Developing novel transgenic reporters to study Lowe syndrome in Zebrafish

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Abstract
Lowe syndrome is a rare X linked disorder, characterized by renal, ocular and cerebral defects, caused by mutation in the protein OCRL1. OCRL1 has been implicated in a plethora of cellular functions, and loss of its catalytic conversion of PtdIns(4,5)P$_2$ into PtdIns(4)P is proposed to underly many of the cellular phenotypes associated with lack of OCRL1. The interaction with other proteins such as IPIP27A are also required for the correct function of OCRL1. Renal tubular dysfunction similar to that seen in Lowe syndrome patients is seen in zebrafish models of ocrl1 and ipip27a mutation.

Using zebrafish as a model of Lowe syndrome, a reduction of increased PtdIns(4,5)P$_2$ levels in ocrl1$^{-/-}$ embryos is shown to alleviate the renal tubular dysfunction. This demonstrates that targeting PtdIns(4,5)P$_2$ is a viable option for therapeutic treatment of Lowe syndrome. Novel transgenic zebrafish lines are also described, that provide megalin specific, fluorescent and luminescent readouts of proximal tubular endocytic function. These will be an important tool to perform high throughput screens for compounds that alleviate the symptoms of Lowe syndrome.

The importance of the binding of IPIP27A to its interaction partners OCRL1 and SH3 containing proteins such as PACSIN2 is demonstrated by rescue of the ipip27a$^{-/-}$ mutant with ipip27a with mutated binding sites. The phenotype of ipip27a$^{-/-}$ mutant embryos is further characterised to demonstrate there is no long term growth defect or defect in tubular polarity, however tubular dilation is seen, suggesting possible mild ciliary defects. In the zebrafish proximal tubule in fish with no functional IPIP27A or OCRL1, a more severe defect in 10 kDa dextran endocytosis is seen, as well as hydrocephaly and curved body axis, cilia impairment related phenotypes. This indicates that IPIP27A and OCRL1 are acting in the same pathway, and therefore depletion of both exacerbates phenotypes. Finally, transgenic lines expressing ubiquitous or pronephric tubule specific fluorescently tagged Rab proteins as markers of membrane compartments in zebrafish are described.
Declaration

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This Thesis is dedicated to a fantastic Grandma, who stayed with us to see me start it and has watched over me from the stars throughout it.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>AMN</td>
<td>Amnionless</td>
</tr>
<tr>
<td>APPL1</td>
<td>Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing</td>
</tr>
<tr>
<td>AP-2</td>
<td>Adapter protein-2</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate solution</td>
</tr>
<tr>
<td>AQP-1</td>
<td>Aquaporin-1</td>
</tr>
<tr>
<td>AQP-2</td>
<td>Aquaporin-2</td>
</tr>
<tr>
<td>ASH</td>
<td>ASPM-SPD2-Hydin domain</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BBS</td>
<td>Bardet Biedl syndrome</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCV</td>
<td>Clathrin coated vesicle</td>
</tr>
<tr>
<td>CCP</td>
<td>Clathrin coated pit</td>
</tr>
<tr>
<td>CDC42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>CUB</td>
<td>complement c1r/C1s, Uegf (epidermal growth factor-related sea urchin protein) and bone morphogenetic protein 1</td>
</tr>
<tr>
<td>DATs</td>
<td>Dense apical tubules</td>
</tr>
<tr>
<td>DE</td>
<td>Distal early tubule</td>
</tr>
<tr>
<td>Dent 2</td>
<td>Dent’s Disease Type 2</td>
</tr>
<tr>
<td>DL</td>
<td>Distal Late tubule</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DPF</td>
<td>Days post fertilisation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetraacetic acid</td>
</tr>
<tr>
<td>EE</td>
<td>Early endosome</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FOAR</td>
<td>Facio-oculo-acoustic-renal syndrome</td>
</tr>
<tr>
<td>GLuc</td>
<td>Gaussia Luciferase</td>
</tr>
</tbody>
</table>
GFP = Green fluorescent protein
GFR = Glomerular filtration rate
HPF = Hours post fertilisation
IL-6 = Interleukin 6
IPIP27 = Inositol polyphosphate phosphatase interacting protein of 27 kDa (A or B)
IPTG = Isopropyl β-D-1-thiogalactopyranoside
LB = Luria-Bertani broth
LDLR = Low density lipoprotein receptor
LE = Late endosome
LMW = Low molecular weight
LRP = Low density lipoprotein receptor related protein
LRP2 = Low density lipoprotein receptor related protein 2
LRPAP = LRP associated protein (aka Receptor associated protein, RAP)
LS = Lowe syndrome
MCS = Multiple cloning site
MVB = Multivesicular body
NHE3 = Sodium-hydrogen exchange protein 3
NL = NanoLuc
OCRL1 = Oculocerebrorenal syndrome of Lowe 1
PBS = Phosphate buffered saline
PBST = Phosphate buffered saline + 0.1% (v/v) Tween-20
PCR = Polymerase chain reaction
PCT = Proximal convoluted tubule
PFA = Paraformaldehyde solution
PH = Pleckstrin homology domain
PHDM = Pleckstrin homology domain mimetic
PKD = Polycystic kidney disease
PST = Proximal straight tubule
PtdIns(4)P = Phosphatidylinositol-4-phosphate
PtdIns(4,5)P_2 = Phosphatidylinositol-(4,5)-biphosphate
PtdIns(3,4,5)P_3 = Phosphatidylinositol-(3,4,5)-trisphosphate
PLC = Phospho-lipase C
PTCs  = Proximal tubular cells
PTU  = N-phenylthiourea
RA   = Retinoic acid
RAC1 = Ras-related C3 botulinum toxin substrate 1
RAP  = Receptor associated protein
RE   = Restriction enzyme
RE   = Recycling endosome
RhoGAP = Rho GTPase activation domain
SDS-PAGE = Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM  = Standard error of the mean
TAE  = Tris-acetate-EDTA buffer
TE   = Tris-EDTA buffer
TEMED = N, N, N', N'- Tetramethylethylenediamine
TGN  = Trans-Golgi network
TGS  = Tris-glycine-SDS buffer
VDBP = Vitamin D Binding Protein
ZO-1 = Zona occludens protein 1
Chapter 1: Introduction
1.1 Lowe Syndrome and Dent’s Disease

Oculocerebrorenal syndrome of Lowe, or Lowe syndrome (LS) is an X-linked disorder with an incidence of 1:500,000, characterised by defects in the eyes, brain and kidney (Lowe et al. 1952; Loi 2006; Bökenkamp and Ludwig 2016). Patients exhibit dense, congenital cataracts, glaucoma, severe mental retardation and behaviour disorders, generalised seizures and selective renal tubular dysfunction with progressive renal failure (Schurman and Scheinman 2009; Walton et al. 2005; Lowe Syndrome Trust 2010; Bockenhauer et al. 2008).

Lowe syndrome is caused by mutations in the OCRL1 gene, which encodes an inositol-5-phosphatase that preferentially hydrolysates the phospholipid phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P₂) to phosphatidylinositol-4-phosphate (PtdIns(4)P) (Lowe 2005). Dent’s Disease Type 2 (Dent 2) is also caused by mutations in OCRL1, and presents with renal tubular dysfunction, but displays milder or absent ocular and cerebral symptoms (Hoopes et al. 2005; Bökenkamp et al. 2009). Mutations in the chloride channel CLC-5 cause Dent’s Disease Type 1, which is phenotypically indistinguishable from Dent 2 (Ludwig et al. 2005). Mutations causing LS occur throughout the gene in the form of mis-sense, nonsense and frameshift mutations, as well as both deletions and insertions (Pirruccello et al. 2011; McCrea et al. 2008). Many mutations affect the stability of the mRNA transcript (as mutations often lead to a reduction or absence of OCRL1 mRNA in patient samples), which in turn results in decreased levels or absence of OCRL1 protein (Attree et al. 1992; Lichter-Konecki et al. 2006). It is also possible that some mutations result in defective 5-phosphatase activity or loss of correct localisation by destruction of protein interactions (McCrea et al. 2008). Mutations causing Dent 2 are also found throughout the gene, however analysis has shown that termination variants causing Dent 2 are only found in the first seven exons, and patients with these mutations may have a truncated form of OCRL via potential alternative initiation codons in exon 7 and 8 (Hichri et al. 2011; Shrimpton et al. 2009). In this way, Dent 2 patients may retain some functionality of the OCRL1 protein, explaining the reduced phenotype compared to LS patients.

1.1.1 The structure of OCRL1

OCRL1 is a 105 kDa protein consisting of 4 functional domains (Figure 1) (Lowe 2005). At the N-terminus of the protein is a pleckstrin homology (PH) domain (Mao et al. 2009). Mao et al propose that this non-classical PH domain lacks the
necessary basic pocket for binding to inositol lipids, and propose instead that it may have a role in interactions with other proteins. There is a central 5-phosphatase domain, characterised by the presence of WXGDXN(F/Y)R and P(A/S)W(C/T)DRIL motifs (Majerus et al. 1999; Zhang et al. 1995). This catalytic domain hydrolyses the 5-position phosphate group in PtdIns(4,5)P₂, and potentially also PtdIns(3,4,5)P₃, which can also serve as a substrate in vitro (Majerus et al. 1999). Deficiency of OCRL1 in either patient derived cell lines or zebrafish models of LS have shown an increase in PtdIns(4,5)P₂ levels, demonstrating that PtdIns(4,5)P₂ is a physiologically relevant in vivo substrate of OCRL1 (Jones et al. 2013; Ramirez et al. 2012; Wenk et al. 2003; Zhang et al. 1998). At the C-terminal end of the protein are ASPM-SPD2-Hydin (ASH) and Rho GTPase activation (RhoGAP) domains (Erdmann et al. 2007; Pirruccello et al. 2011). The RhoGAP domain is catalytically inactive, and these two domains fold to form a single module of the protein, thought to form an interaction platform critical for OCRL1 recruitment to the correct membrane compartments (Pirruccello and De Camilli 2012).

A number of important protein interaction sites have also been identified throughout the OCRL1 protein. There is a clathrin binding motif (LIDIA) present in a loop extending out of the PH domain (Choudhury et al. 2009; Mao et al. 2009), and also a further clathrin binding motif (LIDLE) in a flexible loop protruding from the ASH/RhoGAP domain (Erdmann et al. 2007; Ungewickell et al. 2004). OCRL1 is also able to bind to the clathrin adaptor protein AP-2 via an FEDNF motif in the linker region between the PH and 5-phosphatase domains (Ungewickell et al. 2004). The ability of OCRL1 to bind clathrin suggest a role in clathrin mediated trafficking.

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**Figure 1: Schematic representation of the domain organization and binding sites of OCRL1.**

Numbers correspond to the beginning and end of each individually coloured domain respectively. Labels above schematic show approximate binding sites for the labelled proteins. Red text highlights the 5-phosphatase domain responsible for the catalytic conversion of PtdIns(4,5)P₂.
OCRL1 is also able to interact with several members of the Rab GTPase family (Rab 1, 5, 6, 8, 14, 21, 25 and 35) via its ASH domain which occurs via an atypical mode of Rab effector binding (Fukuda et al. 2008; Hou et al. 2011; Hyvola et al. 2006). Rab GTPases are regulators of membrane trafficking and each Rab is recruited to a unique subset of membranes where they recruit effector proteins required at that particular compartment (Stenmark 2009). In this way, binding of OCRL1 to Rab GTPases is thought to recruit OCRL1 to various membrane compartments, as well as enhance the 5-phosphatase function of OCRL1 (Hyvola et al. 2006). Further, it is able to bind the Rab effector protein APPL1 and endocytic proteins IPIP27A and IPIP27B, which bind to OCRL1 via a conserved F&H motif to the RhoGAP like domain (Erdmann et al. 2007; Noakes et al. 2011; Swan et al. 2010). Through its interaction with IPIP27A, OCRL1 also indirectly binds to the F-bar containing protein PACSIN2, and likely other F-bar containing proteins (Billcliff et al. 2016). Interaction with another endocytic protein, sorting nexin-9 (SNX9) has also been demonstrated via binding of the SNX9 SH3 domain to a PxxP motif located between the PH and catalytic domains of OCRL1 (Nández et al. 2014). Finally, the RhoGAP domain is able to bind to RAC1 and CDC42 which links OCRL1 to actin assembly mechanisms (Erdmann et al. 2007; Faucherre et al. 2003).

OCRL1 has two isoforms in humans, OCRL1a and OCRL1b (Nussbaum et al. 1997). OCRL1a is expressed in all tissues including the brain, while OCRL1b expression is restricted to non-neuronal tissues (Johnson et al. 2003). There is an 8 amino acid region in a loop from the ASH/RhoGAP domain that is only present in the OCRL1a isoform (Nussbaum et al. 1997). This 8 amino acid loop is adjacent to one of the two clathrin binding sites of OCRL1, and is thought to enhance the binding of clathrin to OCRL1 by making the binding motif more accessible (Choudhury et al. 2009). A summary of the domain and binding site structure of OCRL1 is shown in Figure 1.

1.1.2 OCRL1 cellular localisation

OCRL1 localises to many different sub-cellular compartments (Figure 2) (Mehta et al. 2014). Late stage clathrin coated pits, early endosomes and clathrin coated intermediates have all been found to recruit OCRL1 (Cauvin et al. 2016; Choudhury et al. 2005; Erdmann et al. 2007; Nández et al. 2014; Taylor et al. 2011). It is also located at the trans-Golgi network (TGN) (Choudhury et al. 2005; Dressman et al.
membrane ruffles (Faucherre et al. 2005), primary cilia (Coon et al. 2012; Luo et al. 2012; Rbaibi et al. 2012) and the intracellular bridge during cytokinesis (Ben El Kadhi et al. 2011). Localisation has also been reported at the lysosome after lysosomal cargo loading (De Leo et al. 2016; Zhang et al. 1998). The plethora of localisations that OCRL1 can be found in reflect the many functions and interactions of OCRL1 (Mehta et al. 2014).

The targeting of OCRL1 to its many cellular locations is likely due to interaction with its many interaction partners (Mehta et al. 2014). For instance, interaction with Rab1 and Rab6 is required for OCRL1 targeting to the TGN, and interaction with Rab5 is required for the recruitment of OCRL1 to early endosomes (Hyvola et al. 2006). Binding of OCRL1 to Rab8A targets OCRL1 to the primary cilium, an important organelle with signalling, sensing and motility functions (Luo et al. 2012). Rab35 recruits OCRL1 to the midbody during cytokinesis and to the clathrin coated vesicle immediately after scission from the plasma membrane respectively (Cauvin et al. 2016; Dambournet et al. 2011). APPL1 is also recruited by Rab5, and it has been demonstrated that the indirect OCRL1-APPL1-Rab5 association is required for OCRL1 recruitment to phagosomes (Bohdanowicz et al. 2012). The binding of APPL1 or IPIP27A/B to a single motif in the ASH/RhoGAP domain of OCRL1 is required for its localisation to different subsets of early endosomes (Noakes et al. 2011; Swan et al. 2010). How OCRL1 is localised to other locations such as tight and adherens junctions, lysosomes and lamellipodia is currently unknown.

1.1.3 OCRL1 functions in phosphoinositide homeostasis

Phosphoinositides (or inositol lipids) are a group of 7 modified phospholipids found in the cytosolic leaflet of cellular membranes (Figure 3) (Di Paolo and De Camilli 2006; Simonsen et al. 2001). Each of the seven species of phosphoinositide is derived from phosphatidylinositol and is generated by differential phosphorylation of the inositol ring at the 3', 4' or 5' positions. Phosphoinositides can be interconverted from one to the other by addition or removal of phosphates on the inositol ring, which is carried out by specific phosphoinositide kinases or phosphatases respectively (Liu and Bankaitis 2010). The synthesis of phosphoinositides is spatially and temporally restricted, leading to distinct phosphoinositide identities for different membrane compartments, or even subdomains of compartments. Phosphoinositides each recruit a specific subset of effector proteins to the membrane (Choudhury et al. 2005; Di Paolo and De Camilli 2006; Simonsen et al. 2001).
and therefore tight regulation of phosphoinositide identity on a specific membrane is critical. PtdIns(4,5)P$_2$, the preferred substrate for OCRL1 (Zhang et al. 1998), is abundant at the plasma membrane. OCRL1 has a clear role as a 5-phosphatase, and it has been demonstrated in both cell culture and zebrafish that lack of OCRL1 causes an elevation in PtdIns(4,5)P$_2$ levels (Jones et al. 2013; 2001).
Ramirez et al. 2012; Zhang et al. 1998). Further, ectopic PtdIns(4,5)P$_2$ has been shown to accumulate on endosomal membranes (Ben El Kadhi et al. 2011; Vicinanza et al. 2011). Its presence in many different sub-cellular localisations suggests that OCRL1 may represent a housekeeping gene in some of these locations, acting to maintain the phosphoinositide identity of the membrane by hydrolysing any PtdIns(4,5)P$_2$ that may arrive at the compartment (Lowe 2005). Alternatively, it is possible that there are small, but physiologically relevant pools of PtdIns(4,5,)P$_2$ in some or all of these locations that OCRL1 acts upon to perform a given function (Mehta et al. 2014). Expression of catalytically inactive mutants of OCRL1 are unable to rescue cellular phenotypes (Nández et al. 2014; Oltrabella et al. 2015), and therefore it is likely that the lack of hydrolysis of PtdIns(4,5)P$_2$ or PtdIns(3,4,5)P$_3$ is an underlying cause to many of the multitude of phenotypes seen.

1.1.4 OCRL1 is required for membrane trafficking and endocytosis

OCRL1 is a regulator of membrane trafficking both via its regulation of PtdIns(4,5)P$_2$ levels and also by interaction with endocytic proteins such as IPIP27A/B and APPL1 (Bohdanowicz et al. 2012; Erdmann et al. 2007; Nández et al. 2014; Noakes et al. 2011; Swan et al. 2010). The interaction with APPL1 and IPIP27A/B, proteins with a demonstrated role in regulating endocytosis, also links OCRL1 to the endocytic membrane trafficking machinery (Erdmann et al. 2007; Noakes et al. 2011; Swan et al. 2010). OCRL1 is recruited to the clathrin coated pit, where it is thought to dephosphorylate PtdIns(4,5)P$_2$ on the newly forming vesicle (Erdmann et al. 2007).
More recent studies suggest a role for OCRL1 in the uncoating of clathrin coated vesicles coming from the plasma membrane (Nández et al. 2014). Lowe syndrome fibroblasts accumulate clathrin coated pits and vesicles, and the OCRL1 binding partner SNX9 remains associated with these vesicles and promotes actin nucleation, creating actin comets (Nández et al. 2014). The loss of hydrolysis of PtdIns(4,5)P$_2$ by OCRL1 in cells also leads to an accumulation of PtdIns(4,5)P$_2$ on enlarged early endosomes, where it is thought to promote filamentous actin assembly around the endosome via activation of the actin nucleation protein N-WASP (Vicinanza et al. 2011). Actin is known to play a role in carrier formation at the early endosome in trafficking from the early endosome to other compartments and the plasma membrane (Smythe and Ayscough 2006; Anitei and Hoflack 2012). It is possible therefore that OCRL1 is a regulator of the actin dependent formation of intermediate compartments at the early endosome, which causes a defect in the recycling of plasma membrane proteins. In addition to the proposed role in endocytosis, OCRL1 is required for clathrin mediated trafficking between the trans-Golgi network (TGN) and early endosomes, demonstrated by the impaired retrograde trafficking of mannose-6-phosphate receptors (MPRs), causing an accumulation of MPRs in the early endosome and dysfunctional lysosomal hydrolase trafficking (Choudhury et al. 2005; van Rahden et al. 2012).

1.1.5 Other cellular functions of OCRL1

Although the exact mechanism by which mutation of OCRL1 causes Lowe syndrome is not fully understood, much is known about the functions and interactions of OCRL1 at a cellular level. In addition to the roles of OCRL1 in phosphoinositide homeostasis and membrane trafficking, OCRL1 has been shown to be required for many other cellular functions. These are outlined below.

1.1.5.1 Actin dynamics

In addition to the proposed mechanism by which depletion of OCRL1 causes PtdIns(4,5)P$_2$ dependent filamentous actin formation by N-WASP at the early endosome (Vicinanza et al. 2011), OCRL1 may have a role in regulating actin in other subcellular locations (Coon et al. 2009; Dambournet et al. 2011; Cui et al. 2010). It is possible that, as at the early endosome, disruption of physiological actin regulation is a direct consequence of aberrant or mislocalised PtdIns(4,5)P$_2$, as PtdIns(4,5)P$_2$ is a direct regulator of actin nucleases (Hilpelä et al. 2004), but further work is required to fully understand the mechanism. Actin polarisation is controlled
by several classes of actin nucleators, such as ARP2/3 complex, which initiates branching filaments to form from existing filaments (Pollard 2007) and the WASH complex, which likely controls dynamic actin during fission of tubules from early endosomes (David 2010). Alternatively, it is also possible that ectopic accumulation of filamentous actin such as that at the early endosome in OCRL1 depleted cells (Vicinanza et al. 2011) causes a reduction in the dynamic pool of monomeric actin required for actin polymerisation at other cellular locations. Ectopic accumulation and dynamic monomeric actin depletion are not mutually exclusive explanations for the consequences of OCRL1 depletion, and its possible that both scenarios affect different pathways within the cell.

Lowe syndrome patient fibroblasts have demonstrated an increase in filamentous actin, increased sensitivity to compounds that depolymerise actin, and increased numbers of stress fibres (Suchy and Nussbaum 2002). Moreover, an increase in actin comets, likely nucleating from endocytic vesicles, has been described in cells depleted of OCRL1 (Vicinanza et al. 2011). OCRL1 is required for PtdIns(4,5)P$_2$ hydrolysis in phagocytosis, to allow actin remodelling to occur (Bohdanowicz et al. 2012). Actin is also dysregulated in cell migration and cytokinesis, discussed further below (Ben El Kadhi et al. 2011; Grieve et al. 2011; Dambournet et al. 2011). It is possible therefore that local regulation of actin dynamics at many of the subcellular localisations of OCRL1 may be responsible for the many cellular processes affected by Lowe syndrome, however more work is required to understand the exact mechanism.

1.1.5.2 Ciliogenesis

OCRL1 has been implicated in the formation and maintenance of the primary cilium, although the manner in which it affects the cilium has so far proved controversial. Its localisation has been placed either in the cilium proper, or at the basal body (Coon et al. 2012; Luo et al. 2012). The effect of loss of OCRL1 has been reported to cause both a shortening and lengthening of the primary cilium (Rbaibi et al. 2012; Coon et al. 2012; Luo et al. 2012). Coon et al (2012) demonstrated that OCRL1 interaction with Rab8 and the endocytic proteins IPIP27A/B are all required for efficient protein trafficking into the primary cilium. While it is clear that loss of function of OCRL1 effects the primary cilium, Lowe syndrome patients exhibit overlapping but distinct symptoms from other ciliopathies (Schurman and Scheinman 2009; Hildebrandt et al. 2011; Loi 2006). It is currently unclear as to whether OCRL1 is involved in trafficking directly to the primary cilium, or whether
there is a general perturbation of trafficking within the cell, that has an indirect effect on trafficking into the primary cilium.

1.1.5.3 Cell polarity

The formation of tight junctions and adherens junctions is critical to determine epithelial cell polarity, and these junctions define the subdivision between the differentiated apical and basolateral membranes. Tissues in the eyes and kidney, as well as neurons in the brain, affected in Lowe syndrome are all polarized tissues. OCRL1 is required at tight and adherens junctions to mediate correct polarisation of cells (Grieve et al. 2011). In cells lacking OCRL1, this defect in maturation of tight and adherens junctions means cells fail to form a central lumen when grown in 3D culture (Grieve et al. 2011). The author also demonstrates that filamentous actin was redistributed in these cells, lacking assembly at the apical membrane and persisting at the sites of intracellular junctions. Recently, OCRL1 has been shown to be required in 2D cell culture, but not 3D cell culture, for Rab35 dependent trafficking of the apical protein podocalyxin, which requires reorganisation to the apical pole for correct cell polarity (Mrozowska and Fukuda 2016). This is similar to cell polarity reported in animal models of Lowe syndrome, where proximal tubular cells are shown to polarize normally (Oltrabella et al. 2015). Therefore, the relevance in vivo of cell polarity defects seen in cell culture is yet to be fully understood.

1.1.5.4 Cell migration

The localisation of OCRL1 to lamellipodia, along with reduced cell migration in fibroblasts depleted of OCRL1 is consistent with a role in migration (Coon et al. 2009; Faucherre et al. 2005). Furthermore, in zebrafish embryos depleted of ocr1, reduced migration of melanocytes was noted (Coon et al. 2012). It is thought that the defect in cell migration could be caused by misregulation of actin at the lamellipodia due to the incorrect turnover of PtdIns(4,5)P₂, or alternatively it could be due to indirect mistrafficking defects (Mehta et al. 2014). Further work is required to fully understand the role of OCRL1 in cell migration.

1.1.5.5 Cytokinesis

PtdIns(4,5)P₂ turnover at the midbody is critical during cytokinesis to ensure the correct fission of daughter cells. Failed cytokinesis and binucleation has been
reported in *Drosophila* cells depleted of dOCRL1 (Ben El Kadhi et al. 2011). Moreover, in mammalian cells depleted of OCRL1, PtdIns(4,5)P$_2$ accumulates at the intercellular bridge immediately preceding scission, causing a twofold delay in the time taken for OCRL1 depleted cells to undergo abscission (Dambournet et al. 2011). It is likely that the absence of functional OCRL1 from the midbody means PtdIns(4,5)P$_2$ is not turned over, which in turn would lead to the stabilization of actin at this site, inhibiting fission.

1.1.6 Genetic conservation and homologous genes

OCRL1 is a member of a family of phosphatases known as inositol polyphosphate 5-phosphatases, of which 10 types exist in vertebrates (Ooms et al. 2009). Closely related to OCRL1 is INPP5B, another 5-phosphatase that shares 45% similarity with OCRL at the amino acid level, as well as containing the same core PH, 5-phosphatase and ASH/RhoGAP domains (Jänne et al. 1998; Mao et al. 2009; Williams et al. 2007). OCRL1 and INPP5B has similar substrate specificity, both being able to hydrolyse PtdIns(4,5)P$_2$ (Schmid et al. 2004). The expression and localisation of INPP5B overlaps partially with OCRL1. Despite the similarity between INPP5B and OCRL1, INPP5B lacks binding sites for clathrin and AP-2 that OCRL1 has, and both proteins are thought to have distinct roles in the cell (Williams et al. 2007). INPP5B is required for exocytosis in the secretory pathway, as well as being recruited to the early endosome (Shin et al. 2005; Williams et al. 2007). Other examples of this family of proteins are the primary cilium protein INPP5E, mutations of which cause MORM and Joubert syndromes (Jacoby et al. 2009) and Synaptojanin, mutations of which are linked to early onset Alzheimer’s in Downs syndrome (McCrea and Camilli 2009) and early onset Parkinson’s (Quadri et al. 2013). SHIP1 and SHIP2, also members of this family, are thought to have roles in cancer and diabetes (McCrea and Camilli 2009).

In invertebrates such as *Drosophila*, a single orthologue of OCRL1 and INPP5B exists (Ben El Kadhi et al. 2011). The *Drosophila* orthologue contains a catalytic domain as well as a C-terminal RhoGAP like domain (Ben El Kadhi et al. 2011). Both OCRL1 and INPP5B are highly conserved in all vertebrates, and contain the same functional domains and binding sites (Jänne et al. 1998; Ramirez et al. 2012; Swan et al. 2010).

A mouse model of Lowe syndrome was attempted by knocking out the ocrl1 gene, however no phenotype was observed, likely due to compensation for loss of Ocr1 by murine Inpp5b (Jänne et al. 1998). Humanization of the *Inpp5b* gene in
mice, by insertion of human INPP5B into lnpp5b⁻/⁻ mice and crossing into an Ocr1⁻/⁻ background, resulted in proteinuria and aminoaciduria, similar to the renal phenotype of Lowe syndrome and Dent-2 in humans (Bothwell et al. 2011). It is thought that differences in the transcription and splicing between lnpp5b and INPP5B are the reason for this compensation (Bothwell et al. 2010).

In zebrafish, despite a genome duplication prior to the divergence of teleost fish from mammals (Jaillon et al. 2004), there is a single copy of each OCRL1 and INPP5B (Ramirez et al. 2012). Evidence suggests that a model of Lowe syndrome in zebrafish faithfully recapitulates the neural and renal phenotypes of Lowe syndrome in zebrafish (Oltrabella et al. 2015; Ramirez et al. 2012). This will be discussed in more detail in section 1.4.2.

1.2 The vertebrate kidney

1.2.1 Gross structure of the kidney
This thesis will focus primarily on the renal phenotype of Lowe syndrome. The kidney is the main excretory organ in the body, and has a large range of functions such as water and electrolyte homeostasis, the removal of metabolic waste products and the recovery of useful components of the filtrate such as proteins and amino acids (Vize et al. 2003). In humans, a non-functional pronephros forms by the 22nd day of gestation, with a functional mesonephric kidney forming by the 6th week of development, and finally these are replaced with the adult metanephric kidney about 32 weeks into gestation (Carlson 2013). In adult humans, the kidneys are a pair of bean shaped organs located in the abdominal cavity. The human kidney filters around 180L of blood per day, the equivalent of approximately 20% of cardiac output (McMahon 2016). The kidney consists of two main gross structures, the cortex and the medulla (Figure 4A). Blood vessels enter the kidney via the renal hilum in the centre of the kidney and then protrude up to the highly vascularized cortex. Here renal arteries branch further into arterioles, which convolute in the renal capsule of the nephron, the functional unit of the kidney. Filtrate passes through the renal capsule into the nephron, where its content is altered by cells lining the nephron. Finally, the filtrate then passes into the collecting ducts, which carry urine out of the ureter.
1.2.2 The nephron

Nephron number can vary greatly between vertebrate species, and even within species. For instance, mice have between 12000 and 16000 nephrons per kidney (Short et al. 2014), while humans have on average 1 million nephrons per kidney (Bertram et al. 2011). The nephron itself spans both the cortex and medulla, that
have low and high osmolality relative to each other, respectively. This spatial organisation of the nephron through regions of high and low osmolality contributes to the reabsorption of water (Figure 4B) (Sands and Layton 2014).

