INVESTIGATION OF ENDOCYTIC TRAFFICKING IN
THE ZEBRAFISH NEUROEPITHELIUM

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Abstract
OCRL1 is an inositol polyphosphate-5-phosphatase which regulates levels of phosphatidyl-inositol-4,5-bisphosphate, a membrane lipid important for numerous cellular processes. Mutations in OCRL1 are responsible for Lowe syndrome, an X-linked autosomal recessive disorder which specifically affects the eyes, kidneys and CNS. Despite increased understanding of OCRL1’s cellular role, the pathophysiological mechanisms underlying Lowe syndrome are not well understood. Zebrafish are a good organism in which to study OCRL1 function in vivo. In order to investigate whether loss of OCRL1 impairs endocytic trafficking events important for neuronal development and adult CNS homeostasis, the role of zebrafish OCRL1 in endocytosis, early endosome function and lysosomal function was investigated.

This thesis shows that OCRL1 plays a key role in regulating endocytic trafficking within neuroepithelial cells of the developing CNS. OCRL1 is seen to localise to early endosomes and overlap with the endocytic cargo megalin within the neuroepithelium. Consequently, early endosomes are enlarged in OCRL1 mutant zebrafish and trafficking of megalin is impaired, with reduced levels at the apical membrane. Establishment of an assay to test neuroepithelial endocytic uptake revealed that endocytosis is unaffected by loss of OCRL1 function. These results demonstrate that OCRL1 is important for proper functioning of the early endosome within the neuroepithelium and suggest that dysregulated endocytic trafficking may contribute to Lowe syndrome CNS pathology.

The role of OCRL1 in CNS lysosomal function was also investigated based on recent reports implicating OCRL1 in regulating lysosomal levels of PtdIns(4,5)P2 during autophagy. Minimal amounts of OCRL1 localised to neuroepithelial lysosomes at both steady state and following induction of autophagy. Furthermore, absence of OCRL1 in OCRL1 mutant zebrafish did not impair lysosomal homeostasis or autophagosome turnover suggesting the function of OCRL1 at the lysosome may not be relevant to Lowe syndrome pathology within the CNS.

Finally, investigating neuroepithelial lysosome dynamics revealed a function for the vATPase in regulating lysosome morphology. Treatment of embryos with the vATPase inhibitor Bafilomycin A but not the ionophore Nigericin, induced changes in lysosome morphology. Lysosomes were predominantly vacuolar in untreated embryos but assumed a tubular morphology following BafA treatment. Similar to other studies of lysosomal tubulation, tubules were sensitive to treatment with Torin1, indicating a requirement for active mTORC1 signalling. BafA induction of lysosomal tubules may suggest a role for the vATPase in regulating lysosome motility and positioning independent of its known role establishing the lysosomal pH gradient.
Declaration

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Abbreviations Used
Arl8b = ADP Ribosylation Factor Like GTPase 8B
ALR = Autophagic lysosome reformation
AP2 = Adaptor protein-2
ApoER2 = apolipoprotein E receptor 2
APPL1 = Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1
CIMPR = Cation independent mannose phosphate receptor
EEA1 = Early endosomal antigen 1
IKNM = Inter kinetic nuclear migration
INPP5B = Inositol polyphosphate-5-phosphatase 5B
IPIP27A/B = Inositol polyphosphate interacting protein of 27 kDa
KIF5B = Kinesin family member 5B
LAMP1 = Lysosomal associated membrane protein 1
LDLR = Low density lipoprotein receptor
LPS = lipopolysaccharide
LRP1 = Low density lipoprotein receptor-related protein 1
LRP2 = Low density lipoprotein receptor-related protein 2 / megalin
MAP1LC3A / LC3 = Microtubule-associated proteins 1A/1B light chain 3A
mTOR = mammalian target of rapamycin
mTORC1 = mammalian target of rapamycin complex 1
N-WASP = Wiskott Aldrich Syndrome protein
OCRL1 = Ocular-cerebro-renal-syndrome-of-Lowe 1
PIP5K1α / PIP5Kβ = Phosphatidylinositol 4-phosphate 5-kinase alpha / beta
PtdIns(4)P = Phosphatidylinositol-4-phosphate
PtdIns(4,5)P₂ = Phosphatidylinositol-4,5-bisphosphate
PtdIns(3,4,5)P₃ = Phosphatidylinositol-3,4,5-trisphosphate
PcP = Pre chordal plate
RAP = Receptor associated protein of 39 kDa
RDVM = Rostral diencephalon ventral midline
RILP = Rab interacting lysosomal protein
Shh = Sonic hedgehog
SKIP = SifA and kinesin-interacting protein
SNX9 = Sorting nexin 9
TLR4 / 9 = Toll like receptor 9
1. Chapter 1 - Introduction
1.1. Phosphoinositides and membrane trafficking

Phosphoinositides are a class of phospholipid which play a significant role as signalling molecules in a number of cellular processes, ranging from signal transduction to membrane trafficking (Di Paolo and De Camilli, 2006). Seven phosphoinositide species can be derived from the precursor molecule phosphatidylinositol, which is synthesised in the ER from CDP-DAG and myo-inositol (Agranoff et al., 1958). As with other classes of phosphoglyceride, phosphatidylinositol comprises a 3 carbon glycerol backbone linked to two hydrophobic fatty acid tails, allowing insertion into cellular membranes, and a hydrophilic myo-inositol head group, which confers the phosphoinositides with their unique signalling properties. The 3, 4 and 5 positions of the inositol ring can be reversibly phosphorylated on their own or in combination with other positions to give rise to a total of seven species of phosphoinositide (PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂, PtdIns(3,4,5)P₃ (Figure 1).

Figure 1. Interconversion of phosphoinositide species. a) Structure of the phosphoinositide precursor molecule phosphatidylinositol showing the two fatty acid tails attached to the glycerol backbone by diester linkages at carbons 1 and 2 and the inositol headgroup attached at carbon 3 through a phosphate group. b) Species of phosphoinositide found on intracellular membranes. Interconversion between species is shown by solid and dotted lines respectively. Species on which OCRL1 and INPP5B act to remove the 5’phosphate group from are highlighted by red dashed lines. Adapted from (Carlton and Cullen, 2005).
Phosphoinositides are key regulators of trafficking through the endocytic and secretory pathways (Di Paolo and De Camilli, 2006). The endocytic and secretory pathways are organised into a series of distinct membrane enclosed organelles which perform specialised sets of reactions dependent on the presence of specific sets of proteins and lipids (Figure 2b). Organelle function is complicated by the ongoing exchange of membrane between compartments and requires a mechanism for organelles to maintain their identity in order to continue to perform their function. Phosphoinositides are uniquely suited to serve as regulators of membrane traffic due to two properties. First, variations in the inositol head group allow each phosphoinositide species to bind unique subsets of proteins and recruit them to the membrane (Carlton and Cullen, 2005; Lemmon, 2008). A number of phosphoinositide binding modules have been discovered which mediate distinct protein-lipid interactions and drive membrane recruitment or protein activation / inactivation when coupled with binding to additional proteins or lipids. For instance, proteins containing FYVE or PX domains preferentially bind species of phosphoinositide phosphorylated at the 3' position

**Figure 2. Cellular distribution of phosphoinositide species and major roles within the cell.**

a) Major phosphoinositide species found on organelles of the endocytic and secretory pathways. b) Major roles of phosphoinositide species. Phosphoinositide species are colour coded to match those in a). Adapted from (Vicinanza et al., 2008b)
(PtdIns(3)P, PtdIns(3,4)P₂, PtdIns(3,4,5)P₃) (Kanai et al., 2001; Kutateladze, 2006). Specific recruitment or activation of proteins is enhanced by co-operation of phosphoinositides with another key regulator of the endocytic and secretory pathways, the Rab GTPases, which also bind distinct effector proteins (Stenmark, 2009). Second, reversible phosphorylation and dephosphorylation of the inositol ring allows spatial and temporal regulation of phosphoinositide signalling. Spatial regulation contributes to defining organelle identity by enriching distinct phosphoinositide species on specific organelles (Figure 2a) (Behnia and Munro, 2005). This serves to orchestrate organelle function through recruitment of defined sets of phosphoinositide binding proteins. PI(3)P is segregated on early endosomal membranes and recruits proteins involved in cargo sorting and membrane fusion (Dumas et al., 2001; Ellson et al., 2002) whilst PI(4,5)P₂ is predominantly found at the plasma membrane and recruits AP-2 to initiate formation of endocytic vesicles (Honing et al., 2005). Finally, temporal regulation of phosphoinositide signalling ensures that phosphoinositide binding proteins are only recruited to specific locations as and when required and released or inactivated once no longer needed.

1.2 Phosphoinositide metabolising enzymes

Control of phosphoinositide signalling is mediated by phosphoinositide kinases and phosphatases that display specificity towards particular species of phosphoinositide and act by adding or removing a phosphate group from specific positions of the inositol ring (Sasaki et al., 2009). To date 20 phosphoinositide kinases and 35 phosphoinositide phosphatases have been discovered which can be classified based upon their substrate preferences (Vicinanza et al., 2008a). The spatial and temporal profiles of phosphoinositides within the cell are a reflection of the regulation phosphoinositide metabolising enzymes (PMEs) are themselves subject to. This regulation ranges from control of enzyme expression levels to post translational modifications. PMEs often have a number of interaction partners that are also key regulators of PME activity. As critical regulators of phosphoinositide signalling, mutations in PMEs can lead to dysregulation of endocytic trafficking and a number of human diseases. For instance, mutations in the myotubularin family of 3-phosphatases
that dephosphorylate PtdIns(3)P and PtdIns(3,5)P₂ can lead to Charcot Marie Tooth syndrome and monotonic myopathy (Bergmann et al., 2005; Taylor et al., 2000). Conversely, mutations in some of the enzymes responsible for synthesis of PtdIns(3)P, PtdIns(3,5)P₂ and PtdIns(3,4,5)P₃, PIK3CA and PICK3CB, have been implicated in cancer (Wymann and Marone, 2005).

1.2 Lowe Syndrome and Dent-2 disease
OCRL1 is one of ten inositol 5-phosphatases found in vertebrates that act to remove the 5’ phosphate group from PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ (Figure 1) (Billcliff and Lowe, 2014). Mutations in the OCRL1 gene lead to two rare recessive X-linked disorders: Lowe syndrome and Dent-2 disease, which are estimated to affect 1 in 500,000 individuals. Common to both disorders is dysfunction of the renal proximal tubule, characterised by excessive loss of low molecular weight proteins, amino acids and calcium, which ultimately leads to renal failure. Patients with Lowe syndrome frequently present with symptoms affecting the eyes and CNS, however many Dent-2 patients display milder symptoms within these tissues (Bokenkamp et al., 2009). Ocular abnormalities include congenital cataracts, present from birth in the majority of cases, and glaucoma, which develops in at least 50% of infants (Loi, 2006). Symptoms of CNS impairment manifest as severe hypotonia, mental retardation, behavioural problems and in 50% of cases, epileptic seizures (Schurman and Scheinman, 2009). Additionally, a smaller percentage of patients display periventricular cystic lesions (Allmendinger et al., 2014; Charnas et al., 1988; Sener, 2004).

1.4 OCRL1
The OCRL1 gene is located on the X chromosome and is encoded by a 24 exon gene (Nussbaum et al., 1997). Alternate splicing of the OCRL1 transcript can produce two OCRL1 isoforms, OCRL1a and OCRL1b. Inclusion of exon 18a in OCRL1a produces a protein that contains an additional 8 amino acids (Nussbaum et al., 1997). Although OCRL1 is ubiquitously expressed, the relative amount of each splice isoform present between tissues differs. OCRL1b is the main isoform expressed in the
majority of tissues except for the brain, where OCRL1a is the only isoform present (Johnson et al., 2003).

