Adobe Photoshop (all images were modified equally with regards to colour balance). The stria vascularis in the basal cochlear turn of the Boycie heterozygote mice (Aiv-vi) is markedly thinner than in wild-type mice (Ai-iii). Scale bars represent 50 µm, n=6 per genotype, images are representative.
Slides were digitally scanned using a Hammamatsu slide scanner and the total cross-sectional area of the stria vascularis was measured using the NDP.view2 software (www.Hammamatsu.com). Measurements confirmed significant reductions in total cross-sectional stria vascularis area in both the basal (p=0.0003) and mid (p=0.003) cochlear turns, however no difference was observed in the apical cochlear turn (Figure 5.11).

![Figure 5.11 Assessment of stria vascularis total area.](image)

**Figure 5.11 Assessment of stria vascularis total area.** Measurement of the stria vascularis from the basal, mid and apical cochlear turns of wild-type (n=6), and Boycie heterozygote (n=6) mice. The data show a significant reduction in total stria vascularis area in Boycie heterozygote mice in both the basal (p=0.0003) and mid (p=0.003) cochlear turns when compared to wild-type. No difference was seen between the genotypes in the apical turn. Stria vascularis – SV, p values calculated using unpaired students t-test.

### 5.2.6.3. Spiral Ganglion Neurons

Interestingly SGNs in the apical cochlear turn of Boycie heterozygote mice appeared also to be fewer in number than in wild-type control mice at the 5 month time-point; this finding is contradictory to the pattern of hearing loss observed which was predominantly high frequency and related to the more basal regions of the cochlea (Figure 5.12). A predominantly apical loss of SGNs is a fairly uncommon pathological observation in most deafness etiologies, however it has been noted in neuronal ARHL (Ohlemiller and Gagnon, 2004), and also subjects with endolymphatic hydrops, which also display distension of Reissner’s membrane due to impaired potassium recycling altering the endolymphatic
pressure (Megerian et al., 2008; Semaan et al., 2013). It was impossible to reliably check the status of Reissner’s membrane in the paraffin embedded sections as it was not intact in either wild-type or Boycie heterozygote inner ear sections, presumably as an artefact of histological processing. To remedy this, a small number of inner ears from an aged cohort (~19 months of age) of Boycie heterozygote and control mice, were embedded in epoxy-resin for semi-thin sectioning; although time-consuming this technique provides excellent preservation of morphological structures. Examination of the semi-thin sections indicated that Reissner’s membrane was intact and not distended Boycie heterozygote mice even at this aged time-point (Figure 5.13).

Figure 5.12 Histology of the spiral ganglion neurons. H&E stained histology sections showing examples of the SGN from the Basal (A), Mid (B) and Apical (C) cochlear turns of 5 month old wild-type (Ai-vi, Bi-vi and Ci-vi) and Boycie heterozygote (Avi-xii, Bvi-xii and Cvi-xii) mice. No obvious differences in SGN density are observable between wild-type and Boycie heterozygote in the basal or mid cochlear turns, however in 4 of the 6 Boycie heterozygote mice (Cvi, ix, x and xi) SGN density in the apical turn is clearly reduced compared to wild-type (Ci-vi). Scale bars represent 50 µm, n=6 per genotype.
5.2.7 Ultrastructural Study of the Organ of Corti

To investigate the organ of Corti degeneration indicated by the histological examination in finer detail, an SEM study was performed. Analysis of wild-type, Boycie heterozygote and homozygote cochleae aged between 5 and 6 months revealed an interesting pattern of cellular loss. There was sporadic loss of OHC in the very apical region of cochleae of some Boycie homozygote mice, however the normal arrangement of three rows of OHC and one row of IHCs was always present ~90° from the apex. Apart from this, organs of Corti from both Boycie heterozygote and homozygote looked mice normal until 360° – 450° from the apex where the OHC disappeared over a remarkably short length of the cochlear duct in all mice examined (Boycie+/+ n=3, Boycie+/- n=5, Boycie-/- n=2) (Figure 5.14).
It was hard to reliably assess the state of IHCs in all Boycie mutant mice as they were frequently obstructed by the tectorial membrane; in the patches where they were visible, there was occasional evidence of IHC loss towards the hook at the base of the cochlea (Figure 5.14F) but this was not as widespread in the mid or mid-basal regions, and was not consistent in all Boycie cochleae.
The same pattern of cellular loss was also noted in Boycie homozygote mice between 7 and 8 months of age (Figure 5.15), 12 months of age and also in a cohort of Boycie heterozygote mice that were aged to 19 months (Figure 5.16).

![Figure 5.15 Ultrastructural investigation of 7-8 month old Boycie organs of Corti. Representative scanning electron micrographs showing a similar pattern of degeneration of the organ of Corti in the Boycie homozygote (D-F) mice aged between 7 and 8 months to that seen in the 5-6 month old mice. No degeneration of OHC or stereocilia is noted in wild-type cochleae in any of the turns (A-C). All scale bars represent 10 µm.](image)

Figure 5.16 shows lower magnification scanning electron micrographs that highlight the rapid decline in OHC numbers in the mid turn of Boycie mutant mice of both genotypes. This pattern of OHC loss would to some extent agree with the ABR thresholds, however, when taken together with the degeneration of apical SGN is a curious finding; although the very apical regions of Boycie homozygote cochleae display some OHC stereocilia loss at 5-6 months, heterozygote animals similar in age to those in which the SGN density was significantly reduced did not. The abruptness of the organ of Corti changing from the normal organisation of 3 rows of OHC to a near complete loss was quite striking. Inner ears were collected from mice of 5-6 months of age (Boycie+/− n=3, Boycie+/− n=5, Boycie−/− n=2), 7-8 months of age (Boycie+/− n=2, Boycie−/− n=4), 12 months of age (Boycie+/− n=2, Boycie−/− n=4) and 19 months of age (Boycie+/− n=4, Boycie−/− n=4).
Figure 5.16 Scanning electron micrographs highlighting the rapid decline in OHC numbers in the mid turn of Boyce mutant mice. Panel A shows a 100 x magnification overview of a wild-type cochlea at 19 months of age with the OHC highlighted in purple, it is clear that the OHC run all the way to the hook of the base of the cochlea, which is the basal region disappearing from view. Panel B shows an overview of a 5 month old Boyce homozygote cochlea at the same magnification as (A). It is clear that the OHC (purple) rapidly disappear after ~410° from the apex of the cochlea (note that roughly the first quarter turn is missing from this preparation). Panels C-F are 200 x magnification scanning electron micrographs of Boyce 5 month old heterozygote (C), 5 month old homozygote (D) (this is the same cochlea as in (B) at higher magnification), 12 month old homozygote (E) and 19 month old heterozygote (F) cochleae again with OHC highlighted in purple showing the same sudden loss of OHC in the mid turn at all time-point/genotype combinations. Scale bars all represent 100 µm.
Although degeneration of hair cells was observed, where they remained, no obvious differences in stereocilia bundle morphology could be observed between wild-type and Boycie mice (Figure 5.17).

5.2.8. Subcellular Localisation

To investigate possible effects of the Boycie mutation on subcellular localisation of ORAI1, molecular cloning techniques were used to create plasmid constructs expressing either c-Myc or EGFP-tagged wild-type ORAI1, or c-Myc or EGFP-tagged ORAI1(V131A) containing the Boycie mutation. Cos-7 cells were then transiently transfected with each tagged construct before EGFP fluorescence and primary antibodies to c-Myc and ORAI1 were
used to identify the successfully transfected cells and investigate subcellular localisation (Figure 5.18)

![Figure 5.18 Subcellular localisation of ORAI1 constructs.](image)

No obvious differences were observed between the two constructs, either with the EFGP fluorescence or anti-c-Myc or anti-ORAI1 antibodies. All showed correct localisation of ORAI1 to the plasma membrane in transfected cells, consistent with the role ORAI1 has in forming the pore of the CRAC channel. These data suggest that the Boycie mutation does not affect protein localisation on a subcellular level.

5.2.9. Calcium Entry (performed by Prof Anant Parekh and Dr Dan Bakowski)

To elucidate if the subtle change in properties between Valine and Alanine has an effect on Ca^{2+} entry, we collaborated with Prof Anant Parekh’s group at the University of Oxford.
Prof Parekh has been heavily involved with the characterisation of SOCE and the functionality of the CRAC channel (Parekh, 1998; Parekh, 2003; Parekh et al., 1997; Parekh et al., 2002). Channel functionality was investigated by Prof Parekh and Dr Bakowski, using an established calcium imaging technique. The CRAC channel requires both STIM1 and ORAI1 to form and allow Ca\textsuperscript{2+} entry; HEK293 cells were either mock transfected (wild-type) or co-transfected with STIM1 and EGFP-ORAI1, or STIM1 and EGFP-ORAI1(V131A). HEK293 cells are ideal for this experiment as they express low levels of endogenous ORAI1 (Li et al., 2011). The cells were then treated with thapsigargin in a Ca\textsuperscript{2+} free media, to passively deplete ER Ca\textsuperscript{2+} stores, before 2mM Ca\textsuperscript{2+} was added back to the media. Intracellular Ca\textsuperscript{2+} levels were measured using Fura-2 based calcium imaging (Figure 5.19A).

The rate of Ca\textsuperscript{2+} uptake, directly related to the amount of functional CRAC channels, was then calculated from the intracellular Ca\textsuperscript{2+} levels. Both the STIM1 + EGFP-ORAI1 and STIM1 + EGFP-ORAI1(V131A) co-transfected cells displayed greatly increased rates of Ca\textsuperscript{2+} entry.
entry when compared to the wild-type cells, however there were no significant differences between the two constructs, suggesting that the Boycie mutation does not affect the ability of the CRAC channel to replenish Ca\(^{2+}\) stores in the ER (Figure 5.19B).

### 5.2.10. MRC Harwell ENU DNA Archive Screening for Orai1 alleles

Calcium regulation is important for correct auditory function, however, hearing loss has not previously been associated with Orai1, and a role for SOCE in the inner ear has not been reported. As the Boycie mutation appeared not to have significant differences in either sub cellular localisation or CRAC function, additional alleles of Orai1 were identified using a reverse genetics approach. DNA from the Harwell ENU sperm archive was screened using DNA melting analysis on the Lightscanner system (Idaho Technology Ltd.) for G1 male mice harbouring ENU induced mutations in Orai1.

Three alleles were identified with non-synonymous coding ENU induced point mutations: c.560T>A, c.368T>A and c.404T>A that would result in the following amino-acid substitions in the ORAI1 protein: L187Q, V135E and I123N (Figure 5.20).

The identified alleles were investigated using Mutation Taster to see if they would likely be tolerated or deleterious to the protein (Table 5.3).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation Taster score (0-1)</th>
<th>aa change (0-215)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V131A (Boycie)</td>
<td>0.9999999999175600</td>
<td>64</td>
</tr>
<tr>
<td>L187Q</td>
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</tr>
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<td>V135E</td>
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</tr>
<tr>
<td>I123N</td>
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</tr>
</tbody>
</table>

Table 5.3 In silico analysis of the ENU/Sperm archive alleles of Orai1 using Mutation Taster
Figure 5.20 Location of the **Orai1** alleles identified in the MRC Harwell ENU DNA archive. A shows the location within the coding sequence of **Orai1** of the 3 alleles identified in the ENU DNA/Sperm archive screen (T560A, T368A and T404A) in addition to that of the **Boycie** mutation (T392C). Panel B shows the relative position and resulting amino acid substitution in primary structure of **ORA1**. The I123N, V131A (Boycie) and V135E mutations are all located in TM2, whereas L187Q is in TM3. C and D show the position of each amino acid substitution within the tertiary structure (C) and quaternary structures (D), each substitution is colour coded consistently throughout (I123N – green, V131A (Boycie) – yellow, V135E – blue, L187Q – red. Note the quaternary structure is the published hexamer of the crystal structure of dOrai1 (PDB: 4HKR) previously described (Hou et al., 2012). The peptide sequence corresponding to predicted mouse TM domains are highlighted within the structures TM1: – light blue, TM2 – purple, TM3 – dark blue, TM4 – white. Untranslated region - UTR, coding DNA sequence – CDS, transmembrane domain - TM.
As all the alleles identified from the screen were predicted to be highly deleterious to the protein, frozen sperm from the founder G1 males was used to re-derive mice by means of \textit{in vitro} fertilisation. Heterozygote progeny were then inter-crossed in an attempt to produce mice that were wild-type, heterozygote or homozygote for each of the mutations, and underwent auditory assessment by ABR at 6 months of age to determine if these \textit{Orai1} alleles replicated the hearing loss phenotype observed in \textit{Boycie} mice.

\textbf{5.2.10.1. \textit{Orai1}^{L187Q}}

The G1 founder mouse EMRC/323.9h, carried the ENU-induced point mutation c.560T>A in the cDNA sequence of \textit{Orai1}, resulting in a leucine to glutamine substitution (L187Q) in transmembrane-domain 3 of the ORAI1 protein (Figure 5.20). The mutation was predicted to be deleterious to the protein (Mutation Taster score 0.999999999938206/1) and the amino acid substitution returned a score of 113/215 using the Grantham matrix to compare biochemical properties (Table 5.3).

The genotype ratio of progeny was not significantly different to that expected from an inter-crossed heterozygote mating according to Mendelian inheritance (Chi squared p=0.56), indicating that the amino acid substitution was tolerated even in mice homozygote for the allele; however, in accordance with animal welfare guidelines set out in the Home Office guidelines, a total of 15 mice from this line were culled due to failure to thrive. Of these, 8 were homozygote for the c.560T>A mutation, 4 were heterozygote and 3 wild-type.

Assessment of auditory function using ABR at 6 months of age showed no difference in auditory thresholds between any of the genotypes in \textit{Orai1}^{L187Q} mice (Figure 5.21)
Another Orai1 allele, c.404T>A, was identified in DNA extracted from the G1 founder CBMLC/583.10b. This lesion causes a valine to glutamic acid change (V135E) in transmembrane-domain 2 of ORAI1 (Figure 5.20). Using Mutation Taster, the c.404T>A mutation was predicted to be disease causing (0.999999999954807/1) and the Grantham matrix score was 121/215 for the valine to glutamic acid substitution (Table 5.3).