1.2.2.1 The glomerulus

The beginning of the nephron is the glomerulus. Here, highly convoluted arterioles form a knot of leaky capillaries (McMahon 2016). This knot is enclosed within the Bowman’s capsule (Figure 4C). The outside of the capsule is composed of squamous epithelium, however the inside is lined with specialised cells called podocytes, large cells with long protrusions called foot processes, which interdigitate to form a slit diaphragm (Figure 4D). The slit diaphragm forms gaps of around 40 nm which contribute to the size exclusion of the overall barrier structure, and the vast inter-digititation is thought to be critical in controlling flow into the interstitial space of the capsule (Pavenstädt 2000). Loss of this slit diaphragm, for instance by damage or mutation in critical proteins such as CD2AP and Nephrin, results in breakdown of the filtration barrier and massive leakage of large proteins into the nephron (Asanuma et al. 2007; Shih et al. 2001). Between the podocytes and the capillary endothelium is a layer called the glomerular basement membrane. This thick extracellular matrix is a critical third component of the barrier between the interstitial space of the Bowman’s capsule and the nephron, and contributes to the size selective passage of macromolecules into the interstitial space (Jarad and Miner 2009). The size and charge of a molecule has a large effect on its permeability to the basement membrane. Pores in the membrane are small, and therefore there is an inverse relationship between size of the protein and the level of filtration (Jarad and Miner 2009). Structural proteins within the membrane are also negatively charged, therefore plasma proteins with a negative charge themselves are thought to be repelled by the membrane and less likely to pass through. Due to physical restrictions in the size of pores in the membrane, both size and flexibility/deformity of the protein also contribute to how well a protein filters (Venturoli and Rippe 2005). Large globular or inflexible proteins will be retarded in filtration more than small globular or flexible proteins. It has been found that both the podocytes and endothelial cells of the capillaries are required for the correct formation of the basement membrane (Byron et al. 2014).
1.2.2.2 The proximal tubule

The selective filtration of the glomerulus is important in retaining macromolecules in the blood. However low molecular weight (LMW) proteins, amino acids, electrolytes and water all pass freely through this membrane (Haraldsson et al. 2008). Cells along the length of the nephron are specialised for the recovery and homeostasis of all of these filtered components. The first region of tubule encountered by primary filtrate is the proximal convoluted tubule (PCT). This is localised wholly in the cortex of the kidney (McMahon 2016). The single sheet of columnar epithelial cells lining the proximal tubule are polarised cells with an apical brush border of microvilli, which functions to greatly increase the surface area of the apical membrane (Wang 2006). Primary cilia, as well as the microvilli, sense flow in the PCT, and in response to the level of fluid shear stress sensed, can increase the rate of endocytic uptake and ion scavenging by proximal tubular cells when flow increases in the tubule (Raghavan et al. 2014; Wang 2006). The cells of the proximal tubule can be defined by the expression of a variety of transporters and receptors. For instance strong apical expression of megalin and cubilin are required for the endocytosis of filtered proteins (Christensen and Birn 2002). Expression of NHE3 in the PCT is critical for the salvage of filtered Na\(^+\) ions and balancing filtrate pH (Parker et al. 2015). To date, 14 ion transporters and antiporters have been identified on the apical membrane of epithelial cells in the PCT that control the uptake of organic and inorganic ions from the filtrate, as well as glucose (Feric et al. 2011). In addition to their reabsorption role, proximal tubular cells metabolise glutamine to produce ammonium, and unlike most other filtrate solutes that enter the tubule via filtration in the glomerulus, PCT cell secretion is the primary source of urinary ammonia (Weiner and Verlander 2013). Three ATP binding cassettes are expressed in the proximal tubule that are involved in the export of ammonium and other organic ions (Feric et al. 2011). Furthermore, Aquaporin-1 (AQP1), is very highly expressed in the apical brush border, and is responsible for the passive reabsorption of water from the filtrate (Agarwal and Gupta 2008). The high reabsorption of water in the PCT means the volume of fluid leaving the PCT is around one third of the volume that entered the PCT. Overall, the cells of the proximal tubule are highly active cells which are responsible for the recovery of the majority of LMW proteins, glucose, water and electrolytes (Curthoys and Moe 2014).
1.2.2.3 The loop of Henle

Once through the PCT, filtrate then passes into the loop of Henle, a large tubular structure that extends deep into the medulla of the kidney, critical for water retention in terrestrial vertebrates (McMahon 2016). The medulla has a much higher osmolarity than the filtrate, which means that osmotic pressure drives water from the filtrate back into the medulla (Sands and Layton 2014). Cells lining the descending limb of the loop of Henle express AQP1 and are therefore permeable to water (Agarwal and Gupta 2008; Maunsbach et al. 1997) but are impermeable to ions (Sands and Layton 2014). The ascending limb of the loop of Henle is split into a proximal thin limb, and a distal thick limb. The thin limb is impermeable to both water and ions, but passive diffusion of Na⁺ and Cl⁻ ions occurs via the chloride channel CLC-K1 (Imai and Kokko 1976; Liu et al. 2002). The thick ascending loop actively reabsorbs around 30% of filtered Na⁺ and Cl⁻ through the expression of NKCC2 (Ares et al. 2011), as well as bicarbonate and ammonium ions (Mount 2014). NKCC2 expression in the thick ascending limb is increased in response to long term increases in circulating vasopressin, which increases the reabsorption of ions from the filtrate and results in a more dilute filtrate passing into the final part of the nephron, the distal tubule (Kim et al. 1999).

1.2.2.4 Distal tubule and collecting duct

The distal tubule in the cortex makes final modifications to the developing urine (Subramanya and Ellison 2014). Importantly, the reabsorption and excretion of water and electrolytes in the distal tubule is responsive to various circulating hormones, ultimately leading to water and ion homeostasis in the blood. Juxtaglomerular cells situated between the glomerulus and arteriole detect drops in pressure by stretch receptors, and secrete renin in response (Peach 1977; Crowley and Coffman 2012). Renin cleaves the 10 amino acid angiotensin I from the plasma proprotein angiotensinogen. Angiotensin-converting enzyme (ACE) the further processes this by removal of a further two amino acids, to produce angiotensin II, that is responsible for increasing vasoconstriction to increase blood pressure (Fyhrquist et al. 1995). In addition, angiotensin II increases the expression of apical Na⁺/Cl⁻ cotransporters in the distal tubule, to increase the reabsorption of Na⁺ and Cl⁻ ions from the filtrate (San-Cristobal et al. 2009; Sandberg et al. 2007). Circulating vasopressin drives the fusion of intracellular vesicles containing aquaporin-2 with the apical membrane of collecting duct cells, which makes the collecting duct cells permeable to water (Klussmann et al. 2000). The level of ion reabsorption in the
distal tubule regulates the osmolarity of the filtrate, and therefore have a direct effect on how much water is then reabsorbed in the collecting duct. In this way, the distal tubule and collecting duct control the homeostatic regulation of urine content.

1.3 Megalin mediated endocytosis in the renal tubule

Megalin (LRP2) and cubilin are expressed in a variety of epithelial tissues such as those found in the inner ear, the ciliary epithelium of the eye and ependymal cells lining the brain ventricles (Christensen et al. 2012). Very high expression of megalin and cubilin are seen in the proximal tubular epithelium, where these proteins are responsible for the reabsorption of protein within the renal tubule. Over 60 ligands have now been identified that bind to either megalin, cubilin or both proteins to date (Nielsen et al. 2016). Proteins that bind to megalin and cubulin come from several distinct classes, such as vitamin binding proteins like vitamin D binding protein and folate binding protein, carrier proteins such as albumin and lactoferrin, hormones and signalling proteins such as angiotensin II and leptin and plasma proteins such as cystatin C and lysozyme. Once a ligand is bound to megalin, it is internalised via clathrin mediated endocytosis.

1.3.1 Megalin and cubulin structure

Megalin is a 600 kDa, transmembrane protein, with a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail (Figure 5A) (Christensen and Birn 2001) It is a member of the low-density lipoprotein receptor related protein (LRP) family of proteins. LRP proteins are defined by the presence of a large extracellular domain containing cysteine-rich complement-type repeats, epidermal growth factor (EGF) repeats, β-propellar domains, and a cytoplasmic domain (Lillis et al. 2005). A blast search of the megalin protein sequence demonstrates its high level of conservation across many species. Similarity to the human LRP2 protein is 76.7% in mice, 76.4% in rats, 65.7% in zebrafish, 41.2% in Drosophila.
The extracellular domain of megalin contains 36 cysteine rich, complement
like repeats organised into four clusters, and it is thought that these are critical for ligand binding (Nielsen et al. 2016). Each of these repeats contains around 40 amino acids, and three disulphide bridges (Wolf et al. 2007). These ligand binding repeating clusters are separated by EGF precursor homology domains (Christensen and Birn 2001). These domains contain YWTD motif repeats, which are thought to function in the pH dependent release of ligands in the early endosome (Davis et al. 1987). Receptor associated protein (RAP), also known as LRP associated protein (LRPAP), binds with nanomolar affinity to the extracellular domain of megalin in the ER, and chaperones megalin and other LRP proteins between the ER and Golgi (Czekay et al. 1997; Fisher et al. 2006; Lee et al. 2006; Orlando et al. 1997). The 209 amino acid cytoplasmic tail of megalin contains two NPXY motifs, thought to be important for the binding of megalin to endocytic adaptor proteins such as AP-2, DAB-2 and ARH (Nielsen et al. 2016; Shah et al. 2013). A PxxP motif found in the cytoplasmic tail of megalin is thought to bind to the adaptor protein MegBP, however the role of this binding is currently unclear (Petersen et al. 2003). The intracellular domain of megalin also contains PDZ domains however the function of these binding sites in vivo is currently unknown (De et al. 2014). The cytoplasmic tail of megalin can be phosphorylated in a number of positions by protein kinase C, protein kinase A, casein kinase II and glycogen synthase 3 (Baines and Brunskill 2011; Yuseff et al. 2007). Phosphorylation of the cytoplasmic tail of megalin is thought to negatively regulate the recycling of megalin (Yuseff et al. 2007).

Cubilin is a membrane anchored protein of around 460 kDa, comprising a large extracellular domain and transmembrane domain, but with no cytoplasmic tail (Figure 5A) (Nielsen et al. 2016). Blast searches of human cubilin also reveal a high degree of similarity between species, with 70% similarity with mice and rats, 53% with zebrafish. Cubilin homologous genes in invertebrates are less similar, with the cubilin homolog in Drosophila having a similarity score of 28.8%.

The extracellular domain of cubilin contains 27 complement c1r/C1s, Uegf (epidermal growth factor-related sea urchin protein) and bone morphogenetic protein 1 (CUB) domains, which are essential for ligand binding (Christensen and Birn 2001). Importantly, due to the lack of cytoplasmic tail, cubilin relies on interaction with megalin for internalisation. It can bind to megalin via a subset of CUB domains, forming a dimer which can then enter the cell by megalin endocytosis (Ahuja et al. 2008). Another protein, amnionless (AMN) is required for the delivery of cubilin to the plasma membrane, and without it is retained in intracellular structures (He et al. 2005).
1.3.2 Trafficking in the proximal tubule

The organisation and structure of the endocytic pathway in proximal tubule cells is specialised for endocytosis and reabsorption from the tubular lumen, likely due to the highly active nature of these cells (Christensen et al. 1992; Christensen and Willnow 1999; Mattila et al. 2014). Firstly, ligand bound megalin, which is abundant at the plasma membrane of PTCs, is transported from its position on the microvillus to forming clathrin coated pits from at the base of the microvilli (Birn et al. 1993; Nielsen et al. 2016; Rodman et al. 1986). Here it is internalised via clathrin mediated endocytosis, and is trafficked to the large apical vacuolar endosome, a large and specialised early endosome found in PTCs (Christensen and Birn 2002). The cytoplasmic domain is critical in the internalisation of megalin. The first and second NXPY motifs in the cytoplasmic tail have been shown to bind with the clathrin internalization sorting proteins ARH, DAB-2 and GIPC respectively (Nagai et al. 2003; Oleinikov et al. 2000; Traub 2009). DAB-2 and GIPC has also been shown to interact with the motor proteins Myosin VI and nonmuscle myosin heavy chain IIA (NMHC-IIA), which may provide a mechanism for directional travel of megalin towards the clathrin coated pit, as well as movement of megalin containing vesicles to the vacuolar endosome (Hasson 2003; Hosaka et al. 2009). ARH binds during the endocytosis of megalin and accompanies it throughout the endocytic pathway (Nagai et al. 2003). In the apical vacuole, the lower pH driven by the vacuolar ATPase, causes the dissociation of the ligand from megalin. Megalin is then packed into dense apical tubules, a recycling compartment that traffics from the vacuolar endosome to the plasma membrane in a Rab11 and Rab35 dependent manner (Mattila et al. 2014; Perez Bay et al. 2016; Shah et al. 2013). The binding of ARH to the cytoplasmic tail of megalin appears to be more important at recycling stages of megalin cycling (Nagai et al. 2003; Shah et al. 2013). In cultured cells, it has been shown that ARH is critical for the correct sorting and recycling of megalin via the apical sorting endosome (Shah et al. 2013). A summary of the trafficking of megalin can be seen in Figure 5B.

1.3.3 Diseases associated with megalin

Mutations in megalin cause the autosomal disorder Donnai-Barrow syndrome, also known as Facio-occulo-acoustic-renal (FOAR) syndrome (Kantarci et al. 2007). This rare disorder results in facial dysmorphism, ocular defects, hearing loss, LMW proteinuria, diaphragmatic hernia and loss of the corpus callosum. Interestingly, a recently discovered family of patients with a novel mutation in megalin presented
with very few of the typical symptoms of Donnai-Barrow syndrome (Schrauwen et al. 2014). These patients have mild proteinuria, but significant glaucoma and myopia, similar to the symptoms of Stickler syndrome, caused by mutations in Collagen II. This suggests that there may be a phenotypic spectrum of megalin mutations. Mouse and zebrafish megalin knockout models both faithfully recapitulate the proteinuria and ocular myopia seen in Donnai-Barrow syndrome (Kur et al. 2011; Veth et al. 2011; Weyer et al. 2011). While the proximal tubular defects can be described by the lack of megalin internalisation of proteins in Donnai-Barrow syndrome, how mutations in megalin cause the extra-renal symptoms of Donnai-Barrow syndrome is not fully understood. Mutations in either cubilin or AMN result in megaloblastic anaemia 1 (Aminoff et al. 1999). In this disorder, vitamin B12 is not absorbed in the intestine, which in turn causes defects in the formation of blood cells. Subclinical proteinuria is also a symptom.

1.4 Zebrafish as a model organism
The zebrafish (Danio rerio) is a small freshwater teleost belonging to the minnow family (Cyprinidae), and native to the southern Himalayas. Zebrafish are gaining popularity as a model organism of choice for many studies due to a number of advantages over other systems. The relative cost, transparency of the embryo, external and rapid development, ease of manipulation, as well as the amenability to high throughput screens, where one pair can generate hundreds of embryos in one breeding are all attractive properties (Lieschke and Currie 2007; Ingham 2009). Furthermore, the use of zebrafish embryos instead of adult zebrafish or larger vertebrates represents an attractive way to reduce the use of animals in research and meet the target of using the least sentient life form possible (Strähle et al. 2012).

1.4.1 The zebrafish kidney
The zebrafish embryo possesses a functional pronephros in which the nephrons bare remarkable similarity in the morphology and expression of transporters to the mammalian adult (metanephric) kidney (Figure 6) (Drummond and Davidson 2010; Wingert and Davidson 2008). While the mammalian pronephros is not functional (Carlson 2013), the zebrafish develops a functional pronephros during early development, and then a mesonephros at around 11 dpf, which represents the final stage in kidney development in zebrafish (Diep et al. 2015).
The zebrafish pronephros begins to develop during the organogenesis stage of development at around 24 hpf, when two nephron primordia are generated from the third and fourth somite (Drummond et al. 1998). By 32-33 hpf, these nephron primordia become distinct from the coelom and contain a central lumen. By 40 hpf, lateral cells begin to form tubules and polarise, while the cells at the midline destined to be glomeruli are enclosed within a basement membrane. At 50 hpf, the cells at the midline have fused and begun to form a glomerulus, and the pronephric tubule formation is complete. By 72 hpf, the pronephros is functional, with both glomerular filtration and endocytic uptake taking place (Drummond et al. 1998, Kimmel et al. 1995). This gives rise to the gross pronephric structure, consisting of a fused glomeruli at the anterior end, and two lateral pronephric tubules that extend down the midline of the embryo and connect to the cloaca (Drummond and Davidson 2010). A number of genes have been shown to be required for this to occur. Expression of the transcription factors pax2a and lim1 are the earliest markers of nephrogenic cell fate, and expression of pax2a and lim1 is maintained throughout pronephric development (Drummond et al. 1998). Cadherin17 is required for cell adhesion in the renal tubule, and mutations in cadherin17 cause gaps in the tubule as well as a failure of the tubule to fuse at the cloaca (Horsfield et al. 2002).

The developed tubular epithelium is subdivided into functional zones similar to those seen in the mammalian nephron (Wingert et al. 2007). Following the neck region of the tubule is the proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early tubule (DE) and distal late tubule (DL, Figure 6) which is

![Figure 6: Schematic representation of the zebrafish pronephric tubule.](image-url)
connected to the cloaca (Drummond and Davidson 2010). The zebrafish nephron lacks the loop of Henle seen in terrestrial vertebrates, likely because zebrafish have no requirement for the reabsorption of water required of a terrestrial animal (Sands and Layton 2014). The correct formation of these pronephric zones in zebrafish is tightly controlled by the expression of a number of transcription factors. *Pax2a* expression in the tubule is upregulated in the neck segment separating the glomerulus and proximal tubule, and is thought to suppress the expression of the podocyte markers *wt1a, wt1b* and *vegf* (Majumdar et al. 2000). The expression of *wt1a* and *wt1b* regulates the development of podocytes at early and later stages of development respectively (Perner et al. 2007), and in turn *wt1a* and *wt1b* expression in the developing glomeruli is controlled by retinoic acid (RA) signalling (Wingert et al. 2007). RA signalling also regulates the proximal and distal fate of tubular epithelial cells, with high RA doses leading to an expansion of the proximal tubular cell fate into the distal tubule, and inhibition of RA signalling leading to an expansion of the distal tubule (Wingert et al. 2007; Wingert and Davidson 2008).

The epithelial cells of the PCT are columnar, with a well-defined apical/basal polarity and a brush border. The receptors megalin and cubilin are present (Anzenberger et al. 2006), as well as transporters typical of the PCT such as the sodium/hydrogen exchanger *rne1* (a homolog of *NHE3*) (Wingert et al. 2007) and the chloride/bicarbonate exchanger *ae2* (Shmukler et al. 2005). The PST seems to be a subdomain of the proximal tubule, with similar functions to the PCT, and this segment is thought to be involved in the uptake of specific ions due to the expression of the sulfate transporter *slc13a3* and the calcium transporter *trpm7* (Desgrange and Cereghini 2015; Drummond and Davidson 2010; Elizondo et al. 2005). The DE tubule expresses the ortholog of the mammalian NKCC2 symporter, *slc12a1* (Drummond and Davidson 2010). This means the DE, like its mammalian counterpart, is involved in sodium chloride conservation (Igarashi et al. 1995). The DL segment expresses *slc12a3*, a cotransporter that further modulates the sodium and chloride concentration in the developing urine under hormonal regulation, akin to the distal convoluted tubule in the mammalian kidney (Drummond and Davidson 2010; Mastroianni et al. 1996). AQP1 is also expressed in the zebrafish pronephric tubule (Tingaud-Sequeira et al. 2010).

### 1.4.2 Zebrafish kidney models

Due to the similarities between the mammalian nephron and the zebrafish pronephros, it is becoming more popular to model kidney diseases using zebrafish
embryos (Swanhart et al. 2011). The similarity in expression of wt1 and pax2 in controlling the patterning of the tubule makes zebrafish an ideal model for studying nephrogenesis (Drummond et al. 1998; Drummond and Davidson 2010). More crucially to this project, the tubular dysfunction caused by many different factors has also been successfully modelled in zebrafish embryos. Ciliopathies, which often cause dysfunction in the proximal tubule amongst other symptoms, have been successfully modelled in zebrafish. Renal cysts, body axis curvature, hydrocephalus and left-right asymmetry are conserved phenotypes seen in zebrafish ciliopathy models (Kramer-Zucker et al. 2005; Zhao and Malicki 2007). Polycystic kidney disease (PKD) is a genetic disorder in which many cysts form throughout the kidney, ultimately leading to renal failure. Zebrafish models have been used to identify genes that interact with polycystin1 (pkd1), as well as the study of cyst formation in vivo (Sullivan-Brown et al. 2008; Sun et al. 2004). Acute kidney injury (AKI) can be caused by nephrotoxic drugs such as gentamicin and cisplatin, and zebrafish has been established as a valid vertebrate model of such damage (Diep et al. 2011; Hentschel et al. 2005; Zhou et al. 2010; Zhou and Hildebrandt 2012). Proximal tubule protein reabsorption defects such as those in Donnai-Barrow syndrome (megalin mutation) and Lowe syndrome (OCRL1 mutation) have also been modelled in zebrafish (Kur et al. 2011; Oltrabella et al. 2015; Ramirez et al. 2012; Veth et al. 2011).

1.4.3 Assessment of renal function in zebrafish

In humans, clearance of endogenous creatinine from the blood, as well as increased levels of systemic electrolytes and urea are used as a readout of general renal function. Urine tests can also be used to detect increased levels of protein and glucose in the urine. These tests are not easily translated to zebrafish embryos due to the lack of sufficient blood sample sizes, however a number of assays have been established to allow renal function to be studied in zebrafish embryos. The best characterised of these is the use of fluorescent dextran (Christou-Savina et al. 2015; Drummond et al. 1998; Majumdar and Drummond 2000). Injection of a dextran of between 10 and 70 kDa into the zebrafish circulation can then be monitored for the appearance of fluorescent puncta in the renal tubule. This assay can be further extended, by using large fluorescent dextrans (such as 500 kDa dextran) which are too large to filter (Kramer-Zucker et al. 2005). Passage of 500 kDa dextran across the glomerular basement membrane is only possible when the filtration barrier is compromised. More recently, RAP, which binds to megalin in the ER, has been
fluorescently conjugated and used as a readout of renal function in zebrafish (Oltrabella et al. 2015). This assay is similar to the dextran assay in that the conjugated RAP must be injected into the blood stream and the accumulation of puncta in the renal tubule after a period of time can be assessed.

1.4.4 Phenotypic screening in zebrafish

Due to the large numbers of embryos and low cost of maintenance, zebrafish has become a viable phenotypic screening organism. Much like genetic screening is used to identify molecular components of a particular interaction, chemical screens can be used to identify molecular targets that respond to organic chemicals. The overarching principle of phenotypic screening is to apply a chemical library, and look for changes to an established phenotype (Peterson and Fishman 2011). The potential phenotypes to be screened can be hugely diverse, and a number of phenotypic screens using zebrafish have resulted in the identification of novel drug targets (Ablain and Zon 2013; Wang et al. 2015; Zon and Peterson 2005). Screening technology has been developed specifically for zebrafish phenotypic screening assays to aid the data collection and the processing of the volume of data generated (Letamendia et al. 2012; Pulak 2016; Wittbrodt et al. 2014).

1.4.5 Zebrafish as a model of Lowe syndrome

Attempts to make a mouse model of Lowe syndrome have so far been only partially successful (Bothwell et al. 2011; Jänne et al. 1998). The \textit{Ocr1}\textsuperscript{-/-} knockout mouse created has no discernible phenotype (Jänne et al. 1998). This is thought to be due to compensation for the loss of \textit{Ocr1} by \textit{Inpp5b}, as replacement of \textit{Inpp5b} with the human \textit{INPP5B} results in a proteinuria phenotype similar to that of Lowe syndrome and Dent-2 disease (Bothwell et al. 2011). While this recapitulates the renal tubulopathy of Lowe syndrome, no other phenotypes in the eyes or brain consistent with Lowe syndrome are detectable. An alternative to the mouse is to model Lowe syndrome in zebrafish. Zebrafish has one copy of \textit{ocr1}, which is 59\% identical in amino acid sequence to the human protein (Ramirez et al. 2012). Zebrafish have similar patterns of \textit{OCRL1} subcellular localisation, as well as comparable tissue specific expression profiles of the isoforms \textit{OCRL1a} and \textit{OCRL1b} as documented in humans (Ramirez et al. 2012). A zebrafish mutant of \textit{ocr1} has been described, where a retroviral insertion in the promoter for \textit{ocr1} causes a 70\% reduction in the expression of \textit{ocr1} protein (Ramirez et al. 2012), which recapitulates symptoms associated with Lowe Syndrome (Table 1). The authors describe neurological
defects in the ocr1<sup>−/−</sup> embryos such as impaired neural development, cystic brain lesions and an increased propensity to febrile seizures, which recapitulate the symptoms of Lowe syndrome patients (Figure 7A). Further, the substrate of OCRL1, PtdIns(4,5)P<sub>2</sub> is elevated in ocr1<sup>−/−</sup> embryos, consistent with raised PtdIns(4,5)P<sub>2</sub> levels seen in patient derived cell lines (Ramirez et al. 2012; Jones et al. 2013; Wenk et al. 2003). The relative size of the developing brain and eye in ocr1<sup>−/−</sup> mutant embryos is decreased as compared to wild type embryos (Ramirez et al. 2012). More recently, renal defects have been reported in ocr1<sup>−/−</sup> embryos (Oltrabella et al. 2015). Specifically, proximal tubular uptake of the endocytic tracers 10 kDa dextran and Cy3-RAP (a megalin ligand) are defective in ocr1<sup>−/−</sup> mutant embryos (Figure 7B). Also, megalin distribution is altered in the renal tubule of ocr1<sup>−/−</sup> mutant embryos, with a reduced apical localisation and accumulation on sub apical early endosomes (Figure 7C). These data are consistent with the renal endocytosis impairment recorded in ocr1 morpholino studies (Oltrabella et al. 2015; Pietka et al. 2013). The phenotypes in the proximal tubule and the brain of ocr1<sup>−/−</sup> embryos, as well as the high degree of similarity between human and zebrafish ocr1, suggests this model provides an excellent in vivo tool in which we can further characterise the role of ocr1 (Ramirez et al. 2012; Oltrabella et al. 2015).

Table 1: Comparison between the human condition in Lowe Syndrome and the phenotype of ocr1<sup>−/−</sup> hypomorphic zebrafish embryos. All human phenotypic data taken from Loi (2006) and Lowe Syndrome Trust (2010).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Human phenotype</th>
<th>Zebrafish phenotype</th>
<th>Zebrafish References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>Cataracts. Glaucoma in 50% of patients.</td>
<td>Thickening of the lens and a thinning of the retina. Enlarged eyes in adults.</td>
<td>Unpublished observations within the lab.</td>
</tr>
</tbody>
</table>
Zebrafish are highly amenable to genetic manipulation. Alongside large-scale projects to create mutants via ENU mutation, a variety of methods to create targeted mutants of endogenous genes have been described using retroviral insertion,
TALENs, TILLING and CrispR technologies (de Bruijn et al. 2009; Jao et al. 2008; Jao et al. 2013; Huang et al. 2011). It is also possible to create transgenic zebrafish. Initially, injection of linearised plasmid DNA into single cell embryos was used as a way of trying to drive germline integration (Stuart et al. 1988). However, this method of transgenesis often results in <5% germline transmission, and tends to form concatamers at integration sites and therefore risk being silenced in future generations. A much more successful method of genomic integration of transgenic constructs is by the use of transposons. Transgenesis using the Tol2 transposon discovered in Medaka fish drives stable integration into many more embryos (Suster et al. 2009). The ‘tol2 system’ used to introduce transgenes in zebrafish and other organisms consists of two components. Firstly, a plasmid containing the gene of interest is created, with tol2 transposable elements at the 5’ and 3’ end (Urasaki et al. 2006). Importantly, these transposable elements do not contain the transposase coding region, and therefore cannot transpose themselves. Coinjection of mRNA for the transposase region drives the excision of the transgenic gene of interest and the flanking transposon regions from the plasmid, and integrates this into the genomic DNA (Suster et al. 2009). This leads to much higher rates of integration than injection of linearised plasmid DNA alone (Kwan et al. 2007).

The tol2 system has been further improved with the use of Gateway technology (Kwan et al. 2007; Love et al. 2011). Gateway cloning is a modular system of cloning which allows the user to recombine different combinations of plasmids together to create unique expression constructs (Katzen 2007). By flanking your region of interest with att recombination sites modified from the lambda bacteriophage, these regions can be cloned into one of three ‘donor vectors’, which specify the position of the DNA region in the final ‘destination vector’. By creating a destination vector that contains the Tol2 transposon elements flanking the Gateway recombination sites, assembly of complete expression vectors can be performed (Kwan et al. 2007). This has since been developed to also include the human interferon-β scaffold attachment region-chicken β-globin DNase I hypersensitive site 4 (SAR-CH4), which protects integrated transgenes from positional effects (Love et al. 2011; Sekkali et al. 2008; Ramezani et al. 2003).
Chapter 2: Aims
2.1 Aims of the project

The zebrafish model of Lowe syndrome recapitulates neurological, renal and ocular phenotypes associated with Lowe syndrome (see Table 1, page 44) (Oltrabella et al. 2015; Ramirez et al. 2012). Specifically, the renal tubule effects of ocr1\(^{-}\) mutation in zebrafish have now been described, and it is clear that ocr1\(^{-}\) mutants exhibit reduced uptake in the proximal tubule, that is akin to the proteinuria seen in human patients (Oltrabella et al. 2015). The mechanism by which OCRL1 depletion leads to this endocytic defect has been linked to aberrant PtdIns(4,5)P\(_2\) accumulation on endosomes, and modulation of PtdIns(4,5)P\(_2\) to alleviate the phenotype of OCRL1 depletion using genetic tools has been described (Oltrabella et al. 2015; Vicinanza et al. 2011). However, a method of manipulating PtdIns(4,5)P\(_2\) that would be suitable as a clinical treatment for human patients has yet to be established. While PtdIns(4,5)P\(_2\) represents one potential target for clinical use, a high throughput zebrafish screen using chemical libraries represents a more suitable method of searching for novel therapeutic compounds. Currently, no proximal tubular uptake assay exists that is amenable to high throughput study.

Recently, IPIP27A has been identified as an important interaction partner of OCRL1 (Noakes et al. 2011; Oltrabella 2014; Swan et al. 2010). However, its role in vivo is not currently understood, and therefore characterisation of IPIP27A in vivo such as the recently described zebrafish ipip27a\(^{-}\) mutant (Oltrabella 2014) is an important step in understanding the interaction of IPIP27A with OCRL1. While in vivo morphology and uptake studies are important in characterising the interaction of IPIP27A with OCRL1, as well as other OCRL1 interactions, tools to study subcellular morphology in vivo are also required to understand how particular phenotypes, such as the endocytic defect in the proximal tubule, are caused. Fluorescently tagging membrane associated proteins allows visualisation of subcellular compartments, and this can be used in zebrafish to investigate morphological changes to these compartments upon perturbation after mutation of OCRL1, IPIP27A or other endocytic proteins. The specific aims of this thesis are to:

1. Investigate novel therapeutic compounds that target PtdIns(4,5)P\(_2\) in zebrafish to search for compounds that alleviate Lowe syndrome proximal tubular defects

Using the established endocytic phenotype in the ocr1\(^{-}\) zebrafish proximal tubule, the first aim of this project is to investigate whether modulators of PtdIns(4,5)P\(_2\) are able to rescue the defective proximal tubular uptake in zebrafish. The main goals
are to provide further evidence in vivo that the role of ectopic PtdIns(4,5)P$_2$ is important in Lowe syndrome, and to further test the hypothesis that modulation of PtdIns(4,5)P$_2$ is able to alleviate the effects of ocr1$^{-/-}$ mutation in an in vivo setting. Establishing the role of PtdIns(4,5)P$_2$ in vivo is critical to determine whether modulating PtdIns(4,5)P$_2$ could be a potential therapeutic strategy.

The objectives of this are:

- To quantify the renal function of zebrafish embryos to determine the integrity of the currently used qualitative assay accepted by the field.
- Target the PtdIns(4,5)P$_2$ synthesising enzyme PIP5K by genetic and small molecule inhibitor methods to determine if reduction of PIP5K activity can alleviate the renal phenotype in ocr1$^{-/-}$ zebrafish embryos.
- Using an activator of PLC, determine if increased consumption of PthIns(4,5)P$_2$ can alleviate the renal phenotype of ocr1$^{-/-}$ zebrafish.
- ‘Deplete’ available PtdIns(4,5)P$_2$ using a sequestration molecule to prevent interaction with PtdIns(4,5)P$_2$ and determine the effect on renal function in zebrafish embryos.

2. Develop a novel transgenic zebrafish line that reports endocytic function in the proximal tubule for use in high throughput screening of renal function

A second aim of this project is to establish a zebrafish screening assay capable of high throughput determination of proximal tubular function. While a number of methods to study proximal tubular uptake have been described, most require the investigator to inject the tracer into single embryos, which is inappropriate for large scale screening. To address this, transgenic reporter lines where the expressed protein is internalised in the renal tubule by megalin mediated endocytosis will be created. Finally, by utilising the proximal tubular endocytic defect models ocr1$^{-/-}$ and the megalin mutant bugeye, these novel transgenic reporter lines will be characterised to confirm the readout is reliable in preparation for performing high throughput screening.

The objectives for this aim are:

- Assess the ability of the small molecule PT-Yellow (Sander 2015) at correctly reporting renal endocytosis.
- Compare protein based readouts of renal function used in zebrafish, Cy3-RAP and GFP.
Generate secretable fusion proteins combining megalin specificity with sensitive readout such as fluorescence and luminescence, and inject recombinant protein to assess the ability of the fusion protein to give a readout of renal function in zebrafish.