1.4.1 OCRL Structure

The OCRL1 protein is 110kDa in mass and consists of multiple domains (Figure 3). A central 5-phosphatase domain is responsible for the removal of the 5’phosphate group from both soluble and membrane bound inositol lipids. OCRL1 shows activity towards both PtdIns(3,4,5)P$_3$ and Ins(1,4,5)P$_3$ in vitro, but preferentially dephosphorylates PtdIns(4,5)P$_2$ (Schmid et al., 2004; Zhang et al., 1995). Flanking the 5-phosphatase domain are additional domains responsible for the interaction of OCRL1 with a number of binding partners. The N-terminal region of OCRL1 contains a PH domain that lacks a basic patch required for phosphoinositide binding (Mao et al., 2009). A clathrin binding motif, LIDIA, is found on a flexible loop which projects from the PH domain (Mao et al., 2009). Downstream of the PH domain and prior to the 5-phosphatase domain are the motifs REPPPPP and FEDNF that are required for SNX9 and AP2 binding (Choudhury et al., 2009; Mao et al., 2009; Nandez et al., 2014; Ungewickell et al., 2004). All of these proteins are involved in endocytic vesicle generation at the plasma membrane.

C terminal to the 5-phosphatase domain are the ASPM, SPD-2, Hydin (ASH) and RhoGAP like domains. The ASH domain is common to proteins found to localise near cilia and the centrosome (Ponting, 2006). In OCRL1, this region of the protein co-operates with residues from the linker region

Figure 3. Domain architecture of OCRL1 and sites of interaction partner binding. Each domain of OCRL is colour coded with regions shaded grey indicating linker regions not classified as part of any of the four domains. Numbers 1 – 901 represent amino acid number. Approximate locations of interaction partner binding along the length of OCRL1 are indicated.
between the 5-phosphatase and ASH domains to mediate binding to Rab GTPases (Erdmann et al., 2007; Hou et al., 2011; Hyvola et al., 2006), which alongside phosphoinositides are key co-ordinators of membrane traffic (Stenmark, 2009). OCRL1 can engage with multiple Rab proteins including Rab1, Rab5, Rab6, Rab8 and Rab35 (Erdmann et al., 2007; Fukuda et al., 2008; Hyvola et al., 2006).

The RhoGAP like domain of OCRL1 is adjacent to the ASH domain at the C terminus of the protein with the two domains linked by a short flexible linker region within which lies a second clathrin binding motif, LIDLE (Erdmann et al., 2007; Ungewickell et al., 2004). Although the RhoGAP like domain bears significant homology to RhoGAP domains present in other proteins (Lichter-Konecki et al., 2006) and can interact with Rac1, Cdc42 and possibly Arf GTPases (Faucherre et al., 2003; Lichter-Konecki et al., 2006), it lacks critical arginine residues important for GAP activity. Instead, this region of the protein is important for interactions with additional OCRL1 binding partners and constitutes the major structural determinant involved in binding to the F&H domain containing proteins IPIP27A & B and APPL1, both of which localise to endosomal compartments (Erdmann et al., 2007; Noakes et al., 2011; Pirruccello et al., 2011; Swan et al., 2010).

1.4.2 OCRL1 Intracellular localisation

Collectively, these interactions are important for the intracellular localisation of OCRL1, which to date has been shown to localise to multiple locations within the cell including clathrin coated trafficking intermediates, early endosomes and the trans-Golgi network (Figure 4). Targeting to early endosomes is dependent on the interaction of OCRL1 with both Rab5 and APPL1, with the small F&H containing proteins IPIP27A & B being dispensable for OCRL1 endosomal targeting (Hyvola et al., 2006; McCrea et al., 2008; Noakes et al., 2011). Interactions with the Rab proteins are also important for OCRL1 targeting to the Golgi apparatus. Rab1 and Rab6 are responsible for the association of OCRL1 with the trans-Golgi network as mutations in OCRL1 that abolish Rab binding prevent OCRL1 Golgi association (Dressman et al., 2000; Hou et al., 2011; Hyvola et al., 2006). Additional Rab interactions are also crucial for targeting of OCRL1 to the intracellular bridge during cytokinesis.
The interactions of OCRL1 with AP-2 and clathrin recruit OCRL1 to the late stages of clathrin coated vesicle formation at the plasma membrane with further binding to SNX9 and Rab35 contributing to the timing of OCRL1 recruitment (Cauvin et al., 2016; Erdmann et al., 2007; Mao et al., 2009; Nandez et al., 2014). The interactions of OCRL1 with RhoA, Rac1 and Cdc42 (Faucherre et al., 2003) may also

Figure 4. Localisation of OCRL1 along the endocytic and secretory pathways. OCRL1 localises to clathrin coated pits (CCP), early endosomes (EE), the Golgi apparatus (G) the primary cilium (C) and newly forming phagosomes (P) in interphase cells. Under conditions of higher lysosomal load OCRL1 also associates with lysosomes (Lys). OCRL1 interaction partners are shown next to the sites where they interact with OCRL1. Binding partners responsible for recruitment of OCRL1 to each compartment are highlighted in red. Overlapping sites of OCRL1 localisation with INPP5B are also shown. Arrows indicate exchange of material between various intracellular compartments. LE = late endosome, RE = recycling endosome.
contribute to bringing OCRL1 to the plasma membrane to regulate actin dynamics, which are closely coupled to PtdIns(4,5)P$_2$ turnover and endocytic vesicle generation (Saarikangas et al., 2010). Additionally, binding of OCRL1 to clathrin and AP2 regulates the recruitment of OCRL1 to lysosomes under conditions of high lysosomal load (De Leo et al., 2016). Clathrin binding does not contribute to Golgi localisation of OCRL1 as deletion of both clathrin binding boxes does not prevent OCRL1 Golgi association (Choudhury et al., 2009). Interestingly, the different splice variants of OCRL1 show altered association with clathrin coated transport intermediates and as a consequence differ slightly in their intracellular localisation patterns. Isoform-a contains an additional 8 amino acids adjacent to the LIDLE clathrin binding box in the ASH-RhoGAP domain (Choudhury et al., 2009; Nussbaum et al., 1997). This leads to enhanced binding of OCRL1a to clathrin and a more punctate distribution of OCRL1a which displays strong overlap with clathrin (Choudhury et al., 2009). Enhanced clathrin binding may be of relevance to the function of OCRL1 within the brain where OCRL1a is the only splice isoform present.

1.4.3. OCRL1 function

1.4.3.1. Endocytosis and endocytic trafficking

Owing to its varied intracellular localisation, OCRL1 has been implicated in a number of cellular processes. Much evidence exists in favour of a role for OCRL1 in regulating multiple steps along the endocytic pathway. A number of studies have identified that OCRL1 acts in the late stages of clathrin mediated endocytosis at the plasma membrane. Live imaging experiments revealed that OCRL1 is recruited to newly forming clathrin coated pits just prior to a decrease in clathrin fluorescence and at a similar time to SNX9 and Rab35, two additional proteins involved in the later stages of endocytosis (Cauvin et al., 2016; Erdmann et al., 2007; Mao et al., 2009; Nandez et al., 2014; Taylor et al., 2011). Loss of OCRL1 leads to an increase in the number of long lived clathrin coated pits at the plasma membrane and a delay in endocytic uptake of transferrin (Choudhury et al., 2009; Nandez et al., 2014). However, other studies have reported no effect on endocytosis of a number of
other endocytic ligands in the absence of OCRL1 (Cui et al., 2010; van Rahden et al., 2012; Vicinanza et al., 2011).

Downstream of the plasma membrane, OCRL1 is important for early endosome function and the correct trafficking of a number of endosomal cargos. Both overexpression of a dominant negative form of OCRL1 lacking the 5-phosphatase domain (OCRL1-ΔPIP2) and knockdown of OCRL1 lead to redistribution of the cation independent mannose phosphate receptor (CIMPR) from the perinuclear region into enlarged peripheral early endosomes implicating OCRL1 in regulation of retrograde trafficking of CIMPR between endosomes and the Golgi (Cauvin et al., 2016; Choudhury et al., 2005; van Rahden et al., 2012; Vicinanza et al., 2011). A study by Vicinanza et al described impaired trafficking of additional cargos that follow distinct itineraries through the endocytic pathway. Transferrin and megalin recycle back to the plasma membrane following internalisation through fast and slow recycling routes respectively (Maxfield and McGraw, 2004; Perez Bay et al., 2016). In contrast, following its internalisation, the EGFR receptor is trafficked towards degradative compartments. All three cargos were shown to accumulate in a common compartment in the absence of OCRL1, likely to be the early endosome, indicating that OCRL1 plays a crucial role in endosomal trafficking of multiple cargos (Vicinanza et al., 2011).

It is currently unclear whether OCRL1 plays a housekeeping role to remove ectopic accumulation of PtdIns(4,5)P₂ from endosomal membranes which may interfere with endosome function or whether it is recruited to endosomes to regulate pools of PtdIns(4,5)P₂ directly involved in trafficking reactions (Figure 5). A tripartite complex formed between OCRL1, IPIP27A and Pacsin2 on endomembranes suggests that OCRL1 mediated PtdIns(4,5)P₂ hydrolysis may be coupled directly to formation of endosomal carriers which contain CIMPR (Billcliff et al., 2016). IPIP27A and B are key regulators of endocytic trafficking as IPIP27A knockdown leads to enlarged endosomes, missorting of CIMPR and the appearance of aberrant tubular trafficking intermediates (Billcliff et al., 2016; Noakes et al., 2011; Swan et al., 2010). IPIP27A can simultaneously engage with OCRL1 and Pacsin2, an F-
BAR domain containing protein involved in sculpting endosomal membranes and endosomal carrier morphogenesis (Kostan et al., 2014; Quan and Robinson, 2013). Indirect engagement of OCRL1 with Pacsin2 on highly curved membranes stimulates the 5-phosphatase activity of OCRL1 (Billcliff et al., 2016), supporting the idea that the 5-phosphatase activity of OCRL1 is intimately linked to carrier morphogenesis on endosomes.

Figure 5. Receptor recycling from early endosomes in the presence and absence of OCRL1. A) In normal cells, receptors traversing the fast recycling pathway, such as the transferrin receptor (blue), enter early endosomes before being delivered directly back to the plasma membrane following release of bound cargo. Slow recycling receptors such as megalin (red) enter into recycling tubules after internalisation and pass through recycling endosomes before returning to the PM. B) OCRL1 may function to remove ectopic accumulation of PtdIns(4,5)P$_2$ on endosomes which if present leads to aberrant endosomal accumulation of actin (shown as meshed network in (b) + (c)), interfering with trafficking out of this compartment. Cargo may accumulate in the endosomes or ultimately end up being routed to lysosomes and degraded, reducing receptor cell surface levels. C) Alternatively, OCRL may hydrolyse pools of PtdIns(4,5)P$_2$ directly involved in generation of endocytic carriers. Loss of OCRL in this case may lead to failure of tubules to undergo scission, with cargo then retained in EE’s before following the same fate as in (b).