Heterozygote inter-cross matings for this mouse line did not produce large numbers of offspring, and failed to produce any homozygote progeny, suggesting homozygote prenatal lethality. The Mendelian inheritance ratios were obviously significantly different than expected (Chi squared p=0.0095).

There were no differences in ABR thresholds between wild-type and Orai1\textsuperscript{V135E} heterozygote mice at any frequency tested (Figure 5.22)
The c.368T>A mutation in Orai1 was discovered in DNA extracted from the G1 founder male CBMLC/392.7e. This nucleotide substitution results in an isoleucine to asparagin e change (I123N) in the peptide sequence of ORAI1. Like both the Boycie and c.404T>A mutations in Orai1 the affected residue is located in transmembrane-domain 2 (Figure 5.20). In silico analysis of this mutation using Mutation Taster again predicted the substitution to be disease causing, resulting in a score of 0.999999999993661/1, and the Grantham matrix calculated that the isoleucine to asparagin e change resulted in a large difference in biochemical properties of the residue (194/215) (Table 5.3).

The number of homozygote mice recovered from the heterozygote inter-cross mating was very low (4/54), and proved significantly low using Chi squared analysis of Mendelian ratios (p=0.0408), suggesting pre or perinatal lethality.

ABR analysis of 6 month old wild-type and heterozygote Orai1123N mice again showed no difference between any of the genotypes, (Figure 5.23).
5.2.11. Fluorescence-Activated Cell Sorting (FACS) Analysis and Clinical Chemistry.

As previously mentioned Orai1 mutations lead to immune related phenotypes in humans; the same is true for existing mouse models which harbour mutations in Orai1, or those where the gene has been knocked out. The Orai1^{tm1Fesk} allele causes the same residue alteration (p.R93W) seen in patients with the SCID like phenotype, which results from the homologous p.R91W mutation in ORAI1. Mice homozygous for this mutation (Orai1^{R93W/R93W}) all suffer from neonatal lethality.

The loss of CRAC channel activity due to non-functional Orai1 in immune cells does not produce large differences in standard haematological test results; total numbers of B and T cells were normal. However, some patients harbouring loss-of-function mutations in either STIM1 or Orai1 do show defects including B and T cell activation defects, reduced numbers of regulatory T cells (Treg) expressing forkhead box P3 (FOXP3), which is a marker of effector or activated Tregs, and lower numbers of invariant natural killer T cells (iNKT), (reviewed in (Lacruz and Feske, 2015)). Patients with gain-of-function mutations invariably displayed elevated levels of creatine kinase (CK) in clinical chemistry tests. To investigate if the Boycie mutation or any of the Orai1 alleles identified from the ENU DNA archive showed any similar differences, terminal retro-orbital blood samples were taken and spleens harvested from cohorts of age and sex matched mice and sent for FACS, clinical chemistry and haemotology screening. Although all data are presented on the
same box and whisker plots, separate cohorts of mice were compared for analysis, each with a wild-type control; this was done to try and reduce potential variability due to application settings and subtle differences in genetic background between the different alleles. This work was undertaken by the MRC Harwell Institute’s Clinical Pathology core facility using a standardised panel of markers used for end-point screening of mice from the International Mouse Phenotyping Consortium (IMPC) project (http://www.mousephenotype.org/). Data from relevant markers are reported below, for all plots in this section the box represents the interquartile range, the line the median value and the whiskers minimum and maximum value for each dataset.

5.2.11.1. Regulatory T cells

Tregs have a protective function in the immune system; as their name suggests they regulate immune function and can protect against autoimmunity (Sakaguchi et al., 1995). The marker expression profile of Treg cells are identified by our system as cells positive for CD5, CD4 and CD25. As previously mentioned FOXP3 is a marker of effector Treg cells, however our system detects effector Tregs by the markers listed above with the addition of CD44. The subpopulation of Tregs deemed to be resting, or inactive is categorised by all of the markers that effector Tregs express and also CD62L (https://www.mousephenotype.org/impress/protocol/201/8).

No significant differences were seen in either total Treg population, or the proportion of effector/resting Tregs between the genotypes for any of the alleles. Global differences are apparent between the \textit{Boycie/Orai1}^{L187Q} and the \textit{Orai1}^{I123N}/\textit{Orai1}^{V135E} lines, however these are likely due to subtle differences in application settings; these two groups of samples were harvested and processed on different days (Figure 5.24).
5.2.11.2. Natural Killer T cells

The term NKT derives from the fact that these lymphocytes display some properties of both T cells and Natural Killer (NK) cells and specifically bind lipid antigens produced by the glycoprotein CD1d (Brossay et al., 1998). The subpopulation, iNKT have also been linked to a role in autoimmune disease (Yamamura et al., 2007). The markers used to identify NKT cells were CD5, CD161 and CD44, additional markers were used for recognition of resting NKT cells (CD62L), and iNKT cells (CD4), the remaining NKT subpopulation were determined to be effector NKT cells (https://www.mousephenotype.org/impress/protocol/201/8).

No significant differences were seen between genotypes for any of the lines for NKT totals or sub populations. A similar shift due to differences in applications settings was again apparent between the alleles collected and analysed on different days.
Creatine Kinase

The reported gain-of-function mutations in Orai1 all result in increased levels of CK in plasma upon clinical chemistry analysis. Creatine kinase is an enzyme that uses ATP to convert creatine into phosphocreatine and Adenosine diphosphate (ADP); the reaction is reversible so CK can act as both a temporal and spatial buffer to produce ATP where and when it is needed for cells with elevated, rapidly changing energy requirements and the need for a steady supply of ATP (Wallimann et al., 1992). As such one of the most well-known roles of CK is to regulate ATP homeostasis in muscle tissue. There are four isoforms of CK, muscle (M-CK), brain (B-CK) and two mitochondrial (mi-CK) isoforms; these usually form homodimers, although the different isoforms are also known to hetero-dimerise. The homodimer MM-CK is readily detected in skeletal muscle, and BB-CK first detected in brain as the name suggests, is expressed in the brain, nervous system
and many other tissues. Unsurprisingly, given the different isoforms and expression patterns, roles for CK have been described in many tissues including the brain (Hemmer and Wallimann, 1993) and, interestingly, the inner ear, where loss or dysregulation of B-CK results in hearing loss (Lin et al., 2011; Shin et al., 2007). Levels of plasma CK were measured from blood samples collected by terminal retro-orbital bleed (Figure 5.26).

No significant differences were seen in CK levels for any of the alleles between genotypes.

5.2.11.4. CD8 T-cells

Although none of the well described clinical phenotypes related to CRAC channel loss- or gain-of-function were observed in any of the Orai1 mutant lines, some measurements from the battery of clinical pathology did show significant differences. T cells positive for the surface receptor CD8 are known as cytotoxic T cells; these cells are involved with mediating the immune response to intracellular challenges from pathogens such as bacteria and viruses and also protect against cancer. As their name suggests, when presented with an antigen, they can initiate programmed cell death either by secreting cytotoxic granules such as perforin, or by inducing Fas mediated cell death (Berke, 1995).

In Boycie heterozygote mice levels of CD8+ T cells were significantly lower than in the age and genetic background matched wild-type mice (p=0.0414) (Figure 5.27A). The same was
true for the subset of CD8+ also expressing high levels of CD44, which identifies them as CD8 effector T cells \((p=0.0438)\) (Figure 5.27B). Interestingly no significant differences were observed between wild-type and Boycie homozygote mice in either of these cell populations; also the CD8 resting and CD8 naive populations were not significantly different between any of the genotypes, however, the same trend between wild-type and Boycie heterozygote was seen in both (Figure 5.27C and D). No significant differences in CD8+ populations were seen in any of the ENU archive alleles (Figure 5.27A-D).

5.2.11.5. Macrophages

Macrophages are derived from monocytes produced in the bone marrow and are mediators of both the innate and adaptive immune response. They are recruited to sites of infection or inflammation by chemotaxis and phagocytose non healthy cells and cellular debris. The proportion of macrophages in Boycie heterozygote splenocytes was significantly higher \((p=0.0450)\) than in wild-type mice, however, although the mean was still increased in comparison, the same was not true for Boycie homozygotes \((p=0.0954)\) (Figure 5.28).
5.2.11.6. Absolute Eosinophil Count

The haematological analysis only resulted in one significant difference between any of the genotypes in any of the parameters measured; a difference in absolute eosinophil count between \( \text{Orai1}^{L187Q} \) homozygote and wild-type mice (\( p=0.0476 \)) (Figure 5.29A). Whilst this can be a marker of autoimmune disease, when not resulting from another condition it is not necessarily detrimental to health (Chen et al., 2014). The percentage of eosinophils in the total white blood cell population was not significantly different (\( p=0.376 \)) so this finding is likely not to be physiologically relevant.

![Figure 5.28 FACS analysis of macrophage population.](image)

![Figure 5.29 Haematological analysis of eosinophils](image)
5.2.11.7. Clinical Chemistry

The clinical chemical analysis identified 3 parameters which contained significant differences, potassium, albumin and alkaline phosphatase (ALP) levels in the blood.

For potassium (in Boycie) (Figure 5.30A) and albumin (in Orai\textsuperscript{1L187Q}) (Figure 5.30B), in both cases the differences were between heterozygote and homozygote with neither being significantly different from wild-type.

A significant reduction was also identified in the level of ALP activity present in Boycie homozygotes when compared to wild-type, the Boycie heterozygote samples display intermediate levels of ALP activity (Figure 5.30C).

![Figure 5.30 Clinical chemistry analysis](Image)

**Figure 5.30 Clinical chemistry analysis** Significant differences were found between Boycie heterozygote and homozygote mice in plasma potassium levels (A), and Orai\textsuperscript{1L187Q} heterozygote and homzygote mice in albumin levels (B). Panel C shows a significantly lower level of ALP activity in Boycie homozygote mice compared to wild-type with an intermediate heterozygote level. Data were analysed using ANOVA with Tukey post-hoc analysis (Boycie and Orai\textsuperscript{1L187Q}) or unpaired students t-test (Orai\textsuperscript{1V135E} and Orai\textsuperscript{1I123N})

5.2.11.8. Summary of Clinical Pathology Findings

The combination of clinical chemistry, haemotology and FACS analysis did not highlight any of the same clinical markers that have been previously reported for ORAI1 or STIM1 mutations, either loss- or gain-of-function (Lacruz and Feske, 2015).
Although significant differences were observed in a number of clinical chemical and haematological parameters, these would likely lose significance given a larger sample size; all fell well within the normal range that is seen in a large baseline of data collected through screening projects currently in progress at the MRC Harwell Institute. In addition the fact that significance was seen between heterozygote and homozygote animals for potassium and albumin levels with neither being significantly different from respective wild-type measurements makes the clinical importance of these measures questionable.

The FACS findings that macrophage proportion was higher, and CD8+ T cells were lower in Boycie heterozygote mice may also not be relevant given the observed semi-dominant phenotypes seen in the inner ear; if these changes resulted from the mutation, homozygote proportions would likely be more severely affected than the heterozygote.

The ALP data are the only measurement for which Boycie heterozygote mice display a phenotype intermediate between that of wild-type and Boycie homozygote, however the assay is enzymatic and prone to variation. Abnormally low levels of plasma ALP may be tolerated by the animal, but can be related to conditions that would not be typical of a mouse housed in a controlled SPF environment, e.g. malnutrition starvation, ingestion of excess vitamin D, hypothyroidism, achondroplastic dwarfism, deposition of radioactive materials in bone, severe anaemia, celiac disease, Wilson disease, hypomagnesemia, Zn\textsuperscript{2+} deficiency and hypophosphatasia (Lum, 1995; Shaver et al., 1986). Although significantly different, again the values fall well within the normal range gathered from a baseline of C57BL/6NTac IMPC mice (57 -136 U/l, in 16 wk old mice).
5.2.12. Is the c.392T>C Mutation in Orai1 Causative of the Hearing Loss in Boycie Mice?

The mutation in Orai1 was shown to be the only coding ENU lesion in the Boycie candidate interval, however, several aspects of the data gathered during the project have suggested it may not be the causative mutation underlying the hearing loss.

Firstly, although predicted by Mutation Taster to be disease causing, the difference in amino acid properties is relatively low; other in silico prediction platforms, although all predicted the mutation to be deleterious, did so with marginal confidence.

Secondly, none of the Orai1 alleles recovered from the Harwell ENU DNA archive exhibited hearing loss.

Thirdly, the Boycie mutation appeared not to affect CRAC channel function, at least in the rate of Ca$^{2+}$ uptake upon ER store depletion.

In the process of collecting data for the 7-8 month time-point for ABR and SEM examination, a G12 mouse was identified that raised further concerns as to whether the c.392T>C mutation in Orai1 is the causative mutation of the auditory phenotypes in this line. The mouse, BOYCIE-IC/8.2d, was homozygous for the c.392T>C mutation in Orai1, but exhibited no hearing loss upon ABR analysis at 8 months of age (Figure 5.31A and B). In addition, in contrast to all other Boycie mice aged >5 months regardless of whether heterozygote or homozygote, no loss of OHC was observed when investigated using SEM (Figure 5C and D).

It should be noted that as this mouse was the only animal not to exhibit a hearing loss it was not included in the ABR or SEM analysis presented earlier in this chapter. In addition no mice were identified with hearing loss that did not carry the Orai1 mutation, apart
from an occasional reduced click-box response (in excess of 500 mice covering all genotypes).