Create transgenic zebrafish lines that stably express these fusion proteins and, using the bugeye megalin mutant line, test the megalin specificity of the uptake of fusion protein in the renal tubule.

3. Further characterise the ocr1 binding partner ipip27A, using a mutant ipip27a<sup>−/−</sup> zebrafish, in respect to the related proximal tubular phenotype
To further investigate the role of the OCRL1 interacting protein IPIP27A, a mutant ipip27a<sup>−/−</sup> zebrafish has been created by TALENs, that demonstrates a similar proximal tubular uptake defect as that in seen in ocr1<sup>−/−</sup> zebrafish (Oltrabella 2014). While Oltrabella (2014) highlights this proximal tubular uptake defect, further characterisation is required to understand the phenotype of ipip27a<sup>−/−</sup> zebrafish. This thesis aims to further explore the renal defects in ipip27a<sup>−/−</sup> zebrafish, to demonstrate that functional ipip27a is required for endocytosis in the zebrafish proximal tubule.

The gross morphology and development of ipip27a<sup>−/−</sup> embryos will be described. Also, to demonstrate that the reporter proximal tubular defect is specific to endocytic trafficking, ipip27a<sup>−/−</sup> embryos will be screened for other potential causes of loss of function of the proximal tubule, such as tubule polarity and glomerular function.

The objectives of this are:

- To investigate if developmental delay seen in <5 dpf embryos results in a more significant delay over the first 10 weeks of development.
- Describe the morphology of the tubule in respect to the polarity of tubular epithelium, and the size of the tubular lumen.
- Confirm the renal endocytic defect described for 10 kDa dextran using a megalin specific ligand.
- Investigate the role of the interaction of Ipip27a with Ocr1 and SH3 containing proteins in renal endocytosis by rescue of the ipip27a<sup>−/−</sup> mutant with ipip27a constructs containing binding site mutations.
- Determine if there is a compound effect of losing functional Ipip27a and Ocr1 by using morpholino to knock down ipip27a or ocr1 in a mutant background of ocr1<sup>−/−</sup> or ipip27a<sup>−/−</sup> respectively.
Perform a semi-quantitative, preliminary analysis of the expression of known interaction proteins and other proteins likely to be affected in both ocr1<sup>−/−</sup> and ipip27a<sup>−/−</sup> embryos using rt-PCR.

4. Develop a toolbox of transgenic zebrafish lines that stably express fluorescently tagged Rab proteins as markers of endocytic compartments

The final aim of this thesis was to create novel transgenic reporter lines that label different endocytic compartments within the zebrafish, which will ultimately allow the dynamics of endocytosis to be studied in vivo. Unique Rab GTPases associate with specific membranes within the cell (Stenmark 2009), and Rab5c, Rab7 and Rab11a are recruited to the early, late and recycling endosomes respectively. Fluorescently tagged Rab5c, Rab7 and Rab11a expressing zebrafish exist already (Clark et al. 2011), but expression in these is lost after the first two days of development, likely to downregulation of the promoter. In this thesis we developed fluorescently tagged Rab5c, 7 and 11a expressing zebrafish for both whole embryo expression and kidney specific expression using a ubiquitous and proximal tubule specific promoter respectively.

The objectives of this section are:

- Generate fluorescently tagged Rab5c, Rab7 and Rab11a and confirm the correct localisation of these proteins in cell culture.
- Generate zebrafish expression constructs containing fluorescent Rab proteins under either renal specific or constitutively expressed promoters.
- Assess the expression of these fluorescent Rab proteins in stable F2 expressing lines.
Chapter 3: Materials and Methods
### 3.1 List of reagents

Table 2: List of general reagents used in this project

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM dNTP mix</td>
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</tr>
<tr>
<td>Agarose</td>
<td>Lonza</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma</td>
</tr>
<tr>
<td>BL21-CodonPlus (DE3) Cells</td>
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<td>Invitrogen</td>
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<tr>
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<td>Sigma</td>
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<tr>
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<tr>
<td>Hyclone Foetal Bovine Serum</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>HyperLadder 100bp/1kbp</td>
<td>Bioline</td>
</tr>
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<td>Kanamycin</td>
<td>Sigma</td>
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<td>Luria-Bertani (LB) broth/agar</td>
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<td>Oligonucleotide primers</td>
<td>Eurofins MWG Biotech</td>
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<td>Methylene blue</td>
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<td>Supplier</td>
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<td>Ambion</td>
</tr>
<tr>
<td>MS222 (Tricaine)</td>
<td>Sigma</td>
</tr>
<tr>
<td>OPTIMEM</td>
<td>Gibco</td>
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<tr>
<td>Paraformaldehyde solution</td>
<td>Sigma</td>
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<tr>
<td>PCR enzyme: GoTaq Green Master Mix</td>
<td>Promega</td>
</tr>
<tr>
<td>PCR enzyme: Q5 HiFi DNA Polymerase</td>
<td>New England Biolabs</td>
</tr>
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<td>Penicillin-Streptomycin solution</td>
<td>Sigma</td>
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<td>Proteinase K</td>
<td>Sigma</td>
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<tr>
<td>QuickChange Lightning Mutagenesis Kit</td>
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<td>Trizol</td>
<td>Invitrogen</td>
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<td>TOP10 Cells</td>
<td>Invitrogen</td>
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<td>Triton X-100</td>
<td>Sigma</td>
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<tr>
<td>Tween-20</td>
<td>Sigma</td>
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3.2 List of antibodies

Table 3: List of primary antibodies for immunofluorescence microscopy

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<th>Target Protein</th>
<th>Species</th>
<th>Dilution</th>
<th>Source and description</th>
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</thead>
<tbody>
<tr>
<td>3G8</td>
<td>mouse</td>
<td>1:30</td>
<td>Development Studies (Hybridoma Bank, USA)</td>
</tr>
<tr>
<td>α-6F</td>
<td>mouse</td>
<td>1:100</td>
<td>Professor Iain Drummond, Harvard University</td>
</tr>
<tr>
<td>Acetylated Tubulin</td>
<td>mouse</td>
<td>1:200</td>
<td>Sigma (#T7451)</td>
</tr>
<tr>
<td>CD63</td>
<td>mouse</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>EEA1</td>
<td>goat</td>
<td>1:100</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Golgin84</td>
<td>sheep</td>
<td>1:1000</td>
<td>Lowe Lab</td>
</tr>
<tr>
<td>GFP</td>
<td>sheep</td>
<td>1:200</td>
<td>Professor Phillip Woodman, University of Manchester</td>
</tr>
<tr>
<td>Megalin</td>
<td>rabbit</td>
<td>1:100</td>
<td>Dr Michele Marino, University of Pisa</td>
</tr>
<tr>
<td>TfR</td>
<td>mouse</td>
<td>1:100</td>
<td>Development Studies (Hybridoma Bank, USA)</td>
</tr>
<tr>
<td>ZO-1</td>
<td>rabbit</td>
<td>1:300</td>
<td>Karl Matter, UCL</td>
</tr>
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</table>

Table 4: List of secondary antibodies for immunofluorescence microscopy

<table>
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<tr>
<th>Target Protein</th>
<th>Dilution</th>
<th>Source and description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa-488 conjugated anti sheep/mouse/rabbit</td>
<td>1:200</td>
<td>Molecular Probes (Cambridge, UK)</td>
</tr>
<tr>
<td>Alexa-594 conjugated anti sheep/mouse/rabbit</td>
<td>1:600</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Alexa-647 conjugated anti sheep/mouse/rabbit</td>
<td>1:1000</td>
<td>Molecular Probes</td>
</tr>
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</table>
3.3 Molecular cloning methods

3.3.1 Genomic DNA extraction
Adult and juvenile fish were anaesthetised in MS222 (0.2 mg/ml, pH 7, Sigma) and a 1 mm² piece of tissue was cut from the corner of the tail. For genomic DNA extraction from embryos, whole embryos were used. The tissue or embryo was placed in 1.5 ml eppendorf tubes and 100 µl 50 mM NaOH was added to each tube. The samples were heated to 95°C for 20 minutes, and then vortexed to ensure mechanical lysis of the sample. Samples were then cooled on ice for 5 minutes, and 10 µl 1M Tris pH 8.0 was added, before vortexing each sample again. Finally, samples were spun down at 11000 g for 1 minute to pellet debris in the tube. The supernatant was transferred to a fresh tube and stored at 4°C for short term use (<2 weeks) or -20°C for long term storage.

3.3.2 RNA Extraction
To extract RNA, embryos were terminally anaesthetized with an overdose of MS222. Pools of 30 embryos were collected in 1.5 ml eppendorf tubes, and spun down at 3000 g to collect at the bottom of the tube. All the supernatant was removed, and 1 ml Trizol (Invitrogen) was added to each tube of pelleted embryos. Embryos were incubated in Trizol overnight at 4°C. Then embryos were homogenized on ice using an IKA Ultra Turrac homogenizer for 15 seconds at 4000 rpm. Samples were then centrifuged at 12000 g for 10 minutes at 4°C to pellet debris, and the supernatant was then transferred to a fresh tube. After 5 minutes at room temperature, 200 µl chloroform was added to each sample, and each tube was vigorously shaken for 15 seconds to ensure the contents were completely mixed. Samples were then incubated for a further 3 minutes at room temperature, before being spun at 12000 g for 15 minutes at 4°C. The aqueous phase containing the RNA (top) was then removed to a fresh tube, where it was mixed with 0.5 ml of isopropanol to precipitate the RNA. After 10 minutes at room temperature, samples were spun at 12000 g for 10 minutes at 4°C, and the supernatant removed. To the pellet, 1 ml 75% ethanol was added, and after a brief vortex each tube was spun again at 8000 g for 15 minutes at 4°C. Finally, the ethanol was removed from the tube and the pellet allowed to air dry under the hood for 10 minutes, before being resuspended in 20 µl of nuclease-free water.
RNA samples were quantified using a spectrophotometer (BioTek Synergy H1) set to read A260 wavelengths, and diluted into a total volume of 100 μl nuclease free water for further purification. RNA purification was performed using the RNeasy mini kit (Qiagen) following the manufacturer’s instructions. An optional DNAse treatment was also performed as suggested in the instructions. Finally, samples were resuspended in 30 μl of nuclease free water, and quantified using a spectrophotometer (BioTek Synergy H1) to measure the A260 and A280. Samples were stored at -80°C until further use.

3.3.3 cDNA synthesis
A total RNA mass of 1 μg was used to generate cDNA using the SuperScript III First Strand kit (Invitrogen). The RNA was added to 1 μl dNTPs, 1μl OLIGOdt, 1μl random primers and nuclease free water to a total volume of 10 μl in a 0.2 ml PCR tube. The mixture was incubated at 65°C for 5 minutes, and then on ice for 1 minute. Next, a mix of 2 μl 10X reaction buffer, 4 μl 25 mM MgCl₂, 2 μl 0.1 mM DTT, 1 μl RNaseOUT (40 U/μl) and 1 μl SuperScript III RT (200 U/μl) was made, and added to each RNA sample. This was incubated at 25°C for 10 minutes, 50°C for a further 50 minutes and finally 85°C for a further 5 minutes, before being cooled on ice. 1 μL RNase H was added to each tube, and tubes were incubated for 20 minutes at 37°C. Samples were then quantified again to measure cDNA concentration at A260, and diluted with nuclease-free water to give a final concentration of 500 ng/μl. Samples were stored at -20°C until further use.

3.3.4 Polymerase chain reaction
PCR was performed either from plasmid templates, genomic DNA or cDNA to amplify specific regions of DNA. Depending on the required use of the amplified DNA, different polymerases were used. For screening PCR, GoTaq Green master mix (Promega) was used. For high fidelity PCR, Q5 Master Mix (New England Biolabs) was used. For each reaction, conditions were adjusted according to the manufacturers instructions. Examples of PCR protocols are given in Table 5. Primers were designed to have a length of 18-22 bp, GC content within 40-60%, and an annealing temperature of close to 60°C. These were designed using DNA Dynamo (Blue Tractor Software). PCR reactions were performed using a thermal cycler (Techna) with a heated lid.
Table 5: Typical PCR conditions

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of cycles</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>95</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>0.5/kb</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>72</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>4</td>
<td>HOLD</td>
</tr>
</tbody>
</table>

3.3.5 DNA and RNA visualisation
Agarose gels of between 0.7% and 2.5% (depending on the size of bands being visualised) were made by boiling agarose (Lonza) in TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8). To each molten gel, 0.003% of 50 mg/ml Ethidium bromide was added for DNA visualisation. Once gels had set, 6x sample buffer (New England Biolabs) was added to each sample and loaded onto the gel. Electrophoresis was performed in TAE buffer at 100V for 40-60 minutes. Gels were visualised on a UV transilluminator.

3.3.6 Restriction enzyme digestion
Digestion of DNA (PCR product or plasmid vector) was done using appropriate restriction enzymes (RE, New England Biolabs). Reactions were prepared as follows:

Table 6: Digest set ups for plasmid and inserts

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 μl</td>
<td>Purified PCR product</td>
<td>10 μl</td>
<td>0.5 mg/ml plasmid</td>
</tr>
<tr>
<td>5 μl</td>
<td>10X CutSmart Buffer</td>
<td>5 μl</td>
<td>10X CutSmart Buffer</td>
</tr>
<tr>
<td>1 μl</td>
<td>RE 1 (40 U/μl)</td>
<td>1 μl</td>
<td>RE 1 (40 U/μl)</td>
</tr>
<tr>
<td>1 μl</td>
<td>RE 2 (40 U/μl)</td>
<td>1 μl</td>
<td>RE 2 (40 U/μl)</td>
</tr>
<tr>
<td>To 50 μl</td>
<td>MilliQ water</td>
<td>To 50 μl</td>
<td>MilliQ water</td>
</tr>
</tbody>
</table>
Reactions were incubated at 37°C (or the appropriate temperature for the enzyme, if specified as different) for 2 hours. Digestion products were separated on agarose gels, and the expected bands were excised from the gel and purified using the Gel Extraction kit (Qiagen) following the manufacturers' instructions. Samples were eluted into 40 μl of EB buffer.

3.3.7 Vector/Insert ligation
Both vector and insert were quantified using 1 μl of sample on a spectrophotometer (BioTek Synergy H1) and then a ligation reaction was set up using the Quick Ligase kit (New England Biolabs). Following the manufacturer's protocol, 50 ng total vector DNA was mixed with a 3 fold molar excess of insert DNA and made up to a 10 μl volume with MilliQ water.

3.3.8 Bacterial transformation
100 μl aliquots of chemically competent *E coli* Top10 cells (Invitrogen) were thawed on ice before 10 μl of ligation mix was added as per the Quick Ligase kit instructions. For plasmid propagation, around 100 ng of plasmid DNA was used per transformation. The cell-DNA mixture was incubated on ice for 30 minutes, before being heat shocked for 30 seconds at 42°C. Aliquots were then cooled on ice for a further 5 minutes. Next 400 μl LB broth was added to each aliquot, and incubated at 37°C with shaking for 1 hour. After this time, to collect the bacteria, samples were centrifuged at 3000 g for 1 minute at room temperature, and 400 μl LB was removed from the eppendorf tube. The bacteria were resuspended in the remaining 100 μl LB broth, and spread evenly on an LB agar plate containing the required antibiotics for selection. Plates were incubated upside down at 37°C overnight.

3.3.9 Culture Media
Bacterial cultures were grown in Luria-Bertani (LB) broth (Melford) or on LB agar (Melford) plates containing the required antibiotics for selection (100 μg/ml ampicillin or 30 μg/ml kanamycin).

3.3.10 Screening for ligation inserts
Colony PCR was performed using single colonies picked from bacterial plates using a sterile pipette tip. Each colony was resuspended in 10 μl sterile MilliQ water. Eight
µl of this was stored at 4°C ready for miniprep (section 3.3.10). The remaining 2 µl was used for colony PCR. The 2 µl of diluted bacteria was added to GoTaq Green Master Mix (Promega) as per the manufacturer’s instructions. One primer to the vector and one to the insert was used in the PCR reaction. PCR reactions were then separated on an agarose gel, and screened for bands specific to the expected size. Dilutions of the corresponding colonies that gave a positive result in this analysis could then be mini-prepped.

3.3.11 Miniprep of plasmid DNA
Eight µl of the diluted bacteria was then transferred to a universal tube containing 5 ml LB broth and the required antibiotic. Inoculated tubes were incubated at 37°C overnight with 220 rpm shaking. The following day, 500 µl bacterial culture was mixed with 500 µl of sterile 50% (w/v) glycerol and stored at -80°C for long term storage. The remaining 4.5 ml was centrifuged at 4500 rpm for 10 minutes at 4°C to pellet bacteria, and the LB broth removed. Plasmid DNA was then extracted from these bacteria using the QIAprep spin miniprep kit (Qiagen), according to the manufacturers instructions. DNA was eluted into 40 µl EB buffer, and 2 µl used to determine the concentration and purity of DNA in the sample using a spectrophotometer (BioTek Synergy H1). Minipreps were stored at -20°C.

3.3.12 Maxiprep of plasmid DNA
Bacteria was grown in a 200 ml LB broth culture overnight, before the QIAfilter plasmid maxiprep kit (Qiagen) was used according to the manufacturers instructions. DNA was eluted into 500 µl TE buffer, quantified using a spectrophotometer (BioTek Synergy H1) and stored at -20°C.

3.3.12 DNA sequencing
Sequencing of the plasmid DNA was carried out by Source Bioscience. DNA samples of 100 ng/µl were provided alongside the sequencing primers (3.2 pmol/µl).

3.3.13 Gateway Cloning
To create expression constructs for zebrafish, Gateway 3 fragment cloning technology was used (Invitrogen) as described previously (Katzen 2007). This modular system brings 3 DNA fragments (a 5’ element, middle element and 3’ element) together in series to create a full expression construct. Firstly the required
sequence for each fragment was amplified with the corresponding att site primers as specified in the Gateway system manual. The synthesised fragments were gel purified and quantified. Fifty fmol of each PCR product was then mixed with 50 fmol of donor vector specific for the required position. The corresponding mix was made up to 8 μl with TE buffer, before 2 μl BP Clonase II was added. These samples were incubated at 25°C in a PCR machine for 2 hours, before being transformed into TOP10 cells to create entry clones. These were then screened by PCR to identify positive colonies, using primers specific to the donor vector and insert respectively. Positive colonies were then maxiprepped and sequenced to confirm the correct sequence, as described above.

Once all 3 fragments were in entry clones, the entry clones were recombined in an LR reaction. Briefly, 10 fmol of each entry clone was mixed with 20 fmol of destination vector. The mix was made up to 8 μl with TE buffer, and 2 μl of LR Clonase II Plus added to each reaction as per the Gateway cloning manual. The LR reactions were incubated at 25°C in a PCR machine for 16 hours, before being transformed into TOP10 cells. A schematic of this process can be seen in Figure 8

### 3.3.14 Cloning for fusion proteins

Coding sequences for fusion proteins for bacterial expression were generated either by conventional cloning (described in sections 3.3.6 – 3.3.10) or were made by custom gene synthesis (Genscript, USA).

### 3.3.15 Plasmid generation

To generate His-tagged proteins we used the pTrcHis system (Invitrogen). This consists of three plasmids, each containing the lac operator upstream of an ATG start site immediately followed by six histidine residues to form an N-terminal His tag. Immediately downstream of this is a multiple cloning site (MCS). Each of the three plasmids contains the same MCS, but in a different reading frame, meaning that any reading frame for the fusion protein can be directly cloned into one of these plasmids and be in frame.

Coding sequences for fusion proteins were cloned into either pTrcHisA, B or C depending on the reading frame using conventional cloning methods. These plasmids were then transformed into BL21-CodonPlus (DE3) bacteria (Agilent Technologies) ready for protein expression.
Figure 8: Summary of gateway cloning technique.

(A) Typical BP reaction. PCR products are first produced containing the three sequences required, flanked by the appropriate att sites as indicated. The BP reaction then combines these with DONR plasmids to create entry clones containing the required sequence flanked by the correct att sites ready for the LR reaction. (B) Typical LR reaction. The three entry clones required are mixed with the destination vector, which contains the Tol2 expression arms, and LR clonase enzyme recombines these into a single expression clone containing all three fragments in order.
3.4 Bacterial protein expression

3.4.3 Optimization of expression

To test for optimal expression and solubility of the protein, a pilot experiment was performed. A single colony of BL21-CodonPlus (DE3) bacteria containing the expression plasmid of interest was used to inoculate a 20 ml sterile LB culture bottle containing the appropriate antibiotic and grown at 37°C overnight with shaking. The next day, 2 ml of this starter culture was used to inoculate a fresh sterile flask containing 50 ml LB containing antibiotic, three flasks in total. All the flasks were grown at 37°C with shaking until they reached an OD$_{600}$ of 0.6. From the starter culture, 0.5 ml of the remaining bacteria was added to 0.5 ml sterile 50% glycerol and frozen to -80°C for storage and so that in future large scale cultures of the same colony could be used. Upon reaching OD$_{600}$ of 0.6, 1 ml samples of each flask were taken and the bacteria were spun down and the supernatant removed. These uninduced samples were stored at -20°C until required (uninduced sample).

Next, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein production. Each of the three pilot cultures were kept at either 37°C or 30°C for three hours, or 18°C overnight. After incubation for the required time, a further 1 ml sample was taken, spun down and stored at -20°C until required (induced sample).

To test for protein solubility, 2 ml of each flask was removed, spun down and resuspended in 200 μl of bugbuster solution (20 mM HEPES pH 7.4, 0.2 M NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 1 mM DTT, 0.1% (v/v) Triton X-100, 1 mg/ml lysozyme, 10 μg/ml DNAse I, 1X Protease inhibitor cocktail). The cell suspensions were incubated at 37°C for 10 minutes, before being snap frozen in liquid nitrogen. This was repeated as a freeze – thaw cycle three times to lyse the bacteria. Next, samples were sonicated using a Bioruptor (Diagenode) for 5 minutes (30 seconds on, 30 seconds off). Lysates were then spun at maximum speed for 20 minutes at 4°C to pellet the insoluble fraction. The soluble fraction was then transferred to a clean Eppendorf, and both fractions were frozen at -20°C until required. Samples were analysed using SDS-PAGE (see chapter 3.8) to check whether the protein of interest was present in the induced and soluble fractions. The conditions that gave the highest yield in the correct fraction were subsequently used for large scale protein production.
3.4.4 Large scale protein purification

To produce enough recombinant protein for purification, a similar protocol was used as in the pilot experiments but on a larger scale. 50 ml LB starter culture containing antibiotic was grown overnight at 37°C, inoculated from the glycerol stock made in section 3.4.3. The next day, 40 ml of this culture was used to inoculate a 1 L LB flask containing antibiotic, and this was grown at 37°C until it reached an OD$_{600}$ of 0.6. IPTG was added to a final concentration of 1 mM, and then the bacteria were grown in the appropriate conditions for production of soluble protein as determined in section 3.4.3. Bacteria were spun down at 5000 rpm for 15 minutes, and resuspended in 30 ml ice cold lysis buffer (20 mM Tris pH 8.0, 0.3 M NaCl, 5 mM β-mercaptoethanol, 50 μg/ml lysozyme, 1X Protease inhibitor cocktail) and incubated on ice for 10 minutes. Next, samples were sonicated (Qsonica LLC, USA) using a tapered tip at 25% amplitude, 10 seconds on/off cycles for 4 minutes on ice. Lysates were spun down at 12000 g for 20 minutes at 4°C to pellet the insoluble fraction. The soluble fraction was transferred to a clean Falcon tube, and 2 ml of washed nickel beads (Ni-NTA HisBind, Novagen) were added prior to incubation at 4°C for 2 hours to allow binding of the His tagged protein to the nickel beads.

Next, the beads were spun down at 1100 g for 2 minutes and the unbound fraction removed. Ten ml wash buffer (lysis buffer containing 20 mM Imidazole) was added to the beads and incubated for 2 minutes, before being spun down at 1100 g. Washing was repeated 3 times, and during the final wash the bead slurry was transferred into a flow-through protein elution column (BioRad). Protein was eluted by sequentially adding 0.5 ml aliquots of elution buffer (lysis buffer containing 200 mM Imidazole) and collecting the eluate each time. Each fraction was assessed for protein content using a BioRad Protein Assay Kit (BioRad) by adding 2 μl of the eluate to a mixture of 8 μl sterile water and 2 μl BioRad Protein Assay solution and elution continued until protein could no longer be detected in the eluate. The fractions containing the most protein were pooled, and buffer exchanged into 1X PBS using a PD-10 Desalting column (GE healthcare). Protein concentration was measured using a spectrophotometer (BioTek Synergy H1), and where appropriate for downstream applications, proteins were concentrated using a 10,000 kDa filtration column (Amicon Ultra, Millipore). Proteins were aliquoted into single use aliquots and frozen at -80°C until required.
3.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.5.1 Gel Preparation

Gels were prepared using the Mini-PROTEAN III system (Bio-Rad). A Bio-Rad mould was used to make gels. First, into each mould a running gel was poured (filling 80% of the mould), followed by a stacking gel (filling 20% of the mould). A well comb of either 10 or 15 wells was inserted into the stacking gel, and the gel was left to polymerise for 1 hour. The buffers and solutions used are listed in Table 7. Recipes for each percentage gel are shown in Table 8.

Samples of protein were boiled at 95°C for 5 minutes and loaded onto the gel (20 μl per well) and run using a power source at 180 v until the loading dye marker reached the bottom of the gel.

Table 7: SDS-PAGE buffer recipes

<table>
<thead>
<tr>
<th>Solution</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running gel buffer</td>
<td>1.5 M Tris-HCl, pH 8.8, 0.4% (w/v) SDS</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>0.5 M Tris-HCl, pH 6.8, 0.4% (w/v) SDS</td>
</tr>
<tr>
<td>Glycerol solution</td>
<td>50% (v/v) glycerol in ddH₂O</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>10% APS (w/v) in ddH₂O</td>
</tr>
<tr>
<td>solution (APS)</td>
<td></td>
</tr>
<tr>
<td>Acrylamide solution</td>
<td>30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, ProtoGel (National Diagnostics, USA)</td>
</tr>
<tr>
<td>TGS Running buffer</td>
<td>1:10 of 10X TGS (Tris/Glycine/SDS) buffer, (Bio-Rad)</td>
</tr>
</tbody>
</table>

Table 8: SDS-PAGE gel recipes

<table>
<thead>
<tr>
<th>Acrylamide concentration</th>
<th>4%</th>
<th>5%</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
<th>20%</th>
<th>Stacking (3%)</th>
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<tbody>
<tr>
<td>H₂O (ml)</td>
<td>5.95</td>
<td>5.64</td>
<td>5.3</td>
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<td>3</td>
<td>1.8</td>
<td>-</td>
<td>3.25</td>
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<td>Gel buffer (ml)</td>
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<td>0.2</td>
<td>0.5</td>
<td>0.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>
### 3.5.2 Coomassie Staining

The gel was removed from the gel apparatus, rinsed once with distilled water to remove any remaining TGS buffer, and being immersed in Coomassie Blue solution (0.1% [w/v] Coomassie Brilliant Blue [Bio-Rad], 50% [v/v] methanol, 10% [v/v] glacial acetic acid) for 20 minutes with gentle agitation. Next, the gel was washed in destaining solution (40% [v/v] methanol, 10% [v/v] glacial acetic acid) for up to 24 hours or until the background blue staining had dissipated. During this time the destaining solution was changed several times. Once destaining was complete, the gels were washed briefly several times in ddH₂O, and then dried using pre-soaked cellophane on a drying frame, and scanned into a computer.

### 3.6 Cell culture

#### 3.6.1 Maintenance of cells

HelaM cells were incubated at 37°C and 5% CO₂ in DMEM (Sigma) containing 10% (v/v) HyClone Fetal Bovine Serum (Thermo Scientific) and 1% (v/v) penicillin-streptomycin solutions (Sigma). HEK293 EBNA (parental) cells were incubated as above with the addition of 350 μg/ml of Geniticin (Sigma). HEK293 EBNA cells stably expressing mini-megalin were grown in culture medium as above, containing 350 μg/ml of active Geniticin and 1 μg/ml puromycin.

#### 3.6.2 Transfection of DNA

Transfection was carried out using FuGENE-HD (Roche). HelaM cells were cultured in 3cm dishes under standard conditions until 80-90% confluence was established. For each transfection, a 100 μl OPTIMEM (Gibco) solution containing 1 μg DNA was prepared. The mixture was incubated at room temperature for 10 minutes, before the addition of 4.5 μl FuGENE HD and then a further incubation at room temperature.
temperature for 20 minutes, before being added dropwise to the cells. Cells were then grown for 24 – 48 hours at 37°C to allow expression of transfected gene.

### 3.7 Immunofluorescence microscopy

#### 3.7.1 Immunofluorescence microscopy of cultured cells

Cells grown on cover slips were fixed in 3% PFA for 20 minutes at room temperature, before being washed three times in PBS for 5 minutes. The second wash included a single drop of 1 M Glycine (pH 8.5) to quench any remaining PFA in the well. Cells were then incubated in 0.1% Triton X-100 in PBS for 5 minutes to permeabilise cells. Cover slips were washed quickly by sequential dipping into 3 beakers of PBS. An antibody solution containing the appropriate primary antibody diluted in PBS containing 0.5 mg/ml BSA was spotted onto parafilm in 50 μl volumes, and each coverslip was incubated face down on a single spot for 20 minutes at room temperature. Cover slips were dipped in beakers of PBS again 3 times, before being incubated in the appropriate dilution of secondary antibody solution (Antibody diluted in PBS containing 0.5 mg/ml BSA and 0.2 mg/ml Hoescht 33342 DNA Dye) for a further 20 minutes. Finally, coverslips were washed 3 times in PBS by dipping in beakers, excess PBS was blotted from the edge of the coverslip, and it was mounted face down on a 5 μl drop of Mowiol 2-88 on a glass slide. Once dried at room temperature overnight, slides were stored at 4°C until imaged. Cells were imaged using an Olympus BX51 wide field fluorescence microscope and MetaVue imaging software. Images were processed using ImageJ.

#### 3.7.2 Wholemount immunofluorescence of zebrafish embryos

For wholemount immunofluorescence, embryos were fixed at the required developmental timepoint in 4% PFA at 4°C overnight. Embryos were then washed 3 times in 100% methanol for 5 minutes, and then sequentially in 3:1, 2:2 and 1:3 parts methanol diluted in PBS containing 0.1% Tween-20 (PBST) for 5 minutes each. Finally, embryos were washed in PBST containing no methanol three times for 5 minutes each. Embryos were treated with Proteinase K (10 μg/ml in PBST) for 2 minutes at room temperature to increase antibody penetration. Embryos were then incubated with primary antibody at the required dilution, diluted in PBST containing 10% foetal calf serum (FCS), at 4°C overnight. Next, the primary antibody was washed out using six sequential 15 minute washes with PBST at room
temperature, and the secondary antibody was added, diluted in PBST containing 10% FCS, and incubated overnight at 4°C. Finally, six sequential 15 minute washes were performed to remove secondary antibody, and samples were then stored at 4°C in PBST containing 0.04% PFA. For imaging, samples were mounted in 2% low melting point agarose (Flowgen Bioscience) and imaged using a Leica SP5 confocal microscope. Images were analysed and processed using ImageJ software.

3.7.3 Immunofluorescence microscopy of zebrafish cryosections
Frozen cryosections (see section 3.8.5) on microscope slides were dried for 1 hour at room temperature under the fume hood. Once dried, samples were immersed in 100% acetone for 1 minute to dehydrate the samples. While dehydrated, slides were marked with a hydrophobic barrier pen (ImmEdge, Vector Laboratories), and left to air dry for 5 minutes. Slides were rehydrated with PBST by immersion for 2 minutes, and then transferred to a humidity chamber at 4°C. Blocking solution (containing 5% FCS diluted in PBST) was added to the marked area on each slide and incubated on the slide for 1 hour. Next, the blocking solution was poured off the slide, and primary antibody diluted in blocking solution was added to each marked area, and incubated at 4°C overnight. The next morning, slides were washed with PBST three times to remove primary antibody, and secondary antibody diluted in blocking solution was added and incubated at 4°C overnight. Slides were washed three times in PBST, and then mounted in Mowiol 2-88. Sections were imaged using a Leica SP5 confocal microscope. Images were analysed and processed using ImageJ software.