Alternatively, OCRL1 may be recruited to endosomes in a housekeeping role to remove ectopic pools of PtdIns(4,5)P$_2$ on endomembranes not directly involved in carrier morphogenesis (Fig 5). Newly generated endocytic vesicles need to undergo a change in phosphoinositide composition from PtdIns(4,5)P$_2$ to the predominant endosomal phosphoinositide species PI(3)P. This conversion has
been proposed to involve sequential dephosphorylation of PtdIns(4,5)P$_2$ by the 4-phosphatase Sac2 and OCRL1 (Hsu et al., 2015; Nakatsu et al., 2015). Recruitment of OCRL1 to endosomal membranes may act as a homeostatic mechanism to remove residual PtdIns(4,5)P$_2$ brought to the endosome by endocytic carriers or through other sources. A number of studies have shown that loss of OCRL1 leads to an accumulation of actin on early endosomes, at the phagocytic cup during phagocytosis, on newly forming clathrin coated transport intermediates derived from the plasma membrane, and on the intracellular bridge during cytokinesis (Dambournet et al., 2011; Nandez et al., 2014). This has led to the hypothesis that OCRL plays an important role coupling PtdIns(4,5)P$_2$ hydrolysis to actin turnover. PtdIns(4,5)P$_2$ is a key regulator of actin dynamics, serving to promote actin polymerization through activation of the actin nucleation factor N-WASP whilst simultaneously preventing actin disassembly through inhibition of the actin severing protein cofilin (Gorbatyuk et al., 2006; Papayannopoulos et al., 2005). Enhanced accumulation of endosomal PtdIns(4,5)P$_2$ has sporadically been detected in OCRL knockdown cells (Vicinanza et al., 2011). In contrast, sequestration of PtdIns(4,5)P$_2$ binding proteins such as clathrin and AP2 and a number of proteins involved in regulating actin dynamics has consistently been observed on early endosomes and endocytic vesicles (Cauvin et al., 2016; Nandez et al., 2014; Vicinanza et al., 2011). Deficiency of OCRL leads to a reduced number of actin stress fibres and an increase in the number of intracellular actin foci consistent with a role for OCRL1 in regulating actin dynamics (Cui et al., 2010; Nandez et al., 2014; Suchy and Nussbaum, 2002; Vicinanza et al., 2011). Redressing the balance between actin assembly and disassembly can also rescue a number of phenotypes associated with loss of OCRL1 (Dambournet et al., 2011; Vicinanza et al., 2011). Thus, in the absence of OCRL1, elevated PtdIns(4,5)P$_2$ levels on endosomes may lead to aberrant recruitment of PtdIns(4,5)P$_2$ binding proteins including actin, interfering with endosomal function.

1.4.3.2. Lysosomal function and autophagy

OCRL1 has been proposed to play a role in the regulation of lysosomal function. OCRL1 was originally shown to localise to the lysosome following treatment of cells with sucrose, an indigestible
lysosomal substrate (Zhang et al., 1998). This was further supported by work by De Leo et al which showed that OCRL1 is also recruited to the lysosome under conditions of high lysosomal load, such as during starvation induced autophagy (De Leo et al., 2016). Knockdown of OCRL1 resulted in an accumulation of autophagosomes, which was also mirrored in proximal tubule cells taken from kidney biopsies of Lowe syndrome patients. Mechanistically, OCRL1 was proposed to act alongside clathrin and AP2 to maintain rates of autophagic flux, potentially through recycling of SNARE proteins involved in autophagosome – lysosome fusion. OCRL1 was also shown to modulate the activity of mucolipin, a lysosomal calcium channel important for cycles of endosome-lysosome fusion and fission (De Leo et al., 2016; Li et al., 2016). Phosphoinositides control the activation status of mucolipin with PtdIns(3,5)P2 and PtdIns(4,5)P2 activating and inhibiting mucolipin dependent calcium release respectively (Dong et al., 2010; Zhang et al., 2012). As calcium release through mucolipin has been shown to be important for both lysosomal fusion and fission (Venkatachalam et al., 2015), buildup of PtdIns(4,5)P2 on late endocytic compartments in the absence of OCRL1 may inhibit interorganelle fusion or contribute to preventing fission of lysosomal vesicles, which in turn leads to autophagosome accumulation.

1.4.3.3. Ciliogenesis

OCRL1 has been implicated in the biogenesis of the primary cilium. OCRL1 localises to the base of the primary cilium in Lowe syndrome fibroblasts and NIH3T3 cells, and along the length of the primary cilium in number of different cell types (Coon et al., 2012; Luo et al., 2012). Furthermore, OCRL1 could also be seen to localise to the primary cilium in sections taken from human eyes and rat kidney (Luo et al., 2012). Localisation to the cilium is dependent on the interaction of OCRL1 with Rab8, a GTPase involved in targeting of multiple proteins to the primary cilium, and IPIP27A, with clathrin and AP2 not being required for the ciliary localisation of OCRL1 (Coon et al., 2012; Luo et al., 2012). Functionally, OCRL1 appears to participate in trafficking of proteins to the cilium as reduced levels of ciliary rhodopsin can be seen following OCRL knockdown (Coon et al., 2012). This appears to have consequences for cilia formation with two studies reporting shorter or absent cilia (Coon et al.,
2012; Luo et al., 2012) and one reporting elongated cilia in a number of different in vitro and in vivo models (Rbaibi et al., 2012).

1.4.3.4. Phagocytosis and bacterial infection

A number of reports have demonstrated that OCRL1 participates in phagocytosis. Changes in phosphoinositide composition on newly forming phagosomes are critical in co-ordinating actin dynamics with phagocytic cup extension and closure. Depletion of OCRL1 impairs phagocytic cup closure and internalisation of large particles and also leads to changes in phosphoinositide dynamics on phagosomes (Bohdanowicz et al., 2012; Marion et al., 2012). This is linked to accumulation of PtdIns(4,5)P₂ and subsequent accumulation of actin on sealing phagosomes. Invasion of different bacterial species also involves OCRL1. Knockdown of OCRL1 and the Dictyostelium discoideum OCRL1 homolog Dd5P4 increase Listeria monocytogenes and Legionella pneumophila infectivity (Kuhbacher et al., 2012; Weber et al., 2009). Conversely, cellular entry of Yersinia pseudotuberculosis and the ability of Chlamydia to produce infectious progeny are inhibited by OCRL1 knockdown (Moorhead et al., 2010; Sarantis et al., 2012).

1.4.3.5. Cytokinesis

Cytokinesis is the terminal step in cell division and involves the physical separation of two cells. Multiple membrane remodelling events occur during this process and as such there is overlap in the machinery involved in vesicular transport and cytokinesis (Echard, 2008; Skop et al., 2004). Turnover of PtdIns(4,5)P₂ from late cytokinesis bridges has been proposed to be mediated by OCRL1 following its recruitment to this site by binding to Rab35 (Dambournet et al., 2011). Failure to remove PtdIns(4,5)P₂ in the absence of OCRL1 leads to accumulation of actin at the intercellular bridge and defects in cell division, namely either a delay or complete block in abscission. A study by Ben El Kadhi et al also observed defects in cytokinesis upon knockdown of the Drosophila OCRL1 orthologue dOCRL supporting a role for OCRL1 in regulating PtdIns(4,5)P₂ turnover during cytokinesis (Ben El Kadhi et al., 2011).
1.4.3.6. Cell Polarity

The cell types affected in Lowe syndrome, cells of the proximal tubule, neurons, glia and cells of the lens epithelium are highly polarised cell types. Epithelial cells such as those of the proximal tubule or neuroepithelium require junctional complexes to arrange themselves into polarised tissues of varying morphologies. Many studies of OCRL1 function performed in cell culture models have utilised fibroblasts or dedifferentiated epithelial cells, which do not reflect the organisation of tissues in vivo. Investigations into OCRL1 function using cultures of polarised MDCK and CACO2 cells grown in 2-D have shown that OCRL1 participates in the development of polarised epithelial cells (Grieve et al., 2011). OCRL1 localises to junctions in MDCK and CACO2 cells and interacts with the junctional proteins ZO1 and α-catenin. Loss of OCRL1 affects cell shape and number as well as the abundance of apically localised proteins at the plasma membrane. Alterations in apical protein distribution have also been reported by Mrozowska and Fukuda, who demonstrated that depletion of OCRL1 in 2-D MDCK cell cultures led to the accumulation of podocalyxin in intracellular clusters instead of at the apical plasma membrane (Mrozowska and Fukuda, 2016). Two studies have also demonstrated that OCRL1 also impacts upon morphogenesis of 3D cysts, with a failure of cysts to complete lumen opening common to both reports (Grieve et al., 2011; Luo et al., 2012).

1.4.4. Functional redundancy between OCRL1 and INPP5B

Studies of OCRL in cell culture models have revealed a multitude of cellular processes in which OCRL1 participates, however which of these functions is relevant to the symptoms observed in Lowe syndrome is not well understood. This is further complicated by the presence of an additional OCRL1 paralogue in higher eukaryotes, INPP5B, which can potentially compensate for OCRL1 in its absence. INPP5B shows high sequence similarity to OCRL1, has the same domain architecture and interacts with many of the same binding partners including Rab GTPases, APPL1 and IPIP27A & B (Erdmann et al., 2007; Swan et al., 2010; Williams et al., 2007). INPP5B does not interact with clathrin and AP2 however, lacking motifs required for these interactions, suggesting that INPP5B
cannot compensate for OCRL1 in cellular processes which are dependent on OCRL1 recruitment by clathrin and AP2 (Erdmann et al., 2007; Mao et al., 2009). Studies into the subcellular location of INPP5B have revealed that it localises to sites of OCRL1 action including early endosomes and the Golgi apparatus, consistent with the presence of common binding partners (Shin et al., 2005; Williams et al., 2007). However, in comparison to OCRL1, INPP5B shows limited colocalisation with clathrin coated transport intermediates (Erdmann et al., 2007; Williams et al., 2007). Additionally, INPP5B localises to the ER to Golgi intermediate compartment (ERGIC) and cis-Golgi and disrupts trafficking of a number of proteins which cycle between the ER and ERGIC when overexpressed, a function not found for OCRL (Williams et al., 2007).

1.5. Animal models of Lowe syndrome

In order to better understand the physiological role of OCRL1 in relation to the symptoms observed in Lowe syndrome, a number of animal models have been developed. Generation of a mouse lacking OCRL1 surprisingly did not lead to congenital cataracts, Fanconi syndrome or neurological abnormalities (Janne et al., 1998). mRNA levels of INPP5B are higher in adult mouse tissues as compared with humans, including in the brain and kidneys, which may provide an explanation as to why mice are unaffected by loss of OCRL1 (Janne et al., 1998). Additionally, differences in processing of the INPP5B transcript in mice and humans may also affect how each species compensates for loss of OCRL1 function. The human INPP5B transcript undergoes alternate splicing in comparison to mouse INPP5B, producing a protein which lacks 80 amino acids encoded by an additional 240bp incorporated into exon 8 of the mouse INPP5B transcript (Bothwell et al., 2010). This was proposed to be highly relevant to the functional redundancy observed in mice between OCRL1 and INPP5B as humanizing the INPP5B locus in double OCRL / INPP5B knockouts led to low molecular weight proteinurea and aminoaciduria, although ophthalmological and neurological abnormalities were still absent (Bothwell et al., 2011). Selective deletion of INPP5B from the kidneys of OCRL1 knockout
mice also leads to defects in proximal tubule reabsorption further supporting the increased functional overlap between mouse OCRL1 and INPP5B (Inoue et al., 2017).