![Figure 5.31 Analysis of BOYCIE-IC/8.2d](image)

Panel A shows raw ABR traces in response to a broadband click stimulus at decreasing sound pressure levels from 90 – 40 dB SPL as denoted. The stack of traces on the left is from a representative age matched wild-type mouse, and is similar in amplitude and morphology to the 8 month old Boycie homozygote mouse BOYCIE-IC/8.2d (middle), with the response clearly still visible at 40dB SPL. The stack to the right is representative of a Boycie homozygote mouse of the same age, the ABR threshold in this mouse is estimated at 60 dB SPL. Panel B shows an audiogram displaying ABR thresholds for BOYCIE-IC/8.2d (yellow) with the average wild-type (blue) and Boycie homozygote (red) thresholds for mice of similar age. Panels C-F are scanning electron micrographs showing that there is no degeneration of OHC in BOYCIE-IC/8.2d. The cochlear overview (C) has OHC highlighted in purple, there was no loss observed anywhere along the cochlear spiral; the thinner highlighted areas are due to obstruction of the organ of Corti by the tectorial membrane and other structures as a result of the imaging angle. Panels D-F are higher magnification (1000 x) scanning electron micrographs from the apical (A), mid (B) and basal (C) cochlear turns. *Orai1*+/+ and *Orai1*Byc/Byc data presented for reference purpose in B are the mean 7-8 month ABR shown earlier in Figure 5.8 error bars denote standard error of the mean. Scale bars: (C) = 100 µm, (D-F) = 10 µm.
5.3. Discussion

5.3.1. Identification of the Boycie Mutation

A combination of Sanger sequencing and WGS have shown that the c.392T>C Boycie mutation in Orai1 is the only ENU-induced coding lesion in the candidate interval, identified by whole genome linkage analysis and subsequent fine mapping using SNP markers. All of the gaps in WGS coverage have also been ruled out by Sanger sequencing. This strongly suggested that it is the causative mutation in Boycie mice.

5.3.2. Evaluation of Auditory Function

A combination of click-box and ABR analysis shows a progressive predominantly high frequency hearing loss in both Boycie heterozygote and homozygote mice. The click-box data collected from G4 mice, which could only be heterozygous or wild-type for the causative ENU mutation, rules out a recessive mode of inheritance. The data also suggest a progressive hearing loss by the variability of onset of auditory deficit, denoted by the age affected mice lost the response to the click-box, this variability did not appear to be affected by gender.

Time-course ABR analysis from animals nearing congenicity on the C3H background show a delay in onset of hearing loss when compared to mice from earlier generations. At 4 months of age, the mean ABR thresholds of heterozygote mice were not significantly different from wild-type at any frequency tested, although a small number did have slightly elevated thresholds at 32 kHz, as can be seen by the error bars in Figure 5.8A and the appendix. Conversely, mice homozygote for the c.392T>C mutation in Orai1 displayed significant elevations in ABR thresholds over both wild-type and heterozygote animals, at all frequencies, but particularly for the 32 kHz stimulus for which none showed a
response. The time-course shows a steady decline in auditory function, across all frequencies but again predominantly affecting the 32kHz stimulus, for which by 8 months all mice either heterozygous or homozygous for the \textit{Orai1} mutation (with the exception of BOYCIE-IC/8.2d) had ABR thresholds >85 dB SPL. These data show a semi-dominant effect of the mutation.

5.3.3. Histological Analysis

The organ of Corti in a number of the available sections suffered an amount of damage during preparation, as is sometimes the case with paraffin sectioning of adult bisected heads. Regardless, hair cell (and in some sections Deiters’ cell) loss was apparent in the basal and mid turns of 5 month old G4 Boycie heterozygote cochleae, by the absence of nuclei; this observation prompted further investigation using SEM.

The degeneration of apical SGN is an interesting phenotype, given the predominantly high frequency hearing loss. It is possible that this loss of SGN would be tolerated and allow a response to tone burst ABR stimulus; research suggests the cochlea can still elicit a response to sound with only 10% of the SGN remaining (Arvin et al., 2013).

The reduction in the stria vascularis area is somewhat in agreement with the auditory thresholds, and suggests that the hearing loss may result from perhaps a metabolic or vascular deficiency; this would also fit with a progressive hearing loss. Interestingly no difference between Boycie heterozygote and wild-type mice was seen in the stria vascularis in the apical turns of cochleae at 5 months of age, which is somewhat surprising given the degeneration of the SGN in the cochleae of the same Boycie heterozygote animals.
5.3.4. Ultrastructural Investigation

The hair cell phenotype in Boycie mice was very interesting, although stereocilia bundle morphology itself appeared not to be affected. As mentioned it was difficult to assess all mutant cochleae for IHC degeneration due to the obstruction of the non-retracted tectorial membrane, however, from those which were visible it appeared that the IHCs were on the whole unaffected. In very basal regions IHC loss was noted in some samples, but further up the cochlear spiral this was restricted to occasional sporadic loss if at all, suggesting the Boycie mutation mainly affects OHC.

Although the OHC loss in the basal and mid basal regions fits broadly with the high frequency hearing loss observed in Boycie mice, it may not be the primary cause. Previous studies have resulted in place frequency maps of the mouse cochlea (Muller et al., 2005; Ou et al., 2000); using these as a guide, the 32kHz ABR tone burst stimulates the cochlea at roughly the position where the sudden loss of hair cells is observed (~400° from the apex), however the 20kHz click-box stimulus corresponds to an area ~315° from the apex of the cochlea (Figure 5.32). At this position the morphology of the organ of Corti of all Boycie heterozygote or homozygote animals examined by SEM showed the normal one row of IHCs and 3 rows of OHCs. In addition, the moderate increase in ABR threshold of the homozygote mice across all stimuli would also suggest that although OHC function may well be affected, OHC loss is not the underlying, or at least the sole, cause of the hearing loss in Boycie mice.
5.3.5. Additional *Orai1* Alleles

None of the *Orai1* alleles re-derived from the MRC Harwell Institute ENU DNA archive recapitulated the hearing loss seen in Boycie mice. There was some suggestion that homozygote perinatal/prenatal lethality was present in the *Orai1*\textsuperscript{V135E} and the *Orai1*\textsuperscript{I123N} lines due to significantly reduced numbers of homozygote mice. This could fit with the lethality reported in other mice either carrying null alleles of *Orai1* or the p.R91W mutation, however these mice were derived from a G1 founder male’s sperm and consequently would be carrying a large number of ENU induced lesions in addition to the one found in *Orai1*. It is quite possible that these additional lesions may have recessive effects on mortality that would become apparent in the G3 pedigrees that were used in this project. Ideally heterozygote mice would have been backcrossed for a number of
generations before attempting to produce homozygote mice, however time-constraints did not allow this.

5.3.6. Functional Effects of the c.392T>C Mutation in Orai1

Transfection of Cos-7 cells with EGFP and c-myc tagged ORAI1 containing the Boycie mutation showed no difference in subcellular localisation from those transfected with the wild-type ORAI1 constructs fused with the same tags. This suggests that the mutation does not affect the ability of ORAI1 to localise to the plasma membrane where it is known to function.

The Boycie mutation also had no significant effect on the rate of Ca\(^{2+}\) uptake of store depleted HEK293 cells, however, if the mutation had a very subtle effect on channel functionality this could manifest in a phenotype over a period of time. This would, of course, relate to an acquired in vivo effect so would not be possible to investigate with standard in vitro channel function assays.

The clinical pathology findings did not mirror those seen in either loss- or gain-of-function mutations in either STIM1 or Orai1. Differences that were found to be significant fell within the normal ranges for mice of a similar age; no solid conclusions can be drawn from these data as to whether they are physiologically relevant. The data may have returned more interesting findings if the mice were immunologically challenged prior to sampling, however, we could not perform this experiment on the Home Office project licence that these mice were governed by.

5.3.7. Is the c.392T>C Mutation the Cause of the Hearing Loss in Boycie Mice?

Although not all mutations result in complete penetrance of a phenotype, and even though BOYCIE-IC/8.2d is just one animal, the fact that it is homozygous for the mutation
would mean all ORAI1 protein present in this mouse would harbour the p.V131A substitution. Given the fact that ORAI1 forms a multimeric pore of the CRAC channel, if channel function was altered by the mutation, it would have to be affected in this animal as it carries no wild-type Orai1 allele. Even though there is a progressive element to the hearing loss, all other homozygote mice suffered profound high frequency hearing loss by 4 months of age.

This is the only animal either heterozygote or homozygote for the Orai1 mutation from any generation which did not exhibit either of the auditory phenotypes; if this is not the causative mutation it must be in very close proximity as the candidate region is only ~3 Mb. Therefore, DNA from this mouse could be used for sequencing to determine if any of the originally identified ENU-induced lesions have been lost in this animal. There are not many additional informative SNPs available to help narrow the region; as an alternative, genotyping of Boycie mice for these non-coding ENU-induced lesions identified by WGS could be useful in further narrowing the candidate region.

The possibility remains, that the c.392T>C mutation, as with other non-recessive mutations in Orai1/Stim1 has a gain-of-function effect on the channel. Although unlikely, it is possible that this mouse contains a spontaneous mutation that works in opposition to this gain-of-function, resulting in a lack of phenotype. Genetic background was seen to have an effect on the viability of Orai1<sup>tm1Rao/tm1Rao</sup> null mice, which displayed complete perinatal lethality on C57BL/6J background, but when crossed to the outbred mixed ICR strain, around a third of homozygote mice survived past 90 days (Gwack et al., 2008); so if the effect of a null can be modified, it stands to reason that a point mutation resulting in a relatively small difference in amino acid biochemical properties could also be influenced by modifiers. In addition, the semi-dominant mode of inheritance fits very well with a
protein that requires a homomeric configuration to function correctly. In heterozygote 
Boycie animals there is the probability that some functional channels will be formed, 
however in homozygotes no wild-type protein will be present so the oligomerised protein 
will contain only mutant monomers. This could result in a severe homozygote and an 
intermediate heterozygote phenotype, as was observed in Boycie mice.

Unfortunately, due to time constraints imposed by the part-time nature of my PhD 
registration, and given the uncertainty surrounding the genetic basis of the hearing loss in 
Boycie mice, progress on this project was suspended in favour of concentrating on the 
goya project. The only way to categorically prove that the Orai1 c.392T>C mutation is 
causative of the progressive hearing loss and observed degenerative phenotypes in the 
inner ears of Boycie mutant mice, therefore discovering an as yet unreported role for 
Orai1 in the inner ear, would be to replicate the exact point mutation in vivo and assess 
auditory function in mice carrying this substitution. This option is becoming increasingly 
possible with the emergence of CRISPR/Cas9 technology and the accuracy of genome 
editing that it allows; however when the decision was made, access to this technique was 
limited. This situation has changed and the process of producing these mice will likely be 
initiated in the near future.
Chapter 6

General Discussion
6.1. Discussion of Thesis Aims

The aims of this thesis were to identify and characterise new mouse models of deafness. To this end, I have reported two new mouse models of deafness; *goya* which has identified multiple novel roles for *Map3k1* in the auditory system (Parker et al., 2015), and *Boycie* which could in future reveal a role for Orai1 and SOCE in the inner ear. Both of these models were identified using ENU-induced mutagenesis screens, meaning that no *a priori* assumptions were made as to the identity of the genes underlying the observed phenotypes. This approach encourages un-biased analysis of data and has proved a valuable tool for identifying novel genes involved with many diseases, in this case deafness.

The identification of both of these mutants was achieved by assessing auditory function of mice from G3 pedigrees using the standard methods of click-box and ABR screening (Hardisty-Hughes et al., 2010). Subsequently, time-course assessment of auditory function highlighted that both mutants have a progressive element to the hearing loss observed. Various imaging techniques were used to assess the pathological effect of the mutations on the auditory system, with a view to elucidating the cause of hearing loss.

Functional studies were performed to gain insight into the molecular mechanisms underlying the phenotypes in both mutant lines. Whilst no definite mechanisms were found for either mutant, an increase in p38 phosphorylation was observed in the cochlea of *Map3k1*<sup>goya/goya</sup> mice, an observation that is in agreement with previous *in vitro* analysis of human *MAP3K1* mutations (Loke et al., 2014; Pearlman et al., 2010). Investigation relating to the extra OHC observed in MAP3K1 deficient mice did not provide a mechanism, but has eliminated the possibility of the phenotype resulting from dysregulation of proliferation by p27KIP1.
6.2 Characterisation of the *goya* Phenotype

Phenotypic analysis of the *goya* mutation has shown that *Map3k1* is required both for correct development of the organ of Corti, and for maintenance of auditory function. Although very rapid, there is a progression of hearing loss and OHC degeneration, suggesting that MAP3K1 plays a protective role in OHCs, which would fit with its pivotal role in the stress activated MAPK pathway. The OHC loss was apparently the only pathological change in cochleae of *goya* and *Map3k1*\textsuperscript{tm1Yxia/tm1Yxia} mice. In the latter case, a moderate hearing loss was already present at 2 weeks of age, when the mice still clearly possessed 4 rows of OHCs throughout the length of the cochlear spiral. This would suggest that the degeneration of OHC may not be the sole cause of the elevated ABR thresholds in MAP3K1 deficient mice. Notwithstanding this possibility, the fact that both the increased cellular number and the cellular degeneration affected almost exclusively the OHCs, it would appear that MAP3K1 is important mainly for this cell type in the ear.

The MAP3K1-β-Galactosidase fusion protein was expressed widely throughout the epithelial and supporting cells in the cochlear duct, which may be expected given its diverse functionality. A contemporaneous study identified expression of the fusion protein mainly in supporting cells, although they too saw cytoplasmic expression in both IHCs and OHCs (Yousaf et al., 2015). The expression pattern was very similar to that seen in this study, by both the use of an anti-β-Galactosidase antibody and also X-Gal staining. In mid-modiolar cochlear sections the X-Gal staining may appear very diffuse, however punctate expression was also seen in certain orientations using this method (Figure 6.3); the diffuse appearance of the stain in cross-section images may result from the thickness and orientation of the mid-modiolar sections.
One potential explanation for the damage to the OHCs could be the reported involvement of MAP3K1 in mechanical stress response. It has been shown that MAP3K1 is essential for activating JNK in response to microtubule disruption, or stress stimuli which stretch or alter the shape of the cell membrane (Yujiri et al., 1999; Yujiri et al., 2000). The OHCs undergo higher levels of cell shape change than other cell types in the ear due to their role in the mechanoelectric transduction process; as previously described the plasma membrane of OHCs rapidly elongate/contract due to the voltage sensitive motor protein PRESTIN (Liberman et al., 2002). This hypothesis could also explain the apparent lack of cellular pathology in structures such as the stria vascularis and Reissner’s membrane.