3.8 Animal Experiments

3.8.1 Zebrafish strains and husbandry
Zebrafish were raised and maintained at the Biological Services Facility (University of Manchester) under standard conditions as described in the Zebrafish Book (Westerfield 2000) and according to the United Kingdom Animals (Scientific Procedures) Act 1986. Adult fish between 4 and 12 months of age were used for breeding to obtain embryos for further work. Wild type (strain AB Notts) were bred within the University of Manchester. The ocrl<sup>-/-</sup> line was obtained from Znomics inc and maintained in house. All ocrl<sup>1/-</sup> work was performed with this line, which is a hypomorphic line with a 70% reduction in ocrl<sup>1</sup> protein levels. The Bugeye<sup>mut1</sup>
mutant line was obtained from Professor Brian Link and maintained in house and is a full knockout (Kur et al 2011). The ipip27a<sup>−/−</sup> line was made in house by a previous student, and is a full knockout of the ipip27a protein (Oltrabella 2014). After mating, embryos were collected and maintained in clutches of 150 eggs in chorion water (60 μg/ml Instant Ocean Sea Salts [Spectrum Brands], 0.1% Methylene blue [Sigma] in ddH<sub>2</sub>O) and maintained in an incubator at 28°C. After 24-30 hours, any dead embryos were removed, and remaining embryos were moved to PTU medium (60 μg/ml Instant Ocean Sea Salts, 30 μg/ml phenylthiourea [PTU, Sigma] in ddH<sub>2</sub>O) to supress melanocyte formation (Karlsson et al. 2001).

### 3.8.2 Injection into zebrafish embryos

#### 3.8.2.1 Morpholino injection

ATG morpholino oligonucleotides were obtained from GeneTools (LLC, USA) and prepared as a 2 mM stock solution in ddH<sub>2</sub>O, and stored in aliquots at -20°C. To prepare for injection, a fresh aliquot of morpholino was thawed for 10 minutes at 65°C to ensure all morpholino was in solution. The morpholino was then diluted to the required concentration, and an appropriate amount of 50X phenol red (Sigma) solution was added to make a 1X injection solution. Morpholino was injected into the yolk of embryos at the single cell stage using a microinjector (PLI-90 Pico-Injector, Harvard Apparatus). Solutions were prepared fresh on the morning of each injection.

#### 3.8.2.2 DNA injection for transgenesis

Five μl of 80 ng/μl DNA of each transgenesis construct were mixed with 5 μl of 50 ng/μl tol2 transposase mRNA and 1 μl of 10X phenol red. All solutions were prepared freshly immediately before injection. Each single cell stage embryo was oriented to allow access into the cell and 1 nl of this solution was injected directly into the cell using a microinjector.

#### 3.8.2.3 DNA injection for rescue experiments

Two μl of 80 ng/μl DNA of the rescue construct was mixed with 2 μl of 80 ng/μl cmlc2-GFP DNA (GFP heart marker) and 4 μl of 50 ng/μl tol2 transposase mRNA and 1 μl of 10X phenol red. 1 nl of this was injected into the cell of the embryo at the
one cell stage using a micro injector. Each solution was prepared fresh on the day of injection.

3.8.3 Dye filtration experiments
A 2 mg/ml solution of Alexa-488 conjugated 10 kDa dextran (Molecular Probes) was used for dye filtration assays. Anaesthesia was induced in embryos using 0.2 mg/ml MS222 (Tricaine mesylate, Sigma) in chorion water. Embryos were oriented on a 2% agarose mould (2% w/v agarose in chorion water) and injected into the common cardinal vein with the appropriate amount of dye (depending on the specific dye used) using a microinjector. The injected volume was adjusted individually for each dye, based on the fluorescence in the circulation immediately after injection. All embryos were screened immediately after injection to ensure successful delivery to the circulation and to remove any embryos where clots may have formed. Pronephric accumulation of the dye was evaluated between 10 minutes and 2.5 hours after injection, according to the dye used, and the age of the embryo. Embryos were screened and imaged on a Leica MZ10F fluorescent dissecting stereomicroscope and images processed using ImageJ.

3.8.4 Drug treatment of embryos

3.8.4.1 Injection of compounds in embryos
For compounds that required injection into the embryo, the compound was diluted in ddH₂O and made to a 1X solution of phenol red. Embryos were anaesthetised in 0.2 mg/ml MS222 in chorion water, before being oriented on an agarose mould. Specified volumes of the drug were administered to the common cardinal vein at the required dose using a microinjector, and the embryos were transferred to fresh chorion water for recovery.

3.8.4.2 Immersion of embryos in compounds
For compounds that required immersing the embryos in the compound, the following procedure was followed. The required number of embryos were transferred in 2 ml of fresh chorion water to a well in a six well plate. The compound was then prepared to a solution of 2X the required dose in chorion water, and 2 ml of this preparation was added to the required well, to achieve a 1X drug solution. Embryos were maintained in the drug solution for the required time in an incubator at 28°C. Post
injection of fluorescent dye in filtration experiments, embryos were returned to the same drug or vehicle containing medium from which they were removed.

3.8.5 Cryosectioning

Embryos were collected and fixed in 4% PFA overnight at 4°C. Next, embryos were washed in PBS for 10 minutes, before being transferred to a 15% gelatin solution (15% v/v cold water fish gelatin from a 45% stock [Sigma], 15% w/v sucrose [Sigma] made up in PBS) and incubated with rocking at room temperature for 24 hours. At this point, embryos were transferred into an embedding mould and oriented in preparation for sectioning. Once in the correct orientation, moulds were transferred to dry ice for 30 minutes to fix the embryos in position, and then transferred to -80°C for storage. When sectioning, the block was removed from the mould inside a Leica CM3050 S cryotome chamber, and then fixed to a sectioning chuck using OCT (Thermofisher Scientific). 12 μm sections were made through each block, and the resulting sections were mounted onto Super-Frost Plus microscope slides (ThermoScientific) and stored at -80°C until further use.

3.9 Statistical analysis of data

Statistical analysis was performed using GraphPad Prism version 6 (Prism Software Corporation). Categorical data in the study was analysed using a chi-squared test on raw data values. All graphs of categorical data are shown as percentages of the experimental population in each category, using the standard error of the mean (SEM) as a measure of the confidence in the estimated mean as described previously (Oltrabella et al 2015). To account for any type-1 false positive errors, a Bonferroni correction (where the critical P value was divided by the number of comparisons) was used where multiple paired tests were performed on the same data set.

For comparison of two sets of ordinal data, an unpaired T-test was performed using GraphPad Prism 6. All graphs of ordinal data are shown as raw values, overlayed with a mean ± SEM for comparison.

For gene expression data that required multiple comparisons, ANOVA was used using GraphPad Prism 6 to calculate the statistical significance. For comparison within groups, a post hoc Tukeys tests was performed. Graphs of data for gene expression data are percentage change in raw value as compared with the
expression level in the wild type condition. In all data sets throughout this thesis, *= p<0.05, **=p<0.01, ***=p<0.001.
Chapter 4: Identifying novel therapeutic compounds for Lowe syndrome through a targeted approach
4.1 Introduction

In Lowe syndrome, dysfunction of the proximal tubule of the nephron often leads to complete renal failure, which is the main cause of morbidity in patients (Bökenkamp et al. 2009; Loi 2006; Lowe Syndrome Trust 2010). Specifically, Lowe syndrome renal symptoms are defined by low molecular weight (LMW) proteinuria, however there is also a selective defect in the reabsorption of amino acids, calcium, phosphates, sodium and potassium (Bockenhauer et al. 2008). It is not yet understood how this selective reabsorption defect leads to end stage renal failure in patients.

It has been hypothesised that the LMW proteinuria can be accounted for by a defect in the endocytic uptake and recycling of the multi ligand receptor megalin. Experimental support for this hypothesis has been obtained in both tissue culture of HK2 cells and crucially in the renal tubule of our zebrafish model of Lowe syndrome (Vicinanza et al. 2011; Oltrabella et al. 2015). In both studies, lack of functional OCRL1 resulted in a redistribution of megalin from the plasma membrane to endocytic puncta, and an overall lower abundance of megalin, consistent with a mis-sorting defect.

One established method for assessing the endocytic function of the pronephric epithelium in zebrafish embryos is the injection of 10 kDa fluorescent dextran into the cardinal vein, which is then filtered and reabsorbed by cells lining the proximal tubule (Cianciolo Cosentino et al. 2010; Drummond et al. 1998). Consistent with the reduction and redistribution of megalin in the renal tubule of our ocrl1−/− zebrafish, there is also a marked reduction in the accumulation of fluorescent dextran in the renal tubular cells (Oltrabella et al. 2015). Furthermore, in ocrl1−/− embryos, the endosomes of the proximal tubule are fewer in number, and larger (Oltrabella et al. 2015). Interestingly, the defect in uptake of 10 kDa dextran phenocopies the loss of megalin described previously in both zebrafish and mouse models (Anzenberger et al. 2006; Kur et al. 2011; Leheste et al. 1999). Also, both the phosphatase activity and interaction with other trafficking related proteins are required for OCRL1 to function in renal endocytosis, as re-expression of mutants lacking these functions was unable to rescue the phenotype (Oltrabella et al. 2015).

It has been previously postulated that increased levels of the OCRL1 substrate PtdIns(4,5)P₂ may be responsible for some of the clinical manifestations of Lowe syndrome (Suchy and Nussbaum 2002). In HK2 cells, it has been shown that enlarged endosomes seen in OCRL1 depleted cells are enriched for both
PtdIns(4,5)P$_2$, as well as actin (Vicinanza et al. 2011). Downstream PtdIns(4,5)P$_2$ effectors such as N-WASP and Cofilin are important modulators of the actin cytoskeleton, therefore implicating OCRL1 as an indirect regulator of cytoskeletal remodelling (Suchy and Nussbaum 2002; Vicinanza et al. 2011; Gorbatyuk et al. 2006). In zebrafish, global PtdIns(4,5)P$_2$ levels have previously been shown to be increased in ocr1$^{-/-}$ zebrafish (Ramirez et al. 2012; Jones et al. 2013). Transferrin receptor is usually trafficked between the plasma membrane and the early endosome, however in OCRL1 depleted cells it is retained in the early endosome following internalisation, likely due to the aberrant accumulation of PtdIns(4,5)P$_2$ at this compartment (Vicinanza et al 2011). Vicinanza et al (2011) demonstrated that inhibition of the production of PtdIns(4,5)P$_2$ by knock down of PtdIns4P 5-kinase alpha (PIP5K$\alpha$) rescued endocytic recycling of transferrin receptor. Knock down of the zebrafish ortholog of PIP5K$\alpha$, known as PIP5K$\alpha$b, in ocr1$^{-/-}$ embryos at 3 dpf demonstrates a significant restoration of the uptake of fluorescent reporter, as well as rescued megalin distribution and endosome morphology (Oltrabellla et al 2015). The rescue of these phenotypes is consistent with the hypothesis that inhibition of PtdIns(4,5)P$_2$ production by PIP5K leads to a reduction in the global PtdIns(4,5)P$_2$ levels.

The striking rescue of endocytic uptake in ocr1$^{-/-}$ embryos after the knockdown of PIP5K$\alpha$b demonstrates that manipulating PtdIns(4,5)P$_2$ levels in in the renal tubule may be of therapeutic benefit. Therefore, in this chapter, I investigate various ways in which PtdIns(4,5)P$_2$ levels or availability can be targeted in vivo in the zebrafish model system, and describe the effects each has on the ability of the ocr1$^{-/-}$ mutant to accumulate 10 kDa dextran in the renal epithelium.

4.2 Loss of OCRL1 delays and reduces accumulation of fluorescently tagged 10 kDa dextran in 3, 4 and 5 day old embryos.

Previous work has demonstrated impaired uptake of 10 kDa dextran in ocr1$^{-/-}$ embryos at 3 dpf (Oltrabellla et al. 2015). The onset of glomerular filtration in zebrafish embryos is known to be around 40 hpf, with a fully functioning pronephric tubule by around 60 hpf (Drummond et al. 1998). For this reason, 10 kDa fluorescent dextran uptake assays have usually been performed at 3 dpf, as the earliest time point at which the assay can be performed. In ocr1$^{-/-}$ embryos, the uptake of 10 kDa dextran has been assessed by looking at a single time point, two
hours post injection (Oltrabella et al. 2015). Initially, this series of experiments sets out to assess the accumulation of 10 kDa dextran at regular intervals over the first two hours post injection in 3 dpf, 4 dpf and 5 dpf wild type and ocrl1\(^{-/-}\) embryos to test the functionality of the renal tubular endocytic machinery in larvae at different ages. Additionally, this assay was used to determine the most appropriate time point at which to look at embryos in future experiments.

FITC-conjugated 10 kDa fluorescent dextran was injected into the common cardinal vein of wild type and ocrl1\(^{-/-}\) zebrafish embryos of different ages (Figure 9A). Embryos were screened immediately on a stereo fluorescence microscope to confirm successful delivery of the fluorescent dextran to the circulation, and any mis-injected embryos removed. Post injection, embryos were then monitored visually every 15 minutes for accumulation of the 10 kDa dextran in the pronephric epithelium using fluorescence microscopy (Figure 9B, C). As described previously (Oltrabella et al. 2015), and assessed further in section 4.3, at each time point embryos were categorised as showing no uptake, low uptake or high uptake in the pronephros (Figure 9D).

In 3 dpf wild type embryos there are very few embryos with uptake in the first 60 minutes, with a steady increase in those showing high uptake from 60-120 minutes post injection (Figure 10A, E). In contrast, uptake in 4 dpf wild type embryos is much faster, with a lag period of 15 minutes, and the number of embryos showing high uptake reaching a maximum at around 90 minutes (Figure 10B, F). At 5 dpf, the accumulation of fluorescent dextran in wild type pronephric tubules is even faster, showing no lag period and reaching a maximum number of embryos showing high uptake at just 45 minutes (Figure 10C, G). Using these time course data, the earliest time point in each case that the majority of wild type embryos show high uptake was identified. For 3 dpf, 4 dpf and 5 dpf this is 120 minutes, 60 minutes and 15 minutes respectively.

At each time point where some wild type embryos showed high uptake, the number of ocrl1\(^{-/-}\) embryos showing high accumulation of 10 kDa dextran was consistently and significantly lower for embryos tested at 3, 4 and 5 dpf (Figure 10A-C, H-J). Interestingly, while there is a significant impairment in the accumulation of 10 kDa dextran in ocrl1\(^{-/-}\) embryos, it is not a complete abolition of the uptake process. In all cases, there is an increasing trend over the two-hour time course that mirrors the wild type uptake.

It is also apparent in the representative images of wild type that the gross morphology of the renal tubule changes between 3 dpf and 5 dpf (Figure 10D, E, F,
At 3 dpf, the renal tubule is relatively planar, with only a small loop at the proximal end of the tubule. At 4 dpf and 5 dpf, this loop is further extended down the side of the zebrafish body, making it easier to visualise in the sagittal plane. As the aim is to screen embryos for renal fluorescence, 4 dpf embryos represent the best choice for a number of reasons. The interval between injection and observation of 60 minutes is sufficient to mount the batch of embryos in agarose for imaging, while...
at 3 dpf (2 hours) and 5 dpf (15 minutes) this is either excessive or too short. Secondly, the tubule is more obvious in sagittal section at 4 dpf than at 3 dpf. Finally, where longer treatments of drugs may be required such as overnight,
avoiding treatment with drugs at earlier time points like 2 dpf while the tubule is still forming may reduce off target effects on pronephric development.

4.3 Quantification of 10 kDa dextran uptake is consistent with categorised data.

Thus far, the function of the renal tubule in zebrafish embryos has been assayed by injecting 10 kDa fluorescent dextran, and then visually categorising the accumulation of the dye in the renal tubule as either no, low or high after a specified period of time. While in section 4.2 the best time point post injection to look at the uptake for different aged embryos was determined, it is also important to quantify the accumulation of 10 kDa dextran to validate this method of scoring by comparing with measurements of fluorescence intensity, determined using a linescan analysis (Waters 2009).

To assess this, 10 kDa fluorescent dextran was injected into 4 dpf wild type, ocrl1−/− and bugeye embryos. Bugeye embryos are a megalin mutant, and are devoid of any endocytic uptake in the renal tubule (Veth et al. 2011; Kur et al. 2011). To generate images for line scan analysis, the renal tubule was imaged at 80x magnification (Figure 11A). Each embryo was also categorised as either no, low or high uptake as described previously in preparation for later comparison (Figure 11C). Next, images were loaded into ImageJ and the background was subtracted from each image using a rolling pixel average to remove as much non-specific background as possible. A 150 pixel line scan profile of fluorescence intensity was taken, with the tubule centered in the middle of the selection (Figure 11B). This selection allowed us to measure a baseline from tissue either side of the tubule. As dextran accumulates in puncta within the tubule and therefore a single pixel width line selection may pass through varying numbers of puncta, the line scan was set to a width of 30 pixels to remove this source of variation. As a readout of total fluorescence, area under the curve (AUC) was calculated. Finally, this data was accumulated for each genotype and plotted for comparison.

Bugeye embryos that are devoid of renal endocytosis had a significant reduction in AUC of the renal fluorescence as compared to wild type embryos (around 28% of wild type Figure 11D). Ocrl1−/− embryos also showed a significant reduction in renal accumulation as determined by AUC analysis (around 60% of wild type). Finally, the AUC of fluorescence intensity of all embryos was normalised by dividing the value with the average wild type value, giving the average wild type a
Figure 11: Analysis of line plot data to compare accumulation in embryos.

(A) Representative image of a typical wildtype embryo showing accumulation of 10 kDa dextran in the renal tubule. Red box represents selection used to create line plot. Scale bar is 100 µm. (B) Representative line plots for wild type, ocr1<sup>−/−</sup> and bugeye embryos at 4 dpf. Plot is a vertical profile of 300 pixels centered on the tubule. (C) Representative images of the tubules of all three conditions. Red dotted line represents the perimeter of the tubule. Scale bar is 50 µm. (D) Summary data showing the average area under curve (AUC) in each condition. Data are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001. Significance calculated using a t test comparing each condition to the wild type condition. All images have been converted to grey scale and inverted for clarity.

proportion of 1. These values were converted to percentages of the average
wildtype reading, and then categorised as either >75% of the average wildtype, 25-75% of the average wildtype, or less than 25% of the average wildtype, to mimic the categorisation of data as no, low and high. We found that the results for wildtype embryos were 100% identical when analysed using either method (Figure 12A). In \textit{ocrl}^{+/−} embryos, there was a similar phenotype recorded by both methods, however there was a slight shift in the distribution towards higher uptake when assessed by fluorescence intensity (Figure 12B). Finally, with bugeye embryos, where there is a complete defect in renal endocytosis, the fluorescence AUC analysis overestimated the number of low and high embryos (Figure 12C).
4.4 Knockdown of PIP5K in *Ocril1*−/− at 4 dpf rescues the endocytic defect in the renal tubule.

As shown in Oltrabella et al. (2015), knockdown of PIP5Kαb in an *Ocril1*−/− background at 3 dpf using morpholino resulted in a significant rescue of 10 kDa dextran accumulation. To manipulate PtdIns(4,5)P2 levels in 4 dpf embryos, this PIP5Kαb knockdown experiment was repeated in 4 dpf embryos to demonstrate that the rescue of 10 kDa dextran still occurs after PIP5Kαb knockdown.

Wildtype and *Ocril1*−/− embryos were injected into the yolk at the single cell stage with either PIP5Kαb or control morpholino as previously described (Oltrabella et al. 2015). At 4 dpf, embryos were injected with 10 kDa dextran into the common cardinal vein. After 60 minutes, embryos were screened again and categorised into high, low or no uptake. Following this, embryos were terminally anaesthetised, and mRNA was extracted to make cDNA (Chapter 3.3.2 and 3.3.3). RT-PCR was performed to confirm knockdown of the PIP5Kαb in fish injected with the morpholino.

RT-PCR results show that the PIP5Kαb morpholino successfully reduced mRNA levels to 20-25% of wildtype levels in those embryos injected with the PIP5Kαb morpholino, but not in those injected with the control morpholino in one individual experiment (Figure 13A). Injection of the control morpholino had no effect on the ability of either genotype to accumulate dextran (Figure 13B,C). In wild type embryos injected with the PIP5Kαb morpholino, a substantial reduction in the ability of embryos to uptake 10 kDa dextran was evident, with a reduction in the number of embryos showing high uptake at 60 minutes from ~75% to <20%. The percentage of embryos showing low uptake did not significantly change after injection with the PIP5Kαb morpholino, while those showing no uptake significantly increased to similar levels as the *Ocril1*−/− mutants (~60%). This suggests that lowering PtdIns(4,5)P2 levels from wildtype levels causes a severe defect in endocytic uptake, which demonstrates that PtdIns(4,5)P2 is required for endocytosis in the renal tubule. In contrast, *Ocril1*−/− embryos that had been injected with the PIP5Kαb morpholino showed a significant rescue of the phenotype. Numbers of embryos showing high uptake were around 40%, compared to around 15% in the control morpholino injected *Ocril1*−/− embryos. Similarly, the number of *Ocril1*−/− with no uptake after injection with the PIP5Kαb dropped from ~60% to ~25%. In agreement with Oltrabella et al. (2015), these results indicate that PtdIns(4,5)P2 is required for
endocytosis in the renal tubule, and that inhibiting the production of PtdIns(4,5)P$_2$ in the ocr1$^{-/-}$ zebrafish line can restore renal endocytic uptake.

Figure 13: See next page for legend
Figure 13: PIP5K MO rescues the defects in endocytic uptake of 10 kDa dextran in the renal tubule of ocr1<sup>−/−</sup> embryos.

(A) RT-PCR analysis of wild type and ocr1<sup>−/−</sup> embryos at 4dpf from one individual experiment containing pooled sets of 30 embryos. Inset, graph of relative PIP5K mRNA levels measured from the RT-PCR gels adjusted to mRNA levels for eif1α. (B) Graph showing the distribution of no, low and high uptake of 10 kDa dextran in wild type and ocr1<sup>−/−</sup> embryos with or without PIP5K MO. N numbers are total embryos over three independent experiments. Data are mean ± SEM. *** p<0.001. (C) Representative images of renal tubule accumulation of 10 kDa dextran in wild type and ocr1<sup>−/−</sup> embryos injected with either control (Ctrl) or PIP5K MO. Images have been inverted to grey scale for clarity.

4.5 Inhibition of PIP5K using small molecules rescues the endocytic defect in the renal tubule.

Another way to target the synthesis of PtdIns(4,5)P<sub>2</sub> by PIP5Ks is via small molecule inhibition, but until recently no specific PIP5K pharmacological inhibitors have been characterised (Bout and Divecha 2009). To address this, Cancer Research Technologies (CRT, UK) have developed five novel inhibitors specific for PIP5Kα, and performed in vitro inhibition assays to determine their affinity to human PIP5Kα and other phosphoinositide kinases. The IC50 data on these drugs can be found in Table 9.

Table 9: Novel PIP5K from CRT. Table shows IC50 values for CRT compounds for several phosphoinositide kinases for each compound (taken from personal communication with CRT, NT= not tested). Final column in blue shows the concentration of each compound used for ocr1<sup>−/−</sup> zebrafish rescue experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PIP5Kα IC50 (µM)</th>
<th>PIP5Kβ IC50 (µM)</th>
<th>PIP5Kγ IC50 (µM)</th>
<th>PI4KCA IC50 (µM)</th>
<th>PI4KCB IC50 (µM)</th>
<th>PI3Kα IC50 (µM)</th>
<th>Zebrafish test concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT0274630</td>
<td>0.011</td>
<td>0.004</td>
<td>0.001</td>
<td>20.9</td>
<td>5.99</td>
<td>34.6</td>
<td>0.11</td>
</tr>
<tr>
<td>CRT0364349</td>
<td>0.017</td>
<td>0.003</td>
<td>0.001</td>
<td>14.0</td>
<td>4.34</td>
<td>&gt;30</td>
<td>0.17</td>
</tr>
<tr>
<td>CRT0391964</td>
<td>0.0005</td>
<td>0.002</td>
<td>0.001</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>NT</td>
<td>0.005</td>
</tr>
<tr>
<td>CRT0276787</td>
<td>0.100</td>
<td>0.014</td>
<td>0.010</td>
<td>12.8</td>
<td>5.25</td>
<td>&gt;30</td>
<td>1</td>
</tr>
<tr>
<td>CRT0103579</td>
<td>1.54</td>
<td>0.060</td>
<td>0.020</td>
<td>8.81</td>
<td>7.91</td>
<td>5.75</td>
<td>15.4</td>
</tr>
</tbody>
</table>

To determine if any of these inhibitors have an effect on the renal phenotype of Ocr1<sup>−/−</sup> zebrafish, each of the 5 compounds was added to the water containing 4
dpf zebrafish embryos. As these compounds have not been used in zebrafish previously, there is no standardised dose or timescale to use. The inhibitor must penetrate from the water into the zebrafish and reach an effective dose within the renal tubule. To attempt to achieve this effective dose in the renal tubule, we chose to treat with a concentration equivalent to 10 times the IC50 for each compound overnight. Furthermore, the previous experiment in 4 dpf embryos demonstrated that suppression of expression of PIP5Kαb in the ocr11-/- mutant over 4 days was able to restore the phenotype. As PIP5K is required for the production of PtdIns(4,5)P2, it is likely that the effect of inhibiting PIP5K will not immediately lead to the reduction in PtdIns(4,5)P2 on endosomes that is thought to rescue the ocr11-/- phenotype (Vicinanza et al. 2011; Oltrabella et al. 2015). Therefore, an overnight treatment was most appropriate to allow time for the PIP5K inhibition to occur.

To prepare an accurate 10 times IC50 dose of each drug, each compound was first diluted from the stock to 10,000 times the IC50 in DMSO. Then, each drug was further diluted 1 in 1000 with chorion water to a final 0.1% DMSO concentration, that contained the correct concentration of drug as specified in the final column of Table 9. As this is a chronic treatment, 30 mg/L PTU was also added to the chorion water to continue inhibition of melanocyte formation for the duration of the experiment (Milos and Dingle 1978). A vehicle control treatment was made containing 0.1% DMSO. Embryos at 3 dpf were transferred into the treatment and incubated for 16 hours overnight before being injected with 10 kDa dextran. After one hour, embryos were screened for accumulation of the fluorescent dextran in the renal tubule and categorised into no, low or high uptake.

In the Ocr11-/- background, CRT0391964, CRT0276787 and CRT0103579 caused a partial rescue of the Ocr11-/- renal phenotype (Figure 14B). CRT0391964 had the strongest rescue with an increase in embryos that showed any uptake (either high or low) from ~25% to ~55%. After treatment with CRT0391964, the number of embryos showing high uptake increased from <5% to ~25%. Neither CRT0274630 or CRT0364349 were able to rescue the 10 kDa dextran accumulation defect at the trial dose. None of the treatments tested had any significant effect on renal accumulation of 10 kDa dextran in wild type embryos at the concentrations assayed (Figure 14A). As the compound CRT0391964 caused the most significant rescue at the trial dose, next a dose response curve was set up to characterise the rescue of 10 kDa dextran further.
Figure 14: See next page for legend
4.6 Endocytic function rescue in ocr1<sup>+/−</sup> zebrafish by PIP5K inhibition with CRT0391964 is dose dependent.

Embryos were incubated in solution containing varying concentrations of CRT0391964 from 0-40 nM (0-80 times the IC50) to look at the dose response of Ocr1<sup>+/−</sup> embryos to this particular inhibitor. Embryos were incubated overnight and then a dextran uptake assay was performed.

In Ocr1<sup>+/−</sup> fish, a 2.5 nM dose was insufficient to cause a significant rescue, however a 5 nM, 10 nM and 20 nM all gave significant rescue of the phenotype compared with the vehicle control (Figure 15A). In 5 nM, 10 nM and 20 nM doses, the rescue resulted in around 40% of embryos showing no uptake, a reduction from the 70% seen in control treated embryos. The largest effect was seen with a dose of 5 nM, where the number of embryos showing high uptake increased from <10% to around 35% (Figure 15A, B). In 10 nM and 20 nM doses, while the rescue was significant, the majority of rescued embryos showed low uptake. Embryos which received the highest given dose of 40 nM were not significantly different from the Ocr1<sup>+/−</sup> embryos that received the vehicle control, suggesting at this dose the PIP5Kαb inhibition is either causing a reduction in PtdIns(4,5)P<sub>2</sub> levels further than physiologically relevant, or is having off target effects. Surprisingly, none of the doses of inhibitor had a significant effect on the ability of wild type embryos to accumulate 10 kDa dextran in the renal tubule (Figure 16A, B). Based on the PIP5Kαb morpholino results, it was expected that targeting PIP5Kαb with a small molecule inhibitor would have had a negative effect on the ability of wild type embryos to accumulate dextran. These results indicate that the compound CRT0391964 can rescue the ocr1<sup>+/−</sup> mutant phenotype. However, as PtdIns(4,5)P<sub>2</sub> levels were not measured and the wild type embryos were unaffected, we cannot exclude effects on an unrelated pathway resulting in the rescue of these embryos.
The PLC activator m-3m3fbs is able to partially rescue the endocytic defect in ocr11^-/- embryos.

Another potential way to target accumulated PtdIns(4,5)P2 in ocr11^-/- embryos is to activate phospho-lipase C (PLC). PLC is activated when a hormone binds to a...
receptor on the extracellular surface of the plasma membrane, activating a G-protein which in turn activates PLC (Putney and Tomita 2012; Nishizuka 1995). PLC then cleaves the head group of PtdIns(4,5)P$_2$ to create soluble IP$_3$ and membrane bound DAG, important second messengers involved in the release of calcium from the endoplasmic reticulum (ER) and the phosphorylation of proteins by protein

Figure 16: 10 kDa dextran uptake in wild type embryos treated with the PIP5K inhibitor CRT0391964.

(A) Graph showing the distribution of 4 dpf embryos showing no, low and high uptake in wild type embryos treated with CRT0391964 at the specified doses. Data are mean ± SEM. * p<0.05, *** p<0.001. n numbers are total embryos over 3 repeats. (B) Representative images of wild type 4 dpf embryos treated with either vehicle control or 5 nM CRT0391964. Images have been inverted to grey scale for clarity.
kinase C respectively (Hughes and Putney 1988). Therefore, as PtdIns(4,5)P_2 is the substrate for PLC, activating this pathway is an attractive method of regulating PtdIns(4,5)P_2 levels. PLC can be activated artificially by incubation with the drug m-3m3fbs, but not with the inactive analog, o-3m3fbs (Bae et al. 2003).

The *Drosophila* homolog of OCRL1, dOCRL has been previously described (Ben El Kadhi et al. 2012; Ben El Kadhi et al. 2011). By depleting dOCRL in *Drosophila* S2 cells, PtdIns(4,5)P_2 is mislocalised to abnormally large vacuolar endosomes, similar to the findings in mammalian cell culture of Vicinanza et al (2011). Further, these papers show that turnover of PtdIns(4,5)P_2 at the cleavage furrow of *Drosophila* S2 cells is required for successful cytokinesis, and when dOCRL is depleted, proteins normally localised to the cleavage furrow are mislocalised to the enlarged vacuolar endosomes. Current work in the Carreno lab has found that by applying the PLC agonist small molecule, m-3m3fbs, to *Drosophila* S2 cells depleted for dOCRL, both the accumulation of PtdIns(4,5)P_2 on enlarged endosomes and the cytokinesis failures are both alleviated (personal communication). The effect is not seen when one adds the inactive analog of the compound, o-3m3fbs.

To determine if the PLC agonist m-3m3fbs can rescue the endocytic defect seen in the pronephros of *ocr1*^-/-^ embryos, 4 dpf embryos were incubated in water containing either 5 µM m-3m3fbs, 5 µM o-3m3fbs or vehicle control. A 5 µM treatment was chosen as this is the same used in experiments by Sebastien Carreno (personal communication). A relatively short one-hour incubation was chosen to minimise the possibility of effects on other pathways, or the long term raised calcium PLC activation would induce on the whole embryo. Embryos were then injected with 10 kDa fluorescent dextran and incubated for 60 minutes. Embryos were screened and categorised into either high, low or no uptake.