Zebrafish represent an alternative vertebrate organism in which to study OCRL1 function in vivo (Figure 6). The zebrafish genome contains copies of both OCRL1 and INPP5B genes that are highly homologous to their human orthologues, displaying 59% and 58% amino acid identity respectively. Key motifs required for binding to Rab GTPases, clathrin, AP2 and F&H motif containing proteins are also highly conserved and the intracellular localisation of Ocrl1 in zebrafish AB9 or PAC2 fibroblasts mirrors that observed in human cells (Ramirez et al., 2012). Furthermore, the tissue expression of Ocrl1 splice variants closely matches that seen in humans, with zebrafish Ocrl1a being highly enriched in brain tissue (Ramirez et al., 2012). Studies of ocr1 mutant zebrafish, which have attenuated Ocrl1 expression achieved by retroviral insertion into the ocr1 promoter, have revealed that loss of Ocrl1 function leads to abnormalities in all three of the tissues affected in Lowe syndrome (Oltrabella et al., 2015; Ramirez et al., 2012). These findings suggest that Ocrl1 fulfils the same physiological roles in these tissues as it does humans and that zebrafish are a good model in which to study OCRL1 function in vivo.

Figure 6. Comparison of symptoms observed in human patients with Lowe syndrome and zebrafish lacking OCRL1. Defective reabsorption in the proximal tubule is common to both humans and zebrafish and is likely due to impaired recycling of the multiligand scavenger receptor megalin. Zebrafish also show symptoms present in the brain of Lowe syndrome patients including periventricular white matter lesions and an increased susceptibility to febrile seizures. Adapted from (Mehta et al., 2014).
Oltrabella et al. found that loss of Ocrl1 interferes with normal kidney function in the developing zebrafish pronephros (Oltrabella et al., 2015). Endocytic uptake of fluid phase and low molecular weight protein tracers was severely reduced in the proximal tubule of ocrl1 mutants. This was found to be due to reduced levels of the multiligand scavenger receptor megalin at the apical plasma membrane, which mediates the bulk of endocytic uptake in the kidneys (Figure 7) (Christensen and Birn, 2002). Rescue of endocytic uptake by re-expression of various Ocrl1 mutant constructs was dependent on Ocrl1 catalytic activity, binding to F&H domain containing proteins and to a lesser extent interaction with Rab GTPases and clathrin. These data collectively point towards an in vivo role for OCRL1 in regulating endocytic trafficking of megalin within the pronephric tubule and provide a potential explanation for the low molecular weight proteinuria observed in Lowe syndrome patients.

Figure 7. Loss of Ocrl1 in the zebrafish pronephros leads to reduced fluid phase uptake and a reduction in megalin levels. a) Images of megalin immunofluorescence staining in the proximal tubule (dashed line) showing reduced megalin levels at the apical brush border in ocrl1 mutant embryos and accumulation of megalin in sub-apical endosomes. This is likely to be responsible for defective endocytic uptake of fluid phase markers shown in b). Images adapted from (Oltrabella et al., 2015).

Using the same ocrl1 mutant to study the effects of loss of Ocrl1 function in the brain, Ramirez et al. observed an increase in apoptosis of neural cell types at 1 day post fertilisation (dpf) in ocrl1 mutants embryos, leading to a visible reduction in brain size at this stage of development (Ramirez et al., 2012). A reduction in eye size could also be seen at 1dpf. Loss of neural tissue within the brain may potentially underlie the increased susceptibility to febrile seizures seen in 3dpf ocrl1 -/- larvae. Magnetic resonance imaging (MRI) revealed cerebrospinal fluid-filled white matter lesions in the
brain of adult ocrl1 mutants. Lesions showed strong GFAP staining by histological analysis, indicative of gliosis. Both of the latter two CNS abnormalities have been reported in human Lowe syndrome patients (Allmendinger et al., 2014; Loi, 2006). Experiments using various Ocr1 mutant constructs to rescue the increased apoptosis seen at 1dpf found that in addition to the importance of Ocr1 5-phosphatase activity, the enhanced clathrin binding activity of isoform-a was crucial for rescue of apoptosis (Ramirez et al., 2012). Ocr1b, which lacks the additional 8 amino acids adjacent to the LIDLE clathrin binding motif present in isoform-a, was unable to fully return the number of apoptotic cells back to WT levels. These results indicate that the function of OCRL1a within the brain is likely to involve regulation of clathrin dependent trafficking events.

Morpholino (MO) knockdown of ocrl1 also produces phenotypes similar to those described in ocrl1 mutant animals with ocrl1 morphants showing a reduction in brain mass at 1dpf and reduced fluid phase uptake in the pronephros at 3dpf (Oltrabella et al., 2015; Ramirez et al., 2012; Rbaibi et al., 2012). Other studies using zebrafish as a model organism to knockdown ocrl1 have revealed phenotypes consistent with ciliopathies, such as curved body axis, hydrocephaly, altered heart looping and disorganised pronephric cilia, however these phenotypes have not been observed in ocrl1 mutant animals (Coon et al., 2012; Rbaibi et al., 2012).

1.6. Overview of Zebrafish Embryonic Development

Development of a fertilised zebrafish embryo occurs rapidly over the first 24 hours post fertilisation (hpf) and can be broken down into four key stages; cleavage, blastula, gastrulation and segmentation (Figure 8). During the cleavage period, fertilisation of the embryo segregates non-yolky cytoplasm from the underlying yolk to form the first blastomere, which sits atop the yolk cell at the animal pole of the embryo. Subsequent division of the blastomere and its progeny produces a mound of cells which number around 64 by the end of this period (Kimmel et al., 1995). Cell divisions continue to occur through the ensuing blastula period until the formation of the yolk syncytial layer (YSL), a merging of cells which lie at the margin of the bastodisc and yolk cell, signifies the beginning
of epiboly where cells from the blastodisc and YSL thin out and spread down over the yolk cell (Kimmel and Law, 1985; Solnica-Krezel and Driever, 1994). Key morphogenetic movements occur during the gastrula period which begins at 5 hours post fertilisation (hpf). Cells of the spreading blastodisc involute, producing two distinct germ cell layers, the epiblast and hypoblast, which form the upper and lower layers of the germ ring (Warga and Kimmel, 1990). Both layers of cells then begin to stream towards the dorsal side of the germ ring producing a thickening of cells called the embryonic shield. Intercalation of cells from both layers at this point stimulates convergent extension movements to produce a narrowing and elongation of the embryo along the anterior-posterior axis. At 10hpf epiboly is complete, leading into the segmentation period between 10 and 24 hpf where the majority of the primary organs, including the brain and kidneys, begin to take shape and are clearly distinguishable (Kimmel et al., 1995).

Figure 8. Overview of embryonic development and brain organisation at 24hpf. A) Schematic showing different developmental stages during the first 24 hours of zebrafish development from fertilisation through to gastrulation and organogenesis / segmentation. B) Left, drawing of zebrafish embryo at 24 hpf and right, major subdivisions of the brain present at the same time period. Divisions of the forebrain, midbrain and hindbrain are all clearly distinguishable. T = telencephalon, D = diencephalon, E = epiphysis, C = cerebellum, R1-7 = hindbrain rhombomeres 1-7, FP = floorplate. (A) was adapted from www.mun.ca/biology. (B) adapted from (Kimmel et al., 1995).
1.7. Zebrafish Neural Induction and Neurogenesis

Zebrafish CNS development initially begins around 5hpf when involution initiates formation of the primary germ layers (Kimmel et al., 1995). The epiblast layer gives rise to the neuroectoderm in response to signals emanating from the embryonic shield, one of many signalling centres involved in patterning of neuronal cell types along the dorsoventral and anterior posterior axes of the brain. The development of the brain in higher and lower vertebrates progresses to a structure common to both phyla, the neural tube, although the mechanism by which this is achieved differs. Infolding of cells at the midline of the neural plate forms a solid block of cells called the neural keel, an intermediate step not seen in higher vertebrates (Papan and Campos-Ortega, 1994) (Figure 9a). At this point, cells are arranged so that cell bodies and processes lie across the midline of the neural keel (Hong and Brewster, 2006). Formation of the neural tube lumen requires that cells segregate themselves across and clear of the midline and polarise, forming an apical pole lining what will eventually become the brain ventricles (Ciruna et al., 2006; Clarke, 2009). Rather than in higher vertebrates where tissue polarisation is inherited from cells within the epiblast, in zebrafish, cell divisions are thought to drive polarization of the neuroepithelium and lumen formation (Tawk et al., 2007). Division of cells aligned across the midline generates daughter cells with mirror symmetric apical basal polarity and deposits them either side of the newly formed neural rod by 17 hpf. Apical protein markers such as Par3 can be observed either side of the cleavage furrow prior to abscission, indicating formation of apico-basal polarity in adjacent cells (Tawk et al., 2007). By 20 hpf the end feet of these cells push apart with the apical poles still facing one another to form the lumen of the neural tube.

Within the zebrafish neural tube, neuroepithelial cells comprise the predominant cell type at this timepoint and serve as the pool of cells from which neurons and further progenitors are derived (Gotz and Huttner, 2005). Neuroepithelial cells are bipolar and adopt an elongated spindle like morphology with thin attachments to both basal and apical surfaces. Due to interkinetic nuclear migration (IKNM) of cell nuclei at different stages of the cell cycle along the apico-basal axis, the
neuroepithelium has a pseudostratified appearance but is in fact a monolayer (Figure 9b-e) (Norden, 2017).

To drive neurogenesis, progenitor divisions in the neuroepithelium are split between asymmetric divisions which generate neurons and symmetric divisions which replenish precursor pools, with the decision to commit to either fate determined by numerous cell fate determinants (Willardsen and Link, 2011). Initially, neuroepithelial cells undergo symmetric, proliferative divisions to generate two identical daughter cells. This expands and maintains neuronal precursor pools for subsequent asymmetric cell divisions from which neurons are derived (Gotz and Huttner, 2005). Patterning of neuronal cell types along the dorsoventral and anterior-posterior axis of the CNS is accomplished by morphogens such as Wnt, FGF and Shh, which act over long distances to impart positional information to cells in a concentration dependant manner and thereby specify cell fate (Le Dreau and Marti, 2012; Schmidt et al., 2013). The proneural genes *ash* and *neurog* respond to the cues provided by morphogen gradients and other factors to up regulate production of the Notch ligand Delta, inhibiting differentiation of neighbouring cells (a process called ‘lateral inhibition’) whilst simultaneously initiating a positive feedback loop which commits the signal-receiving cell to a neuronal fate (Bertrand et al., 2002). Morphogens have also been shown to have a role as mitogenic or cell survival signals, sustaining proliferation of neuronal precursors (Ulloa and Briscoe, 2007). Proliferation and differentiation of neuronal tissue has occurred to such a degree by 24 hours that prominent subdivisions of the brain are already visible (Figure 8b) and the first neuronal circuits are well established (Kimmel et al., 1995).
Figure 9. Tissue architecture of the neuroepithelium at 24 hpf. A) Transverse view of the developing zebrafish brain showing stages of neuroepithelium development. The apical marker Par3 is shown in green. To highlight the eventual fate of cells within the developing neuroepithelium, cells from the left and right hand sides of the neural plate are shown in light and dark grey respectively. Adapted from (Clarke, 2009). B) Dorsal view of the zebrafish brain at 24 hpf with the fore, mid and hindbrain ventricles labelled with F, M and H respectively. C) Single plane confocal micrograph showing the arrangement of neuroepithelial cells within the boxed region in (B). Scale bar = 10 µm. Multiple layers appear to be visible. Cell membranes have been labelled with membrane-GFP. D) Schematic showing that the stratified appearance of the neuroepithelium is due to differing positions of cell nuclei along the apico-basal axis. Nuclei migrate along this axis depending upon cell cycle stage. Cells round up to undergo division at the apical membrane (for example see asterisk in (C)). Adapted from (Norden, 2017). E) Confocal micrograph of a tissue section from a 1dpf zebrafish brain, showing positioning of differentiated neurons within the midbrain hindbrain boundary region of the neuroepithelium. Differentiated neurons are labelled with an antibody to HuC, an RNA binding protein important for post mitotic neuronal differentiation (Park et al., 2000). Cell nuclei are stained with Hoechst. Following division, newly born neurons migrate towards the basolateral surface. Scale bar = 20µm.
1.8. Endocytic trafficking and neuronal development

As described in the previous sections, OCRL1 has been implicated in numerous cellular processes, many of which are crucial for CNS development. Signalling through the primary cilium is important for Shh signalling and progenitor proliferation (Goetz and Anderson, 2010; Han and Alvarez-Buylla, 2010), whilst junctional complexes are critical for neuroepithelial tissue organisation (Hong and Brewster, 2006). OCRL1 may directly participate in PtdIns(4,5)P2 dependent processes at these compartments. However an indirect role for OCRL1 regulating trafficking of components to the primary cilium or junctional complexes may also contribute to cellular phenotypes caused by dysfunction of cilia or junctional complexes in vitro and in vivo.