Another stress that OHC in particular are exposed to is oxidative stress. Metabolic rates in OHC are very high in comparison to many other cell types; as such mitochondria in OHC produce a large amount of reactive oxygen species (ROS). Although OHC electromotility is not dependent on ATP itself, maintenance of membrane integrity most likely is. Accumulation of ROS connected to mitochondrial dysfunction has been credited with preferential loss of OHC over IHC in both noise and aminoglycoside damaged cochleae (Griffiths et al., 2001; Jensen-Smith et al., 2012; Meyer zum Gottesberge et al., 2012;
Ohinata et al., 2000; Ohlemiller and Dugan, 1999). MAP3K1 is known to mediate the cellular response to oxidative stress in certain cell types, notably in plants (Ichimura et al., 2006; Nakagami et al., 2006) but also potentially in the mammalian heart (Minamino et al., 1999). MAP3K1 deficient embryonic stem cell derived cardiomyocytes (ESCM), undergo high levels of apoptosis upon stimulation with H$_2$O$_2$. In these cells it was shown that MAP3K1 was required for activation of the JNK pathway in response to ROS stimulation. Interestingly apoptosis was shown to result from increased p38 activity leading to accumulation of tumour necrosis factor alpha (TNF$\alpha$), a pro-apoptotic cytokine, in the cell. The study concluded that the JNK and p38 pathways differentially regulate production of TNF$\alpha$ in response to oxidative stress, JNK playing a repressive role mediated by MAP3K1, and p38 activity leading to increased levels of TNF$\alpha$ and subsequent apoptosis (Figure 6.2) (Minamino et al., 1999).

**Figure 6.2 Proposed mechanism of TNF$\alpha$ regulation in ESCM.** A model for the opposing effects of JNK and p38 MAPK regulation of the pro-apoptotic cytokine TNF$\alpha$. MAP3K1 activation of JNK inhibits TNF$\alpha$ expression, whereas p38 activity increases TNF$\alpha$ production. Adapted from (Minamino et al., 1999)
This in an interesting observation, increased levels of phosphorylated p38 were seen throughout the cochleae of goya homozygote mice. However, it should be noted that the studies mentioned above were *in vitro* observations of cell types that are drastically different from the specialised epithelia in the cochlea, and may not well represent the *in vivo* situation in the inner ear. Increased p38 expression was also observed in cell types other than OHC, so if the degeneration were due to increased p38 activity leading to accumulation of TNFα it might be surprising not to see more widespread cellular loss. That said there were small regions of both Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> cochleae where extensive organ of Corti degeneration was observed. It may be that global increases in p38 phosphorylation arise from a similar MAP3K1 inhibition of JNK in responses regulated by a balancing of opposing JNK or p38 activity, but from stimuli which do not ultimately elicit apoptotic responses. This may make sense as the increase in p38 phosphorylation was already evident at P1, before the onset of hearing and subsequent increased mitochondrial load resulting from electromotility.

Beyond the involvement in cell survival and apoptosis through the classical ERK/JNK/p38 MAPK pathways, a number of other possibilities exist as to why MAP3K1 deficiency may lead to a loss of auditory function.

Tight junctions are very important in the inner ear for conservation of the barrier between endolymph in the scala media and perilymph in the scala tympani/scala vestibule, and also for the electrical isolation of the intrastrial space. As described in the Introduction (1.3.1, 1.3.4), this separation is essential for the maintenance of the endocochlear potential required for cochlea function (Kitajiri et al., 2004). The MARVEL (MAL and related proteins for vesicle trafficking and membrane link) are a family of tight junction associated proteins that share a common multi-transmembrane domain. The MARVEL family consists of three proteins, OCCLUDIN, TRICELLULIN (MARVELD2) and
MARVELD3, interestingly mutations in two of the genes that code these proteins are known to result in hearing loss (Raleigh et al., 2010). TRICELLULIN is the causative gene underlying the hearing loss in DFNB49 families (Riazuddin et al., 2006), mice carrying mutations in the homologous Marveld2 gene display early onset progressive hearing loss and degeneration of the organ of Corti, predominantly the OHC (Nayak et al., 2013). Mice deficient for Occludin also display a similar phenotype, which the researcher’s attribute to perturbed localisation of TRICELLULIN in mutant cochleae (Kitajiri et al., 2014). The most recently discovered member of the MARVEL protein family, MARVELD3, has been shown to couple MAP3K1 to tight junctions during osmotic stress (Steed et al., 2014). Although no link has currently been made between MARVELD3 and hearing loss, given the overlapping functions of the family members, and the similarities between the observed phenotypes and that of MAP3K1 deficient mice, it may warrant further investigation.

There is also the suggestion from both whole-mount immunofluorescence labelling and also X-gal staining that MAP3K1 may be present in the stereocilia, however caution should be used when interpreting these findings; stereocilia tips are often the site of non-specific binding of antibodies, anecdotally many researchers deem them to be adhesive. The X-gal staining does not arise from a binding of antibodies; it is a chemical reaction therefore should not be affected in the same way, and no staining was observed in the wild-type controls. It is still unclear as to whether this labelling is real, however, it is an intriguing finding that may warrant future studies. Research has shown that cell division control protein 42 homolog (CDC42) is important for apical junction complexes and maintenance of stereocilia in hair cells by fine regulation of actin polymerisation and turnover (Ueyama et al., 2014). MAP3K1 is known to bind both CDC42 and RAC, and inactivation of the kinase domain of MAP3K1 blocks CDC42/RAC induced JNK activity in transfected COS cells (Fanger et al., 1997). Interestingly, similar to MAP3K1 deficient
mice, conditional Atoh1–Cre;Cdc42^floxflox knockout mice harbouring a hair cell specific inactivation of Cdc42 also show an early onset, rapid progressing hearing loss. They also show a degeneration of cochlear hair cells over a comparable timescale, however in contrast to Map3k1 mutants the IHCs appear to be more severely affected than OHCs (Ueyama et al., 2014). Another consideration is that although goya mice have extra OHC which undergo rapid degeneration, they do not display any obvious stereocilia abnormalities.

It is possible there may be other pathological effects of MAP3K1 deficiency not detectable by the methods utilised in this thesis. Loss of SGN was not observed in goya homozygote mice, although it was reported in a parallel study of Map3k1^tm1Yxia/tm1Yxia mice (Yousaf et al., 2015). This could be due to differences in the nature of the alleles, or possibly due to a genetic modifier in the C3H background; whereas the mice in the parallel study were solely maintained on C57BL/6J (Yousaf et al., 2015); this strain is known to carry the age related hearing loss (ahl) allele of Cdh23 and are known to exhibit SGN degeneration (White et al., 2000). It would be useful to use transmission electron microscopy to study structures such as tight junctions and the stria vascularis in greater detail.

6.3. Functional Characterisation of the goya Mutation and Map3k1 Deficient Mice.

The true effects of the goya mutation in Map3k1 structure and function are yet to be fully understood. On the surface of it the mutation appears to result in a functional null, in that it mirrors the phenotype of the kinase deficient Map3k1^tm1Yxia/tm1Yxia allele. This might be true, although the capability of MAP3K1 to act as both a kinase and an E3 Ubiquitin Ligase (Lu et al., 2002) leaves an alternative explanation. It is possible that the observed auditory phenotype in both alleles may not in fact result from a lack of the enzymatic kinase
activity, but altered function of the full-length MAP3K1, as both the N-terminal ZnF RING and kinase domain are known to be required for E3 Ubiquitin Ligase activity (Lu et al., 2002). It has also been proposed that 6 different heterozygous MAP3K1 mutations in human patients with 46 XY DSD result in a gain-of-function due to increased binding of RHOA, and MAP3K4 (Loke et al., 2014). The suggested gain-of-function is inferred from the fact that RHOA promotes kinase activity of MAP3K1 (Gallagher et al., 2004); increased binding of RHOA could potentially cause MAP3K1 mediated hyper-phosphorylation of p38 (and ERK1/2). One of the mutations in question, c.2180-2A>G (human) resulted in either skipping of exon 13 or the use of a cryptic splice acceptor site which results in the deletion of the first 35 amino acids of exon 13 (Loke et al., 2014); this is very similar to the goya mutation which could result in exon 13 skipping, or deletion of the last 21 amino acids of exon 13. This raises an interesting question, does the goya mutation result in a gain of RHOA mediated MAP3K1 kinase activity which results in the observed increase in p38 phosphorylation in the cochlea? The argument against this would clearly be that both goya and Map3k1<sup>tm1Yxia</sup> mutants display similar phenotypes, and it would be impossible for the latter to be the result of increased MAP3K1 kinase activity.

The <i>in silico</i> analysis using predicted 3-D models of wild-type MAP3K1 and the goya MAP3K1<sup>CS</sup> indicated that the tertiary structure of the kinase domain is largely unaffected by the use of the cryptic splice site. The main structural differences between the two predictions were, as one might expect from the location of the missing amino acids, in and around the ARM domain, in close proximity to the ZnF RING domain. Consultation with a structural biologist may help interpret these data more accurately and this would be a likely future direction for this project.

Immunodetection of total and phosphorylated p38 in inner ear total protein lysates using the Peggy™ system and immunohistochemistry, showed increased phosphorylation of
p38 MAPK in goya homozygote mice. The data from Peggy™ did not show a significant difference between wild-type and goya homozygote lysates, however as discussed this is likely due to a wild-type lysate, identified as an outlier, displaying a very high ratio of phosphorylated p38 when compared with total p38. This experiment was performed on whole inner ear total protein lysates; a more accurate indication of the situation in the organ of Corti may have been possible by either sub-dissecting the organ of Corti, or using FACS to separate certain cell types. Unfortunately the FACS analyser I had access to only allowed counting of cells, they could not be collected after sorting. Removal of the cochlear duct was considered, however concerns arose about the amount of total protein that would be recovered, and therefore the amount of mice required to extract sufficient to run the experiments. The physiological relevance of the increase in p38 phosphorylation in relation to either additional OHCs or their subsequent degeneration and related hearing loss is unclear. However, it is interesting given the opposing effects of MAP3K1 activated JNK and p38 activity in relation to TNFα regulation during oxidative stress, as previously discussed (Minamino et al., 1999).

The fact that MAP3K1 is involved with a large number of pathways (see Table 6.1) makes it very easy to hypothesise a mechanism whereby a Map3k1 mutation may result in a given phenotype. Unfortunately, however, it also makes it extremely difficult for those hypotheses to be proved.

It is clear that MAP3K1 regulates a huge range of genes, proteins and cellular processes, many of which are stimulus, cell type or organism specific. For this reason I decided instead to investigate further the mechanism behind the additional row of OHC in Map3k1 mutants. Previous research regarding Map3k1 deficiency in the retina, combined with the phenotypes observed in p27kip1 and Rb1 null mice, potentially supported the hypothesis that dysregulation of the p27KIP1/RB pathway could result in increased
proliferation of cochlear progenitor cells and subsequently lead to the presence of supernumerary OHCs (Chen and Segil, 1999; Kil et al., 2011; Lee et al., 2006; Lowenheim et al., 1999; Mongan et al., 2011; Sage et al., 2005; Sage et al., 2006b). However, assessment of proliferation in the cochlea of Map3k1tm1Yxia/tm1Yxia mice using EdU and expression of p27KIP1 concluded that this is not the case.

Investigation of other proteins involved in the tightly co-ordinated process of cellular differentiation and patterning in the cochlea may help elucidate the mechanism that is responsible. When the genes involved with an increased OHC number phenotype are placed in a STRING interaction network (http://string-db.org/) (Szklarczyk et al., 2015), it is clear that the NOTCH signalling pathway would be an ideal starting point (Figure 6.3).

Table 6.1 Pathways involving MAP3K1. Lists of Biosystems, Reactome, GeneGo, KEGG and Qiagen pathways that involve human MAP3K1. (Source GeneCards)
As mentioned in chapter 4 (4.4.1.3) there are six members of the NOTCH pathway associated with this phenotype. Investigation of this pathway was considered before commencing the EdU/p27KIP1 study, however, I felt the evidence from the literature given the retinal \( \text{Map}3k1^{tm1Yxia/tm1Yxia} \) phenotype (Mongan et al., 2011) was compelling enough to pursue this line of investigation. In addition, the only direct interaction represented in the network for \( \text{Map}3k1 \) is that with \( Rb \).
The study by Yousaf et al. reported downregulation of Fgfr3, fibroblast growth-factors (Fgf8, Fgf10) and activating transcription factor 3 (Atf3) in the inner ear of Map3k1\textsuperscript{tm1Yxia/tm1Yxia} mice (Yousaf et al., 2015). This is interesting as an extra row of OHC is found in Fgfr3 null mice that at first glance closely resembles that of Map3k1 mutants (Hayashi et al., 2007). However the Fgfr3 null mice also show defects in pillar cells which are not observed in either goya or the tm1Yxia alleles of Map3k1 described in this thesis.