Neither the active or inactive analog had any effect on the renal accumulation of 10 kDa dextran in wild type embryos (Figure 17). However, in *ocr1*^-/-^ embryos, the m-3m3fbs activator partially rescued the phenotype, while the inactive analog failed to rescue.
Figure 17: Pronephric uptake of tracer dye in zebrafish embryos following treatment with PLC activator.

(A) Graph shows quantification of pronephric accumulation in each of the indicated treatments of 4 dpf embryos. Data presented are mean ± SEM. *** p<0.001, * p<0.05. (B) Representative images of 10 kDa dextran accumulation in wild type and ocr1/-/ embryos treated for one hour with either DMSO control, inactive o-3m3fbs or active m-3m3fbs. Red dashed line represents tubules. Images have been converted to grayscale and inverted for clarity.
4.8 Sequestration of PtdIns(4,5)P$_2$ using the small molecule PHDM is able to partially rescue the renal phenotype in ocr1/−/− embryos

Another potential way of reducing the available PtdIns(4,5)P$_2$ in the renal tubule is to use low levels of a compound that would sequester the head group of PtdIns(4,5)P$_2$ to prevent the lipid interacting with downstream effector proteins. A synthetic compound called pleckstrin homology domain mimetic (PHDM), designed to specifically bind to PtdIns(4,5)P$_2$ is capable of binding to PtdIns(4,5)P$_2$ both in vitro and in cultured cells (Mak et al. 2011). The addition of 50 µM PHDM to fibroblasts results in a near total loss of stress fibres and also impairs transferrin endocytosis, consistent with PHDM blocking the binding of PtdIns(4,5)P$_2$ to effectors responsible for the control of actin dynamics and endocytosis respectively (Mak et al. 2011). As the work of Mak et al. has demonstrated that PHDM can successfully manipulate these pathways by binding to PtdIns(4,5)P$_2$, it is important to determine if PHDM is able to rescue the renal tubular endocytic defect in ocr1/−/− zebrafish embryos.

Because of the poor solubility of PHDM in water, attempts to treat zebrafish embryos by soaking in PHDM containing water failed (data not shown). To reach a dose of 50 µM as used in Mak et al. (2011) would give a solution of 1% DMSO. While previous work has shown that zebrafish can tolerate up to 2% DMSO without detectable abnormalities (Maes et al. 2012), our own work suggests that incubation in water with a concentration of DMSO above 0.5% reduces renal accumulation of 10 kDa dextran (data not shown). As we wanted to assess a range of doses both higher and lower than this 50 µM dose to establish a dose response curve, we therefore chose to deliver PHDM via intravenous injection, limiting the exposure to DMSO. Either wild type or Ocr1/− embryos were injected with 5 nl of PHDM at the required concentration in the common cardinal vein.

As the injection gave a finite dose of PHDM, instead of the drug being readily available in the water, incubation overnight as performed with the PIP5Kα inhibitors is not appropriate. Mak et al. (2011) used a treatment time of 20 minutes to treat cells in culture. After injection in the cardinal vein, PHDM must be absorbed into the renal tubule to reach the target cells, therefore an intermediate incubation of three hours was chosen to allow PHDM sufficient time to access the tubule. After three hours, embryos were injected with 10 kDa fluorescent dextran. Sixty minutes post injection, embryos were screened for uptake and the accumulation was categorised
as high, low or no uptake. For ease of comparison, data was analysed in two groups; those that showed only uptake categorised as high, and those that showed any uptake (high and low uptake combined).

In wild type embryos, at each increasing dose of PHDM there was a proportional decrease in the number of embryos showing high 10 kDa dextran (Figure 18B). At the highest tested dose, only 10% of embryos exhibited high uptake, whereas it was around 70% for the vehicle control embryos. However, the total number of wild type embryos showing any uptake at doses ≤ 40 µM PHDM was not different from the vehicle control, suggesting that at these lower doses the ability for the majority of wild type embryos to uptake 10 kDa dextran was reduced but not completely lost (Figure 18A). At higher doses, 60-100 µM PHDM, significantly less wild type embryos were able to take up any 10 kDa dextran. This result is consistent with the results of Mak et al. (2011) when looking at the graded response of stress fibres in wild type fibroblasts to the concentration of PHDM.

In contrast, ocr1/- embryos injected with 40 µM, 60 µM or 80 µM PHDM showed a significant increase in the number of embryos showing any uptake (Figure 18A). A corresponding significant increase in the number of embryos showing high uptake was also seen at these doses (Figure 18B). At 100 µM, there was still a significant increase in embryos showing uptake but the number of fish showing high uptake has returned to be comparable to ocr1/- embryos treated with vehicle control. In this dose response assay, a dose of 60 µM gave the best rescue, increasing the number of ocr1/- embryos that show any uptake from 30% to around 65% (Figure 18C,D). Correspondingly, the number of embryos showing high uptake increased from around 10% to 35%.

4.9 Discussion

In both Lowe syndrome and Dent-2 Disease, endocytic trafficking defects have been proposed to be the primary cause of proximal tubule dysfunction (Norden et al. 2002; Vicinanza et al. 2011; Mehta et al. 2014; Oltrabella et al. 2015). PtdIns(4,5)P$_2$ levels are elevated in cells depleted of OCRL1, and also in our ocr1/- zebrafish model (Vicinanza et al. 2011; Ramirez et al. 2012). The OCRL1 depleted cells also have an enrichment of PtdIns(4,5)P$_2$ on the endosomal membrane, indicating ectopic PtdIns(4,5)P$_2$ accumulation on these membranes (Vicinanza et al. 2011). The mis-localisation of PtdIns(4,5)P$_2$ to the incorrect membrane could lead to the defects in endocytic recycling seen in OCRL1 depleted cells (Vicinanza et al. 2011),
and therefore be responsible for the reduction in megalin mediated endocytosis. Therefore in this chapter we sought to investigate whether targeting of PtdIns(4,5)P$_2$ could rescue renal endocytic defects due to the loss of OCRL1 in vivo, looking at a relevant tissue within a vertebrate organism. To do this we adopted several strategies – targeting the major enzyme responsible for PtdIns(4,5)P$_2$ synthesis, treatment with a small compound known to activate hydrolysis of PtdIns(4,5)P$_2$ or treatment with a synthetic compound previously shown to sequester PtdIns(4,5)P$_2$ on the membrane, preventing interaction.

Figure 18: Quantification of pronephric uptake of 10 kDa fluorescent dextran in wild type and ocrl$^{1/-}$ zebrafish larvae after injection with PHDM.

(A) Graph showing the number of embryos showing any uptake after treatment with the specified dose of PHDM. Error bars represent SEM. N numbers are total embryos used in each condition over 3 repeats. Significance was calculated using one way ANOVA with a Dunnetts post hoc test, comparing all means to the 0 μM data for each genotype. ns = not significant, * = p > 0.05, ** = p > 0.01, *** = p > 0.001, **** = p > 0.0001. Significance values shown are only for ocrl$^{1/-}$ data. (C) Graph directly comparing the number of no, low and high embryos scored for each condition, comparing the vehicle control with the 60 μM dose which gave the strongest rescue in ocrl$^{1/-}$ embryos. Data are mean ± SEM. (D) Representative images of pronephric tubules 1 hour post injection of 10 kDa dextran in wild type and ocrl$^{1/-}$ embryos treated with vehicle control or 60 μM PHDM. Scale bar is 50 μm. Images have been converted to grey scale and inverted for clarity.
4.9.1 Analysis of 10 kDa uptake as a suitable reporter of renal endocytosis in zebrafish embryos

Thus far, 10 kDa dextran injection to study renal uptake has been performed in 3 dpf zebrafish, observing the accumulation 2-2.5 hours post injection (Drummond et al. 1998; Kur et al. 2011; Oltrabella et al. 2015). 10 kDa dextran injection has also been described in adult zebrafish, however this is injected into the abdominal cavity and uptake in the mesonephros is assessed between 8 hours and 3 days after injection (McCampbell et al. 2014). We have now described the increased speed of accumulation of 10 kDa dextran in the pronephros over the developmental time point between early filtration (3 dpf) and the latest time point before protection of the animal under UK law (5 dpf). As development of the pronephros is complete before 40 hpf (Drummond et al. 1998), and mesonephric expansion of the renal tubules occurs around 11 dpf (Diep et al. 2015), we hypothesise that this increase in speed of accumulation of 10 kDa dextran in the renal tubule with age of the fish is likely to be due to an increased glomerular filtration rate (GFR) as well as an increase in the activity of endocytic uptake in the proximal tubule. While it is not yet possible to measure GFR precisely in zebrafish, it can be estimated by measuring the time taken for a defined volume of fluorescent dextran to be completely cleared from the vasculature by glomerular filtration (Kotb et al. 2014; Rider et al. 2012). For example, Kotb et al (2014) describes the reduction of 10 kDa dextran fluorescence in wild type embryos being complete after 24 hours. Although we did not quantitatively measure clearance in our experiments, the rate of clearance appeared comparable between wild type and mutant embryos at all time points, as indicated by similarly reduced dextran fluorescence in the vasculature in both wild type and ocr1l−/− embryos at 6 hpi and 24 hpi of the dextran injection (data not shown).

We have chosen to work with 4 dpf embryos for our further work on PtdIns(4,5)P₂ modulation in zebrafish for a number of reasons. Assay duration is halved for 4 dpf embryos as compared to 10 kDa dextran uptake assays in 3 dpf zebrafish. Embryos naturally tend to lie on their side, and at 4dpf the tubule is more convoluted and therefore easier to see in sagittal plane, meaning less manipulation of the embryo is required when imaging. Finally, using 4 dpf zebrafish allows us to perform overnight treatments with small compounds before injection, without disrupting the tubule development that is complete by around 48 hpf.

In previous publications, a semi-quantitative scoring system of categorising data into groups by the presence of visible uptake has been used (Anzenberger et al. 2006; Kur et al. 2011; Oltrabella et al. 2015). By comparing this method with
fluorescence intensity measurements taken from embryos injected with 10 kDa dextran, we have shown that this semi-quantitative scoring system is valid. We have found that this method is accurate where embryos show strong or weak uptake phenotypes. However, when there is a severe endocytic uptake defect such as the one we have reported in the *bugeye* mutant, quantification by line plot will provide false positive results due to background fluorescence around the vasculature, that in other embryos is usually masked by the renal tubule fluorescence. In our experiments, we chose to continue using the semi-quantitative scoring method of categorising 10 kDa dextran uptake, as it is more tractable and avoids inaccurate measurement due to dextran in the blood stream. However, this system of analysis could be prone to investigator bias, and therefore where possible this should be performed blind by multiple investigators to eliminate this source of bias.

4.9.2 Previously reported endocytic defects in the pronephros of *ocrl1*<sup>−/−</sup> embryos are present throughout later stages of embryonic development.

The endocytic defect caused by loss of OCRL1 has been demonstrated both in cell culture models and also now in a zebrafish model of Lowe syndrome (Vicinanza et al. 2011; Oltrabella et al. 2015). Both of these reports demonstrate that upon loss of OCRL1, receptor mediated endocytosis is disrupted, causing a mislocalisation of megalin to endosomes. It is likely that this mislocalisation is caused by megalin being internalised correctly, but accumulation of mislocalised proteins due to aberrant PtdIns(4,5)P<sub>2</sub> at the endosome is blocking the recycling of megalin to the plasma membrane. While megalin protein levels are reduced, mRNA levels are not, suggesting that mistrafficked megalin is being degraded, likely in the lysosome (Oltrabella et al. 2015). We have shown that there is a consistent defect in renal uptake of 10 kDa dextran in *ocrl1*<sup>−/−</sup> embryos at 3 dpf, 4 dpf and 5 dpf. Interestingly, our results show that this is not a complete loss of uptake as seen in *bugeye* embryos (Kur et al. 2011), but delayed and reduced uptake. It is possible that the low level of uptake seen in our *ocrl1*<sup>−/−</sup> embryos is due to the presence of a reduced pool of functional megalin at the cell surface driving internalisation, which cannot be replenished by recycling.

By knocking down PIP5Kαb, we were able to partially rescue the endocytic defect in *ocrl1*<sup>−/−</sup> embryos to similar levels as reported in Oltrabella et al. (2015), suggesting that PtdIns(4,5)P<sub>2</sub> levels have been reduced to physiological levels. The rtPCR data presented here demonstrated the effect of the PIP5Kαb morpholino on PIP5Kαb
expression levels, but requires repeating to confirm this. Unfortunately due to the availability of equipment we were not able to measure PtdIns(4,5)P₂ levels for these experiments, but at 3 dpf the morpholino results in knock down back to physiological levels (Oltrabella et al. 2015) therefore this is also likely true at 4 dpf.

4.9.3 Targeting PtdIns(4,5)P₂ is able to rescue the renal reabsorption defects in ocrl1⁻⁄⁻ embryos.

The seven species of phosphoinositides present in the cell are involved in a plethora of cellular functions. The conversion between these species by kinases and phosphatases control the phosphorylation of the lipid. Therefore inhibition of PI kinase and phosphatase enzymes is an important tool for studying the mechanism by which these phosphoinositides recruit effector proteins to carry out their functions as well as a useful clinical tool for treating dysfunctional phosphoinositides such as Lowe syndrome, and inhibitors of most phosphoinositide kinases and phosphatases are available. For example, in some cancers with PI3K activation, inhibitors have now been approved for use as treatment to reduce PtdIns(3,4,5)P₃ levels that activate akt signalling pathways in cancer cells, and thus stop uncontrolled growth and promote apoptosis (Fruman and Cantley 2014; Costa et al. 2015). Another example is the use of lithium to inhibit the production of the phosphoinositide precursor myoinositol to treat bipolar disorder (Detera-Wadleigh 2001). Bipolar disorder can be treated by reducing PtdIns(4,5)P₂ levels, and lithium is used in patients to globally suppress the production of phosphoinositides to achieve this (Soares et al. 2000). We have now shown that inhibition of PIP5Kαb in zebrafish successfully rescues the endocytic defect in the renal tubule associated with ocrl1⁻⁄⁻ mutants. Interestingly, the inhibitor which showed the most effect on ocrl1⁻⁄⁻ embryos at the trial dose, CRT0391964, also demonstrated the highest affinity for human PIP5Kα in the in vitro trials (Table 9). This suggests this compound may have clinical potential, although further experiments are needed to confirm this. While it is possible to measure PtdIns(4,5)P₂ levels directly (Jones et al. 2013), unfortunately we lack data on PtdIns(4,5)P₂ levels for our experiments. Previous reports have described the increased levels of PtdIns(4,5)P₂ in the ocrl1⁻⁄⁻ background (Ramirez et al. 2012; Jones et al. 2013; Oltrabella et al. 2015). Therefore all explanations for this data is based upon the assumption that each reagent used is reducing PtdIns(4,5)P₂ levels as expected.

We demonstrated that the rescue from the inhibitor in ocrl1⁻⁄⁻ embryos is dose responsive, and too high a dose of inhibitor in the ocrl1⁻⁄⁻ background results in
loss of the rescue. This is consistent with the results seen with PIP5Kαb morpholino. Surprisingly however, our data shows no effect of the inhibitor on wild type embryos. Measurement of PtdIns(4,5)P₂ levels after treatment with the inhibitor will help to establish in these experiments that PtdIns(4,5)P₂ levels have been affected, to verify activity of the inhibitor in vivo. As yet we have no established data demonstrating how the drug might reach the tubule, nor whether a meaningful concentration of drug is achieved within the tubule. Potentially, the different level of activity in the renal tubule in wild type and ocr1ț- embryos may alter the availability and delivery of the drug to the renal tubule, accounting for the difference in response.

This work has shown that treating ocr1ț- embryos with a PLC agonist, aiming to upregulate the conversion of PtdIns(4,5)P₂ to IP₃ and DAG, is able to rescue the defects seen in our ocr1ț- zebrafish model. This is the first demonstration that this approach may be possible in an in vivo setting. Most endogenous activation of PLC is achieved via the interaction of G proteins with mitogenic receptors on the cell surface (Kadamur and Ross 2013). It has also been noted that Phosphatase and tensin homolog (PTEN), a phosphatase that converts PtdIns(3,4,5)P₃ into PtdIns(4,5)P₂, can activate PLC (Alvarez-Breckenridge et al. 2007). In unpublished data, Sebastien Carreno has shown that this activation is driven by the C2 domain of PTEN in Drosophila S2 cells, and that expression of PTEN without the catalytic domain is sufficient to activate PLC and reduce PtdIns(4,5)P₂ levels in dOCRL1 depleted S2 cells. Following the success of m-3m3fbs in rescuing the endocytic defect in the renal tubule of ocr1ț- embryos, it will be interesting to see if expression of the C2 domain of zebrafish PTEN is also able to rescue this defect. Also, confirmation that PtdIns(4,5)P₂ levels are reduced after m-3m3fbs treatment in ocr1ț- embryos is important to verify that the resulting rescue is due to rebalancing of PtdIns(4,5)P₂ levels. If so, this demonstrates another potential therapeutic target in the search for alleviators of Lowe syndrome. However, it is important to note that IP₃ and DAG are both active second messengers (Putney and Tomita 2012). The release of calcium from the ER in response to IP₃ activates a plethora of other pathways within the cell, and often elicits tissue specific responses. While the experiment reported in section 4.7 has demonstrated a rescue of the renal uptake defect in ocr1ț- embryos, the effects of both acute and chronic IP₃ and DAG production may have effects on unrelated pathways by altering the correct functioning of other pathways within the cell. Likewise, it is possible that activation of PtdIns(4,5)P₂ hydrolysis in response to m-3m3fbs could act to ‘mute’ incoming
signals from the extracellular environment, affecting the cells ability to respond to environmental changes.

Sequestration of lipids has been described as a method of modulating the available lipid for normal activity. For example, sequestration of cholesterol has been shown in vitro with a β-cyclodextrin-dextran polymer, which is currently being tested for use in treating elevated cholesterol (Stelzl et al. 2015). Binding of a sequestration agent to PtdIns(4,5)P$_2$ should prevent the interaction with downstream effectors. It is an attractive idea that by sequestering aberrant PtdIns(4,5)P$_2$ in the ocr1$^{-/-}$ zebrafish, we could stop aberrant PtdIns(4,5)P$_2$ interactions, without having to inhibit or activate other enzymes and thus change other pathways within the cell. The PH domain from PLCδ can be used as a reporter of PtdIns(4,5)P$_2$ due to its highly specific binding (Lemmon et al. 1995). The binding of PLCδ-PH domain to PtdIns(4,5)P$_2$ however is competitive and the delivery of the PH domain to the renal tubule may be difficult without transgenic expression, therefore synthetic PHDM is an attractive alternative (Mak et al. 2011). Our results show that PHDM is able to alter the uptake of 10 kDa dextran in zebrafish when injected into the renal bloodstream. It also represents the first use of PHDM in an in vivo setting. The profile of the dose response curve is consistent with the theory put forward by Oltrabella et al (2015) that PtdIns(4,5)P$_2$ levels are required to be at physiological levels for endocytic uptake of 10 kDa dextran to occur.

Delivery of these compounds specifically to the renal tubule also represents a significant challenge. While the modulation of PtdIns(4,5)P$_2$ in the renal tubule may provide clinical benefit, it is unknown what effect altering PtdIns(4,5)P$_2$ may have on other tissues. Conjugation to LMW proteins such as lysozyme is one possible mechanism for targeted delivery of compounds to the renal tubule, as demonstrated in the accumulation of naproxen-lysozyme conjugates (Franssen et al. 1991; Zhou et al. 2014). Naproxen-lysozyme conjugates are internalised in the proximal tubular cells, where lysosomal hydrolysis of the lysozyme releases the bioactive metabolite resulting in a 70 fold increase in naproxen accumulation in the kidney as compared with standard naproxen treatment (Haas et al. 1997). However, the mechanism of export from the lysosome by transporters and channels on the lysosomal membrane (Xu and Ren 2015) for a particular drug must be quantified. Further, lack of OCRL1 results in a defect in megalin mediated endocytosis (Vicinanza et al. 2011; Oltrabella et al. 2015), which would likely result in reduced drug targeting. For this reason, LMW protein conjugation and other endocytic uptake mechanisms for drug delivery such as conjugation to LMW chitosan, folate, small
peptides and amino acids are also likely unsuited to treatment of Lowe syndrome (Zhou et al. 2014). Recently, nanoparticles have been demonstrated to be able to target the proximal tubular epithelium, however the author suggests this is also likely to be via an endocytic route (Williams et al. 2015).

While it is clear from the literature that the accumulation of ectopic PtdIns(4,5)P$_2$ on endosomes is the likely cause resulting in endocytic and recycling defects in proximal tubular cells (Vicinanza et al. 2011; Oltrabella et al. 2015), the mechanism by which this excess PtdIns(4,5)P$_2$ causes this defect is less clear. In cell culture models, excess PtdIns(4,5)P$_2$ that accumulates on endosomes following OCRL1 depletion results in excess filamentous actin accumulating around the endosome (Vicinanza et al. 2011). This may result in stabilisation of the endosomal structure, thus inhibiting the ability of recycling tubules to form due to the role of actin in carrier biogenesis (Anitei and Hoflack 2012). This role of actin is consistent with altered cytoskeletal actin reported in Lowe syndrome fibroblasts (Suchy and Nussbaum 2002). The series of experiments performed in this thesis supports the hypothesis that PtdIns(4,5)P$_2$ accumulation may be the cause of the endocytic defect, however as PtdIns(4,5)P$_2$ levels were not measured or visualised this provides only anecdotal support to this theory. The fact that targeting PtdIns(4,5)P$_2$ in multiple ways all led to an increase in renal uptake gives further support to this theory, and measurement and localisation of PtdIns(4,5)P$_2$ in zebrafish should be a priority to provide stronger evidence. Further work is required to understand fully how the accumulation of PtdIns(4,5)P$_2$ may result in the endocytic phenotype. If the accumulation of actin is indeed the mechanism by which recycling is impaired, this identifies new drug targets for the alleviation of Lowe syndrome. The formation of filamentous actin can be inhibited by latrunculin A, which binds to actin monomers (Coué et al. 1987). Furthermore, overexpression of a constitutively active form of the actin depolymerisation protein cofilin successfully inhibited the formation of ectopic actin on endosomes in OCRL1 depleted cells (Vicinanza et al. 2011), demonstrating that activation of cofilin may also represent a novel therapeutic target.
Chapter 5: Developing novel transgenic lines to study renal endocytosis in zebrafish
5.1 Introduction

As described in the previous chapter, the uptake of injected 10 kDa fluorescent dextran into the pronephric tubule can be used as a readout of renal function. This labelled polysaccharide however is not a specific ligand for megalin mediated endocytosis, and therefore is passively taken up into proximal tubule cells via fluid phase uptake. The key objective of this work is to generate a genetically encoded reporter, that will allow the throughput of the assay to be increased. The use of a megalin ligand as a reporter would be a more specific indicator of endocytosis in the renal tubule. Furthermore, as megalin binds to LMW proteins, the use of a LMW protein as a reporter will allow the generation of a transgenic reporter line. At present, all embryos must be individually injected with tracer, and a transgenic reporter line will avoid the necessity to inject tracer into the embryos, vastly improving throughput.

A number of alternative reporter of proximal tubular uptake have been described. Receptor Associated Protein (RAP) is a 39 kDa protein which is endogenously expressed in the endoplasmic reticulum (ER), where it binds with nanomolar affinity to megalin to chaperone megalin during its transit to the Golgi apparatus (Lee et al. 2006). Recombinant RAP has been used in numerous studies as a specific megalin ligand to monitor endocytosis (Anzenberger et al. 2006; Czekay et al. 1997; Oltrabella et al. 2015; Vicinanza et al. 2011). RAP has been used both in cell culture and in zebrafish embryos where it can be added to the cell culture medium or injected into the bloodstream respectively. Another protein reporter for endocytosis is GFP, which has been reported to accumulate in the renal tubule of injected rats and frogs (Prutskova and Seliverstova 2011). It is currently unclear whether GFP is taken up by megalin or whether it is acting as a fluid phase reporter in the renal tubule. More recently, an intrinsically fluorescent aromatic compound known as PT-Yellow has been identified in a zebrafish target screen, and is proposed to report endocytosis in the renal tubule, following simply soaking the embryo in a low concentration of the compound for 20 minutes (Sander et al. 2015).

In this chapter, the relative merits and shortcomings of the current alternatives to 10 kDa dextran uptake as a readout of renal function were assessed. Then, using these conclusions, a suitable renal uptake reporter that can be genetically encoded and expressed within the zebrafish embryo was designed. A major objective of this study is to create a genetically encoded reporter that can be expressed within the zebrafish and used to monitor proximal tubular endocytosis.
5.2 PT-Yellow does not report classical megalin mediated endocytosis in the renal tubule

PT-Yellow is a small (MW=591.74) fluorescent compound (Excitation 548 nm; Emmission 564 nm), which has been shown to label the renal tubule in zebrafish following incubation with a low concentration in the water (Sander et al. 2015). Staining in treated embryos is localised to apical punctate vesicles, and the authors propose that these may be endosomes, which the compound may have reached via megalin mediated endocytosis or fluid phase uptake. They further show that knockout of hnf1ba and hnf1bb (transcription factors critical for kidney tubule differentiation and cardiac function), or treatment with butanedione monoxime (a drug which stops cardiac function) resulted in pericardial oedema and loss of renal uptake of PT-Yellow. It is likely that if uptake is from the apical membrane of proximal tubular cells, filtration of PT-Yellow through the glomerulus and into the tubule is a prerequisite for this tubular uptake. In stopping cardiac function, it is likely that Sander et al (2015) will reduce the filtration in the glomerulus, which requires hydrostatic pressure through the vasculature of the glomerulus to drive filtration across the glomerular barrier (Jarad and Miner 2009). We set out to test the use of PT-Yellow in the ocr11⁻/⁻ and bugeye lines, two lines with an established defect in megalin mediated endocytosis.

Following the treatment regime reported in Sander et al. (2015), 4 dpf embryos were incubated in 100 nM PT-Yellow. After 20 minutes, the compound was removed by washing, and the embryos maintained in fresh chorion water for 24 hours, before embryos were imaged using a stereomicroscope (Figure 19A). The number of embryos showing uptake was counted, and images collected for further analysis. We saw highly reliable uptake of the PT-Yellow into punctate structures within the proximal tubule of 5 dpf wild type embryos (Figure 19C), in agreement with the findings of Sander et al. (2015). However, both the ocr11⁻/⁻ and bugeye embryos also showed strong accumulation of the PT-Yellow into renal puncta (Figure 19B, C). As we know that the ocr11⁻/⁻ line has reduced endocytic uptake of 10 kDa dextran and reduced numbers of endosomes in the proximal tubule, and the bugeye line has no endocytic or pinocytic uptake of 10 kDa dextran and very few endosomes in the proximal tubule, this means that it is likely that PT-Yellow is not a true reporter of endocytic uptake in the renal tubule.
Figure 19: PT-Yellow uptake is not reduced in renal endocytosis defect models.

(A) A low magnification showing the location of the renal tubule (red arrow) after 20 minute 100 nM PT-Yellow treatment and 24 hour incubation in 5 dpf embryos. Also note the intense PT-Yellow visible in the gut (blue arrow). Scale bar is 100 µm. (B) A high magnification image of the renal tubule after PT-Yellow treatment. Red box marks the area used to make horizontal line plots for fluorescence intensity.
5.3 **GFP is a poor reporter of renal endocytosis in zebrafish embryos**

Previous work in rats and frogs has shown that GFP can be internalised into the renal tubule (Prutskova and Seliverstova 2011). This would make GFP an attractive genetic reporter of endocytic uptake in the renal tubule in zebrafish as it can be both genetically encoded and detected easily by direct fluorescence. We therefore decided to assess the uptake capacity of zebrafish embryos after injection of recombinant GFP. Purified recombinant GFP was injected into the cardinal vein of 4 dpf wild type, ocr1/ocr1 and bugeye embryos and accumulation in the renal tubules was assessed at 40 minutes post injection.

The uptake of GFP was very poor in wild type embryos, where 80% of embryos had no visible uptake of GFP in the renal tubule (Figure 20A, B). A small number of wild type fish showed some low uptake of the GFP, an example of which can be seen in Figure 20C. In ocr1/ocr1 there was similarly very low levels of uptake, around 90% of ocr1/ocr1 embryos showing no uptake. In bugeye mutants, no uptake was detected in any of the injected embryos. This result demonstrates that, in zebrafish embryos, GFP is not endocytosed efficiently into the proximal tubular epithelium, and therefore is a poor reporter of endocytic function in the renal tubule.

5.4 **Cy3 conjugated RAP is a good reporter of megalin mediated endocytosis in the renal tubule.**

The binding of the protein RAP to LRP receptors, such as megalin, has been well documented in cell culture models, and the crystal structure of a RAP-LRP interaction has been solved (Medved et al. 1999; Fisher et al. 2006; Lee et al. 2006). RAP is usually restricted to the ER, where its binding is thought to prevent the interaction of megalin with other proteins. Upon delivery to the golgi, RAP is targeted for retrograde transport back to the ER via its KDEL motif (Stornaiuolo et
Cy3-RAP has previously been used as a reporter for megalin mediated renal endocytosis in zebrafish studies of ocrl1-/- at 3 dpf (Oltrabella et al. 2015).

We injected Cy3-RAP into either wild type, ocrl1-/- or bugeye 4 dpf embryos. After 40 minutes, we assessed uptake of the Cy3-RAP in the renal tubule. In wildtype embryos, we saw around 70% of embryos showed some punctate RAP staining in the renal tubule, and around 40% of the total showed high uptake or RAP (Figure 21A). RAP staining was restricted to the proximal most region of the tubule (Figure 21B). In ocrl1-/- embryos uptake was severely impaired, with around 80% of
embryos showing no evidence of Cy3-RAP accumulation. In bugeye embryos, there were no embryos showing any uptake of the Cy3-RAP.

5.5 Generating a novel reporter for use in zebrafish as a genetically encoded reporter.

To create a novel genetically encoded reporter, a combination of the specificity of the RAP protein with either fluorescent or luminescent proteins to provide easily quantifiable measurements seems an attractive option. The domain structure of
RAP has been solved (Medved et al. 1999)(Figure 22A), and past work has demonstrated that the 14 kDa D3 domain of RAP, which binds with high affinity to LRP receptors, can fold and function independently of the rest of the protein (Fisher et al. 2006). Fusion of the full length RAP protein to GFP would create a protein of around 66 kDa, meaning that as a larger protein, it is more likely to be retained in the blood by the glomerular filtration barrier. The D3 domain however, could be tagged to a reporter protein and still retain a similar size to the full length RAP protein (Figure 22B). Therefore two novel reporters were designed by tagging this D3 domain with either the fluorescent reporter GFP (27 kDa), or the luminescent reporter NanoLuc (NL, 21 kDa, Figure 22B).

Figure 22: RAP-D3 domain is a suitable ligand for fluorescent and luminescent tagging.

(A) Schematic representation of full length RAP domains. (B) Schematic representations of alternatively tagged variations of RAP and RAP-D3 domain, against a 70 kDa scale marker.
5.6 Recombinant GFP-D3 and NL-D3 is taken up by endocytosis in cell culture in a megalin specific manner.

To test whether these D3 fusion proteins were suitable to determine megalin specific uptake, pilot experiments were performed using recombinant bacterially expressed GFP-D3 and NL-D3. To express these fusion proteins in bacteria, coding sequences for each part were cloned in frame into the bacterial expression vector pTrcHis, which contains an N-terminal His tag, expressed and purified as described in Section 3.4. To confirm the purity of the final recombinant protein preparations, an aliquot of each protein was analysed by SDS-PAGE. Each preparation had a strong band at the expected size, with little background staining, showing that the protein preparations are good (Figure 23A).