Endocytic trafficking directly co-ordinates many aspects of developmental signalling, from control of receptor internalisation and the length of time an activated receptor signals for (Nowak et al., 2011), to ligand secretion and recycling of signalling components (Shilo and Schejter, 2011). Multiple signals are sent, received and interpreted by neuroepithelial cells which influence cellular behaviours ranging from tissue morphogenesis to cell fate decisions. Key morphogen pathways such as the Wnt, Shh, TGFβ and Notch signalling pathways are all at some level regulated by endocytosis or intracellular trafficking of signalling pathway components (Bokel and Brand, 2014) and many proteins involved in development signalling in the CNS utilise trafficking pathways regulated by OCRL1.

Megalin has been shown to interact directly with Shh (McCarthy et al., 2002) and influence Shh signalling in the CNS, playing opposing roles in different tissues. In the mouse retinal margin, megalin antagonises morphogen action by enhancing endocytic clearance of Shh, preventing over-proliferation of retinal progenitor cells (Christ et al., 2015). In contrast, in neuroepithelial cells of the mouse rostral diencephalon ventral midline (RDVM), megalin sequesters Shh in the target field and
regulates its intracellular trafficking, expanding Shh signalling range (Christ et al., 2012). Deficiency of megalin in mice therefore leads to forebrain malformations due to loss of Shh signalling from areas of the forebrain (Christ et al., 2012). In cells of the RDVM, megalin forms a complex with Ptc and Shh that traffics through Rab4 and Rab11 dependent recycling pathways to further increase local Shh concentrations. This contrasts with the retinal margin where following binding to megalin, Shh is instead trafficked towards the lysosome for degradation (Figure 10). This example serves to highlight the importance of receptor recycling in CNS development. However, the function of Shh in patterning the ventral forebrain does not appear to be conserved in zebrafish as megalin knockout embryos do not display forebrain defects (Kur et al., 2011).
Other members of the low density lipoprotein (LDL) family of receptors that megalin belongs to are also important for development of the CNS (Auderset et al., 2016b). Both megalin and LRP1 are expressed in growth cones required for axon guidance and innervation of target cells by newly born neurons. Here they act as chemotactic receptors through binding to metallothionein which stimulates chemotaxis towards the source (Landowski et al., 2016). Additionally, selective deletion of mouse Lrp1 from neurons causes motor dysfunction and behavioural abnormalities amongst a plethora of other symptoms affecting neuronal tissue, highlighting a key role for Lrp1 in the CNS (May et al., 2004; Mulder et al., 2004).

Due to variations in the sorting motifs found within the cytoplasmic tails of LDL receptors (LDLR) that dictate their intracellular trafficking, many of these receptors reside basolaterally in polarised cell types, rather than apically as with megalin (Marzolo et al., 2003; Takeda et al., 2003). However similar recycling pathways participate in routing receptors to the basolateral membrane, with receptors being trafficked through basolateral sorting endosomes and a common perinuclear recycling endosome on route to the plasma membrane (Apodaca et al., 2012). Similar machinery likely participates in regulating trafficking to both the apical and basolateral membranes. OCRL1 therefore has the potential to affect cargos trafficking to apical and basolateral domains within CNS cell types including members of the LDLR family.
1.9. Aims / Purposes of study

Whilst much has been discovered about the cellular roles of OCRL1, the pathogenesis of Lowe syndrome remains unclear. Studies in zebrafish have provided some insight into the causative mechanisms, particularly in the kidneys where abnormalities in the endocytic pathway and reduction in megalin levels point towards dysregulation of endocytic trafficking in the absence of OCRL1. Additionally, OCRL1 has been implicated in regulating lysosomal function and autophagosome clearance within the kidneys which may also contribute to disease pathology. In the brain, characterisation of ocrl1 -/- mutant zebrafish has revealed that increased apoptosis of neural tissue during early development is linked to OCRL1 mediated regulation of clathrin dependent trafficking, suggesting OCRL1 also plays an important role in endocytic trafficking within the CNS. However, no in depth analysis of endocytosis or endocytic compartments has been performed within this tissue.

This study therefore set out to test the hypothesis that OCRL1 regulates endocytic trafficking within the developing zebrafish brain, with dysfunction of trafficking pathways due to the loss of OCRL1 function being a potential causative mechanism for the symptoms affecting the CNS observed in Lowe syndrome patients. To achieve this, an initial aim was to characterise trafficking of megalin and associated ligands within the neuroepithelium for use as a readout of OCRL1 dependent endocytic trafficking, followed by functional analysis of loss of OCRL1 on the endocytic pathway.

Based on studies proposing a role for OCRL1 in lysosome function and autophagosome turnover, a second aim was to utilise OCRL1 knockout zebrafish to investigate whether the loss of OCRL1 leads to impairment of lysosome function and autophagosome clearance in the brains of OCRL1 mutant zebrafish, which may also contribute to CNS abnormalities in Lowe syndrome.
2. Chapter 2 - Materials and methods
2.1. Materials

mMESSAGE machine T7 / SP6 mRNA synthesis kit (Ambion)
MEGAclear transcription cleanup kit (Thermofisher)
GoTaq (Promega)
Q5 (New England Biolabs)
Primers (MWG)
Restriction enzymes (New England Biolabs)
Tris-acetate-EDTA (TAE) buffer (made in house)
Phosphate buffered saline (PBS) (made in house)
Ethidium bromide
Agarose (Lonza)
Low melting point agarose (Clontech)
Superfrost plus slides (Thermofisher)
Gelatin (Sigma-aldrich)
Sucrose (Fisher scientific)
Donkey serum (Thermofisher)
Mowiol 4-88 (Sigma-aldrich)
Tween-20 (Sigma-aldrich)
Triton X-100 (Fisher scientific)
NP-40 substitute (Sigma-aldrich)
Sodium hydroxide tablets (Sigma-aldrich)
Organic solvents – Methanol / ethanol (Fisher scientific)
Acetone (Millipore)
Dimethyl sulfoxide (Sigma-aldrich)
Sodium dodecyl sulfate (SDS) (Fisher scientific)
Glycerol (Fisher scientific)
Methylene blue (Sigma Aldrich)
N-phenylthiourea (Sigma Aldrich)

Ethyl 3-aminobenzoate methanesulfonate (Sigma-aldrich)

Acrylamide (Protogel, National diagnostics USA)

Ammonium persulphate (APS) (Sigma-aldrich)

Tetramethylenediamine (BioRad)

Tris(hydroxymethyl)aminomethane (Tris) (Fisher scientific)

10X Tris-glycine-SDS (TGS) buffer (Biorad)

Nitrocellulose (Merck)

Polyvinylidene fluoride membrane (PVDF) (Merck)

Molecular weight markers (New England Biolabs)

Supersignal west pico / femto chemiluminescent substrate (Thermofisher)

Gel purification kits, PCR clean up kits, miniprep and maxiprep kits (Bioline)

Glass cover slipped bottomed 35mm dishes (Corning)

Torin1 (1mM stock in DMSO) (Merck)

Dyngo4a (Abcam)

BafilomycinA1 (1mM stock in DMSO) (Sigma-aldrich)

Luria-bertani (LB) broth and agar (Melford laboratories)

Cy3 (GE lifesciences)
2.2. Antibodies

Table 1 – Primary antibodies used for western blotting (WB) and immunofluorescence (IF)

<table>
<thead>
<tr>
<th>Name</th>
<th>Species Raised in</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMP1</td>
<td>Rabbit</td>
<td>1:200 (IF)</td>
<td>Abcam (ab24170)</td>
</tr>
<tr>
<td>Golgin 84</td>
<td>Sheep</td>
<td>1:1000 (IF)</td>
<td>Custom made, Professor Martin Lowe, University of Manchester</td>
</tr>
<tr>
<td>EEA1</td>
<td>Sheep</td>
<td>1:400 (IF)</td>
<td>Santa cruz (SC-6415)</td>
</tr>
<tr>
<td>Megalin</td>
<td>Rabbit</td>
<td>1:100 (IF)</td>
<td>Dr Michella Marino, University of Pisa</td>
</tr>
<tr>
<td>HuC</td>
<td>Mouse</td>
<td>1:200 (IF)</td>
<td>Thermofisher, (A-21271)</td>
</tr>
<tr>
<td>LC3</td>
<td>Rabbit</td>
<td>1:1000 (WB)</td>
<td>Abcam (ab51520)</td>
</tr>
<tr>
<td>Gabarap</td>
<td>Rabbit</td>
<td>1:400 (WB)</td>
<td>Abgent (AP1821)</td>
</tr>
<tr>
<td>OCRL1</td>
<td>Sheep</td>
<td>1:200 (WB)</td>
<td>Custom made, Professor Martin Lowe, University of Manchester</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Mouse</td>
<td>1:2000 (WB)</td>
<td>Keith Gull</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rabbit</td>
<td>1:2000 (WB)</td>
<td>Santa cruz (SC-25778)</td>
</tr>
<tr>
<td>p-P70 S6K</td>
<td>Rabbit</td>
<td>1:1000 (WB)</td>
<td>Cell signalling (9234S)</td>
</tr>
<tr>
<td>P70 S6K</td>
<td>Rabbit</td>
<td>1:1000 (WB)</td>
<td>Cell signalling (2708S)</td>
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</table>
### Table 2 — Secondary antibodies used for western blotting and immunofluorescence

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-rabbit horseradish peroxidase</td>
<td>Goat</td>
<td>1:2000 (WB)</td>
<td>Jackson immuno</td>
</tr>
<tr>
<td>Donkey anti-goat horseradish peroxidase</td>
<td>Donkey</td>
<td>1:2000 (WB)</td>
<td>Jackson immuno</td>
</tr>
<tr>
<td>Goat anti-mouse horseradish peroxidase</td>
<td>Goat</td>
<td>1:2000 (WB)</td>
<td>Jackson immuno</td>
</tr>
<tr>
<td>Donkey anti-Rabbit / Goat / mouse Alexafluor488</td>
<td>Donkey</td>
<td>1:200 (IF)</td>
<td>Jackson immuno</td>
</tr>
<tr>
<td>Donkey anti-Rabbit / Goat / mouse Alexafluor594</td>
<td>Donkey</td>
<td>1:600 (IF)</td>
<td>Jackson immuno</td>
</tr>
<tr>
<td>Donkey anti-Rabbit / Goat Cy3</td>
<td>Donkey</td>
<td>1:200 (IF)</td>
<td>Jackson immuno</td>
</tr>
<tr>
<td>Donkey anti-Rabbit / Goat Cy5</td>
<td>Donkey</td>
<td>1:600 (IF)</td>
<td>Jackson immuno</td>
</tr>
</tbody>
</table>

#### 2.3. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blotting

##### 2.3.1. Preparation of zebrafish protein extracts

Embryos at the appropriate stage were culled in MS222. To remove yolk proteins, 500µl Ginsburg fish Ringer’s solution (110mM NaCl, 3.5mM KCl, 2.7mM CaCl$_2$·2H$_2$O, 2.3mM NaHCO$_3$, 10mM Tris pH 8.5) was added to embryos in a 1.5ml Eppendorf and a 200 µl pipette used to draw up this solution repeatedly (x20), disrupting the yolk sac. Following two washes in Ginsburg, embryos were pelleted at 300 x g for 1 minute and as much of the Ringer’s solution as possible removed. An appropriate volume of RIPA buffer (20mM Tis-HCl, 150mM NaCl, 1mM EGTA, 1% (v/v) NP-40 substitute, 1% (w/v) sodium deoxycholate) was then added and embryos homogenised using a pestle. Lysates were centrifuged for five minutes at 15,000 x g to pellet debris and transferred to a fresh 1.5ml tube. 6 µl of lysate was removed and protein concentration determined using the BCA assay. Lysates were snap frozen in liquid nitrogen and stored at -80°C. All steps prior to quantification were performed at 4°C.