At around E15.5, FGFR3, is observed in a subset of supporting cell progenitors expressing PROX1, which become either pillar cells or Deiters’ cells (Hayashi et al., 2007). The FGFR3 ligand, FGF8, is secreted from immature IHCs and is thought to interact with the receptor; the cells nearest the IHCs contain the highest levels of FGF8, which results in their differentiation into pillar cells, whereas those expressing lower levels become Deiters’ cells (Hayashi et al., 2007; Shim et al., 2005). The extra row of OHC in Fgfr3 null mice are present with a corresponding extra row of Deiters’ cells, but no outer pillar cells; in the basal third of the cochlea a normal arrangement of inner and outer pillar cells and 3 rows of OHCs and Deiters’ cells are present (Hayashi et al., 2007). This suggests that the extra row of OHC (and Deiters’ cells) differentiate at the cost of the outer pillar cells in the mid and apical regions of the cochlea of Fgr3 null mice. This does not appear to be the case in goya mice, which as can be seen by the histology images in Chapter 3 (Figure 3.6), have both inner and outer pillar cells present. This would suggest the extra OHC in Map3k1 mutant mice do not result from the same mechanism and by association dysregulation of FGFR3/FGF8 is likely not the molecular basis of the phenotype. However, as both were found to be downregulated in the inner ear of Map3k1\textsuperscript{tm1Yxia/tm1Yxia} mice (Yousaf et al., 2015), it would certainly be worth investigating the localisation of these genes and protein products in the cochlea of Map3k1 mutants.
6.4 Characterisation of the Boycie phenotype

The phenotype observed in Boycie mice highlighted a progressive, predominantly high frequency hearing loss. In all G4 mice heterozygous for the c.392T>C mutation in Orai1, a reduced or lack of response to a 20 kHz click-box stimulus was observed between 4 and 11 weeks of age. When the Boycie mutation was considered to be congenic on the C3H inbred strain (G10+), the onset of hearing loss was delayed significantly. At 4 months of age Orai1Byc/+ mice still exhibit ABR thresholds not significantly different from wild-type, whereas Orai1Byc/Byc mice show significantly elevated thresholds when compared to wild-type at all stimuli tested (with the exception of BOYCIEIC/8.2d). This shows (1) that the mutation has a semi-dominant effect, and (2) that the C3H genetic background appears to offer a protective effect over mice with a more mixed background involving C57BL/6J. The pathological findings of OHC loss, thinning of the stria vascularis and loss of SGN in the inner ear, combined with the progressive hearing loss make Boycie an excellent model of presbycusis.

6.5. Does ORAI1 have a Function in the Inner ear?

Although ORAI1 and CRAC have not previously been reported to be involved with auditory function, there is emerging evidence for store related Ca$^{2+}$ activity in hair cells. Research has shown that Ryanodine receptors (RyR) are expressed in hair cells in the developing and adult inner ear, and may play multiple roles in auditory function.

RyRs, like InsP$_3$R are a mediator of store Ca$^{2+}$ release, first discovered as channels in the sarcoplasmic reticulum (SR) of muscle cells, which is analogous with the ER in other cell types (Fill and Coronado, 1988). The role of RyRs in skeletal and cardiac muscle is well described; RyRs release Ca$^{2+}$ from the SR when activated, causing a localised intracellular calcium spike, which results in muscle contraction, however activation mechanisms are
different. In skeletal muscle, RyRs are activated through direct coupling to the voltage gated Ca\(^{2+}\) channel dihydropyridine receptor DHPR (Ca\(_{\text{v1.1}}\)) in the transverse tubule (t-tubule) of the myocyte sarcolemma; when the DHPR is stimulated by an action potential it alters conformation and activates the RyR (Schneider, 1994). In cardiac muscle, however, this is achieved through the mechanism known as calcium induced calcium release (CICR) whereby influx of Ca\(^{2+}\) through voltage gated channels in the sarcolemma activate the RyR to release ER Ca\(^{2+}\) (Fabiato, 1983). In contrast to the InsP\(_3\)R which needs to be primed by InsP\(_3\) before activation by Ca\(^{2+}\), RyRs can be activated solely by localised increases in cytosolic Ca\(^{2+}\).

The exact role(s) of RyRs in the inner ear is still somewhat unclear, however a number of hypotheses have been proposed. It has been shown that RyRs are widely, but differentially, expressed in mammalian inner ear structures and SGN both throughout development and in adults (Liang et al., 2009; Morton-Jones et al., 2006); this could suggest numerous roles for CICR in correct auditory function.

In mature IHCs, the large conductance, voltage- and Ca\(^{2+}\) activated potassium channels known as ‘Big K’ (BK) channels have been shown to be responsible for the main outward K\(^+\) current, \(I_{K,f}\) (Kros and Crawford, 1990; Marcotti et al., 2004). This current is required for precise temporal high frequency tuning of IHC neurotransmission by rapidly hyperpolarising the membrane following depolarisation from K\(^+\) influx through the MET channels in the stereocilia. This periodicity is thought to be important for both high frequency sound transduction and more centrally discrimination and recognition (Kurt et al., 2012; Oliver et al., 2006; Ramanathan et al., 1999). The \(I_{K,f}\) current has been shown to be reliant on activation by Ca\(^{2+}\) from intracellular stores by dual depletion of the IHC Ca\(^{2+}\) stores with the RyR agonist caffeine and inhibition of SERCA with thapsigargin significantly reduced the current (Marcotti et al., 2003). Further evidence that RyR mediated CICR
regulates $I_{K_f}$ comes from the fact that ryanodine, in concentrations that block the RyR, also decreases currents derived from BK channels in the IHCs; stimulation with caffeine produces the opposite effect (Beurg et al., 2005). These data together implicate CICR mediated by RyRs in the regulation of BK channels, consequently membrane potential, and therefore neurotransmitter release (Beurg et al., 2005).

Induced release of Ca$^{2+}$ from intracellular stores in vivo affects compound action potentials and DPOAEs in mice, but interestingly not the EP (Beurg et al., 2005; Bobbin, 2002; Bobbin et al., 2003); this highlights a potentially important role for CICR in OHC function but indicates that strial function is unaffected. Also it is suggestive that CICR can work post-synaptically to inhibit OHC function in response to incoming signals through cholinergic efferent synapses of the OHC (Sridhar et al., 1997). A presynaptic role for RyR in hair cell afferent ribbon synapses has also been proposed, although not well defined as yet (reviewed in (Castellano-Munoz and Ricci, 2014)).

Although CICR is involved with SOCE in that CRAC channels are opened in response to low levels of Ca$^{2+}$ in the ER, it should be noted that they are distinct mechanisms. Ionic balance is ever changing in cochlear hair cells and with it the receptor potential of the membrane. It may be that enough Ca$^{2+}$ is able to be sequestered from the cytoplasm by SERCA pumps to replenish stores, however a role for SOCE is a distinct possibility, particularly given the near ubiquitous expression of both STIM proteins and ORAI proteins. Indeed SOCE via STIM1 and ORAI1 has been shown to replenish ER Ca$^{2+}$ in photoreceptors (Molnar et al., 2012). As previously mentioned, ORAI1 and ORAI3 form a heteromeric pore for a channel gated by STIM1 and TRPC1; TRPC channels are known to play a role in the cochlea, so this is another possibility as to the requirement of functional ORAI1 in the inner ear.
6.6. Is the Pattern of OHC loss in Boycie Mice Significant?

The abrupt loss of OHC at a remarkably similar position from the apex in all Boycie mice regardless of whether they were heterozygote or homozygote for the mutation (BOYCIE-IC/8.2d excluded) suggests that Orai1, if indeed it is the mutation, is differentially required along the cochlear duct. It is known that OHC in the basal and mid regions of the cochlea differ from those found towards the apex, not only in physical size but also in the channels present in their membrane. It has been shown that there are opposing gradients of expression of BKα and Ca_v1.3 along the cochlear spiral, with higher expression of BKα in the basal and mid basal regions, and Ca_v1.3 higher in the more apical cochlear regions (Engel et al., 2006). Interestingly although BK has mainly been discussed in relation to clearing the K⁺ from IHCs, in BKα null mice it is OHCs that degenerate, and consistently with the differential expression, exclusively in the basal and mid basal regions. The OHC loss has been attributed to the loss of KCNQ4 channels in this population of OHC, although exactly why they are lost remains a mystery. Kcnq4 null mice exhibit a very similar phenotype to BKα, DPOAE’s in both of these mutants are reduced even before OHC degeneration, most likely due to electro-motility deficits resulting from constantly depolarised membrane potential (Kharkovets et al., 2006). In addition Ca_v1.3 null mice also show a progressive hearing loss with a degeneration of OHCs, although hearing loss in these mice has been attributed to a lack of presynaptic Ca^{2+} currents in the IHCs. In these mice it is the lower frequency OHC that degenerate, again consistent with the elevated expression of Ca_v1.3 in the apex of the cochlea (Engel et al., 2006).

Taken together these mutants highlight an important role for Ca^{2+} regulation in OHCs; deficits in a number of channels that mediate this can result in progressive auditory dysfunction and a loss of OHCs. In addition it is clear that ion channels are differentially
expressed along the tonotopic axis of the cochlear spiral; both of these observations and
the high level of similarity with the Boycie phenotype could point towards the
involvement of Orai1 in the maintenance of intracellular Ca\(^{2+}\) flux essential for OHC function in the sub-population that exhibit high levels of BK\(\alpha\).


I have fulfilled the aims of my thesis by using the forward genetics approach of ENU mutagenesis to identify two novel mouse models of deafness, goya and Boycie. The goya project has revealed that the serine threonine kinase Map3k1 has distinct roles in auditory development and maintenance of correct auditory function. My findings during the course of this project were recently published in Disease Models and Mechanisms (Parker et al., 2015). The Boycie project has identified a potential novel role for the CRAC channel pore forming sub-unit Orai1 in the inner ear. There is the suggestion from a number of aspects of the data that this is not the causative mutation, however, it is the only ENU-induced non-synonymous coding lesion in the candidate interval and all mice carrying the allele, apart from the one homozygote animal, develop a progressive hearing loss. The semi-dominant phenotypic effects of the mutation would also potentially support the involvement of homomultimer. It is clear that before further studies are undertaken into the Boycie mutant, the identity of the mutation should be corroborated.

As additional ENU–induced Orai1 alleles failed to replicate the hearing loss in Boycie mice, CRISPR/Cas9 technology should be used to recreate the c.392T>C mutation. This would ultimately confirm or refute the involvement of Orai1 in the observed phenotype.

Both the goya and Boycie mouse models highlight the benefits of ENU mutagenesis screens. The goya mutation in Map3k1 was originally identified from the EOB phenotype. Reverse genetic approaches i.e the generation of kinase null Map3k1\(^{tm1Yxia}\) mice, had
previously established that Map3k1 deficiency resulted in this phenotype (Yujiri et al., 2000). The hearing loss, which was clearly present in these mice, was not discovered for over a decade, until the goya mutant was identified during the ENU mutagenesis screen facilitated by the use of multiple phenotyping platforms to investigate a range of disease areas. The Boycie mutant, if Orai1 does indeed prove to be the mutation, also potentially highlights another advantage of ENU mutagenesis. The fact that dominant mutations in Orai1 result in a gain-of-function (Endo et al., 2015; Nesin et al., 2014), raises the possibility that if the c.392T>C mutation in Orai1 could also be a gain-of-function. This would not have been discovered using traditional knockout strategies, and although now it would be possible to recreate using CRISPR/Cas9 technology, it is unlikely that reverse genetics approaches would have been used to investigate Orai1 function in relation to deafness. This project, however, also highlights one of the disadvantages of forward genetics screens; identity of the causative mutation. In order for reports of novel genes in any disease model to be credible, it is critical that solid evidence is obtained to prove beyond doubt that they are the underlying cause of the observed phenotype. In the case of recessive mutations, as was shown in the goya project, this can be fairly straightforward by generating compound heterozygote animal using a null allele of the candidate gene; recapitulation of the phenotype will indicate that the correct mutation has been identified. For dominant mutations, however, it is not quite as simple; irrefutable phenotypic or functional evidence or additional alleles displaying the same phenotype are required to remove doubt. It is likely that with the rapid developments with regard to CRISPR/Cas9 confirming the identity of these mutations will become much easier in the future, as is the plan for the Boycie model.

Often when a powerful new technique emerges, older methods become redundant. At first glance the ease and accuracy of genome editing possible with CRISPR/Cas9 may
suggest it will render technologies such as random mutagenesis obsolete. It is a fact though that random mutagenesis, in particular ENU, is an excellent way to identify novel disease genes, because of the unbiased nature of the phenotyping screens. The systematic approach taken by the International Mouse Phenotyping Consortium (IMPC) to create conditional knockouts and functionally annotate every gene in the mouse, will undoubtedly uncover countless novel genes and pathways in a wide range of disease systems, and will become a hugely valuable resource to researchers in the years to come. The resource will, however, only be functionally annotated in relation to ablation of gene function, the use of ENU mutagenesis to facilitate investigation of pathogenic point mutations which can closely mirror human mutations is still highly useful to researchers. It is likely that the resource created by the IMPC both in functional data and availability of established knockout and conditional knockout mice, combined with advances in CRISPR/Cas9 will result in ENU mutagenesis becoming more efficient in that confirming the causative mutation is becoming more straightforward than has been the case in recent years.
Chapter 7

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Chapter 8

Appendix
Figure 8.1 Analysis of 9 week old female and male $\text{Map3k1}^{\text{goya/goya}}$ ABR thresholds. No sexual dimorphism was evident in $\text{goya}$ homozygote ABR thresholds at 9 weeks of age.
Figure 8.2 Individual ABR thresholds for wild-type and Boycie heterozygote and homozygote mice at 4 months of age
Figure 8.4 Individual ABR thresholds for wild-type and Boycie heterozygote and homozygote mice at 6 months of age.
Figure 8.5 Individual ABR thresholds for wild-type and Boycie heterozygote and homozygote mice at 8 months of age
Figure 8.6 Individual ABR thresholds for wild-type and Boycie heterozygote and homozygote mice at 12 months of age
Publications Relating to the

Work Presented in this Thesis
RESEARCH ARTICLE

The goya mouse mutant reveals distinct newly identified roles for MAP3K1 in the development and survival of cochlear sensory hair cells

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ABSTRACT

Mitogen-activated protein kinase, MAP3K1, plays an important role in a number of cellular processes, including epithelial migration during eye organogenesis. In addition, studies in keratinocytes indicate that MAP3K1 signalling through JNK is important for actin stress fibre formation and cell migration. However, MAP3K1 can also act independently of JNK in the regulation of cell proliferation and apoptosis. We have identified a mouse mutant, goya, which exhibits the eyes-open-at-birth and microphthalmia phenotypes. In addition, these mice also have hearing loss. The goya mice carry a splice site mutation in the Map3k1 gene. We show that goya and kinase-deficient Map3k1 homozygotes initially develop supernumerary cochlear outer hair cells (OHCs) that subsequently degenerate, and a progressive profound hearing loss is observed by 9 weeks of age. Heterozygote mice also develop supernumerary OHCs, but no cellular degeneration or hearing loss is observed. MAP3K1 is expressed in a number of inner ear cell types, including outer and inner hair cells, stria vascularis and spiral ganglion. Investigation of targets downstream of MAP3K1 identified an increase in p38 phosphorylation (Thr180/Tyr182) in multiple cochlear tissues. We also show that the extra OHCs do not arise from aberrant control of proliferation via p27KIP1. The identification of the goya mutant reveals a signalling molecule involved with hair-cell development and survival. Mammalian hair cells do not have the ability to regenerate after damage, which can lead to irreversible sensorineural hearing loss. Given the observed goya phenotype, and the many diverse cellular processes that MAP3K1 is known to act upon, further investigation of this model might help to elaborate upon the mechanisms underlying sensory hair cell specification, and pathways important for their survival. In addition, MAP3K1 is revealed as a new candidate gene for human sensorineural hearing loss.