Uptake experiments were performed with HEK293 cells expressing mini-megalin (a truncated version of megalin containing the cytoplasmic tail, transmembrane domain and one of four large extracellular repeats, named MmR1-MmR4) obtained from Thomas Willnow at the MDC, Berlin. Similar results were obtained with all four clones, and so only results with MmR4 are shown here. Recombinant NL-D3 or the NL protein lacking D3 were incubated with parental HEK293 cells lacking mini-megalin or the MmR4 line for 30 minutes, and uptake monitored by lysing the cells and measuring luciferase activity in the resulting cell lysates. There was approximately 10-fold more NL-D3 internalisation in MmR4 cells compared to the parental control (Figure 23B). There was negligible uptake of the NL alone in both the MmR4 and parental cells. These results indicate that NL-D3 is competent to function as a megalin ligand in vitro.

Next, to test GFP-D3, the same assay as above was performed adding either GFP, GFP-D3 or Cy3-RAP (as an uptake control). For this analysis, we fixed cells in and stained them for megalin and DNA. We found that in parental cells, which do not express the mini-megalin construct, there was no visible accumulation of GFP-D3 (Figure 24). Likewise, in MmR4 mini-megalin expressing cells, there was no uptake of GFP without the D3 fusion. However, megalin expressing cells that were exposed to Cy3-RAP (full length) or GFP-D3 showed internalisation of the reporter into punctate structures. This demonstrates that GFP-D3 is a ligand of megalin in vitro. It is likely that the visible puncta are endosomes.
5.7 Recombinant GFP-D3 functions as a renal endocytosis reporter in zebrafish embryos.

To determine whether GFP-D3 can faithfully report proximal tubular endocytosis in zebrafish, GFP-D3 was injected into the common cardinal vein of 5 dpf wild type or ocr11−/− embryos and imaging performed at 10 minute intervals using a fluorescence stereomicroscope for a total of 40 minutes, and uptake categorised as done previously in 10 kDa dextran uptake experiments.

To examine the data, we plotted a graph comparing between wild type and ocr11−/− genotypes those that showed any uptake (those categorised as low or high) or those embryos that showed high uptake. Uptake was detectable in the majority of wild type embryos (Figure 25A, B). When the data was plotted as ‘any uptake’, it is clear there is a small but significant reduction in the uptake of GFP-D3 in ocr11−/− embryos during the time course (Figure 25A). When we look at those only categorised as high uptake, we can see a substantial difference between wild type and ocr11−/− embryos (Figure 25B, C). This is similar to the phenotype seen when 10 kDa dextran is injected into ocr11−/− embryos. This tells us that at 5 dpf embryos, GFP-D3 is taken up efficiently into the pronephric tubule.
Uptake of NL-D3 was also assessed in zebrafish by injection of recombinant protein. Unfortunately, reliable luminescence readings could not be taken for water samples from injected embryos. It is likely that this is due to excess luciferase being deposited on the body of the fish during microinjection, which could then be leaching into the water samples.

Figure 24: GFP-D3 is internalised by megalin expressing HEK293 cells.
Representative images of MmR4 mini megalin expressing or parental cells showing the uptake of GFP-D3 and Cy3-RAP, but not GFP. Scale bar is 25 µm.
Clearance of GFP-D3 is not affected in *ocrl1*<sup>−/−</sup> embryos

Clearance of a reporter from the blood stream is an important consideration when looking at a defect in renal accumulation. If the reporter is not being cleared efficiently by the glomerulus, then less of the reporter will pass into the tubule to be available for reabsorption, thus giving the impression that the proximal tubule is not functioning correctly. Clearance of 10 kDa dextran has been measured previously in embryos treated with a nephrotoxic compound that blocks filtration, by measuring the fluorescence intensity of the cardinal vein over time (Kotb et al. 2014). While observations in the lab have shown that dextran is cleared from 4 dpf wild type and *ocrl1*<sup>−/−</sup> embryos by 24 hours post injection of 10 kDa dextran (data not shown), the clearance of a reporter in *ocrl1*<sup>−/−</sup> embryos has not yet been documented in the
literature. Additionally, in developing NL-D3 and GFP-D3, it is also important to confirm that these proteins are able to efficiently filter as expected, and are not retained in the blood by the filtration barrier.

To assess the clearance of GFP-D3 in wild type and ocr11⁻/⁻ embryos, we used an assay similar to that of Kotb et al. (2014). We injected 5 dpf embryos with recombinant GFP-D3 into the common cardinal vein. Next, embryos were imaged immediately, and again after 20 and 40 minutes using a fluorescence stereomicroscope, focusing on the area of the tail that contains the cardinal vein immediately behind the cloaca (Figure 26A, B, E). Once complete, images were analysed by placing a 150 x 30 pixel box over the cardinal vein in the same position on each of the three images for each fish (Figure 26B). Vertical fluorescence intensity line plots were then made for each one selection (Figure 26D) and the AUC was then compared between each genotype to give a measure of fluorescence in the bloodstream.

We found in both wild type and ocr11⁻/⁻ embryos that around 60% of the fluorescence in the vein was lost in the first 20 minutes, and intensity continued to decrease between 20 and 40 minutes at a slower rate (Figure 26C). This confirmed that most of the GFP-D3 is being filtered and cleared from the bloodstream within this time frame. Further, it confirmed that there is no defect in the clearance of GFP-D3 in ocr11⁻/⁻ embryos.

5.9 Building expression vectors to drive secretion of NL-D3 and GFP-D3 in zebrafish embryos

Now that the megalin mediated uptake of GFP-D3 and NL-D3 proteins has been characterised in cells and GFP-D3 in zebrafish and shown that they are potentially viable reporters of proximal tubular endocytosis, transgenic construct to express in zebrafish can now be created. Glomerular studies have been done previously using GFP fused with the secreted VDBP and expressing it in the liver (Zhou and Hildebrandt 2012) under the expression of the l-fabp10 promoter that drives liver specific expression (Her et al. 2003). The liver is the site of albumin production and secretion, therefore secretion of proteins into the bloodstream by the liver is already known. Therefore GFP-D3/NL-D3 will be fused with a signal sequence to target the protein for secretion, and be produced and secreted from the liver into the bloodstream, where it will then be filtered by the glomerulus and reabsorbed in the renal tubule (Figure 27). With the GFP-D3 transgenic fish, we expect this to be a
Figure 26: Wild type and ocr11<sup>−/−</sup> embryos show similar clearance of injected recombinant GFP-D3.

(A) Schematic representation of an injected GFP-D3 embryo (black) with GFP-D3 fluorescence throughout the vasculature (green). Inset, a close up diagram of the area used for measuring the fluorescence intensity. Red box represents the selection used to make a fluorescence line plot. (B) Representative image of the cardinal vein and the area used for quantification. (C) Graph showing the total fluorescence (area under the curve) of fluorescence line plots for individual wild type and ocr11<sup>−/−</sup> embryos.
embryos. Values are relative to an average wild type intensity at 0 minutes of 1. Summary of 11 ocrl1−/− and 8 wild type animals in a single experiment. (D) An example fluorescence intensity line plot taken through the vasculature of a single embryo at the point shown in (B). (E) Representative images of wild type and ocrl1−/− embryos focused on the region clearance was assessed. MPI = Minutes post injection. Images have been converted to grayscale and inverted for clarity. Scale bar is 50 µm.

Figure 27: Schematic representations of the assay design for NL-D3 and GFP-D3 transgenic zebrafish embryos.

(A) Shows the expected assay for NL-D3 expression. Blue vasculature and liver represents circulating and newly synthesised NL-D3 respectively. Blue ovals represent excreted protein. (B) Shows the expected assay for GFP-D3 transgenic embryos. Green vasculature and liver represent circulating and newly synthesised GFP-D3 respectively.

visual readout, where the presence of GFP-D3 accumulating in the renal tubule can be used to detect renal function (Figure 27B). In transgenic NL-D3 embryos the NL-D3 is expected to be largely reabsorbed into the renal tubule, resulting in relatively little being excreted into the water (Figure 27A). In embryos with a proximal tubular defect, much more NL-D3 is expected to be excreted into the water. The readout of
this assay will be luciferase measurement from water samples, providing a completely non-invasive readout.

A plasmid containing the Interleukin-6 (IL-6) signal sequence fused in frame with the NanoLuc protein was obtained from Promega (UK) (Hall et al. 2012). PCR was used to amplify the IL-6 signal sequence and NL sequence, without its stop codon at the C terminus. Next, conventional cloning was used to fuse this in frame with the D3 domain of RAP followed by a stop codon. This was then cloned into the ‘p3’ position of the gateway cloning system (see section 3.3.13). Into the middle position ‘p2’ the liver specific Ifabp10 promoter was cloned. As an easy marker to follow transgenic animals a γ-Crystallin promoter driving mCherry expression was placed in the ‘p1’ position as previously published (Offield et al. 2000). This is summarised in Figure 28A, B.

We also obtained a GFP plasmid containing the human Calreticulin (hCal) signal sequence upstream of GFP, followed at the end of the protein by an ER retention KDEL sequence and a stop codon as used previously (Losfeld et al. 2012). We amplified the hCal-GFP fusion, without the KDEL motif or stop codon, and used conventional cloning to fuse this with the sequence for D3. This was then cloned into the ‘p3’ position of the gateway cloning system (Figure 29A, B).

To generate expression constructs for use in zebrafish, p1, p2 and p3 (either GFP-D3 or NL-D3) plasmids were combined with the destination vector ‘p4-l-Scel SAR-CH4 Tol2’ described in Love et al. (2011), and construct sequences were confirmed using DNA sequencing. These expression constructs were mixed with a with Tol2 mRNA, and injected into wild type embryos at the single cell stage. Embryos were then screened for mCherry expression from the γ-crystallin reporter in the lens at 5 dpf to identify animals in which the construct had successfully integrated, and embryos with a fluorescent lens were grown in the nursery to adulthood. Additionally, GFP-D3 F0 transgenic animals had GFP-D3 expression in the liver (Figure 28C), and NL-D3 F0 transgenic animals were both positive for γ-crystallin expression in the lens (Figure 29C) and also whole lysates of transgenic animals contained active luciferase (Figure 29D), demonstrating that the NL-D3 was being produced in these animals.
5.10 GFP-D3 and NL-D3 are stably expressed in F2 transgenic lines

GFP-D3 and NL-D3 founders were crossed to wild type fish, and the offspring screened for mCherry expression in the lens from the γ-crystallin reporter gene. Embryos positive for mCherry expression were grown to adulthood, and in-crossed
to generate F2 transgenic fish (Figure 30). The expression of GFP-D3 and NL-D3 was then characterised in the transgenic fish to understand how this reporter is functioning in the embryos.

**Figure 29 Generating transgenic fish expressing NL-D3.**

(A) Schematic representations of the three donor vectors used to create the γ-Cry-mCherry:lfabp10-NL-D3 expression vector. (B) Schematic of the final γ-Cry-mCherry:lfabp10-NL-D3 expression vector. (C) Representative images of 5 dpf F0 zebrafish embryos, either wild type or after injection with the transgenic construct. Yellow region on the image is autofluorescence from the yolk. (D) Relative luminescence comparison of whole embryo lysates of wild type and NL-D3 5 dpf embryos.

was then characterised in the transgenic fish to understand how this reporter is functioning in the embryos.
First, NL-D3 expression was confirmed in adult F2 fish positive for mCherry expression in the lens (Figure 31A). Adult transgenic and wildtype fish were euthanised, before the tail was removed at the base with a razor blade (Figure 31B). Blood was collected from the wound, and diluted in PBS. NanoGlo luciferase substrate was added to the blood sample before being read on a luminescence plate reader. Basal levels of luciferase activity in wild type embryos was around 10 relative luminescence units (Figure 31C). In contrast, the blood from NL-D3 transgenic zebrafish gave a reading of around 100,000 relative luminescence units. This demonstrates that the NL-D3 is being sufficiently secreted from the liver in these fish.

Next, adult F2 NL-D3 transgenic fish were in-crossed to generate transgenic embryos. To establish whether there was a detectable increase in NL-D3 excretion
when proximal tubular uptake was defective, pools of embryos were injected with either control morpholino or a morpholino to knock down megalin expression, as previously described (Kur et al. 2011). Embryos were grown to 3 dpf when renal function is known to be active and transgenic embryos were selected by the presence of mCherry in the lens. These were transferred in groups of 5 embryos to a 96 well plate in 100 µl of clean chorion water to collect excreted NL-D3 in a small volume of water. After 48 hours, 50 µl samples were taken from each well for luminescence reading. To confirm the wild type and morpholino injected embryos demonstrated renal uptake and lack of renal uptake respectively, all embryos from each well were injected with 10 kDa dextran and assessed to confirm the presence of uptake in wild type embryos, and the absence of uptake in megalin morpholino injected embryos. To identify embryos where clearance may be affected, gross morphology was also assessed to identify embryos with oedema. In each well, if more than two of the five embryos did not fit the established phenotype for that genotype, the well was discarded as this would not give a reliable readout of renal function of the group of embryos. A summary is shown in Table 10. Finally, embryos were lysed in NanoLuc lysis buffer, and whole embryo lysate was tested for luciferase concentration.

**Table 10: Phenotypic screening of NL-D3 embryos**

<table>
<thead>
<tr>
<th>Zebrafish</th>
<th>Expected 10 kDa dextran uptake</th>
<th>Expected morphology</th>
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</thead>
<tbody>
<tr>
<td>NL-D3 uninjected</td>
<td>High 10 kDa dextran uptake</td>
<td></td>
</tr>
<tr>
<td>NL-D3 Ctrl Mo injected</td>
<td>High 10 kDa dextran uptake</td>
<td>Normal – no oedema</td>
</tr>
<tr>
<td>NL-D3 Megalin Mo injected</td>
<td>No 10 kDa dextran uptake</td>
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Luminescence readings for these embryos demonstrate that similar levels of NL-D3 are excreted into the water in uninjected and control morphant embryos (Figure 31D). However, in megalin morphant NL-D3 embryos, there is around a 7 fold increase in the excretion of NL-D3 into the water, consistent with reduced endocytic uptake in the renal tubule.
Next, adult F2 GFP-D3 transgenic zebrafish which express mCherry in the lens (Figure 32A) were crossed to generate transgenic embryos. Zebrafish were assessed for visible accumulation of GFP-D3 in the renal tubule at 3, 4 and 5 dpf, which found that 5 dpf embryos demonstrated the most renal uptake of GFP-D3 (Figure 32B). At 5 dpf, GFP-D3 can be detected both in the liver, and in the renal tubule by stereomicroscopy (Figure 32C,F-first panel). In confocal section, it is
Figure 32: GFP-D3 expression and uptake in F2 transgenic zebrafish.

(A) Images of 50 dpf wild type and GFP-D3 transgenic zebrafish. (B) Graph showing the uptake of GFP-D3 in 3 dpf, 4 dpf and 5 dpf F2 embryos. Data are mean ± SEM from 3 repeats. (C) Schematic representation of a GFP-D3 expressing embryo in frontal and sagittal plane. Red arrow shows direction of view of representative images. Red dashed box shows region of interest. (D) Confocal section through the renal tubule of a GFP-D3 expressing embryo. Red dashed region marks the limits of the renal tubule. Scale bar is 10 µm. (E) Graph showing the proportion of GFP-D3 F2 embryos showing uptake of GFP-D3 in the renal tubule ± megalin morpholino in 5 dpf embryos. (F) Representative images of GFP-D3 embryos ± megalin morpholino. Red dashed line represents approximate limits of the renal tubule. P = pronephros, L = liver. Bottom, Confocal sections through the renal tubule of embryos ± megalin morpholino. Red dashed line marks the edges of the renal tubule. Scale bar is 5 µm.
clear that GFP-D3 is accumulating in punctate structures within the proximal tubular cells, likely endosomes (Figure 32D). To confirm the uptake of GFP-D3 is by megalin dependent endocytosis, GFP-D3 embryos were injected with either morpholino against megalin or a control morpholino, and the accumulation of GFP-D3 in renal tubules observed at 5 dpf using fluorescence microscopy. Uptake in GFP-D3 embryos injected with control morpholino was as expected, however uptake in GFP-D3 megalin morphants was impaired (Figure 32E,F).

5.11 GFP-D3 from transgenic fish is not taken up in the bugeye mutant line

Lack of visible uptake in GFP-D3 megalin morphants suggests the GFP-D3 is working correctly. To confirm that GFP-D3 uptake in zebrafish is megalin dependent, GFP-D3 fish were crossed with bugeye mutants, and embryos were selected by the presence of red lenses and green livers at 5 dpf. These embryos were all bugeye<sup>−/−</sup>, and were grown to adulthood. Next, these animals were incrossed, to generate segregating offspring. A 1:2:1 ratio of wild type, heterozygotes and homozygotes were expected from this cross. Embryos were screened for red eyes and green livers at 5 dpf and individual embryos were assessed for GFP-D3 uptake in the renal tubule. Embryos were then put into individual tubes, and genomic DNA extracted for genotype analysis. Results for GFP-D3 uptake were then organised by genotype.

Bugeye<sup>+/−</sup> and bugeye<sup>−/−</sup> embryos were both able to reabsorb the GFP-D3 and accumulate the protein in the renal tubules (Figure 33). However, in bugeye<sup>−/−</sup> embryos, there was a complete defect in the ability of embryos to take up the GFP-D3.

At the time of writing, NL-D3 fish are being crossed with bugeye mutants to confirm the megalin dependent uptake of NL-D3.

5.12 Discussion

To study renal endocytosis in zebrafish, accumulation of injected reporters has been used as a readout. As already discussed, 10 kDa dextran can be used to assess uptake in the proximal tubule (Drummond et al. 1998). Similarly, large dextrans such as 500 kDa fluorescent dextran can be used to assess glomerular integrity (Kramer-Zucker, Wiessner, et al. 2005). For small scale screening or colocalisation studies,
the use of dextrans to assess both of these functions is attractive, due to the range of fluorescent conjugant variants that span the entire size spectrum. For larger screening experiments, a genetically encoded reporter is required. A VDBP-GFP glomerular integrity reporter has already been designed (Hentschel et al. 2007) that is unable to filter unless the glomerular barrier has been compromised, however to date no such proximal tubular endocytosis reporter exists.

In this chapter, the use of CY3-RAP, PT-Yellow and GFP as renal reporters has been assessed to determine their suitability as a non-invasive reporter. Based on the suitability of the D3 domain of RAP in its megalin specificity as well as its relatively small size, novel genetically encoded reporters of D3 fused with fluorescent and luminescent proteins have then been designed, and their specificity

Figure 33: Uptake of expressed GFP-D3 in bugeye+/+ and bugeye−/− embryos.

(A) Representative images of 5 dpf GFP-D3,bugeye+/+ (left) and GFP-D3,bugeye−/− (right) liver and proximal tubule regions. L liver; red dotted line represent the proximal tubule. Scale bar is 50 µm. (B) Genotyping results for GFP-D3 embryos, either wild type, heterozygous or homozygous for bugeye mutation as previously described in Kur et al (2011). (C) A graph showing percentage of embryos with visible accumulation of GFP-D3 in transgenic embryos with or without bugeye mutation. Data are mean ± SEM. GFP-D3; bugeye+/+ n= 24, GFP-D3:bugeye−/− n= 15, over 2 separate experiments.
to megalin and suitability for use as a tubular uptake reporter in zebrafish characterised.

5.12.1 Assessing alternative renal uptake reporters to 10 kDa dextran

The use of PT-Yellow to assess endocytosis in the renal tubule is an attractive idea. A compound which can be applied by brief incubation in the water would allow high throughput studies to be performed, without the invasive need to inject individual embryos. We know that in ocrl1−/− embryos there is reduced numbers of apical endosomes (Oltrabella et al. 2015), and in bugeye embryos the endocytic apparatus, including apical endosomes, are almost absent (Kur et al. 2011). However, PT-Yellow still accumulates in punctate structures within the tubule of both ocrl1−/− and bugeye embryos. There also appears to be no difference in the level of uptake in each of these mutants compared with wild type embryos. This suggests that PT-Yellow is not being reabsorbed via tubular cells by megalin mediated endocytosis. Sanders et al. (2015) proposed that PT-Yellow was being taken up be endocytosis in the proximal tubule, as when filtration was blocked by inhibiting cardiac function, the uptake of PT-Yellow was prevented, suggesting apical entry into the proximal tubule cell. The zebrafish proximal tubule has been shown to contain apical transporters similar to mammalian kidneys, which can internalise a variety of ions and other small molecules from the filtrate (Drummond and Davidson 2010). It remains possible that the accumulation of PT-Yellow from the apical membrane may be through such a transporter, rather than megalin mediated endocytosis. Until a clear mechanism is in place, PT-Yellow is not of use as a renal uptake reporter, although it is still possible to give a readout of glomerular filtration.

GFP represented another possible renal endocytic tracer, due to its reported uptake in previous work with rats and frogs (Prutskova and Seliverstova 2011). However, in our work, the uptake of GFP in wild type fish was very poor. Megalin is known as a promiscuous receptor, known to bind to at least 50 different ligands (Marzolo and Farfán 2011). While it is not fully understood how megalin is able to bind ligands, it is thought that the presence of basic residues is required (Moestrup and Verroust 2001). While GFP does contain some basic residues, it may be that the barrel shape of GFP (Tsien 1998) prevents the interaction of these motifs with megalin.

Cy3-RAP is a megalin specific reporter which, when injected into the blood stream of zebrafish embryos, is accumulated in the renal tubules (Anzenberger et
al. 2006; Oltrabella et al. 2015). We have found in this work that there is no uptake of RAP in *bugeye* embryos, which is consistent with this megalin mutation. We have also found that there is a significant reduction in the ability of *ocr17−/−* embryos at 4 dpf to take up RAP, which reproduces the findings of Oltrabella et al. (2015). Overall, the ~70% uptake seen in wildtype embryos suggests that RAP is a good reporter of megalin mediated uptake in the proximal tubule. Megalin is most strongly expressed in the most proximal part of the renal tubule (Kur et al. 2011), and this matches the proximal pattern of uptake in Cy3-RAP injected embryos. It is unlikely that full length RAP tagged with GFP would be likely to filter efficiently through the glomerulus, due to its estimated size of 66 kDa. The typical mammalian glomerulus is size, charge and molecular shape selective, with an estimated cut off size of around 70 kDa (Haraldsson et al. 2008). Although the exact selectivity of the zebrafish glomerulus has not been determined, it has been shown that VDBP-GFP, a protein of 79.6 kDa, is not filtered in zebrafish (Zhou and Hildebrandt 2012). One caveat to the use of Cy3-RAP, is that embryos die after around 12 – 24 hours post injection (data now shown). This could be due to toxicity of the Cy3 conjugate, or interaction of the RAP protein itself.

5.12.2 GFP-D3 and NL-D3 are good reporters of megalin function.

Full length RAP, at 39 kDa, is known to be filtered and taken up efficiently in the renal tubule (Anzenberger et al. 2006; Oltrabella et al. 2015). By fusing the megalin binding D3 domain of RAP to either GFP or NL, we have designed fluorescent and luminescent proteins with predicted sizes of 42 kDa and 35 kDa respectively, similar to the size of the full length protein, and therefore likely to filter. The binding of RAP is determined by the interaction of D3 with two cysteine rich repeat modules in the extracellular domain of megalin, multiples of which are found in all four repeat regions of megalin (Andersen et al. 2000). Due to the presence of these repeats in all four extracellular regions, it is unsurprising that D3 fusion proteins were internalised by cell lines expressing any one of the four repeat regions.

The uptake of NL-D3 we have reported in megalin cells demonstrates the specificity of the protein for megalin. Megalin is a member of a family of 7 LRP proteins, and interaction of D3 with the other main family member, LRP1, has been previously demonstrated (Fisher et al. 2006). A basal level of NL-D3 uptake we have shown in parental cells not expressing mini-megalin is likely due to the binding of NL-D3 to another LRP receptor such as LRP1. The poor endocytosis of NL without a D3 fusion into MmR4 cells that we have reported demonstrated further
that the D3 domain is responsible for interaction with megalin and internalisation of the protein. The uptake of GFP-D3 in megalin expressing cells confirms that the D3 domain is responsible for megalin endocytosis of the GFP protein.

The accumulation of GFP-D3 in the renal tubule after injection into zebrafish embryos demonstrates a number of properties of the protein. Firstly, GFP-D3 accumulation is restricted to the proximal tubule, the main site of megalin expression. Megalin is strongly expressed in the proximal tubule of zebrafish, as well as the neural ventricle (Kur et al. 2011). Importantly, no visible accumulation of GFP-D3 in cells other than the proximal tubule was identified, suggesting any uptake of GFP-D3 by other LRP receptors is not of significant consequence in zebrafish. Although injection of Cy3-RAP causes death in 4 dpf embryos within 24 hours, embryos injected with GFP-D3 are not affected in this way (data not shown). It is likely therefore that the lethality associated with injection of Cy3-RAP is due to either interactions of the RAP domains D1 and D2, or the Cy3 conjugate. The uptake of GFP-D3 in the proximal tubule in wild type fish is similar to that of 10 kDa fluorescent dextran (see Figure 9 for example of dextran). Encouragingly, the accumulation defect in ocr11−/− embryos is consistent with the defects in Cy3-RAP uptake reported in Oltrabella et al. (2015).

Uptake in the renal tubule is closely coupled to the glomerular filtration rate, and thus clearance from the bloodstream (Hentschel et al. 2007; Kotb et al. 2014). Full clearance of 10 kDa dextran takes place over 8 – 24 hours in 4 dpf embryos (Kotb et al. 2014). Our results show that the clearance of injected GFP-D3 in 5dpf zebrafish embryos is much faster than this, with around a 70% reduction in vascular fluorescence within 40 minutes. It is likely that this increase in speed of clearance of GFP-D3 compared with 10 kDa dextran is due both to the increased age of the zebrafish embryo (see section 4.2 for quantification of the effects of embryo age on renal uptake) and also the type of reporter. Fluorescent dextran is able to stick to lipid membranes (Diamond and DeMaggio 2012), likely slowing its movement from blood stream to ultrafiltrate. Taken together, the speed of clearance and speed of reabsorption of GFP-D3 is similar to that observed in Cy3-RAP injected embryos.

The integrity of the glomerular barrier is not compromised in ocr11−/− embryos as determined by the retention of 500 kDa dextran in the vasculature (Oltrabella et al. 2015), however later onset glomerular defects have been noted in human patients with Lowe syndrome (Lowe Syndrome Trust 2010; Loi 2006). While it is not fully understood, it has been shown that cross talk between proximal tubular cells and the endothelial cells in the glomerulus promotes better maintenance and repair
of these tissues (Tasnim and Zink 2012). While the integrity to stop very large solutes crossing the glomerular barrier is intact, the data presented here shows no difference in the clearance of the filtered protein GFP-D3 from \textit{ocrl}^{-/-} embryos as compared with wild type embryos.

5.12.3 Design considerations for GFP-D3 and NL-D3 zebrafish expression constructs
There are a number of inducible experimental systems that have been designed for use in zebrafish as an alternative to constitutive expression. GFP-D3 and NL-D3 were expressed constitutively, as disadvantages of inducible systems outlined below meant that no currently available inducible system was appropriate for this study. Spatial and temporal control of gene expression using the GAL4-UAS system has been adapted for use in zebrafish (Halpern et al. 2008, 4). However leaky expression using the GAL4-UAS system has been documented (Rodríguez et al. 2011), and the requirement for crossing multiple transgenic lines to induce protein production is more suited to the expression of harmful genes. Alternatively, a mifepristone inducible system has been devised in zebrafish (Emelyanov and Parinov 2008), where the addition of mifepristone induces protein expression after about 9 hours. However, expression can still be seen over 5 days after removal of the mifepristone signal. Loose temporal control such as this is unlikely to be of any consequential benefit in expressing our renal transporter, as the time taken to induce expression is too high. Further, as the ultimate goal is to create a drug screening protocol, the addition of mifepristone as well as other compounds may interfere in unknown ways, and therefore is to be avoided. A zebrafish heatshock promoter is also available, which gives much more rapid induction than the mifepristone system (Shoji and Sato-Maeda, 2008). With the heatshock promoter, induction of a transgenic protein can be seen immediately post treatment, and persists for at least 6 hours. After 15 hours, there is still significant amounts of protein present. Heatshock promoters in zebrafish have also been shown to be expressed under non-stressed conditions (Krone et al, 2003). Due to this leaky expression, heatshock is unsuitable for use for the GFP-D3 and NL-D3 lines, as any non-induced expression of the protein could alter the results of the screen.

We decided to use the modular Gateway system of expression (Katzen 2007; Kwan et al. 2007; Love et al. 2011), under the Ifabp10 promoter which has been described previously (Her et al. 2003; Zhou and Hildebrandt 2012). While this does not allow temporal control to the user, the Ifabp10 promoter is known to begin
expressing around 32-40 hpf and continue throughout embryonic development (Mesens et al. 2015; Her et al. 2003), timings which coincide with the onset of glomerular filtration and renal reabsorption. An additional benefit of the Gateway system is its modularity (Love et al. 2011). If future requirements change, each of the three modular components can be recombined with other modules (for instance, to express GFP-D3 under a different promoter, or to express a different reporter under the Ifabp10 promoter) to quickly generate new expression plasmids for injection.
Chapter 6: Characterisation of the OCRL1 interacting protein IPIP27A in zebrafish
6.1 Introduction

To understand the function of OCRL1 in vivo, it is important to consider the role of its interaction partners to fully elucidate the mechanisms by which OCRL1 activity is regulated within the cell. One such interaction partner is IPIP27A, a recently identified binding partner of OCRL1 (Noakes et al. 2011; Swan et al. 2010). IPIP27A binds to OCRL1 via a conserved F&H motif near the C terminus of IPIP27A, and is localised to early endosomes, recycling endosomes and the TGN, indicating a role in endocytosis (Noakes et al. 2011; Pirruccello et al. 2011; Swan et al. 2010). Indeed, transferrin receptor recycling to the plasma membrane, and CIMPR trafficking from the recycling endosome to the TGN are defective in cells depleted of IPIP27A (Noakes et al. 2011). A related protein, IPIP27B has also been identified that can bind to OCRL1 and IPIP27A, however its function is currently unknown (Noakes et al. 2011; Swan et al. 2010) A zebrafish mutant of IPIP27A, ipip27a<sup>−/−</sup>, has been generated using TALENS to disrupt exon 1 of the ipip27a gene that demonstrates reduced endocytic uptake of 10 kDa dextran and redistribution of megalin from the apical membrane to endosomes, similar to that seen in ocr1<sup>−/−</sup> zebrafish (Oltrabella 2014). IPIP27A has also been shown to bind via a proline-rich motif to the SH3 containing proteins ABI1, CD2AP, MYOSIN1E and PACSIN2 (Noakes 2011; Billcliff et al. 2016), all of which function in the endocytic pathway and associate with the actin machinery (Cheng et al. 2012; Kirsch et al. 1999; Kostan et al. 2014; Ouderkirk and Krendel 2014). Therefore, IPIP27A represents an excellent candidate for directly linking OCRL1 to the actin machinery that functions in endocytic trafficking.

Thus far, the ipip27a<sup>−/−</sup> mutant zebrafish pronephric tubule has demonstrated reduced pronephric uptake of 10 kDa dextran, altered megalin distribution to the vacuolar endosome, a disorganisation of pronephric cilia and an atypical association of enlarged vacuolar endosomes with lysosomes (Oltrabella 2014). However, further characterisation of the ipip27a<sup>−/−</sup> mutant zebrafish is required to determine if the phenotypes displayed are caused by perturbation of endocytic trafficking in the proximal tubule, or whether the phenotype is related instead to the defective cilia. Therefore, this chapter investigates further the role of ipip27A in zebrafish in relation to its renal phenotype.
6.2 General morphology of *ipip27a*<sup>−/−</sup> mutant embryos

*ipip27a*<sup>−/−</sup> mutants at 24 hpf displayed a 20% reduction in size compared to wild type embryos at 24 hpf (Oltrabella 2014), which has also been observed in this study (data not shown). To determine whether this reduction in size is present only in early development or whether this is a more sustained developmental delay in growth of the embryo into an adult zebrafish, juvenile *ipip27a*<sup>−/−</sup> and wild type fish were grown over 10 weeks and their length from the nose to the base of the tail was measured (Figure 34A) at week five and week ten of development. There was no significant difference in the growth of *ipip27a*<sup>−/−</sup> mutant zebrafish at either five or 10 weeks post fertilisation to wild type fish (Figure 34B, C), demonstrating that the reduction in size evident in *ipip27a*<sup>−/−</sup> mutant embryos at 24 hpf in Oltrabella (2014) is not a sustained developmental delay through the juvenile phase of development.