For 2dpf head lysate preparation, embryos were anaesthetised in MS222, held in position with a pair of forceps and the head removed at approximately the 5th rhombomere using a stainless steel blade. Lysates were prepared as described above using 0.5µl RIPA buffer added per head.
For adult brain lysate preparation, adults were euthanised in MS222, quickly transferred into a dish of cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and the brain dissected as previously described (Gupta and Mullins, 2010). Dissected tissue was transferred into a 1.5ml Eppendorf tube and as much PBS as possible removed. 100 µl RIPA buffer was then added and tissue homogenised on ice with a pestle. Lysates were centrifuged for five minutes at 15,000 x g to pellet debris and transferred to a fresh 1.5ml tube. 6 µl from each sample was used to quantify protein concentration using the BCA assay.

2.3.2. SDS-PAGE

Tris-based gels were used to separate proteins by SDS-PAGE using the BioRad Mini-Protean system. Gels consisted of two parts - a 3% stacking gel and a running gel of variable %. Ingredients for running gels of various percentages are shown in Table 3. The volumes of acrylamide and ddH₂O were adjusted in the running gel to achieve the desired final percentage gel.

Table 3 – SDS-PAGE running buffer recipes used

<table>
<thead>
<tr>
<th></th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>14%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O (ml)</td>
<td>4.85</td>
<td>4.15</td>
<td>3.2</td>
<td>2.8</td>
</tr>
<tr>
<td>1.5M Tris buffer, 0.4% (w/v) SDS, pH 8.8 (ml)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>30% Acrylamide (ml)</td>
<td>2.65</td>
<td>3.35</td>
<td>4</td>
<td>4.7</td>
</tr>
<tr>
<td>APS (ml)</td>
<td>0.036</td>
<td>0.036</td>
<td>0.036</td>
<td>0.036</td>
</tr>
<tr>
<td>TEMED (ml)</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
</tr>
</tbody>
</table>

For stacking gels, 0.5M Tris buffer, pH 6.8 was mixed with 30% (v/v) polyacrylamide and MQ water to a final volume of 10ml. 15µl APS and 7µl TEMED were then added and gels allowed to set for 20 minutes at room temperature. Prior to running lysates, an appropriate volume of 5X Laemml buffer (0.5M Tris HCl pH 6.8, 50% (v/v) glycerol, 5% (v/v) SDS, 0.25% (w/v) bromophenol blue, 12.5% (v/v)
β-mercaptoethanol) was added to quantified zebrafish lysates and lysates heated to 95°C for 5-10 minutes. Samples were then loaded as desired into the wells of an appropriate % Tris-glycine gel depending on the protein of interest, as shown in Table 4. For SDS-PAGE, gels were run in 1X TGS buffer (25mM Tris, 0.19M Glycine, 0.1% (v/v) SDS) for 90 minutes at a voltage of 120V. Following separation of proteins, gels were then removed from their casts and incubated in Towbin buffer (1X, 25mM Tris, 192mM glycine, 20% (v/v) methanol) for 5 minutes prior to transfer.

Table 4 - Molecular weights of proteins blotted, % gels and membrane types used for each protein

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Molecular weight (kDa)</th>
<th>% gel</th>
<th>Membrane used to blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCRL</td>
<td>110</td>
<td>8</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>Tubulin</td>
<td>55</td>
<td>10</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>GAPDH</td>
<td>36</td>
<td>12</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>LC3II</td>
<td>12</td>
<td>14</td>
<td>PVDF</td>
</tr>
<tr>
<td>p-p70-s6k</td>
<td>70</td>
<td>10</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>p70 s6k</td>
<td>70</td>
<td>10</td>
<td>nitrocellulose</td>
</tr>
</tbody>
</table>

2.3.3. Western blotting

Following separation of proteins by gel electrophoresis, proteins were transferred to either nitrocellulose or PVDF membrane by wet transfer in 1x Towbin buffer for 2 and a half hours at a constant current of 300 mAmps. Following transfer, membranes were blocked for 1 hour in 5% (w/v) milk in TBST (1X TBS (50mM Tris-Cl pH 7.5, 150mM NaCl), + 0.1% (v/v) Tween-20). Primary antibody incubations were then performed in 5% milk in TBST for 2 hours at room temperature or overnight at 4°C. After primary antibody incubation, membranes were washed in containers on a shaker for 3 x 5 minutes in TBST. Secondary antibodies conjugated to horseradish peroxidase were added to 5% milk in TBST and membranes typically incubated for one hour at room temperature before three 5 minute washes in TBST to remove unbound antibody. West Pico or Femto ECL substrate (Thermofisher) was then added onto membranes and bands visualised using MR film. For quantification of protein levels on western blots, a Bio-Rad gel doc imaging station was used to
automatically calibrate emitted luminescence within the linear range for each protein. Protein amount for each sample were calculated by normalising the luminescence value for the protein of interest (POI) to an internal loading control (tubulin or GAPDH) and values presented as POI luminescence value divided by internal loading control luminescence value.

2.4 Molecular biology

2.4.1. Polymerase chain reaction

For subcloning of LAMP1-GFP and LAMP1 RFP into pcGlobin, primers flanking the sequence of interest with appropriate restriction enzyme sites were added to a reaction containing 100 ng of template plasmid DNA and Q5 mastermix (New England Biolabs). 12.5 µl of 2X Q5 mastermix was added to 1 µl each of 10 µM forward and reverse primer stocks, an appropriate volume of DNA template and the final volume adjusted to 25 µl with ddH₂O. Cycling conditions for reactions involving Q5 mastermix are as shown in Table 5.

For ocrl121582 genotyping, adult fish were anaesthetised in 0.4mg / ml MS222 solution and a small piece of tissue clipped from the tip of the dorsal tail fin. This was subsequently dissolved in 100 µl of 50 mM NaOH by incubating at 95 °C for 20 minutes. After 5 minutes incubation on ice, 10 µl 1M TRIS (pH 8) was added, samples vortexed and centrifuged at 15,000 x g for 1 minute to pellet cellular debris. 1 µl of this crude genomic DNA mixture was then added to a reaction containing 1 µl each of 10 µM forward and reverse primers flanking the region of interest, 12.5 µl 1x GoTaq green mastermix (Promega) and adjusted to a final volume of 24 µl with ddH₂O. Representative cycling conditions for reactions using GoTaq are as shown in Table 6. Following PCR on genomic DNA, 1 µl of SpeI enzyme (20,000 units / ml) was added to the PCR reaction mixture and incubated at 37 °C for 2 hours before visualisation of digestion products on a 1.5% (w/v) agarose gel.
Table 5 – Thermocycler program for PCR using Q5 mastermix

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>30 seconds</td>
<td>1</td>
</tr>
<tr>
<td>98</td>
<td>10 seconds</td>
<td>30</td>
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<tr>
<td>50-55</td>
<td>20 seconds</td>
<td></td>
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<tr>
<td>72</td>
<td>20 s / kb</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>2 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 6 - Thermocycler program for PCR using GoTaq green mastermix

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2 minutes</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>30 seconds</td>
<td>30</td>
</tr>
<tr>
<td>50-55</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1 minute / kb</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>5 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

2.4.2. Ligation of amplified inserts

For ligation of amplified PCR products into recipient vectors, insert and vector were combined at a molar ratio of 1:3 containing no more than 100 ng DNA, in a reaction mixture consisting of 2 µl 10X T4 DNA ligation buffer and ddH2O to 20µl. 1µl of T4 (400,000 units / ml, New England Biolabs) was then added to this mixture and the solution mixed before being incubated at room temperature for 5 minutes.
2.4.3. Bacterial transformation

1 μl of ligation mixture was incubated on ice in 100 μl of Top10 bacterial cells for 30 minutes before being heat shocked for 90 seconds at 42°C. Heat shocked bacteria were incubated for a further 5 minutes on ice. 400 μl chilled LB broth was added to bacteria and the mixtures incubated at 37°C for 40 minutes to 1 hour to allow expression of ampicillin resistance genes. Transformants were then centrifuged at 3000 x g for 1 minute to pellet cells and resuspended in 200 μl LB broth. All 200 μl was then plated up onto LB agar plates containing 100 μg/ml ampicillin and incubated at 37°C overnight. The next day, transformed colonies were picked with a 2 μl pipette tip, placed into 5 ml of LB supplemented with 100 μg/ml ampicillin and left to grow at 37°C overnight. To confirm successful ligation of insert into donor vector, colony PCR was performed using a forward primer within the insert of interest and a reverse primer, typically BGH reverse, within the plasmid backbone. Products were separated by gel electrophoresis on a 1% (w/v) agarose TAE (40 mM Tris pH 7.6, 20 mM acetic acid, 1mM EDTA) gel supplemented with ethidium bromide and visualised with an ultraviolet (UV) transilluminator.

2.4.4. Miniprep and maxiprep of plasmid DNA

For minipreps, overnight cultures of transformed bacteria were centrifuged at 3,000 x g 4 °C for 10 minutes in order to pellet bacteria. For maxipreps, culture volumes were scaled up as required and incubated overnight at 37°C. In both cases plasmid DNA was then isolated as per the manufacturer’s instructions for mini and maxi prep kits (Qiagen). Quantity and quality of purified DNA was measured by spectrophotometry. 40-100 ng/μl of purified plasmid DNA was then sent for sequencing by GATC Biotech.

2.5. Animal experiments

2.5.1. Animal husbandry

AB Notts, ocrl<sup>1sa11582</sup>, ocrl<sup>umc5Tg</sup><sup>umc5Tg</sup>, and transgenic adult zebrafish (section 2.6) were maintained by the University of Manchester Biological Services Facility as previously described (Westerfield, 2000).
and in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. To facilitate live imaging, N-phenylthiourea (PTU) was added to the water at 24 hpf to inhibit melanin synthesis.