KEY WORDS: MAP3K1, Supernumerary outer hair cells, Cochlear development, Sensory hair cell survival, Hearing loss

INTRODUCTION

The signalling pathways underlying epithelial sheet movements are well studied, and have identified the mitogen-activated protein kinase (MAPK) MAP3K1 as having an important role in this process (Takatori et al., 2008; Xia and Kao, 2004; Zhang et al., 2005, 2003). In mice, loss-of-function mutations in the Map3k1 gene lead to defects in epithelial migration that manifest as an eyes-open-at-birth (EOB) phenotype (Xia and Kao, 2004; Zhang et al., 2003), due to defects in actin polymerisation and c-JUN phosphorylation. Studies in keratinocytes demonstrate that activation of c-Jun N-terminal kinase (JNK) by TGF-β and activin requires MAP3K1, leading to c-JUN phosphorylation, actin-stress-fibre formation and cell migration (Zhang et al., 2005, 2003). Although it is clear that a MAP3K1-JNK cascade is crucial for epithelial-sheet movements during eye organogenesis, it might also be expected to have a role in the development of other epithelia. Indeed, MAP3K1 is required during wound healing, where injury upregulates MAP3K1 and leads to changes in the expression of genes associated with extracellular matrix homeostasis. Conversely, knockdown of MAP3K1 impairs wound healing (Deng et al., 2006). MAP3K1 has also been shown to act independently of JNK during the regulation of cell proliferation and apoptosis in the retina (Mongan et al., 2011).

In humans, MAP3K1 mutations have been shown to cause 46,XY disorders of sexual development (DSD) (Loke et al., 2014; Pearlman et al., 2010). A number of these mutations have been studied, and they all result in the increased phosphorylation of the downstream MAPK proteins p38 MAPK and ERK1/2. As part of an N-ethyl-N-nitrosourea (ENU)-mutagenesis recessive screen, we have identified a mouse mutant, goya, which carries a mutation in the Map3k1 gene. The mutant was identified by its EOB phenotype, and also by the reduction or absence of a response to a click-box test, indicating hearing loss. Homozygous goya mice initially develop supernumerary outer hair cells (OHCs) in the inner ear; widespread OHC degeneration is observed by 4 weeks of age, and the mice are profoundly deaf by 9 weeks of age. This identifies a previously unknown role for MAP3K1 in auditory hair cell development and survival. Characterisation of the goya mutant provides an opportunity to elaborate upon the requirement of this MAPK in cochlear organogenesis and maintenance.

RESULTS

Identification of a mouse mutant with eye, vision and auditory defects

The goya mouse mutant was identified, from a recessive ENU-mutagenesis phenotype-driven screen, as having EOB (see Fig. S1A). In the adult EOB mice, eye pathology is highly variable, ranging from microphthalmic, to apparently normal, to bulging (see Fig. S1B). However, all EOB mice failed to respond in an optokinetic drum visual-function assay (data not shown). Rosetting was observed in the retinal layers, although, at postnatal day 0 (P0), the structure of tight junctions and the outline of retinal
There is a strong candidate, Map3k1, with mice deficient for this gene having previously been shown to display EOB (Juriloff et al., 2004; Yujiri et al., 2000; Zhang et al., 2003). There have been no reports of hearing loss in Map3k1 mouse models. Map3k1 is highly expressed in the migrating leading edge of the eyelid epithelium and it is thought that, in its absence, the migration of these cells is impaired, leading to the failure of eyelid closure during embryogenesis (Xia and Kao, 2004; Zhang et al., 2005, 2003). Investigation of a kinase-deficient Map3k1 in Map3k1 allele showed that retinal phenotypes, including increased proliferation and subsequent apoptosis of Müller glial cells, were due to a pathway that is separate to eyelid closure, highlighting the multiple roles of MAP3K1 in cellular development and survival (Mongan et al., 2011).

Sanger sequencing of the Map3k1 gene exons and intron/exon boundaries revealed a single-nucleotide change in the intron 13 splice donor site (IVS13+2T>C) of affected mice (Fig. 1A). To ascertain the effect of the goya mutation on splicing, we performed reverse-transcriptase PCR (RT-PCR) analysis of RNA isolated from postnatal day 1 (P1) organ of Corti dissected from goya littermate mice. For Map3k1, the expected product of 341 bp was obtained (Fig. 1B). However, for Map3k1, the wild-type product was absent and two smaller products could be seen (Fig. 1B). Sanger sequencing of the Map3k1 RT-PCR product showed that exons 12, 13 and 14 were correctly spliced, whereas sequencing of the two Map3k1 products showed aberrant splicing (Fig. 1C). The more abundant mutant product (Map3k1) demonstrates the use of a cryptic splice donor site within exon 13 (Fig. 1C). In this case, 81 nucleotides are skipped, leaving the transcript in-frame but producing a protein with an internal deletion of 27 amino acids. The less abundant mutant product (Map3k1) showed complete skipping of exon 13 (190 nucleotides), with exon 12 spliced directly to exon 14 (Fig. 1C). Translation of this transcript would lead to the production of a protein containing the first 723 amino acids of MAP3K1 followed by a frame-shift and the incorporation of seven novel amino acids before a stop codon is encountered. If produced, this truncated protein would lack the C-terminal 770 amino acids of MAP3K1, including the kinase domain.

The goya mutant is severely deaf

To confirm Map3k1 as the causative gene, we mated mice carrying the goya mutation to mice carrying the Map3k1 allele. We performed auditory brainstem response (ABR) analysis on both Map3k1 and Map3k1 heterozygote and homozygote mice as well as compound heterozygote and wild-type mice. At 9 weeks of age, Map3k1 and Map3k1 heterozygous mice had normal auditory thresholds, similar to wild-type (Map3k1) mice, whereas both Map3k1 and Map3k1 homozygotes showed a significant hearing loss with average ABR thresholds of ~80 decibels sound pressure level (dB SPL) across all frequencies tested (P<0.0001) (Fig. 2C). Importantly, the compound heterozygous mice (Map3k1) also have elevated ABR thresholds, similar to those of homozygote mice. Failure of these models to complement confirms that the ENU-induced Map3k1 mutation underlies the goya phenotype. To investigate the onset of hearing loss, we performed ABR testing of homozygote and wild-type mice at 2 and 4 weeks of age. At 2 weeks of age, Map3k1 homozygote mice had ABR thresholds similar to those of wild-type mice at all but the highest frequency tested (26 kHz), whereas Map3k1 homozygote mice showed elevated thresholds at all frequencies tested (Fig. 2A). At 4 weeks of age, both homozygous mutants exhibited significantly elevated ABR thresholds (+30-40 dB) at all frequencies tested when compared to wild-type controls (Fig. 2B).
To determine the longitudinal effects of the goya mutation on hearing function, we performed ABR on mice at 1 year of age. Map3k1^{goya/+} mice had ABR thresholds similar to those of wild-type mice, demonstrating that goya heterozygotes do not develop late-onset hearing loss (Fig. 2D). Moreover, there is no further decline in auditory function of Map3k1^{goya/goya} animals between 9 weeks and 1 year.

Fig. 1. Map3k1 is mutated in goya mice. (A) Genomic DNA sequence traces showing the Map3k1 exon 13 donor splice site in the parental strains (C57BL/6 and C3H), and homozygous mutant (Map3k1^{goya/goya}) mice. The affected nucleotide is boxed (T in parental strains and C in Map3k1^{goya/goya}) and the exon/intron border is indicated by the dashed line. (B) RT-PCR analysis of RNA extracted from the organ of Corti. For Map3k1^{+/+}, a single 341-bp amplicon corresponding to the expected wild-type Map3k1 sequence was observed. For Map3k1^{goya/goya}, no wild-type amplicon was observed; instead, two smaller amplicons were identified (denoted by blue and red lines). All three amplicons were found for Map3k1^{goya/+}. (C) Sanger sequencing reveals aberrant splicing in Map3k1^{goya/goya}. (Top) Sequencing of the single Map3k1^{+/+} product confirms normal splicing of exons 12/13/14; (middle) sequencing of the larger Map3k1^{goya/goya} product (blue) reveals the use of a cryptic splice site within exon 13, resulting in an in-frame deletion of 81 nucleotides; (bottom) sequencing of the smaller Map3k1^{goya/goya} product (red) reveals exon 12 splicing directly to exon 14, with complete skipping of exon 13. (D) Cartoon depicting the aberrant splicing events occurring in Map3k1^{goya/goya} mice. Exons are depicted as numbered boxes, and the ‘cryptic’ and ‘exon 13 skip’ splicing events are shown as blue and red lines, respectively. The location of the goya mutation is shown.
To investigate vestibular effects of the mutation, we performed swim tests on 1-year-old Map3k1goya/goya mice, and no overt vestibular dysfunction was observed.

Hair-cell abnormalities in the Map3k1 mutants

Given the elevated auditory thresholds of Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia mice, we proceeded to examine in detail the ears of these mice. Hematoxylin and eosin (H&E) staining of cochlear sections suggested cellular degeneration within the organ of Corti of 9-week-old homozygote mice; however, other inner-ear structures such as Reissner’s membrane, stria vascularis and spiral ganglion neurons (SGNs) appeared normal (data not shown). Additionally, no defects in middle-ear morphology were observed. To further assess the organ of Corti, we used scanning electron microscopy (SEM) to examine the ultrastructure of the sensory epithelium. At 2 weeks of age, Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia mice showed a normal cellular arrangement of the sensory epithelium with the exception that both mutants had more OHCs than did wild type (Fig. 3A). The additional OHCs were organised as an extra row that extended largely throughout the cochlear regions examined (Fig. 3A). However, by 4 weeks of age both mutants showed degeneration of OHCs with an increasing severity from apex-to-base (Fig. 3B). By 9 weeks of age both mutants showed further degeneration. Conversely, no OHC loss was observed in wild-type mice by 9 weeks of age (Fig. 3C). At 2, 4 and 9 weeks of age, inner hair cell (IHC) morphology appeared normal in all except Map3k1tm1Yxia/tm1Yxia cochlea at 9 weeks, which showed a slight reduction in number again with an apical-to-basal gradient (Fig. 3C). Although at 9 weeks of age the majority of IHCs were unaffected, the extent of degeneration in small patches of the Map3k1tm1Yxia/tm1Yxia organ of Corti was very severe. IHCs, OHCs and pillar cells had disappeared and rosette-like formations of what seemed to be Claudius and Hensen cells had formed in their place (Fig. 3D). A similar pattern of cellular remodelling has been previously noted, in post-aminoglycoside-
Fig. 3. Mice lacking functional MAP3K1 develop supernumerary OHCs and show progressive degeneration of OHCs. (A-C) Representative scanning electron micrographs from the apical (A), mid-apical (MA), mid (M) and mid-basal (MB) turns of the organ of Corti from Map3k1+/+, Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice at 2, 4 and 9 weeks of age. Both Map3k1<sup>goya/goya</sup> and Map3k1<sup>tm1Yxia/tm1Yxia</sup> mice have an extra row of OHCs at 2 weeks of age. A progressive loss of OHCs is seen between 2 and 9 weeks of age in both homozygous mutants, but not in wild-type cochleae. An apical-to-basal increase in severity of degeneration was also observed. (D) Scanning electron micrograph demonstrating the rosette-like cellular formation in a 9-week-old Map3k1<sup>tm1Yxia/tm1Yxia</sup> mouse. The remains of some IHC stereocilia bundles can be seen (IHC), all OHC are missing (*) and pillar cells (PC) have been replaced in the rosette formation with Hensen (HC)-like and Claudius (CC)-like cells (all scale bars represent 10 µm). (E) Scanning electron micrograph showing an extra row of OHCs in the mid-basal region of a 9-week-old Map3k1<sup>goya/goya</sup> cochlea. (F) Clustered histogram representing the percentage of total images captured from Map3k1<sup>+/+</sup> (n=54), Map3k1<sup>goya/</sup>goya (n=11) and Map3k1<sup>tm1Yxia/+</sup> (n=30) mice containing three rows of OHCs (normal), extra OHCs (1-4 OHCs in addition to the three normal rows), or extra rows of OHCs (≥5 OHCs in a continuous line, in addition to the three normal rows). Images of cochleae from both heterozygote alleles and Map3k1<sup>+/+</sup> mice showed isolated extra OHCs; however, 27% of Map3k1<sup>goya/goya</sup> and 43% of Map3k1<sup>tm1Yxia/+</sup> heterozygote images contained extra rows of OHCs, significantly higher than the 4% of Map3k1<sup>+/+</sup> images. The percentage of Map3k1<sup>tm1Yxia/+</sup> images containing the normal three rows of OHCs was also significantly lower when compared to Map3k1<sup>+/+</sup> (*P<0.05, ***P<0.0001, Fisher’s exact test, see Materials and Methods and Table S3 for estimates confidence intervals and P-values).
damaged cochlea (Taylor et al., 2012). Interestingly, similar to homozygous mutants, Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia mice also displayed extra OHCs compared to wild-type mice, but, unlike homozygous mutants, no OHC degeneration was observed at any time point (Fig. 3E). Further investigation shows that, similar to wild type, the heterozygote mutants have episodic patches of extra OHCs. However, unlike wild type, the heterozygote mutants show an increase in the occurrence of extra
rows of OHCs, which are present in 43%, 27% and 4% of images from Map3k1tm1Yxia/+ , Map3k1goya/+ and wild-type mice, respectively (Fig. 3F). These data suggest roles for MAP3K1 in sensory-hair-cell development and survival.