The defect in proximal tubular uptake of 10 kDa dextran reported in *ipip27a*<sup>−/−</sup> mutant embryos at 3 dpf is indicative of an endocytic defect, however it may also be caused by glomerular filtration defects. Furthermore, incorrect polarity of the tubule epithelium would result in the incorrect localisation of apical proteins such as megalin, which would also abrogate endocytic uptake. It is also possible that defective development or maintenance of the primary cilium may cause a reduction in fluid flow sensing by the primary cilium, which can reduce endocytosis rates (Raghavan et al. 2014). An established assay of glomerular integrity can be performed in which 500 kDa dextran, which is too large to filter, is injected into the cardinal vein, and the level of fluorescence in the embryo measured 24 hours later (Kramer-Zucker, Wiessner, et al. 2005). In embryos were glomerular integrity is compromised, leakage of the high molecular weight dextran results in a reduction in vascular fluorescence and, if uptake is functional in the proximal tubule, visible fluorescent puncta accumulating in the pronephric duct. To demonstrate that the glomerular basement membrane integrity was not compromised, wild type and *ipip27a*<sup>−/−</sup> embryos were injected with 500 kDa (Figure 35A) fluorescent dextran and imaged 24 hours later to measure the level of fluorescence in the vasculature. Both wild type and *ipip27a*<sup>−/−</sup> embryos retained the 500 kDa dextran in the blood stream for 24 hours, and in neither wildtype nor *ipip27a*<sup>−/−</sup> embryos was there any dextran puncta visible in the renal tubule, demonstrating that the glomerular filtration barrier is not compromised in *ipip27a*<sup>−/−</sup> embryos (Figure 35B).
To investigate whether tubule polarity is disrupted in ipip27a<sup>−/−</sup> embryos, transverse sections through the pronephros of 3 dpf wild type and mutant embryos were stained for the apical membrane marker zona occuldens-1 (ZO-1), and the basolateral marker α6F, an antibody that recognises sodium/potassium-transporting ATPase subunit alpha-1. In wild type sections, the ZO-1 staining successfully marks the apical border enclosing the lumen of the proximal tubule, and the α6F clone successfully marks the basolateral membrane of the proximal tubular cells. In ipip27a<sup>−/−</sup> mutants, the staining pattern matches that of the wild type indicating that proximal tubule cell polarity is not altered by ipip27a mutation (Figure 36).

6.3 Proximal tubules are dilated in ipip27a<sup>−/−</sup> embryos

The disorganisation of the primary cilia reported in ipip27a<sup>−/−</sup> mutants suggests that mutation of ipip27a may disrupt the function of the cilia (Oltraballa 2014). In
zebrafish embryos with ciliation impairment, tubular dilation can be observed, likely due to an overproliferation of tubular epithelial cells (Sullivan-Brown et al. 2008). To determine whether loss of IPIP27A leads to dilation of the proximal tubules, 3 dpf wild type and ipip27a⁻/⁻ embryos were fixed and wholemount staining was performed for the kidney specific marker 3G8. Embryos were then imaged using confocal microscopy to generate a maximum projection through the renal tubule in a frontal plane (Figure 37A). Proximal tubule lumen diameter was measured using ImageJ by measuring the width of the 3G8 staining in the tubule immediately behind the first loop in the tubule (Red arrow, Figure 37A, B). Distal tubule lumen diameter was ascertained by measuring the diameter of the tubule at the most proximal straight portion at the midline of the body (Blue arrow, Figure 37A, B).

Figure 35: Glomerular integrity is not compromised in ipip27a⁻/⁻ mutant embryos.

(A) Zebrafish injected with 500 kDa fluorescent dextran at 72 hpf. (B) Wild type and (C) ipip27a⁻/⁻ embryos were imaged 24 hours post injection using a fluorescence dissecting microscope.
Proximal tubule lumen diameter in wild type fish was on average 18.95 µm ± 2.9 µm (n=26, Figure 37C). In ipip27a⁻/⁻, the proximal tubule diameter was increased significantly, with an average diameter of 26.92 µm ± 3.7 µm (n=22). Diameter of the distal tubule in wild type embryos was 14.36 µm ± 1.59 µm (Figure 37D). In ipip27a⁻/⁻ mutants, there was no difference in distal tubular diameter, with an average diameter of 14.26 µm ± 2.63 µm. This result suggests that there is dilation of the proximal tubule of the ipip27a⁻/⁻ mutants, which is consistent with the possible cilia disorganisation phenotype recorded in Oltrabella (2014). The dilation is restricted to the proximal tubule.

6.4 Uptake of Cy3-RAP is defective in the pronephric tubules of ipip27a⁻/⁻ embryos

A reduction in the proximal tubular uptake of 10 kDa dextran has been reported in ipip27a⁻/⁻ embryos (Oltrabella 2014). As megalin is also redistributed to vacuolar endosomes in ipip27a⁻/⁻ embryos (Oltrabella 2014), the megalin specific ligand Cy3-RAP was injected into zebrafish embryos to determine if the uptake of Cy3-RAP was impaired in the renal tubule, thus testing a specific impairment of the uptake of LMW proteins. Four dpf wild type and ipip27a⁻/⁻ embryos were injected with Cy3-RAP into the cardinal vein, and imaged 40 minutes post injection to assess
renal tubular uptake. In wild type embryos, around 60% of embryos demonstrated uptake of Cy3-RAP into the proximal tubule (Figure 38), which is similar to results for Cy3-RAP uptake in wild type embryos reporter in section 5.4. In ipip27a<sup>−/−</sup> mutant embryos, Cy3-RAP uptake was reduced, and out of 63 embryos uptake was seen in only 1 embryo. This clearly demonstrates that megalin mediated endocytosis in the proximal tubule is strongly reduced in ipip27a<sup>−/−</sup> embryos, demonstrating the critical role that ipip27a plays in the apical endocytosis of LMW proteins.
6.5 Interaction with OCRL1 and SH3 containing proteins is required for IPIP27A function in zebrafish.

The interactions of IPIP27A with OCRL1 and SH3 containing proteins such as PACSIN2 are likely to be critical to its correct function, however how the interaction of IPIP27A with its binding partners contributes to its function is not yet understood. To determine whether the binding of OCRL1 or SH3 containing proteins is required for IPIP27A function in endocytosis in the renal tubule, the F&H motif and proline rich motif, responsible for OCRl1 and PACSIN2 binding respectively, were mutated (Figure 39) and re-expressed in ipip27a−/− mutant embryos under the pronephric tubule specific promoter enpep. Constructs where the F&H motif and PPRR motif responsible for binding to OCRL1 and PACSIN2 respectively, were produced by

![Figure 38: Quantification of Cy3-RAP uptake in wild type and IPIP27A−/− embryos.](image)

(A) Graph shows the distribution of percentage of animals able to accumulate high, low and no Cy3 for each condition at 4 dpf. N numbers are total number of embryos of the specified genotype used over 3 in total over 3 independent experiments. Significance was calculated using chi squared test. Data are mean ± SEM. *** = p > 0.001. (A) Representative images of pronephric tubules 1 hour post injection of Cy3-RAP in each condition. Images have been colour inverted for clarity. Red dashed lines represent renal tubule. Scale bar is 50 µm.
mutating critical amino acids in these motifs to alanine (Figure 39B, C). As the *ipip27a*−/− mutant zebrafish has a strong endocytic impairment in the renal tubule, this phenotype was used as a readout of function of IPIP27A. The *ipip27a*−/− mutant fish was rescued by re-expressing wild type IPIP27A protein or a mutant that lacks either a functional F&H motif (ΔF&H) or a PPRR motif (ΔPPRR) under a renal specific promoter by injection of DNA constructs and Tol2 RNA. Expression of the construct in injected embryos was confirmed by rtPCR from cDNA synthesised from whole embryo mRNA extraction (Figure 40). The mutation in the *ipip27a*−/− mutant line destroys an MwoI restriction site in exon one of the *ipip27a* gene. Therefore, digestion of the PCR product from this rtPCR can be used to identify the wild type
mRNA samples from wild type, ipip27a<sup>−/−</sup> mutants, and mutants with pronephric tubule specific re-expression of an ipip27a construct was collected and rtPCR used to determine the presence or absence of wild type mRNA. Genomic DNA was also run alongside as a control for the size of ipip27a mRNA cDNA products (right). Expression levels of the mutant mRNA (top band, 400 bp and 376 bp) were equivalent for both ipip27a in ipip27a<sup>−/−</sup> mutants, and mutants with re-expressed ipip27a. The loading control ef1α was expressed at equivalent levels in all cDNA samples. Data from only one experiment, therefore needs repeating. Weak lower band from whole embryo lysates in rescued embryos is a result of expression of the rescue construct only in the renal tubule.

(cut) cDNA in a distinct band from the mutant (uncut) cDNA. In each rescue experiment we detected a weaker additional band running at the same size as wild type ipip27a in whole embryo mRNA extraction, consistent with re-expression of this construct in the renal tubule. Importantly, the intensity of this band was similar in all three rescue conditions, demonstrating an equivalent level of expression of each construct.

Re-expression of wild type ipip27a demonstrated a rescue of uptake of 10 kDa dextran to near wild type levels (Figure 41), confirming the specificity of the endocytic phenotype in the ipip27a<sup>−/−</sup> mutant. Strikingly, neither re-expression of ΔF&H or ΔPPRR mutants of ipip27a were able to rescue the endocytic defect in the renal tubule. This strongly suggests that endocytosis in the proximal tubule requires IPIP27A interaction with both OCRL1 and its SH3 domain containing binding partners, most likely PACSIN2 which is known to function with IPIP27A in endocytic recycling in mammalian cells (Billcliff et al. 2016).
Figure 41: See next page for legend
6.6 Removal of functional OCRL1 and IPIP27A in zebrafish results in a more severe phenotype than either mutation alone.

Mutation of OCRL1 and IPIP27A both give similar phenotypes of reduced pronephric uptake of 10 kDa dextran and reduced megalin expression at the apical membrane and mislocalisation of megalin to apical endosomes (Oltrabella 2014; Oltrabella et al. 2015), and interaction of these proteins is critical to their function (section 6.5). Neither of these mutations block endocytosis completely, so if they act together in the endocytic pathway its likely that a reduction in both of these proteins may result in a more severe impairment of proximal tubular endocytosis. To explore this further, wild type, ocrl1\(^{-/-}\) and ipip27a\(^{-/-}\) embryos were injected with either control morpholino, or morpholino to either ipip27a or ocrl as described in Table 11, as a preliminary study to mimic the effects of a double knockout.

Table 11: Morpholino injection used in each condition.

<table>
<thead>
<tr>
<th>Embryo strain</th>
<th>Morpholino injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Control</td>
</tr>
<tr>
<td>Wild type</td>
<td>OCRL1</td>
</tr>
<tr>
<td>Wild type</td>
<td>IPIP27A</td>
</tr>
<tr>
<td>ipip27a(^{-/-})</td>
<td>Control</td>
</tr>
<tr>
<td>ipip27a(^{-/-})</td>
<td>OCRL1</td>
</tr>
<tr>
<td>ocrl1(^{-/-})</td>
<td>Control</td>
</tr>
<tr>
<td>ocrl1(^{-/-})</td>
<td>IPIP27A</td>
</tr>
</tbody>
</table>

Figure 41: Re-expression of wild type ipip27a, but not ΔF&H ipip27a or ΔPPRR ipip27a, is able to rescue the endocytic impairment of 10 kDa dextran in ipip27a\(^{-/-}\) embryos.

(A) Graph showing the percentage of embryos showing either no, low or high uptake in each of the experimental and control conditions. Re-expression of wild type ipip27a in the pronephric tubule restored proximal tubular endocytosis of 10 kDa dextran in 4 dpf ipip27a\(^{-/-}\) mutant embryos. Expression of neither binding site mutation in ipip27a\(^{-/-}\) mutant embryos was able to restore 10 kDa dextran endocytosis. N numbers are total embryos used for each condition over 3 repeats. Data are mean ± SEM. *** p<0.001. (B) Representative images showing the accumulation of 10 kDa dextran, or lack of accumulation of 10 kDa dextran, in the tubule (white outline) in each condition. Scale bar is 50 µm.
Developing embryos were monitored for gross morphological phenotypes at 24 hpf and 72 hpf. At 24 hpf, mutant embryos depleted of either ocr11 and ipip27a with morpholino demonstrated a developmental delay as compared to the mutant embryos injected with control morpholino (Figure 42). At 72 hpf, there was hindbrain oedema in both morpholino injected mutant conditions, but not in ocr11<sup>−/−</sup> or ipip27a<sup>−/−</sup> mutants injected with control morpholino (Figure 43A, C). The body axis of morpholino injected mutant embryos was also disrupted (Figure 43B, C).

At 4 dpf, embryos of all conditions were injected with 10 kDa dextran to assess the proximal tubular uptake. Ocr11<sup>−/−</sup> and ipip27a<sup>−/−</sup> mutants were defective in proximal tubular uptake of 10 kDa dextran as previously described (Oltrabella 2014; Oltrabella et al. 2015). Similarly, wild type embryos injected with either ocr11 or ipip27a morpholino displayed a comparable defect in uptake of 10 kDa dextran (Figure 43D). Morpholino injected mutant embryos displayed a more severe loss of 10 kDa dextran uptake than either mutant on its own, with significantly fewer embryos able to accumulate any 10 kDa dextran in the renal tubule. This demonstrates that reduction of both proteins leads to a more significant impairment of renal endocytosis, consistent with both proteins act together in endocytic trafficking in the renal tubule.

### 6.7 Trafficking and lysosomal gene expression is altered in ipip27a<sup>−/−</sup> and ocr11<sup>−/−</sup> embryos.

The association of enlarged apical vacuolar endosomes with lysosomes in ipip27a<sup>−/−</sup> mutant embryos observed in electron microscopy is a particularly striking phenotype, which suggests a role for ipip27a at the lysosome (Oltrabella 2014). Furthermore, it has been demonstrated that ipip27a is co-fractionated on trafficking intermediates containing CIMPR and lysosomal hydrolases (Borner et al. 2014), and IPIP27A depleted cells have impaired trafficking of CIMPR and lysosomal hydrolases (Noakes et al. 2011), suggesting ipip27a is required for the trafficking of lysosomal hydrolases to the lysosome. In both ocr11<sup>−/−</sup> and ipip27a<sup>−/−</sup> mutant zebrafish embryos, an increase in both size and quantity of lysosomes has been reported (Oltrabella 2014). In addition to this, a role for OCRL1 has recently been proposed in autophagosome-lysosome fusion (De Leo et al. 2016). De Leo et al (2016) shows that PtdIns(4,5)P<sub>2</sub> production at the lysosomal membrane is required for fusion of autophagosomes, and OCRL1 is recruited in an AP-2/Cathrin dependent manner to...
remove this PtdIns(4,5)P₂, to spatially and temporally control its presence at the lysosome. Depletion of OCRL1 causes PtdIns(4,5)P₂ accumulation on the lysosome, which inhibits the lysosomal calcium channel mucolipin-1 (MCOLN1), which is also required for lysosomal fusion. In addition to this direct role in lysosomal fusion, loss of OCRL1 (either by depletion in cells, in Lowe syndrome fibroblasts or in ocrl¹⁺⁻ mutant embryos) results in upregulation of the TFEB transcription factor, which controls lysosomal biogenesis (De Leo et al. 2016).

To determine if lysosome biogenesis is affected in ipip27a⁻⁻ and ocrl¹⁻⁻ mutant zebrafish at a transcriptional level, the relative expression of genes encoding lysosomal hydrolases and lysosomal transporters that co-fractionate with IPIP27A on endosomes in Borner et al (2014) was determined by rtPCR. In addition to this, to identify possible compensatory responses of zebrafish cells to the loss of ipip27a or ocrl1, the effect on expression of relevant endocytic machinery proteins was also assessed by rtPCR. Genes assessed are listed in Table 12.

Figure 42: Developmental delay at 24 hpf in ocrl¹ morphant ipip27a⁻⁻ mutant embryos, and ipip27a morphant ocrl¹⁺⁻ embryos.

Representative images of 24 hpf embryos, either ipip27a⁻⁻ or ocrl¹⁻⁻ mutants injected with control morpholino (top row), or ipip27a⁻⁻ embryos injected with ocrl morpholino (bottom left) or ocrl¹⁻⁻ mutant embryos injected with ipip27a morpholino (bottom right). Scale bar is 500 µm.
Figure 43: Zebrafish embryos lacking function ipip27a and ocr11 demonstrate hindbrain oedema, curved tails and a more severe impairment of 10 kDa dextran reabsorption in the renal tubule.

(A) Representative images showing the hindbrain oedema (red dotted line) in ipip27a\(^{-/-}\) mutants and ocr11\(^{-/-}\) mutants injected with ocr1 or ipip27a morpholino respectively. Scale bar is 100 µm. (B)
Showing the curved body axis present in \textit{ipip27a}\textsuperscript{-/-} mutants and \textit{ocr11}\textsuperscript{-/-} mutants injected with \textit{ocr11} or \textit{ipip27a} morpholino respectively, but not in either mutant injected with control morpholino. Scale bar is 500 µm. (C) Graph showing the percentage of embryos with hindbrain oedema or curved tail in each condition in one individual experiment. (D) Graph showing the percentage of embryos showing no, low or high uptake of 10 kDa dextran in each condition. Data are mean ± SEM. * \( p<0.05 \), ** \( p<0.01 \). N numbers are total embryos over 2 repeats.

**Table 12: Lysosomal hydrolase and membrane trafficking related proteins assayed for altered expression in \textit{ipip27a}\textsuperscript{-/-} and \textit{ocr11}\textsuperscript{-/-} mutant embryos**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
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</thead>
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<tr>
<td></td>
<td><strong>Lysosomal hydrolase genes</strong></td>
<td></td>
</tr>
<tr>
<td>\textit{abca2}</td>
<td>ATP binding cassette A2</td>
<td>ATP binding cassette transporter</td>
</tr>
<tr>
<td>\textit{ctsc}</td>
<td>Cathepsin C</td>
<td>Lysosomal cysteine proteinase</td>
</tr>
<tr>
<td>\textit{dnase2}</td>
<td>Deoxyribonuclease 2</td>
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</tr>
<tr>
<td>\textit{hexa1}</td>
<td>Hexosaminidase subunit 1 alpha</td>
<td>Lysosomal glycosyl hydrolase</td>
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<tr>
<td>\textit{tpp1}</td>
<td>Tripeptidyl peptidase 1</td>
<td>Lysosomal exopeptidase</td>
</tr>
<tr>
<td></td>
<td><strong>membrane-trafficking associated genes</strong></td>
<td></td>
</tr>
<tr>
<td>\textit{dennd1a}</td>
<td>DENN domain containing protein 1A</td>
<td>Rab35 GEF</td>
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<tr>
<td>\textit{igf2r} (\textit{cimpr})</td>
<td>Insulin like growth factor 2 receptor</td>
<td>Intracellular trafficking of lysosomal proteins</td>
</tr>
<tr>
<td>\textit{inpp5b}</td>
<td>Inositol polyphosphate-5-phosphatase B</td>
<td>PtdIns(4,5)P\textsubscript{2} 5-phosphatase</td>
</tr>
<tr>
<td>\textit{ipip27a}</td>
<td>Inositol polyphosphate interacting protein of 27 kDa A</td>
<td>Endocytic protein that binds to OCRL1</td>
</tr>
<tr>
<td>\textit{ipip27b}</td>
<td>Inositol polyphosphate interacting protein of 27 kDa A</td>
<td>Endocytic protein</td>
</tr>
<tr>
<td>\textit{lrp2}</td>
<td>Low density lipoprotein-related protein 2 (Megalin)</td>
<td>Scavenger receptor for plasma membrane – endosome transport</td>
</tr>
<tr>
<td>\textit{m6pr}</td>
<td>Mannose-6-phosphate receptor</td>
<td>Golgi-lysosome trafficking protein</td>
</tr>
<tr>
<td>\textit{pacsin2}</td>
<td>Protein kinase C and casein kinase substrate in neurons 2</td>
<td>Links actin polymerisation to tubule formation in endocytosis</td>
</tr>
</tbody>
</table>
Batches of 30 4dpf wild type, *ipip27a*−/− and *ocr1*−/− embryos were collected from three individual crosses of the parent fish. cDNA was synthesised from mRNA extracted from each clutch and PCR for each gene was performed using the primers outlined in Appendix 1. Semi-quantitative analysis was performed by measuring the band intensities of the rtPCR products using ImageJ. For each gene, expression in wild type batches was normalised to 100%. Then, for each mutant batch, percentage change from wild type was calculated. In the three batches of wild type embryos, gene expression did not vary between batches by more than 10% for any gene (Figure 44C, 45C). In both *ipip27a*−/− and *ocr1*−/− embryos, levels of the control gene, *ef1α*, were comparable to the expression in wild type embryos (Figure 44A, B).

The lysosomal hydrolase *dnase2* was significantly increased in *ocr1*−/− mutant embryos compared to wild type control embryos (Figure 44A). Smaller increases in the expression of other lysosomal hydrolases *ctsc, hexa1* and *tpp1* were also present, however these increases were not large enough to reach significance in this experiment. Expression of the lysosome-associated transporter *abca2* was also significantly upregulated in *ocr1*−/− mutants, which is consistent with the upregulation of lysosome biogenesis described in OCRL1 depleted cells (De Leo et al. 2016). Increases in the expression of *abca2* and *dnase2* were also found in *ipip27a*−/− mutant embryos (Figure 44B), which suggests lysosomal biogenesis may also be upregulated in *ipip27a*−/− mutant
embryos. The level of increase in expression of abca2 and dnase2 in ipip27a<sup>−/−</sup> and ocr1<sup>−/−</sup> mutant embryos is similar (Figure 44D), suggesting that there is likely a
common mechanism leading to the increased expression of these proteins in both mutant lines.

The lysosomal hydrolase trafficking receptor \textit{igf2r} expression is significantly upregulated in \textit{ocrl1}−/− (Figure 45A) and \textit{ipip27a}−/− (Figure 45B) mutant embryos. This is consistent with the defect in lysosomal hydrolase delivery previously described in both OCRL1 and IPIP27A depleted cells (Choudhury et al. 2005; Noakes et al. 2011). Interestingly, expression of \textit{ipip27a} is upregulated in both \textit{ocrl1}−/− and \textit{ipip27a}−/− mutants. In \textit{ipip27a}−/− mutants, there is also a significant increase in the expression of \textit{stx8}. STX8 is a component of the SNARE complex required for fusion of late
endosomes (Prekeris et al. 1999), suggesting that homotypic fusion of late endosomes may also be impaired in *ipip27a*⁻/⁻ mutant embryos. The expression of many of the other membrane trafficking genes assessed appeared to be increased or decreased compared to wild type levels, however did not reach significance (Figure 45A, B). This suggests there may be subtle transcriptional changes to many membrane trafficking associated genes in response to either *ocrl1* or *ipip27a* mutation. Comparison of the expression of the genes quantified between *ocrl1*⁻/⁻ and *ipip27a*⁻/⁻ embryos demonstrates that many of membrane trafficking associated genes are upregulated to similar levels in both mutants (Figure 45D). Interestingly, it appears that *ipip27b* is upregulated in *ocrl1*⁻/⁻ mutants, but down regulated in *ipip27a*⁻/⁻ mutants, however the reason for this is not clear. Similarly, *Pacsin2* is also upregulated in *ocrl1*⁻/⁻ mutants but downregulated in *ipip27a*⁻/⁻ mutants.

6.8 Discussion

An approximately 20% reduction in size of *ipip27a*⁻/⁻ mutant zebrafish has been recorded up to 3 dpf (Oltrabella 2014). The mechanism behind this developmental delay is currently not known. The reduction in size of embryos reported in Oltrabella (2014) has also been seen during this work (data not shown). This chapter has demonstrated that while a reduction in size exist at ≤ 3 dpf, there is no effect on the length of embryos in juvenile development at five weeks or 10 weeks of age. Oltrabella (2014) reports a potential cytokinesis delay in early cell divisions of *ipip27a*⁻/⁻ embryos. Potentially, cytokinesis delay at this early stage may result in delayed development of the early embryo, which could account for the reduced size in ≤ 3dpf *ipip27a*⁻/⁻ mutants. Additionally, some *ipip27a*⁻/⁻ mutant embryos may not be able to overcome this cytokinesis phenotype, which would explain the ~25% increase in death of embryos before 24 hpf seen in *ipip27a*⁻/⁻ embryos (data not shown). Why this delay does not translate into a reduced length in five and 10 week old juvenile fish is not clear, however it could be that surviving mutants are able to compensate somehow for the lack of *ipip27a* and therefore catch up to their wild type counterparts.

Typically, defects in the development or function of primary cilia in zebrafish result in a curved body axis, hydrocephalus, oedema, renal cysts, dilated pronephric tubules and dysregulated left-right symmetry (Austin-Tse et al. 2013; Tobin and Beales 2009; Zhao and Malicki 2007). In contrast to mammals, zebrafish pronephric tubular cells possess motile cilia, beating of which is required for fluid flow through the pronephric duct (Kramer-Zucker et al. 2005; Zhao and Malicki 2007). In *ipip27a*⁻/⁻
mutant fish, primary cilia in the pronephric tubule are disorganised, and there is an increase in the size of the brain ventricle at 24 hpf consistent with hydrocephaly (Oltrabella 2014). The hindbrain oedema at 24 hpf was also evident in ipip27a^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^
Cilia function display hydrocephaly due to loss of fluid flow in the brain ventricle (Kramer-Zucker et al. 2005). Knowing that mutations in ocr1 and ipip27a in zebrafish result in disorganisation of primary cilia (Oltrabella 2014; Oltrabella et al. 2015), defective cilia in the hindbrain is therefore the most likely explanation for the hydrocephaly observed in embryos lacking functional OCRL1 and IPIP27A. Further study of primary cilium morphology and function in embryos lacking both OCRL1 and IPIP27A is required to confirm this hypothesis. Zebrafish lacking both OCRL1 and IPIP27A also display a more extreme impairment in proximal tubular endocytosis of 10 kDa dextran. Proximal tubular dilation and renal cysts in zebrafish ciliopathies are thought to result in reduced flow through the renal tubule (Tobin and Beales 2009). It is possible that reduced flow caused by tubular dilation may be a possible explanation for the severe impairment of 10 kDa dextran uptake in the renal tubule of embryos lacking both functional OCRL1 and IPIP27A. Establishing if there is delayed clearance of a fluorescence reporter, alongside staining for acetylated tubulin to measure the length and organisation of the cilia in the renal tubule in embryos lacking functional OCRL1 and IPIP27A would demonstrate if this hypothesis is correct. A more likely hypothesis is that the compound effect of removing OCRL1 and IPIP27A from proximal tubular cells results in a more substantial loss of megalin mediated endocytosis than loss of either protein alone. This could be supported by measuring the megalin subcellular distribution and presence of other endocytic machinery and compartments in the proximal tubular cells. These experiments were performed using a hypomorphic ocr1\(^{-/-}\) mutant line and morpholinos, which do not result in complete knockdown of the target protein. Therefore this interpretation is based on the assumption that the hypomorphic ocr1\(^{-/-}\) line does indeed display true symptoms of Lowe Syndrome, as has been described previously (Ramirez et al 2012, Oltrabella et al 2015). Based on this preliminary work, crossing zebrafish to create offspring that are null for both ocr1 and ipip27a and performing the same analysis may generate more conclusive data.

Uptake of both 10 kDa dextran and Cy3-RAP are impaired in ipip27a\(^{-/-}\) mutant embryos at 4 dpf. This phenotype is consistent with the impaired uptake of 10 kDa dextran and Cy3-RAP in ocr1\(^{-/-}\) mutant embryos (Oltrabella et al. 2015), indicating that OCRL1 and IPIP27A are both required for endocytosis in the renal tubule. Further, this thesis has shown that ipip27a mutant protein that is unable to bind OCRL1 or SH3 containing proteins such as PACSIN2, cannot rescue the endocytic defect in the zebrafish proximal tubule, in agreement with cell culture data from Noakes et al (2011). While clearance was not measured during this study,
confirming that glomerular clearance of 10 kDa dextran or other reporters is efficient is an important step in confirming that this is a tubular reabsorption defect. Based on the assumption of efficient glomerular filtration, this demonstrates that IPIP27 must bind OCRL1 and SH3 containing proteins such as PACSIN2, which has been shown to be required in trafficking carrier biogenesis (Billcliff et al. 2016). Based on the data in Billcliff et al (2016) and the data presented in this thesis, it could be proposed that IPIP27A, OCRL1 and PACSIN2 associate to make recycling trafficking intermediates containing megalin. Alternatively, it is possible that IPIP27A, OCRL1 and PACSIN2 may also be associating to function in endocytosis at the plasma membrane. Further investigation is required to identify the exact mechanism by which this interaction regulates membrane trafficking.

Defects in sorting of lysosomal hydrolases in IPIP27A knockdown cells (Noakes et al. 2011), and the association of lysosomes with apical vacuolar endosomes in ipip27a−/− mutant embryos (Oltrabella et al. 2015) strongly suggests a role for IPIP27A in lysosomal cargo sorting. Indeed, proteomic analysis of endosome fractions containing IPIP27A demonstrates a large number of lysosomal hydrolases closely associated with IPIP27A (Borner et al. 2014). In the proximal tubule, lysosomal hydrolases are internalised via megalin mediated endocytosis from the plasma membrane (Nielsen et al. 2007). It is possible therefore that IPIP27A plays a direct role in the delivery of hydrolases to the lysosome. By semi-quantitative rtPCR, two of the five lysosomal hydrolase genes tested show significantly increased expression levels in ipip27a−/− embryos. This is potentially due to the cells upregulating expression of these genes in an effort to compensate for the defective delivery of hydrolases to the lysosome. Consistent with this defect, ipip27a−/− mutant embryos have over 2.5-fold higher expression of igf2r which is a major component of the TGN-endosome/lysosome transport pathway for lysosomal hydrolases. In cells depleted of IPIP27A, retrograde trafficking of IFG2R is defective (Noakes et al. 2011). Interestingly, the expression of another closely related TGN-endosome trafficking protein involved in the delivery of lysosomal hydrolases, M6PR, is not significantly upregulated in ipip27a−/− mutant embryos. IGF2R is a larger protein than M6PR and bind to more lysosomal hydrolases than its smaller counterpart (Varki and Kornfeld 2009), therefore it may be that the increased function of IGF2R is the reason for igf2r but not m6pr upregulation in ipip27a−/− mutant embryos. There is also an increase of around 50% in the expression of the early endosome to late endosome trafficking gene stx8. The delivery of lysosomal hydrolases to the early endosome by IFG2R then requires either maturation of the
endosome or trafficking from the early endosome to a maturing late endosome. STX8 is required as part of the SNARE complex for homotypic fusion of late endosomes (Kasai et al. 2007). The upregulation of *stx8* in *ipip27α−/−* mutant embryos could therefore also be in response to the defect in hydrolase delivery to the early endosome. Expression of *pacsin2*, that has recently been proposed to have a role in M6PR and IFG2R carrier biogenesis alongside OCRL1 and IPIP27A (Billcliff et al. 2016), is decreased slightly in *ipip27α−/−* embryos. Whether the variation in expression levels of these genes translates to altered protein levels in embryos remains to be elucidated. The many changes recorded in this analysis of *ipip27α−/−* embryos to the expression of lysosomal hydrolase and membrane trafficking genes suggest that the mutation in *ipip27α−/−* zebrafish does have an effect on the transcriptome of related proteins. To confirm that these genes are truly upregulated, validation with qPCR is required. A much wider range of genes could also be studied using full transcriptome analysis by RNAseq.