2.5.2. mRNA synthesis and embryo injections

Plasmid DNA containing the transcript of interest was linearised using Xbal (pcGlobin) or NotI (pCS2) for 1 hr at 37°C and purified using a PCR clean up kit (Qiagen). mRNA was then synthesised using an mMESSAGE mMACHINE transcription kit (Ambion) containing either T7 or SP6 enzymes. Between 0.6 – 1 µg of linearised plasmid DNA was added to a reaction containing 10 µl dNTPs, 2 µl 10x reaction buffer and 2 µl T7/SP6 enzyme mix and adjusted to a final volume of 20 µl with ddH₂O. Following 2 hour incubation at 37°C the reaction was treated with 1µl Turbo DNAse (Ambion) to digest remaining template DNA. Synthesised mRNA was purified using a MEGAgclear transcription clean up kit (Ambion) and quantified by spectrophotometry.

For one cell stage injections, adult breeding pairs were set up the night prior to injection. Fresh mRNA solutions were typically prepared on the day, although solutions were on occasion reused a further 1-2 times after storage at -80°C. For fresh solutions, mRNA was mixed with a small volume of phenol red and KCl to a final concentration of 0.1 M. mRNA concentration was then adjusted to the desired final concentration using RNAsé free water. Microinjection needles were prepared from glass rods with a 1 mm outer diameter and 0.75 mm internal diameter using a P97 microinjection needle puller (Sutter Instruments). Dividers were removed from adult breeding pairs the next morning after setting up and eggs collected, rinsed in ddH₂O, and mounted in agarose moulds to hold eggs in place for injection. 1 nl of mRNA solution (See section 2.7 for mRNA concentration used for each transcript) was injected into blastocytes prior to the first cellular division. Embryos were then incubated at 28°C overnight before being screened for fluorescence the next morning on a Leica M165FC stereomicroscope.
2.5.3. Cryosectioning

Embryos were fixed in 4% (v/v) formaldehyde in PBS (pH 7.4) at 4°C overnight or at room temperature for 2-3 hours before being rinsed three times in PBST (PBS & 0.1% (v/v) Triton X-100) and dehydrated for a minimum of 30 minutes in methanol at -20 °C. Embryos were then rehydrated in descending 75%, 50% and 25% concentrations of methanol with PBST, rinsed three times in PBST and embedded overnight in gelatin (15% (v/v) fish skin gelatin, 30% (w/v) sucrose in PBS). 12 µm sections were then cut using a Leica CM3050 cryostat (object temperature -35°C, chamber temperature -25°C), tissue sections collected on coated Superfrost plus slides (Thermofisher), dried at room temperature for 5 minutes to allow adherence to slides and then frozen and stored at -80 °C until further use.

2.5.4. Immunofluorescence microscopy

Sectioned material was dried for 1 hour at room temperature before being submerged in 100% acetone at room temperature for 2 minutes and rehydrated in PBS for 10 minutes. To reduce non-specific binding of antibodies, slides were incubated with blocking solution (PBST & 10% (v/v) donkey serum) at room temperature in a humidified atmosphere. Following blocking, primary antibodies in blocking solution were added to slides and typically incubated for a minimum of 2 hours at room temperature, although the precise incubation conditions and contents of the antibody solution varied depending on the antibody. After primary antibody incubation, slides were rinsed in PBST 6 times for five minutes each. Secondary antibodies in blocking solution with or with Hoechst 33342 (1/1000) were then added to slides for 2 hours at room temperature or 4°C overnight. Slides were again washed 6 times in PBST for 5 mins each before excess liquid was drained and slides dried for two minutes at room temperature. Cover slips were then mounted on top of slides using Mowiol 4-88 and left to dry overnight.
2.5.5. Embryo mounting and hindbrain ventricle injections

For experiments where embryos were not to be injected with any endocytic tracer, embryos were anaesthetised in MS222 solution (0.4 mg/ml MS222 in embryo water), transferred to a 35mm coverslip bottomed cell culture dish and 1% (w/v) low melt agarose containing MS222 (0.4 mg/ml). Whilst the agarose was setting, embryos were orientated so as to be mounted laterally. Once the agarose had set, embryo water supplemented with MS222 (0.4 mg/ml) was then added onto embryos.

For experiments where embryos were to be injected with endocytic tracer, embryos were mounted dorsally. A 35mm cell culture dish was filled with 1% (w/v) liquid agarose, an Eppendorf lid added to the centre of the molten agarose to create a depression in the agarose and then left to set. Once set, the Eppendorf lid was removed from the solidified agarose with forceps and a series of holes made using a 2 µl pipette tip. Anaesthetised embryos were then transferred onto the plate and using a set of forceps manoeuvred into the newly made holes so that embryos were then stood on their tails. To hold embryos in place, excess liquid was removed and 100 µl of 1% (w/v) low melt agarose was then added on top of embryos until set and embryos anaesthetised with 0.4mg MS222 per ml chorion water. For hindbrain ventricle injections, a microinjection needle was loaded with the relevant endocytic tracer (Table 7) and 1 nl of the solution injected caudal to the midbrain hindbrain border as shown in Chapter 3, Figure 18.

Table 7 – Endocytic tracers and vital stains used and working concentrations

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Working concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAP-Cy3</td>
<td>2.45 mg / ml</td>
<td>Miss Guanhua Yan, University of Manchester</td>
</tr>
<tr>
<td>DQ-BSA</td>
<td>2 mg / ml</td>
<td>Thermofisher</td>
</tr>
<tr>
<td>FM4-64</td>
<td>1 mg / ml</td>
<td>Thermofisher</td>
</tr>
<tr>
<td>Lysotracker</td>
<td>10 µM</td>
<td>Thermofisher</td>
</tr>
</tbody>
</table>
2.5.6. Confocal microscopy and image analysis

For RAP-Cy3 endocytic uptake experiments, a maximum of 7 embryos were injected at 1 minute 15 second intervals over a ten minute period before being incubated at 28°C prior to imaging on a Leica SP8 upright confocal microscope at 30 minutes post injection. For each embryo, images from an approximately 50 µm volume of tissue were collected at 1.5 µm intervals with a 25x (NA 0.95) water immersible dipping objective. For live imaging of lysosomes, a similar volume of tissue was imaged in laterally mounted embryos. A 552 nm laserline set at 30% power (gain 545) was used for Cy3 excitation and emitted light between wavelengths 560 – 680 nm collected. Pinhole settings for all live imaging of RAP-Cy3 were fixed at 1 Airy unit.

After collection of images quantification of RAP-Cy3 uptake was performed by selecting equivalent areas of tissue measuring approximately 50 µm x 50 µm from 1 side of the MHB for analysis. Tracer within the ventricle was manually obscured in FIJI to prevent inclusion within any subsequent analysis. Images were then thresholded to exclude all pixel values below 40. Using the ‘analyse particles’ function in FIJI, endosomal puncta highlighted by thresholding were identified by the software and a series measurements on each identified puncta performed automatically by the analyse particles function. These measurements included particle area, average fluorescent intensity and total fluorescence intensity.

For imaging of cryosections, sequential dual channel imaging was used for dual labelling experiments where more than one fluorophore or fluorescent probe was used. Cryosections were imaged using a Leica SP5 upright confocal microscope with either a 40x (NA 1.25) or 63x (NA 1.4) oil immersion objective. For each cryosection, an approximately 20 µm volume was imaged at 0.5 µm intervals (pinhole setting 1 Airy unit, 95.48µm). A digital zoom of 2.5 – 4x was used depending on the experiment.
Colocalisation between proteins was measured manually. The total number of puncta for each protein were counted by hand and the number positive for both markers tallied before being expressed as the % of the protein of interest positive for the corresponding marker.
2.6. Transgenic lines used in this study

<table>
<thead>
<tr>
<th>Line</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<tr>
<td>Tg(h2afx:EGFP-Rab11)</td>
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2.7. mRNAs used in this study

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2.8. Primers used in this study

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OCRL1<sup>sa11582</sup> geno R  GCTGCTTTGACTAATCTCAGCTTTAC GTAAAGCTGAGATTAGTCAAAGCAGC
3. Chapter 3 - Investigating the role of Ocr1 in endocytic trafficking within the neuroepithelium
3.1. Characterisation of *ocrl*<sup>sa11582</sup>

Neurodevelopmental defects have been reported previously in mutant zebrafish containing a retroviral insertion upstream of the *ocrl1* start codon, reducing *ocrl1* gene expression by 70-80% (Ramirez et al., 2012). As part of the zebrafish mutation project (Kettleborough et al., 2013), additional mutations in the *ocrl1* gene have been isolated, induced by treatment of adult zebrafish with N-ethyl-n-nitrosourea (ENU). A mutant zebrafish line generated by this method, *ocrl*<sup>sa11582</sup>, was used in this study to examine the role of Ocr1 within the brain. *ocrl*<sup>sa11582</sup> contains a single G>T mutation in a splice acceptor site at the boundary of intron 16-17 and exon 17 which also alters a SpeI restriction enzyme site (Figure 11a).

This mutation would be predicted to disrupt Ocr1 function either through production of a truncated form of the protein lacking the ASH-RhoGAP domain or total ablation of *ocrl1* gene expression.

![Figure 11. Characterisation of *ocrl1*<sup>sa11582</sup>. A) Zebrafish *ocrl1* gene structure and regions of the protein encoded by each exon. Dashed box and blow up of DNA sequence showing the region in intron16-17 with the single base pair mutation and its position in relation to the SpeI restriction enzyme site (b). Intron sequence is coloured blue, exon sequence is black and the mutated residue is highlighted in red. C) Representative image of different *ocrl1*<sup>sa11582</sup> genotypes identified following amplification of exon16 – 17 from genomic DNA extracts and digestion with SpeI.](image)
through nonsense mediated decay of a faulty ocr1 transcript. Digestion of a 720 base pair (bp) DNA fragment amplified from a region spanning part of exon16 through to exon 17 with SpeI was able to accurately identify animals heterozygous and homozygous for the mutant allele (Figure 11b-c). Homozygous sa11582 mutants displayed a single 720 bp band resistant to digestion with SpeI (Figure 11c lane 4), whilst this fragment was fully digested in wild type animals, yielding DNA fragments of 444 bp and 276 bp respectively (Figure 11c, lane 2).

Western blotting for Ocr1 in lysates from homozygous ocr1\(^{sa11582/-}\) mutant embryos confirmed that the mutation led to a complete loss of the protein (Figure 12a). This contrasted with levels of Ocr1 in ocr1\(^{umc5Tg\text{umc5Tg}}\) (Retro) embryos, which exhibited only a partial reduction in Ocr1 levels as previously described (Ramirez et al., 2012). To control for off target effects caused by ENU mutagenesis, adult heterozygous ocr1\(^{sa11582+/-}\) zebrafish were incrossed and ocr1\(^{sa11582+/-}\) offspring retained alongside ocr1\(^{sa11582/-}\) embryos. ocr1\(^{sa11582+/-}\) zebrafish were used as controls for all subsequent experiments. Morphologically, ocr1\(^{sa11582/-}\) displayed a significant reduction in brain size at 1dpf which was not seen in ocr1\(^{sa11582+/-}\) embryos, (Figure 12b). In comparison to ocr1\(^{+/-}\) embryos, hindbrain, midbrain and forebrain regions are all less well defined at equivalent stages of development in ocr1\(^{-/-}\) mutants.