To assess the progressive nature of cell loss in the different regions of the cochlear spiral, and to allow comparison between genotypes, sensory-cell counts were performed. At 2 and 4 weeks of age, IHC numbers were similar between Map3k1+/+ , Map3k1goya/goya and Map3k1tm1Yxia/+ mice. At 9 weeks of age, IHC numbers were similar between Map3k1+/+ and Map3k1goya/goya mice, but a trend for reduced numbers of IHCs in the basal region of Map3k1tm1Yxia/+ cochlea was observed (Fig. 4A-C). Up to 9 weeks of age, Map3k1+/+ mice showed consistent numbers of OHCs across the different regions of the cochlea (Fig. 4D). At 2 weeks of age, Map3k1goya/goya and Map3k1tm1Yxia/+ mice had increased numbers of OHCs compared to wild type, which was
significant for most of the regions assessed (Fig. 4D-F). At 4 weeks of age, degeneration of OHCs progressed in Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia mice. In the apical, mid-apical, mid and mid-basal regions of the cochlear spiral, Map3k1goya/goya mutants showed an average reduction in OHC numbers of 26%, 24%, 70% and 95%, respectively, whereas Map3k1tm1Yxia/tm1Yxia mutants showed reductions of 20%, 43%, 67% and 96%, respectively (Fig. 4E,F). A similar apical-to-basal increase in severity was also observed in 9-week-old mutants (Fig. 4).

To determine statistical significance, the rate of hair-cell loss was estimated under a Poisson generalised linear model (see Materials and Methods). There was no statistical support for IHC loss in any genotypic group (see Tables S1, S2). There was strong statistical support for OHC loss in the Map3k1goya/goya and...
Fig. 7. The inner ears of P1 Map3k1<sup>gyalcgyal</sup> mice exhibit increased levels of p38 phosphorylation. (A) Box plot of the ratio of phosphorylated p38 to total p38 in goya inner-ear total protein lysates from Map3k1<sup>+/+</sup>, Map3k1<sup>gyalgyal</sup> and Map3k1<sup>gyalgyal</sup> mice. Three ears (one each from separate mice) were pooled for each lysate (number of lysates: Map3k1<sup>+/+</sup> n=8, Map3k1<sup>gyalgyal</sup> n=7, Map3k1<sup>gyalgyal</sup> n=8). A trend of increased p38 phosphorylation is observed in Map3k1<sup>gyalgyal</sup> inner-ear lysates when compared to Map3k1<sup>+/+</sup> (P=0.282). Average Map3k1<sup>gyalgyal</sup> p38 phosphorylation levels are intermediate between Map3k1<sup>+/+</sup> and Map3k1<sup>gyalgyal</sup>. (B-E) Immunohistochemical analysis of P1 Map3k1<sup>gyalgyal</sup> cochlea confirms the increased level of p38 phosphorylation observed in the Peggy Western data. Panels Bi and iii show phosphorylated p38 expression in the mid turn of the cochlea of P1 Map3k1<sup>+/+</sup> (i) and Map3k1<sup>gyalgyal</sup> (iii) mice. Widespread nuclear expression is seen in both genotypes; however, the depth of staining is greatly increased in Map3k1<sup>gyalgyal</sup>. Some nuclei in spiral ligament, spiral limbus and basilar membrane in the Map3k1<sup>+/+</sup> organ of Corti remained unstained with this length of chromogenic exposure. These results were consistent for littermate controls (Map3k1<sup>+/+</sup> n=2, Map3k1<sup>gyalgyal</sup> n=3). (C) Immunoratio was used to quantify the percentage of nuclei stained in each mid turn image. This analysis showed that the Map3k1<sup>gyalgyal</sup> mice (n=3) had significantly increased numbers of positively stained cells when compared to Map3k1<sup>+/+</sup> (n=2). Bii and iv show increased positive staining for phosphorylated p38 in the spiral ganglion neurons of Map3k1<sup>gyalgyal</sup> (iv) mice compared to Map3k1<sup>+/+</sup> (ii). (D,E) Immunostaining of P1 organ of Corti using anti-phospho JNK and anti-phospho ERK1/2 antibodies, respectively. No differences can be seen between Map3k1<sup>+/+</sup> (Di,Ei) or Map3k1<sup>gyalgyal</sup> (Dii,Eii) mice, although, interestingly, expression of both proteins is mainly observed below the basal surface of inner and outer hair cells, in contrast to the widespread nuclear expression of phosphorylated p38.
Map3k1tm1Yxia/m1Yxia genotypic groups (but no evidence in the wild-type group; see Table S2). The rate of OHC loss in the mutant groups increased consistently from apical-to-basal turns (see Table S1). The rate of OHC loss did not differ significantly between the two mutant groups. These statistical analyses quantify and corroborate the obvious effects visible in Fig. 3.

Localisation of MAP3K1 to the inner ear

Commercially available anti-MAP3K1 antibodies have poor specificity; therefore, we utilized the Map3k1tm1Yxia allele, which produces a MAP3K1–β-galactosidase fusion protein (Xia et al., 2000; Zhang et al., 2003). Using X-Gal staining, widespread expression of Map3k1–β-galactosidase was observed in Map3k1tm1Yxia/m1Yxia cochleae (Fig. 5A). Closer examination of the cochlear duct showed staining in IHCs and OHCs, border cells of the internal spiral sulcus, Claudius and Hensen cells, as well as SGNs (Fig. 5C). No labelling was observed in wild-type cochleae (Fig. 5B,D). Histological sections confirm the whole-mount localisation data and also reveal expression in cell types such as Deiters’ cells, pillar cells, Reissner’s membrane, marginal cells in the stria vasularis and the tympanic border cells of the basilar membrane (Fig. 5E,G,O,P). A transverse section of the organ of Corti also highlighted the expression of the fusion protein in IHCs and OHCs (Fig. 5Q). In addition to the expression in the cochlea, staining was observed in the vestibular system, including the apical surface of supporting cells and hair cells in both otolithic organs and the cristae of the semi-circular canals (Fig. 5L,K,M). Positive staining in all Map3k1tm1Yxia/+ samples mirrored that of homozygotes, whereas no staining was seen in either the vestibular system or cochlea of Map3k1+/- mice (Fig. 5F,H,J,L,N).

Ablation proliferation is not the cause of supernumerary OHCs in Map3k1 mutant mice

The cyclin-dependent kinase inhibitor p27Kip1 is a key regulator that arrests the cell cycle at G1. Its expression in the developing cochlea produces a zone of non-proliferation (ZNP), and these cells subsequently differentiate into sensory hair cells and supporting cells. It is known that these ZNP cells undergo their final division by embryonic day 14.5 (E14.5). However, in p27Kip1 homozygous-null mice, there is an extended period of pro-sensory precursor-cell proliferation leading to increased numbers of hair cells and supporting cells (Lowenheim et al., 1999). We used 5-ethyl-2'-deoxyuridine (EdU) to investigate whether proliferation was increased or extended in the developing Map3k1tm1Yxia/m1Yxia mutant cochlea. In addition, we used an anti-p27KIP1 antibody to investigate for possible regulatory defects associated with a MAP3K1 deficiency. Fig. 6 shows that p27KIP1 localisation is unaffected in the Map3k1tm1Yxia/m1Yxia cochlea at E14.5 (Fig. 6A,B), and the lack of EdU-positive nuclei in the region of the cochlea duct that positively immunolabelled for p27KIP1 indicates that the ZNP is established correctly. At E18.5, we again found no difference in localisation of p27KIP1 in mutant cochleae. Moreover, no increase in the number of proliferating cells was seen in the cochlear duct of Map3k1tm1Yxia/m1Yxia mice compared to Map3k1+/- littermate controls (Fig. 6C-H). Together, these data suggest that increased or extended proliferation of pro-sensory precursor cells is not the cause of extra OHCs in Map3k1 mutant mice.

The goya mutation in Map3k1 results in an increase of p38 phosphorylation in P1 mouse inner ears

To investigate the effects of the goya mutation on MAPK pathway targets, we used both gene expression and immunodetection strategies. We extracted RNA from isolated P1 cochlear ducts and performed qRT-PCR to investigate the expression of Map3k1 and genes likely to be involved in MAP3K1 signalling [Ccnd1 (cyclin A1), Ccne1 (cyclin D1), Lgr5, Dhr, Axin2, Cnbbl (β-catenin), E2f1, Rbl1] (Loke et al., 2014; Mongan et al., 2011). In addition, expression of JNK targets, Jun and Fos, were also analysed. Although some differences were observed between genotypes, we did not see any notable fold changes in expression of any of the genes investigated (see Fig. S2A).

Because MAP3K1 has been shown to be involved with activation of all three major MAPK pathways – ERK, JNK and p38 MAPK – we investigated the phosphorylation of these downstream target proteins using the capillary based Simple Western Peggy system (Protein Simple). Protein lysates from P1 inner ears were analysed for both total and phosphorylated ERK1/2, JNK and p38 MAPK. Moreover, given the effects in the eye of Map3k1 mutations on RB phosphorylation (Mongan et al., 2011), we also assessed total and phospho-RB levels. No differences in phosphorylation were detectable in ERK1/2, JNK or RB. However, a trend towards increased p38 phosphorylation in Map3k1goya/goya inner-ear lysates was observed (Fig. 7A).

To further investigate p38 MAPK phosphorylation in Map3k1goya/goya inner ears, we performed immunohistochemistry on sections from P1 mice (Fig. 7B). Anti-phospho-p38 labelling of Map3k1goya/goya homozygotes showed intense nuclear staining of all cell types in the cochlea duct and SGNs (Fig. 7Bi,Biv). The majority of nuclei in the surrounding structures, such as the spiral ligament and spiral limbus, were also intensely stained. Labelling of Map3k1+/− mice displayed a similar pattern of nuclear expression; however, under identical experimental conditions, the staining was much weaker and fewer nuclei in the surrounding structures stained positive for phospho-p38 MAPK (Fig. 7Bii,Biv).

We quantified the difference in anti-phospho-p38 staining using the ImageJ plugin, ImmunoRatio, analysing mid-cochlear sections from Map3k1goya/goya and Map3k1+/− mice. ImmunoRatio has been designed to diagnostically assess the percentage area of positively DAB-stained nuclei in a given sample; however, the analysis does not take into account intensity of stain. The results show a significant (P=0.0126) increase in positive nuclear area in Map3k1goya/goya mice (n=3) when compared with Map3k1+/− (n=2) (Fig. 7C).

We also performed immunohistochemistry with anti-phospho-JNK and anti-phospho-ERK1/2 antibodies (Fig. 7D,E), and, consistent with our Simple Western assay data, no obvious differences in intensity or expression pattern were observed between Map3k1goya/goya and Map3k1+/− mice. It is worth noting that these antibodies showed strong labelling beneath the basal surface of the HICs and OHCs, consistent with the location of SGN neurite extensions at the P1 time point. In addition, low-level anti-phospho-JNK was detected in the cytoplasm and nucleus of cells throughout the organ of Corti of both Map3k1goya/goya and Map3k1+/− mice.

DISCUSSION

We report that an IVS13+2T>C ENU-induced lesion in Map3k1 is the causative mutation underlying both the EOB and auditory phenotypes in the goya mutant. These findings demonstrate a newly identified role for MAP3K1 in auditory function. Similar findings are reported in the parallel study (Yousaf et al., 2015). Mice homozygous for the goya mutation and Map3k1-null mice each develop supernumerary OHCs, and both show a progressive decline in auditory function resulting in severe hearing loss by 9 weeks of
The goya mutant showed a slower rate of auditory decline, but this is likely due to the different genetic backgrounds of the two mutants, with goya crossed to C3H and tm1Yxia crossed to C57BL/6J. Apart from the early auditory thresholds, no major differences in phenotype were noted between the goya and the tm1Yxia alleles. Ultrastructural examination of Map3k1goya/goya and Map3k1tm1Yxia/tm1Yxia mutant cochleae uncovered a progressive cellular degeneration in the organ of Corti with an apical-to-basal increase in severity. Cell loss was first seen in the OHCs, although, by 9 weeks, IHCs and pillar cells were also missing in some mid and mid-basal regions, although not statistically significantly. Mice heterozygous for either the Map3k1goya or the Map3k1tm1Yxia allele also developed extra OHCs but, interestingly, they do not show progressive cellular degeneration as seen in the respective homozygotes. Indeed, at 1 year of age, Map3k1goya+ and wild-type mice had similar auditory thresholds. These data suggest that, within the organ of Corti, MAP3K1 plays multiple roles in cellular development and survival.

We show that MAP3K1 is widely expressed in the inner ear. The expression in OHCs and IHCs, along with Deiters’ cells, in the organ of Corti is consistent with the observed phenotype of additional rows of OHCs and OHC degeneration that we observe in Map3k1 mutants. Our findings of MAP3K1 expression in the cochlea are consistent with the observations of Yousaf et al. (2015) in that they too report expression in Deiters’ cells, Reissner’s membrane and the stria vascularis. However, we identified some additional sites of expression, including Claudius cells, Hensen cells and Border cells, as well as the basilar membrane. We also observed expression at the apical surface of the vestibular sensory epithelia; however, no overt vestibular dysfunction was detected in either homozygous mutant. It is possible that the normal vestibular function in these mice is a consequence of functional redundancy between MAP3K1 and other MAP3Ks. For example, MAP3K4 is known to activate the same downstream pathways as MAP3K1 (Morrison, 2012). However, a previous study investigating the role of MAP3Ks in testis determination failed to uncover functional redundancy between MAP3K1 and MAP3K4 (Warr et al., 2011).