Interestingly, many of the genes upregulated in *ipip27α−/−* embryos are also upregulated to similar levels in *ocr1−/−* embryos too, supporting the hypothesis that OCRL1 and IPIP27A interaction are both for correct membrane trafficking, in agreement with reported defects in CI-MPR trafficking in Lowe syndrome fibroblasts and depleted cells (Billcliff et al. 2016; Choudhury et al. 2005; van Rahden et al. 2012). The upregulation of lysosomal related genes in both of these lines also supports the observations of De Leo et al (2016) that OCRL1 is required at the lysosome. De Leo and colleagues show that loss of OCRL1 results in an upregulation of the transcription factor TFEB, which is a master regulator of lysosomal gene expression by recognition of CLEAR motifs in the promoter region of genes (Sardiello et al. 2009). Interestingly, *igf2r, abca2* and *dnase2*, that are upregulated in both *ipip27α−/−* mutants and *ocr1−/−* are all TFEB target genes in humans (Brozzi et al. 2013; Sardiello et al. 2009), showing that TFEB may also be upregulated in *ocr1−/−* and *ipip27α−/−* mutant embryos. However, due to the lack of significant upregulation of other TFEB targets *ctsc, hexa1* and *tpp1* in either *ocr1−/−* or *ipip27α−/−* embryos, it remains possible that an alternative mechanism to TFEB upregulation is responsible for the upregulation of *igf2r, abca2* and *dnase2*. This data suggests that the requirement for OCRL1 at the lysosome may be IPIP27A dependent, however further work is required to establish the exact mechanism.
Chapter 7: Generating transgenic zebrafish lines expressing the endocytic compartment markers Rab5c, Rab7 and Rab11a
7.1 Introduction

Rab proteins (RAS oncogene family), are a family of >75 small GTPase proteins that regulate membrane trafficking, each with distinct functions, interaction partners and membrane localisation (Galvez et al. 2012; Stenmark 2009; Zhang et al. 2007). Recruitment of the correct subset of Rabs to a membrane is critical in the recruitment of effector proteins for the maturation, fission and fusion of intracellular compartments (Stenmark 2009). Due to this subcellular specificity, Rab proteins are very good markers of membrane compartments. Of particular interest for study of endocytosis, Rab5c, Rab7 and Rab11a are Rab proteins that localise to early, late and recycling endosomes respectively (Galvez et al. 2012).

Fluorescently tagged Rabs have been used in vivo in Drosophila and zebrafish to identify endocytic compartments (Zhang et al. 2007; Clark et al. 2011). Specifically in zebrafish, transgenic lines expressing N-terminally tagged GFP-Rab5c, Rab7 and Rab11a under the control of the ubiquitous histone promoter h2afx have been created and characterised in embryos up to 30 hpf (Clark et al. 2011). The h2afx promoter is only active during proliferation (Thisse and Thisse 2004), and at later stages of embryonic development (> 2dpf) expression of transgenic GFP-Rab proteins in these lines is downregulated (Clark et al. 2011).

To overcome this down regulation issue and generate transgenic Rab fusions to follow vesicle trafficking in embryos >2 dpf, transgenic lines expressing fluorescent Rab fusion proteins under the control of alternative promoters were generated. Like Clarke et al (2011), Rab5c was used as an early endosomal marker, as Rab5a is duplicated in zebrafish, and Rab5b is only expressed in a subset of tissues, while Rab5c is ubiquitously expressed (Thisse and Thisse 2004). Similarly, Rab7 and Rab11a also show ubiquitous expression in zebrafish (Thisse and Thisse 2004). To prepare for future experiments where multiple Rab transgenic fish may be crossed to study two or more endosomal compartments in the same fish, GFP and mApple tagged Rabs will be used. Two separate promoters were used to generate kidney specific and ubiquitous expression respectively. The enpep promoter has been shown to drive kidney specific expression in embryonic and adult zebrafish (Seiler and Pack 2011), and this was therefore used to express Rab proteins in the renal tubule. The Xenopus derived ef1α promoter has been used in zebrafish for ubiquitous expression of transgenes (Moon et al. 2013; Thummel et al. 2006). Our collaborator has cloned the zebrafish ef1α promoter and demonstrated strong ubiquitous expression of GFP throughout development up to 5 dpf (Adam
Hurlstone, personal communication), therefore to drive ubiquitous Rab expression the zebrafish *ef1a* promoter will be used.

### 7.2 mApple-tagged Rab5c, 7 and 11a mark the correct endosomal compartments in cell culture

Zebrafish Rab5c, 7 and 11a sequences were sub-cloned from vectors obtained from Clark et al (2011) into the mApple N1 vector previously published (Shaner et al. 2008). To determine whether these constructs localise to the correct compartments, either the parental mApple plasmid, or plasmids encoding mApple-Rab5c, mApple-Rab7 or mApple-Rab11a were transiently transfected into HeLaM cells. Fixed cells were then co-stained with antibodies against either EEA1 (early endosomal marker), CD63 (late endosomal marker) or TfR (recycling endosomal marker).

mApple alone, expressed from the parental vector was diffuse in the cytosol with some nuclear localisation, as expected (Figure 46, top row). Cells expressing mApple-Rab5c demonstrated a punctate staining with a high degree of colocalisation with EEA1 (Figure 46, second row). Partial overlap of mApple-Rab7 was seen with staining for CD63 in HeLaM cells (Figure 46, third row). There was also a high degree of colocalisation between TfR staining and mApple-Rab11a (Figure 46, bottom row). These data indicate that the mApple tagged Rab proteins are correctly localising to the expected compartments. This experiment was not performed for GFP-Rab fusions, because Clark et al (2011) has already determined that tagging with GFP does not affect the localisation of the zebrafish Rab proteins.

### 7.3 Generation of GFP and mApple tagged Rab5c, 7 and 11a expression constructs for use in zebrafish

To generate mApple and GFP tagged Rab5c, Rab7 and Rab11a constructs for expression in zebrafish, N-terminally mApple and GFP tagged coding sequences for each Rab was subcloned into the p3 plasmid of the Gateway system (Figure 47A, C). To generate a transgenic line with ubiquitous GFP-Rab expression, the GFP-Rab coding sequence was recombined into a zebrafish expression vector with a plasmid containing the *ef1a* promoter, and a plasmid containing the *γ-crytallin*:mCherry transgenesis marker gene, to identify transgenic animals (Offield et al. 2000), using Gateway recombination (section 3.3.13, Figure 47B). For mApple
tagged Rab expression, the kidney specific promoter enpep promoter, described previously (Seiler and Pack 2011), was cloned into the middle fragment p2 plasmid (Figure 47D). Finally, constructs were then injected into single cell stage zebrafish embryos alongside tol2 mRNA to drive integration. Adult founders were selected and bred with wild type fish to generate transgenic offspring. These offspring were then crossed again to wild type fish, and fluorescent embryos selected, to produce an adult transgenic F2 population. A summary of the lines created can be found in Table 13.

Figure 46: Fluorescently tagged Rab5c, Rab7 and Rab11a mark the expected compartments in HeLaM cells.

Representative images of cells expressing either unfused mApple, mApple-Rab5c, mApple-Rab7 or mApple-Rab11a. In each row, left hand image shows a compartmental marker for the early, late or recycling endosome. Middle image of each row shows the mApple-Rab expression, and the right hand image shows a merge to demonstrate colocalisation. Scale bar is 10 µm
Figure 47: Schematic representation of the donor and expression plasmids created for expression of fluorescently tagged Rab5c, Rab7 and Rab11a in zebrafish.

(A) Diagram of the three donor plasmids used to create ubiquitous GFP-Rab expression in zebrafish under the efta promoter. P3- plasmids contained either GFP-Rab5c, GFP-Rab7 or GFP-Rab11a cDNA. (B) A schematic representation of the final expression vector, containing all the components in series, as well as the SAR-CH4-I-SceI region for transgenic stabilisation and the Tol2 homology arms for genomic integration in zebrafish. (C and D) As above, for kidney specific expression of mApple-Rab5c, Rab7 and Rab11a under the control of the enpep promoter.
Table 13: Summary of transgenic endocytic marker lines made. Table shows the strain, fluorescent marker colour, expression pattern and number of independent lines studied for reference. For all lines, further founder fish are available to generate more independent lines where necessary.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colour</th>
<th>Expression</th>
<th>Rab</th>
<th>Number of lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>eif1a:GFP-Rab5c</td>
<td>Green</td>
<td>Ubiquitous</td>
<td>Rab5c</td>
<td>1</td>
</tr>
<tr>
<td>eif1a:GFP-Rab7</td>
<td>Green</td>
<td>Ubiquitous</td>
<td>Rab7</td>
<td>2</td>
</tr>
<tr>
<td>eif1a:GFP-Rab11a</td>
<td>Green</td>
<td>Ubiquitous</td>
<td>Rab11a</td>
<td>2</td>
</tr>
<tr>
<td>enpep:mApple-Rab5c</td>
<td>Red</td>
<td>Kidney only</td>
<td>Rab5c</td>
<td>1</td>
</tr>
<tr>
<td>enpep:mApple-Rab7</td>
<td>Red</td>
<td>Kidney only</td>
<td>Rab7</td>
<td>1</td>
</tr>
<tr>
<td>enpep:mApple-Rab11a</td>
<td>Red</td>
<td>Kidney only</td>
<td>Rab11a</td>
<td>1</td>
</tr>
</tbody>
</table>

7.4 Ubiquitously expressed GFP-tagged Rab5c, Rab7 and Rab11a mark punctate structures in zebrafish embryos

To begin characterising the expression of eif1a:GFP-Rab5c, eif1a:GFP-Rab7 and eif1a:GFP-Rab11a in zebrafish embryos fluorescence was assessed at 2 dpf and 4 dpf using stereomicroscopy. Stereomicroscopy was used to generate low magnification images of transgenic zebrafish embryos showing the expression throughout the embryo. Confocal microscopy was then used to visualise higher magnification images of punctate structures. Eif1a:GFP-Rab5c embryos at 2 dpf and 4 dpf show diffuse GFP staining throughout the embryo (Figure 48A,B). At 4 dpf, stronger staining is visible in the midbrain (Figure 48C), with small puncta visible in confocal sections. This demonstrates that GFP-Rab5c is being expressed in tissues throughout the embryo, and at higher magnification puncta consistent with GFP-Rab5c being on early endosomes can be seen.

In eif1a:GFP-Rab7 embryos, strong GFP-Rab7 expression throughout the whole embryo can be seen at both 2 dpf and 4 dpf (Figures 49A, B), and puncta are visible in the location and shape of the proximal tubule (Figure 49C). Expression of GFP-Rab7 is high in the notochord of 4 dpf transgenic embryos, with a strong
punctate pattern visible throughout the notochord (Figure 49D). Puncta in the

![Figure 48: Expression of GFP-Rab5c under the control of the *ef1α* promoter in 2 dpf and 4 dpf transgenic zebrafish embryos.](image)

(A) Stereomicroscope image of a 2 dpf transgenic *ef1α-GFP-Rab5c* embryo (scale bar 500 µm) with magnified views (scale bars 200 µm) of the mid-tail regions (lower left) and head region (right) of the embryo, showing diffuse GFP-Rab5c expression. (B) Same as above for 4 dpf embryos. Scale bars-upper left 1 mm, lower left and right, 200 µm (C) Confocal maximum projection through region of the midbrain, likely the optic tectum (white arrow), showing punctate GFP-Rab5c expression at the apical membrane. Scale bar 50 µm. (D) Magnified view showing the apical puncta (white arrow heads) in the midbrain. Scale bar 10 µm.
Figure 49: Expression of GFP-Rab7 under the control of the ef1α promoter in 2 dpf and 4 dpf transgenic zebrafish embryos.

(A) Stereomicroscope image of a 2 dpf transgenic ef1α-GFP-Rab7 embryo (scale bar 500 µm) with magnified views of the mid-tail regions (lower left) and head region (right) of the embryo, showing diffuse GFP-Rab7 expression (scale bars 200 µm). (B) Same as above for 4 dpf embryos. Scale bars top left 1 mm, bottom left and right 200 µm. (C) Magnified stereomicroscope view of the region of the renal tubule, showing puncta consistent with being expressed in the renal tubule (renal tubule shape shown in red dashed line). Scale bar 50 µm (D) Confocal image showing expression of GFP-Rab7, visible most prominently in the notochord (blue arrow), cloaca (white arrow) and distal tubule (red arrow). Scale bar 100 µm. Inset, magnified confocal image showing punctate GFP-Rab7 expression (white arrowheads) in the notochord. Scale bar 10 µm.
Figure 50: Expression of GFP-Rab11a under the control of the eflα promoter in 2 dpf and 4 dpf transgenic zebrafish embryos.

(A) Stereomicroscope image of a 2 dpf transgenic eflα-GFP-Rab11a embryo (scale bar 500 µm) with magnified views of the mid-tail regions (lower left) and head region (right) of the embryo, showing diffuse GFP-Rab11a expression (scale bars 200 µm). (B) Same as above for 4 dpf embryos. Scale bars top left 1 mm, bottom left and right 200 µm. (C) Magnified stereomicroscope view of the region of the renal tubule, showing puncta consistent with being expressed in the renal tubule. (renal tubule shape shown in red dashed line). Scale bar 50 µm (D) Confocal image showing expression of GFP-Ra11a7, visible most prominently in the notochord (blue arrow), cloaca (white arrow) and distal tubule (red arrow). Scale bar is 100 µm.
notochord of GFP-Rab7 transgenic embryos are approximately 2 µm in size (Figure 47D, inset), and are therefore likely late endosomes. High expression can also be seen in the DL segment of the proximal tubule and cloaca (Figure 49D). Expression in the alternative independent line showed similar pattern of expression, but lower fluorescence (data not shown).

_Ef1a::GFP-Rab11a_ transgenic embryos demonstrate a similar fluorescence pattern in 2 dpf and 4 dpf embryos as the GFP-Rab7 embryos. GFP-Rab7 fluorescence throughout the whole embryo is detectable via stereomicroscopy in both 2 dpf and 4 dpf embryos (Figure 50A, B), and punctate staining is detectable in the region of the proximal tubule (Figure 50C). GFP-Rab11a is also strongly expressed in the cells of the notochord, DL pronephric tubule and the cloaca (Figure 50D). Expression in the alternative independent line showed similar pattern of expression, but lower fluorescence (data not shown).

### 7.5 Kidney specific expression of mApple-tagged Rab5c, Rab7 and Rab11a is visible in proximal tubular cells

_Enpep::mApple-Rab5c, enpep::mApple-Rab7 and enpep::mApple-Rab11a_ expressing zebrafish were assessed for fluorescence at 4 dpf using confocal microscopy. The _enpep _promoter expression drives expression of proteins under the control of _enpep _from around 48 hpf (Thisse and Thisse 2004), so there will be no detectable fluorescence before this. To obtain high resolution images of the renal tubule of transgenic embryos, imaging using a confocal microscope to create maximum projections through the renal tubule was performed.

Expression of all three mApple-Rabs are detectable in the renal tubule of 4 dpf transgenic embryos (Figure 51A). In maximum projections through sagittal sections, _Enpep::mApple-Rab5c_ embryos show diffuse staining throughout the proximal tubular cells, as well as an apical enrichment of fluorescence. This apical enrichment is likely due to the presence of mApple-Rab5c on subapical vesicles, however in the maximum projection it is not possible to see individual puncta. Colocalisation of early endosomal markers or 10 kDa dextran in transverse sections is needed to determine if the expression is marking Rab5c positive membranes. Maximum projections through the proximal tubule of _enpep::mApple-Rab7_ (Figure 51B) and Rab11a (Figure 51C) show kidney specific expression as expected. The
expression of mApple-Rab7 and mApple-Rab11 appears to be strongly apical in the renal tubular cells, likely marking sub-apical late and recycling endosomes.

7.6 Discussion

Rab proteins are ideal compartmental markers in zebrafish that allow endomembrane identity to be determined by the presence of a particular Rab protein or subset of several Rab proteins, depending on the compartment (Stenmark 2009; Galvez et al. 2012). Until now, transgenic markers of endocytic compartments...
have only been described in zebrafish < 2 dpf (Clark et al. 2011). This chapter provides initial insight into the expression of six novel transgenic lines, that tag the endocytic early, late and recycling endosomes with fluorescently tagged Rab5c, Rab7 or Rab11a respectively, either throughout the whole embryo or under the control of the kidney specific *enpep* promoter.

GFP tagged Rab5C, Rab7 and Rab11a have been expressed under the *ef1a* zebrafish promoter. In the literature, the *Xenopus ef1a* promoter has been used in zebrafish to drive expression in most tissues of zebrafish embryos of up to at least 10 dpf (Kague et al. 2012). As the zebrafish *ef1a* promoter has now been cloned and the expression confirmed as equivalent to the *Xenopus ef1a* promoter (Adam Hurlstone, personal communication), the zebrafish *ef1a* was used. In our GFP-Rab lines, expression of the transgene appears to be higher in the midbrain, notochord, renal tubule and cloaca than in other tissues where a more diffuse fluorescence is seen. This could potentially indicate variation in the tissue specific expression of the *ef1a* promoter. Alternatively, it could be that these tissues have more Rab positive endosomal structures than others, and therefore there are more visible puncta present, giving the impression of more intense staining. The renal tubule and notochord are all endocytically active tissues (Essner et al. 2005; Oltrabella et al. 2015; Stemple 2005), therefore the hypothesis that these tissues have more endocytic machinery is possible. Further characterisation is required to determine if this tissue specific expression is due to promoter activity or increased presence of endocytic machinery. The high level of expression seen in the midbrain and notochord in these transgenic embryos suggest this will be an excellent tool for studying endosomal morphology in these tissues. Interestingly, In 30 hpf zebrafish, GFP-Rab proteins under the control of the *h2afx* promoter are also seen in the otic vesicle (Clark et al. 2011), as well as the retinal epithelium and hindbrain epithelium. This manuscript however does not elude to more general expression of the GFP-Rab proteins for comparison in the pronephric tubule, notochord or any other tissues. Transient expression of GFP-Rab7 in the notochord gave strong, mosaic expression throughout the notochord (Ellis et al. 2013), however as this was transient expression it cannot be directly compared to stable expression. It remains to be elucidated whether there is reliable expression of these reporters specifically in the proximal tubule, and whether the punctate structures seen are truly endosomes. To describe these punctate structures further, co-localisation with internalised cargo or endosomal markers in each tissue is required, which is the next objective for these fish.
As alternative, pronephric tubule specific endocytic markers, mApple tagged Rab5c, Rab7 and Rab11a transgenic fish have been generated, with expression of the mApple-Rab fusion under the control of the kidney specific enpep promoter. The enpep promoter is a strong pronephric tubule specific promoter (Seiler and Pack 2011), and expression of enpep begins around 40-48 hpf (Thisse and Thisse 2004). Expression of these Rabs is limited to the apical membrane of the renal tubule in F2 embryos, which is consistent with the subapical localisation of the early endosomes and recycling tubules in proximal tubular cells in vivo (Christensen et al. 1992; Christensen and Willnow 1999; Mattila et al. 2014). In the maximum projections obtained in this thesis, mApple-Rab staining appears continual on the apical membrane. This is likely an artefact of stacking many images into one projection, which was required to visualise the mApple fluorescence in wholemount sagittal images. It is possible that in transverse sections through the tubule, specific puncta may be visible, which will further corroborate that the mApple-Rabs are localising correctly. As with GFP-Rab transgenic lines, further characterisation is required to determine that the fluorescent Rab proteins are localising to the correct compartments.

The purpose of generating these transgenic fluorescently tagged Rab5c, Rab7 and Rab11a transgenic lines is to study the morphology, number, localisation and dynamics of these compartments when endocytosis is impaired, such as in the ipip27a$^{-/-}$ and ocr1$^{-/-}$ mutant zebrafish lines. Once characterised fully, these lines can be crossed into a mutant zebrafish line to create a transgenic Rab -tagged line in a homozygous mutant background. In addition to the Rab5c, Rab7 and Rab11a lines already created, fluorescently tagged Rab35 may also be an informative marker. Rab35 localises to recycling endosomes in the fast recycling pathway (Galvez et al. 2012; Shah et al. 2013), which is proposed to be used by megalin for recycling in some cell types (Shah et al. 2013). In addition, Rab22, which mediates trafficking between the TGN and the early endosome (Stenmark 2009), may be of interest in studying the role of OCRL1 and IPIP27A in lysosomal hydrolase trafficking via the M6PR and CIMPR receptors. Further work is required to fully utilise these lines in the characterisation of the fluorescence to ensure that the localisation is correct, the level of expression is not too high (as to not interfere with the normal functioning of Rab proteins) and to ensure there are no detrimental positional effects from gene insertion. To do this, more of the founders can be bred to establish alternative lines for all transgenics, and the gene insertion locations identified by inverse PCR to
ensure that no critical genes or regulatory sequences have been disrupted in each individual line.
Chapter 8: Final Conclusions
8.1 The role of PtdIns(4,5)P$_2$ in Lowe syndrome

Aberrant accumulation of PtdIns(4,5)P$_2$, and dysregulation of the actin cytoskeleton has been proposed to explain many of the symptoms of Lowe syndrome, as well as specific cellular phenotypes noted in OCRL1 deficient cultured cells and ocr1$^{-/-}$ mutant zebrafish (Ben El Kadhi et al. 2011; Bohdanowicz et al. 2012; Dambournet et al. 2011; Jones et al. 2013; Mak et al. 2011; Mehta et al. 2014; Oltrabella et al. 2015; Ramirez et al. 2012; Suchy and Nussbaum 2002; Vicinanza et al. 2011). PtdIns(4,5)P$_2$ is therefore an attractive target for therapeutic modulation to attempt to alleviate the symptoms of Lowe syndrome. In addition, by modulating PtdIns(4,5)P$_2$ levels, it may be possible to dissect the phenotypes caused by dysregulation of PtdIns(4,5)P$_2$ levels, and what phenotypes may have an alternative underlying mechanism. In this thesis, ways of manipulating PtdIns(4,5)P$_2$ have been explored to determine whether targeting PtdIns(4,5)P$_2$ in embryos is able to rescue the impaired endocytosis of 10 kDa dextran in the renal tubule of ocr1$^{-/-}$ mutant embryos. Morpholino knockdown and small molecule inhibitors have demonstrated two ways in which the PtdIns(4,5)P$_2$ synthesizing enzyme PIP5K can be targeted to restore dextran uptake in the renal tubule. In addition, sequestration of PtdIns(4,5)P$_2$ and treatment with a small molecule known to enhance the hydrolysis of PtdIns(4,5)P$_2$ PLC also rescues the 10 kDa dextran uptake defect in ocr1$^{-/-}$ zebrafish.

The rescue or partial rescues reported after these various ways of targeting PtdIns(4,5)P$_2$ indicate that the endocytic defect in the ocr1$^{-/-}$ zebrafish renal tubule may be PtdIns(4,5)P$_2$ dependent. An important future consideration for this work is whether the methods of disrupting PtdIns(4,5)P$_2$ explored in this thesis are indeed causing a reduction in PtdIns(4,5)P$_2$ levels as suspected. Measurement of whole embryo PtdIns(4,5)P$_2$ using previously defined methods will answer this question (Jones et al. 2013). In addition, PHDM has recently been further developed, to generate a fluorescently conjugated variant that binds to PtdIns(4,5)P$_2$ in a 1:1 ratio (Rudiger Woscholski, personal communication), which can be developed as a biosensor to analyse PtdIns(4,5)P$_2$ levels in vivo. In addition, if restoring physiological PtdIns(4,5)P$_2$ levels is indeed responsible for restoring endocytic uptake of 10 kDa dextran in the proximal tubule, then it is reasonable to expect that the altered cellular distribution of endocytic proteins like megalin will be restored to the wild type condition.
8.2 GFP-D3 and NL-D3 as a readout of proximal tubular function in zebrafish embryos in a high throughput phenotypic screen.

This thesis has also described two novel reporters of endocytic function in the pronephric tubule of zebrafish embryos based on a fluorescence and luminescence readout respectively. Further validation of these transgenic reporter lines and establishment of a reliable, optimised high throughput protocol for recording the effect of drugs will need to be established. For example, parameters such as volume of water and number of larvae per well will need to be optimized, as well as incubation time and the best day post fertilisation to start/finish the assay. This assay can be validated using nephrotoxic drugs that block renal tubular uptake such as cisplatin and gentamycin (Hentschel et al. 2005; Hentschel et al. 2007).

By crossing the newly generated transgenic reporter lines into the ocr1l−/− mutant high throughput drug screening can be carried out to identify novel compounds that alleviate the renal endocytic defect in 10 kDa dextran uptake in ocr1l−/− embryos. The design of a dual set of proximal tubular uptake reporters utilising both fluorescent and luminescent proteins is a deliberate step to maximise the number of on target hits that can be identified in a chemical screen. In a primary screen, NL-D3 expressing embryos with or without ocr1 mutation could be assessed for compounds that cause a reduction in the luciferase excreted from the cloaca (Figure 52). Hits from this first round of screening can then be validated in a second screen, using GFP-D3 embryos. In GFP-D3;ocr1l−/− embryos, where uptake is restored by application of a specific compound, uptake of the GFP-D3 into visible puncta in the renal tubule will be observed. This secondary screen will allow compounds that affect excretion of NL-D3 in other ways (such as by blocking synthesis in the liver or glomerular filtration) to be discarded from the final number.

Selection of a compound library for a screen will be key to developing novel therapeutics. A particularly attractive library of compounds for initial testing is the MicroSource Spectrum Library (MicroSource Discovery Systems Inc, USA), which contains 2560 FDA approved compounds, which has previously been used in zebrafish phenotypic screening assays (Novodvorsky et al. 2013; Rennekamp and Peterson 2015; Saydmohammed et al. 2011). A clear advantage of using an FDA approved library is that the target of each compound is already known, and therefore this will accelerate the pathway from initial testing to use in the clinic. Secondly, extensive drug safety testing can be avoided, as any use of an FDA
approved compound will be a repurposing of an existing drug approved for use in humans. As an alternative compound library, it would also be advantageous to screen libraries of compounds known to affect kinases and phosphatases, as a targeted approach.

Any hits identified in a compound screen would then require further validation to determine both the mechanism of action, and any ability to rescue other phenotypes observed in ocr1<sup>−/−</sup> zebrafish. To do this, a more in depth analysis of the compounds ability to rescue megalin localisation and endosome morphology in ocr1<sup>−/−</sup> zebrafish could be performed, as well the compounds ability to rescue other reported phenotypes in ocr1<sup>−/−</sup> mutant embryos such as disorganised primary cilia.
and neurological impairment. Where the target of a compound is known, validation of the compounds mechanism of action could be assessed by using other known activators or inhibitors of the relevant pathway, as well as genetic methods such as morpholino knockdown of target genes. These validation steps will be critical to assessing the potential of a specific compound for therapeutic use.

A further potential use for the GFP-D3 and NL-D3 transgenic zebrafish would be to further develop them to create dual reporters of both glomerular integrity and proximal tubular uptake. When identifying compounds in a screen that rescue proximal tubular function, assessing glomerular integrity in response to the compound could identify compounds that cause damage to glomerular barrier, which would cause leaking of protein into the ultrafiltrate and ultimately lead to false positive results that are instead due to an influx of protein after glomerular damage. Furthermore, combined proximal tubule and glomerular reporter lines may provide a tool to further study the role of OCRL1 in the glomerulus. CD2AP and Myosin 1E, are both required for slit diaphragm integrity in podocytes (Krendel et al. 2009; Li et al. 2000; Shih et al. 2001), and both have been shown to bind to the OCRL1 interacting partner IPIP27A (Noakes 2011). Progressive glomerular damage has been demonstrated in Lowe syndrome patients with renal disease (Lowe Syndrome Trust 2010), and it is possible that lack of OCRL1 may have an unidentified function in the glomerulus. Additionally, glomerular damage could be caused by crosstalk between the proximal tubule and the glomerulus. Proteinuria has been shown to elicit an inflammatory response in the nephron, which causes progressive damage to the glomerular filter (Abbate et al. 2006). A dual glomerular/proximal tubule reporter fish would allow investigation of both tubular function and glomerular integrity simultaneously, which may help to determine how OCRL1 mutation leads to renal failure.

To do this, transgenic GFP-D3 embryos could be further injected with a construct encoding for liver specific expression of secreted VitDBP-RFP, similar to the VitDBP-GFP protein used in Zhou and Hildebrandt (2012). This protein would be retained in the vasculature in fish where glomerular integrity is in tact, providing a double transgenic system able to report glomerular integrity and proximal tubular function simultaneously (Figure 53A). Likewise, NL-D3 fish could be injected with a construct that encodes Gaussia luciferase fused to VitDBP (VitDBP-GLuc). Like VitDBP-GFP, This VitDBP fused luciferase would also be too large to filter, and therefore remain in the vasculature unless glomerular integrity is compromised (Figure 53B). NL and GLuc have different substrates and emission spectra, and
therefore the activity of both proteins in the water could be assessed simultaneously (Hall et al. 2012; Tannous 2009).

8.3 OCRL1 and IPIP27A interaction is required for endocytic function in the zebrafish renal tubule.

Evidence has shown that IPIP27A is an important binding partner for OCRL1, and is required for endocytic uptake of 10 kDa dextran and RAP, as well as for CIMPR trafficking and the generation of recycling trafficking intermediates (Billcliff et al. 2016; Noakes et al. 2011; Oltrabella 2014; Swan et al. 2010). It has been proposed that OCRL1, IPIP27A and PACSIN2 are required in the formation of endosomal
trafficking intermediates in cultured cells (Billcliff et al. 2016). Re-expression of IPIP27A containing mutations abolishing binding of either OCRL1 or PACSIN2 in this thesis demonstrates that the binding of IPIP27A to SH3 containing proteins such as PACSIN2 is critical to its function in endocytosis from the renal tubule, suggesting that the OCRL1-IPIP27A-PACSIN2 interaction may be required for megalin mediated endocytosis. The accumulation of megalin in endosomes in ipip27a−/− mutants (Oltrabella 2014), suggests that the role of IPIP27A may be in the recycling of megalin from the endosome to the plasma membrane, however further work is required to elucidate the mechanism.

While the most likely proposed mechanism leading to cellular phenotypes of ocrl1−/− and ipip27a−/− zebrafish is impaired endocytosis, when embryos lack both functional ocrl1 and ipip27a, the presence of hydrocephaly, impaired development of the correct body axis and severe developmental delay is consistent with impairment of ciliary function (Kramer-Zucker et al. 2005). Both ocrl1−/− and ipip27a−/− mutant embryos display mild ciliary disorganisation (Coon et al. 2012; Oltrabella 2014), and it is important now to assess the cilia morphology in embryos lacking ocrl1 and ipip27a, to determine if these phenotypes are due to a more significant impairment in cilia formation or maintenance. It is plausible that any affect on cilia is secondary to a more severe membrane trafficking defect, which could abrogate trafficking to the cilium more in embryos lacking functional ocrl1 and ipip27a than either protein alone.

The defective trafficking of CI-MPR in IPIP27A depleted cells (Noakes et al. 2011) and the association of lysosomes with endosomes in ipip27a−/− mutant zebrafish embryos (Oltrabella 2014) suggests a role for IPIP27A in lysosome function. In addition, OCRL1 has also been implicated in CI-MPR trafficking and lysosomal biogenesis, as well as a direct role in hydrolysing PtdIns(4,5)P2 on the lysosome (Choudhury et al. 2005; De Leo et al. 2016). The data in this thesis demonstrating upregulation of some genes required for lysosome function, suggests lysosomal impairment in the zebrafish mutants also. The impairment of lysosomes in ipip27a−/− and ocrl1−/− zebrafish pronephric tubules could be caused by either a direct role for OCRL1 in PtdIns(4,5)P2 hydrolysis at the lysosome or upregulation of TFEB, as proposed in De Leo et al (2014). It is also possible that this is caused by impaired endocytosis of lysosomal hydrolases from the renal filtrate (Nielsen et al. 2007), or impaired trafficking of lysosomal hydrolases from the TGN (Choudhury et al. 2005; Billcliff et al. 2016). These effects are not mutually exclusive, and it is likely that a combination of these effects are responsible for lysosomal impairment in
ocr1<sup>−/−</sup> and ipip27a<sup>−/−</sup> embryos. RNASeq of ipip27a<sup>−/−</sup> and ocr1<sup>−/−</sup> zebrafish would provide a comprehensive assessment of gene regulation in these mutants, and quantitatively determine the role of TFEB activation in lysosomal impairment in these embryos.
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Appendices
Appendix 1: Primers for rtPCR to determine \textit{ipip27a^{-/-}}
effects on expression of lysosomal genes

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