The goya mutation has led to the identification of a new sensorineural deafness locus and it is important to consider Map3k1 as a candidate gene for both dominant and recessive human deafness loci. Human mutations in MAP3K1 have been shown to cause 46,XY DSD (Pearlman et al., 2010). Two mutations, including a splice-acceptor mutation and a missense mutation, were identified in two families with 46,XY DSD. Moreover, a further two missense mutations were found in 11 sporadic cases examined. For three of these mutations, MAP3K1 function was studied, including phosphorylation of the downstream targets p38, ERK1/2 and JNK. Two of the mutations increased activation of p38 and ERK, possibly resulting from enhanced binding of RHOA to MAP3K1. In conclusion, we show that, in addition to previously reported eye phenotypes resulting from MAP3K1 deficiency, both heterozygous and homozygous goya and Map3k1-null mutant mice initially develop supernumerary cochlear OHCs. In homozygous, but not heterozygous, mutants, OHCs progressively degenerate and mice are severely deaf by 9 weeks of age. These phenotypic differences indicate that MAP3K1 might play distinct roles in cochlear development and hair-cell survival. We show increased p38 phosphorylation in the cochleae of goya homozygote mice, and EdU studies reveal that the extra OHCs result from a mechanism other than aberrant proliferation. Characterisation of goya reveals a signalling molecule that was previously unknown to be involved with mammalian audition, and identifies a candidate gene for human sensorineural hearing loss.

**MATERIALS AND METHODS**

**Mice**

All animals were housed and maintained under specific pathogen-free (SPF) conditions in individually ventilated cages in the Mary Lyon Centre, MRC Harwell, in adherence to environmental conditions as outlined in the Home Office Code of Practice. Animal procedures were carried out in line with Home Office regulations, and mice were euthanized by Home Office Schedule 1 methods.

The goya mutant line was identified from the collaborative ENU mutagenesis vision screen undertaken by MRC MGU Harwell and MRC HGU Edinburgh. ENU-treated G0 C57BL/6 male mice were mated to C3H. C-Pde6b+ female mice to produce G1 progeny. G1 males were mated to C3H.C-Pde6b− female mice to produce G2 progeny. Female G1 mice were backcrossed to the G1 fathers to produce G3 mice that were screened for
recessively inherited phenotypes. The goya line was maintained on a C3H genetic background by outcrossing and intercrossing successive generations. Map3k1-null mice (Map3k1<sup>tm1Yxia/tm1Yxia</sup>) were imported from Ying Xía’s group at the University of Cincinnati College of Medicine (Cincinnati, USA) and rederived by in vitro fertilisation by the FESA core in the Mary Lyon Centre to maintain SPF status. The null mice were backcrossed to C57BL/6J.

**Histological analysis**

Animals were euthanized and eyes fixed in Davidson’s fixative. Fixed specimens were decalcified, dehydrated and embedded in paraffin wax, and 5-µm sagittal sections were obtained and H&E-stained using standard protocols. For inner ears, heads were removed, skinned and bisected along the midline before fixation in 10% neutral buffered formalin (Surgipath), and subsequent processing as above. See Fig. S1.

**Linkage analysis**

DNA from the parental strain (C57BL/6J and C3H.C-Pde6b<sup>+</sup>) and five affected G3 mice were scanned using an Illumina mouse low-density linkage array employing 271 informative SNPs.

**Mutational analysis of Map3k1**

Exons and the immediate flanking sequences of Map3k1 were amplified from goya, C57BL/6J and C3H.C-Pde6b<sup>+</sup> genomic DNA employing oligonucleotides that were also used for Sanger sequencing.

**RT-PCR**

Total RNA was extracted from microdissected P1 organ of Corti using the RNeasy miKit (Qiagen). For each sample, RNA was pooled from four ears from two mice. First-strand cDNA was synthesised using a high-capacity cDNA reverse-transcription kit (Life Technologies) using a combination of oligo-(dT), random-hexamer and Map3k1-specific primers. The cDNA was then used as a template for PCR amplification using a forward primer spanning the end of exon 11 and the beginning of exon 12 and a reverse primer from exon 14 (primer sequences on request). PCR products were separated by gel electrophoresis, bands excised, cloned into pGEM-T vector, and sequenced using SP6 and T7 primers.

**Gene expression**

RNA extractions and cDNA synthesis were performed as described above, except that only random hexamers were used to prime the cDNA synthesis reactions. TaqMan<sup>®</sup> (Life Technologies) assays for Ccnal (cyclin A1), Ccdn1 (cyclin D1), Dhhfr, Map3k1, Rb1, Colla1, E2f1, Fos, Jun, Axin2, CTNNB1 (β-catenin), Lgr5 and Gapdh were run on a 7500 Fast real-time PCR machine (Applied Biosystems) as per the manufacturer’s recommended instructions. See Fig. S2.

**Auditory brainstem response**

ABR testing was performed as previously described by Hardisty-Hughes et al. (2010). Tone-burst stimuli were presented free-field at 8 kHz, 12 kHz, 20 kHz and 26 kHz to the right ear of the mouse. TDT system III hardware and software (Tucker Davis Technology) was used for stimulus presentation and response averaging, starting at the highest level (90 dB SPL) and reducing in 5 or 10 dB increments until no response trace could be observed. Mice that displayed no response to a 90 dB SPL stimulus were recorded as 100 dB SPL for subsequent analysis.

**Scanning electron microscopy (SEM)**

Animals were euthanised and excised inner ears were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer (Sigma-Aldrich), then decalcified for 48 h in 4.3% EDTA in 0.1 M phosphate buffer (Sigma-Aldrich) and then critical point dried using an Emitech K850 (EM Technologies Ltd). Specimens were then mounted on stubs using silver paint (Agar Scientific) and sputter coated with platinum using a Quorum Q150T sputter coater (Quorum Technologies). Prepared cochleae were visualised with a JEOL LSM-6010 (Jeol Ltd) scanning electron microscope. Hair-cell counts were performed by counting the number of adjacent IHCs and OHCs to 20 pillar cells; for the analysis, the cochlea was divided into four separate regions (turns): apical (<90° from apex), mid-apical (90-180° from apex), mid (180-360° from apex) and mid-basal (360-540° from apex). Ears from at least three mice were analysed for each genotype at each turn and time point.

**X-Gal staining**

Mice were euthanized and inner ears removed and fixed for 2 h at 4°C in 0.1 M phosphate buffer containing 1% paraformaldehyde (Sigma-Aldrich), 2 mM MgCl<sub>2</sub> (Sigma-Aldrich), 0.25% glutaraldehyde (Sigma-Aldrich) and 5 mM EGTA (Merck Millipore). Ears were then washed in 0.1 M phosphate buffer containing 2 mM MgCl<sub>2</sub> (Sigma-Aldrich) and 0.02% NP-40 (Fluka). Staining was performed overnight at room temperature (RT) in a solution of 0.1 M phosphate buffer containing 2 mM MgCl<sub>2</sub> (Sigma-Aldrich), 5 mM potassium ferrocyanide (Sigma-Aldrich), 0.02% NP-40 and 1 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) (Sigma-Aldrich). Post-staining, ears were decalcified in 4.3% EDTA in 0.1 M phosphate buffer (Sigma-Aldrich) for 48 h at 4°C, before paraffin embedding and sectioning at 10 µm. Sections and whole-mount dissected cochlea were imaged on a Zeiss Axio Observer Z-1 microscope using extended focus image capture.

**Peggy Simple Western size assay**

Whole inner ears from three P1 mice (one ear per mouse) were pooled and lysed in 20 mM Bicine with 0.6% Chaps supplemented with phosSTOP™ and cOmplete mini™ inhibitor cocktails (Roche), using a Precellys 24 homogeniser with a soft tissue kit (Precellys). Capillary-based immunodetection was performed using the automated Peggy™ system (Simple Western™) as described previously (Siggers et al., 2014). Briefly, lysates were mixed with Simple Western™ sample dilution buffer (Protein Simple) containing reducing agent and fluorescent standards, and denatured at 95°C for 5 min. Samples were then loaded into the 384-well assay-plate and proteins were separated through size-resolving matrix, immobilized to the inner capillary wall, incubated with p38 MAPK (CST9212), phospho-p38 MAPK (Thr180/Tyr182) (CST9215), p44/p42 MAPK (ERK1/2) (CST9102), phospho-p44/p42 MAPK (ERK1/2) (Th202/Tyr204) (CST4377), SAPK/JNK (CST9252) and phospho-SAPK/JNK (Thr183/Tyr185) (CST9251) primary antibodies and HRP-conjugated secondary antibodies before detection using chemiluminescence.

**Immunohistochemistry**

Phospho-p38, phospho-JNK and phospho-ERK1/2

P1 mice were euthanized by decapitation and bisected heads fixed in 4% PFA in PBS for 1 h at 4°C. The bisected heads were then dehydrated and embedded in paraffin wax and 5-µm sections collected onto charged slides. Sections were de-paraffinised, endogenous peroxidase activity quenched by submersion in 3% H<sub>2</sub>O<sub>2</sub>, washed in 1× TBST and blocked in 1× TBST containing normal goat serum. Sections were then incubated overnight at 4°C with rabbit monoclonal anti-phospho p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology) at 1:5000 dilution, phospho-p44/42 MAPK (ERK1/2) (Cell Signaling Technology) at 1:2000 dilution or phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology) at 1:5000 dilution. The VECTASTAIN® Elite ABC rabbit IgG avidin biotin kit (Vector Laboratories) and DAB+ Chromagen (Dako) were used for detection. For anti-phospho-p38-abelled images, the ImmunoRatio plugin (http://jvsmicroscope.uta.fi/immunoratio/) for ImageJ (http://imagej.nih.gov/ij/) was used to quantify the percentage of positively stained nuclei.

**Proliferation detection and p27KIP1 immunofluorescence**

The Click-iT Plus™ EdU Alexa-Fluor-594 Imaging Kit (Life Technologies) was used to identify proliferating cells in embryonic
cochlea. Pregnant females were injected with 100 μg 5-ethyl-2′-deoxyuridine (EdU) in 200 μl PBS twice, at 2 h intervals, before embryos were harvested 2 h after final injection at either E14.5 or E18.5. Embryonic heads were fixed in 4% PFA in PBS for 1 h at 4°C and tail collected for genotyping. Fixed heads were then dehydrated and embedded in paraffin wax and 5-μm sections collected onto charged slides. The copper-azide ‘click’ Alexa-Fluor-594 reaction for detection of EdU was performed as per the manufacturer’s instructions, and processed slides were washed in PBS containing 3% BSA at RT. Slides were then blocked with 1× PBS containing 5% donkey serum and 0.5% Triton X-100 (Sigma), before incubation with rabbit polyclonal anti-p27KIP1 (Cell Signaling Technology) at 1:200 dilution overnight at 4°C. Slides were washed in PBS before incubation with Alexa-Fluor-488-conjugated donkey anti-rabbit secondary antibody at 1:200 dilution. Fluorescent confocal images were collected using a Zeiss LSM 700 inverted microscope.

Statistical analysis

Within-genotype rates of hair-cell loss across weeks 2, 4 and 9

Counts were split into six distinct datasets for model fitting, according to hair-cell type (inner or outer) and genotypic group. A Poisson generalised linear model was fitted to each dataset separately. The model was specified as:

\[
\Pr(y_{twi} = k) = \frac{\exp(-\lambda_{twi})^{k}}{k!}, \quad k = 0, 1, 2 \ldots
\]

(i.e. with a turn-specific, log-linear relationship between mean hair-cell count and week), where

- \(t \in \{1, 2, 3, 4\}\) indexes turn (1=apical, 2=mid-apical, 3=mid, 4=mid-basal),
- \(w \in \{2, 4, 9\}\) denotes week,
- \(i \in \{1, 2, \ldots, n_j\}\) indexes mouse within (turn, week) group,
- \(\lambda_{twi}\) is the observed hair-cell count in week \(w\) at turn \(t\), in mouse \(i\), and
- \(\lambda_{twi}\) is the mean hair-cell count in week \(w\) at turn \(t\), in mouse \(i\).

For estimates and confidence intervals for the weekly percentage change in mean hair-cell count at each turn \(t\) (i.e. 100\% [exp(\(\beta_t\)) – 1]), see Table S1. Table S2 displays \(P\)-values from testing the null hypothesis that the weekly percentage change is zero (i.e. \(H_0: \beta_t = 0\)). Application of a variety of diagnostic tools suggested that the model provided a reasonably good fit to the data.

Model fitting, diagnostic plots and hypothesis tests were performed using the glm()-based functionality of the package ‘stats’ in R (R Core Team, 2013).

Inter-genotype comparison of hair-cell counts at week 2

For each cell type (inner and outer), and for each turn, cell counts were compared pair-wise between genotypic groups; \(P\)-values result from a Welch \(t\)-test applied to log-transformed hair-cell counts.

Comparison of qualitative phenotypes across genotypic groups

The proportion of mice carrying each particular qualitative phenotype (Normal, Extra row or Extra OHC) was estimated in each genotypic group (\(\text{Map3k1}^{+/+}\), \(\text{Map3k1}^{+\text{Yxia}}\) and \(\text{Map3k1}^{+\text{Yxia}+\text{Yxia}+}\)). Phenotype proportions were compared pair-wise across genotypes to determine whether mice with a particular phenotype were over-represented in some genotypic groups relative to others. Specifically, estimates and exact binomial confidence intervals were obtained for the proportion of mice of a particular genotype carrying a particular phenotype (Brown et al., 2001). Fisher’s exact test was used to test the null hypothesis of equality of phenotypic proportion across a pair of genotypic groups (for estimates, confidence intervals and \(P\)-values, see Table S3).

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Competing interests

The authors declare no competing or financial interests.

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Supplementary information


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