To examine levels of cell death in the brain at 1dpf, mutant embryos were crossed with transgenic zebrafish expressing secreted AnnexinV-mVenus under the control of a ubiquitous promoter (Tg(ubi:secAnnexinV-mVenus)) (van Ham et al., 2010). Cells undergoing apoptosis expose phosphatidylserine on the outer leaflet of their plasma membrane which acts as an ‘eat me’ signal for professional and non-professional phagocytes (Hochreiter-Hufford and Ravichandran, 2013). Annexin-V can bind phosphatidylserine and thereby act as a marker for apoptotic cells. ocr1\(^{-/-}\) embryos displayed higher levels of AnnexinV-mVenus positive cells in all regions of the brain, with the forebrain showing the greatest difference (Figure 12c-e).
Figure 12. Increased apoptotic cell death in OCRL1 mutant embryos. A) Western blot for Ocrl1 levels in ocrl1\textsuperscript{sa11582+/+}, ocrl1\textsuperscript{sa11582-/-} and ocrl1\textsuperscript{umc5Tg/umc5Tg} (Retro) (Ramirez et al, 2012) zebrafish lines. B) Brightfield images of laterally mounted ocrl1\textsuperscript{sa11582+/+} and ocrl1\textsuperscript{sa11582-/-} embryos showing head morphology at 1dpf. Scale bar = 50 µm C) Confocal z-projections of the head region of 1dpf Tg(ubi:secAnnexinV-mVenus);ocrl1\textsuperscript{sa11582+/+} or Tg(ubi:secAnnexinV-mVenus);ocrl1\textsuperscript{-/-} embryos. Right hand images are zoom ins of boxed areas in main image. Scale bar in main image = 50 µm. Scale bar right hand images = 20 µm. D) Quantification of the total number of AnnexinV-mVenus positive cells per head. E) Quantification of the number of AnnexinV-mVenus positive cells per region of the brain. Data in (d) and (e) are from 1 experiment, error bars represent mean ± s.e.m. *** p < 0.0001.
To determine which cell types are undergoing apoptosis in the developing brain, Tg(ubi:secAnnexinV-mVenus);ocrl1-/- embryos were fixed at 1dpf, sectioned and stained with an antibody to HuC, which labels differentiated neurons. No overlap could be seen between AnnexinV-mVenus and HuC suggesting that the dying cells are not neurons and are likely to be neuronal precursors (Figure 13). Dying cells are unlikely to be glia, as these cell types do not begin to colonise the brain until approximately 35 hpf (Herbomel et al., 2001).

In summary, increased cell death in the brain of ocrl1sa11582/- embryos is indicative of impaired neurodevelopment due to loss of Ocr1 and is consistent with the neurodevelopmental defects observed in a previously described Lowe syndrome zebrafish model (Ramirez et al., 2012). Furthermore, the lack of overlap between apoptotic and neuronal markers at this stage of development suggests that Ocr1 appears to be critical for survival of neuronal precursors.

3.2. Localisation of Ocr1 within the neuroepithelium

Loss of function studies in cultured cells have revealed that OCRL1 participates in both the late stages of endocytosis and intracellular trafficking events (Billcliff et al., 2016; Choudhury et al., 2005; Erdmann et al., 2007; Nandez et al., 2014; van Rahden et al., 2012; Vicinanza et al., 2011). Loss of OCRL1 in fibroblasts impairs uncoating of clathrin coated endocytic intermediates and leads to the
aberrant association of a number of PIP2 binding proteins with newly formed endocytic vesicles and endosomes (Nandez et al., 2014). Downstream of the plasma membrane, trafficking to and from endosomes is altered upon loss of OCRL1; CIMPR is typically found to accumulate in endosomes in OCRL knockdown cells (Billcliff et al., 2016; Cauvin et al., 2016; Choudhury et al., 2005; van Rahden et al., 2012; Vicinanza et al., 2011), consistent with reduced retrograde transport to the TGN. Trafficking of endocytic cargoes such as megalin and the transferrin receptor, which follow both slow and fast recycling pathways back to the plasma membrane, have also been shown to be affected by loss of OCRL1 (Choudhury et al., 2009; Vicinanza et al., 2011). Much evidence from in vitro studies therefore exists implicating OCRL1 in the regulation of both endocytosis and endocytic trafficking. In vivo, work in the developing zebrafish kidney also supports the view that OCRL1 plays a key role in endocytic trafficking, which likely underlies some of the symptoms of Lowe syndrome (Oltрабella et al., 2015). Endosomes of the proximal tubular epithelium enlarge in the absence of OCRL1 and levels of megalin are reduced, leading to a reduction in endocytic uptake from the glomerular filtrate. Studies in the CNS of ocr1 knockout embryos have revealed the importance of OCRL1 interaction with components of the trafficking machinery as mutant forms of OCRL1 deficient in clathrin binding are unable to rescue the observed neurodevelopmental phenotype (Ramirez et al., 2012). However in this tissue, a more in depth analysis of endocytic trafficking in the absence of OCRL1 has not been performed.

To gain further insight into the role of Ocrl1 within the neuroepithelium, the localisation of Ocrl1a within neuroepithelial cells was examined. As antibodies to the endogenous protein were unable to detect Ocrl1 in immunofluorescence experiments, a GFP tagged form of Ocrl1a was used. OCRL1a is the only OCRL1 isoform expressed in human brain tissue, and contains an additional 8 amino acids which enhances clathrin binding (Johnson et al., 2003; Nussbaum et al., 1997). Fixed cryosections through the brain of 1dpf embryos transiently expressing EGFP-Ocrl1a were labelled with antibodies to a number of compartments where OCRL1 has previously been shown to localise in cultured cells. Experiments in cell culture have shown OCRL1 to predominantly be on the trans-Golgi and early
endosomes (Choudhury et al., 2005; Dressman et al., 2000). EGFP-Ocr1a is present at the Golgi, as indicated by staining with the cis-Golgi marker Golgin-84 (Figure 14a, 60.3% ± 7.7%). The proteins are in close proximity but do not overlap directly, consistent with OCRL1 localisation to the trans-side of the Golgi (Figure 14a). A proportion of neuroepithelial EGFP-Ocr1a is also found on early endosomes as marked with an antibody to early endosomal antigen 1 (EEA1) (Figure 14b, 14.2% ± 0.96%). Other sites of action for OCRL1 include late endocytic compartments, particularly when traffic to the lysosome is high (De Leo et al., 2016; Zhang et al., 1998). However at steady state in this cell type, only a small percentage of Ocr1 co-localised with the late endosomal / lysosomal marker LAMP1 (Figure 14c bottom panel, 6.1% ± 2.4%).

Figure 14. Localisation of Ocr1a in neuroepithelial cells. Representative confocal images of coronal cryosections through the brain of 1dpf embryos transiently expressing EGFP-Ocr1a and stained with antibodies to Golgin 84 (a), EEA1 (b) or LAMP1 (c). d) Mean % of EGFP-Ocr1a positive for each marker. Between 200-300 puncta were counted per marker from a minimum of three embryos. Scale bars = 5 µm. Error bars in (d) = s.d.
3.3. Analysis of Ocr11 function within the neuroepithelium

Megalin belongs to the LDL family of receptors that play important roles as multi-ligand receptors in a number of tissues, including the CNS (Auderset et al., 2016b; Christensen and Birn, 2002). In mice, loss of megalin impairs forebrain development through alterations in a number of morphogen pathways - most prominently those involving sonic hedgehog (Shh) (Christ et al., 2012; Spoelgen et al., 2005). In mouse forebrain neuroepithelial cells, megalin was shown to act as an apical Shh binding protein, sequestering Shh released from the pre chordal plate (PcP) within a target field to help pattern the rostral diencephalon ventral midline (RDVM) (Christ et al., 2012). Following Shh binding to megalin, this complex subsequently recycles back to the plasma membrane where Shh is released into the extracellular space, potentially increasing local Shh concentrations to facilitate RVDM patterning (Christ et al., 2012). However this function does not appear to be conserved in zebrafish as megalin knockout animals show no defects in forebrain development (Kur et al., 2011).

Despite megalin playing no apparent role in zebrafish brain development, alterations in megalin localisation or abundance in the absence of Ocr11 would suggest impairment of endocytic pathways likely to be traversed by other receptors and their cargos. As Ocr11 and Ipip27a are key regulators of megalin recycling in the pronephros (Oltrabella et al., 2015), they may also participate in trafficking of megalin within neuroepithelial cells, where the receptor follows a similar recycling route back to the plasma membrane (Christ et al., 2012). Megalin and its associated ligands were therefore used as a paradigm for endocytosis and receptor recycling in neuroepithelial cells.

From studies in forebrain neuroepithelial cells of E8.5 mouse embryos and explants from the retinal margin, megalin was found to reside in Rab4 positive early endosomes and Rab11 recycling endosomes in addition to the plasma membrane (Christ et al., 2015; Christ et al., 2012). To determine where megalin localises in the zebrafish neuroepithelium, control embryos were injected with mRNA encoding either EGFP tagged Rab5c, Rab11a or LAMP1, fixed and processed for sectioning before being stained with an antibody against megalin. In agreement with its localisation
in mice, megalin is found predominantly in Rab5c positive early endosomes and Rab11a positive recycling endosomes (Figure 15a-c) (Rab5c/megalin 25% ± 2.5%, Rab11a/megalin 80% ± 5%), with very little co-localisation seen between megalin and LAMP1 (5% ± 2%). To examine whether Ocrl1

![Confocal images](image.png)

**Figure 15. Ocrl1a partially colocalises with megalin in the neuroepithelium.** Confocal images of coronal sections through the dorsal midbrain hindbrain boundary (MHB) region of WT control embryos transiently expressing either EGFP-Rab5c (a), EGFP-Rab11a (b) or LAMP1 GFP (c) and stained with anti-megalin. d) Mean % of marker positive for megalin. A minimum of 300 puncta from 3 embryos were counted. e) Coronal sections through the MHB of embryos expressing EGFP-OCRL1a and stained with anti-megalin. f) Mean % of EGFP-Ocrl1a puncta positive for megalin. Approximately 1000 EGFP-OCRL1a puncta from 3 embryos were counted. Data in (a)-(c) and (d) are from 1 experiment. Scale bars = 5 µm. Error bars in (d) and (f) represent means ± s.d.
directly colocalises with megalin, EGFP-Ocr1a expressing embryos were fixed and stained with anti-megalin antibodies (Figure 15e-f). Approximately 20% of total Ocr1a co-localised with megalin, a figure similar to that for Ocr1 with the early endosome marker EEA1 (Figure 14).

The presence of Ocr1 on EEA1 positive early endosomes would suggest that it is actively recruited to this compartment to control PtdIns(4,5)P₂ levels. To examine the functional consequences of loss of Ocr1 on the neuroepithelial endocytic pathway, early endosomes were visualised by staining for EEA1 in ocr1+/+ and ocr1 -/- embryos. Strikingly, in comparison to WT controls, EEA1 positive endosomes in ocr1/-/- embryos appeared to be noticeably enlarged (Figure 16a). Quantification of endosome size revealed a 1.3 fold increase (Figure 16b, ocr1+/+; mean = 0.137µm² ± 0.02, ocr1-/-; mean = 0.178 µm² ± 0.06) suggesting that Ocr1 plays an important role in regulating endosome function within neuronal precursors.

As megalin transits through early endosomes and also co-localises with EGFP-Ocr1a, likely on early endosomes, megalin abundance and distribution in the brains of 1dpf ocr1+/+ and ocr1-/- embryos were assessed by immunofluorescence microscopy. A noticeable reduction in megalin intensity could be observed in ocr1-/- embryos (Figure 17a-d) (Mean fluorescence intensity: ocr1+/+; 113.1 ± 4.7, ocr1-/-; 105.6 ± 4.8. Total fluorescence intensity / µm membrane: ocr1+/+; 1982 ± 334, ocr1-/-; 701 ± 151, Total fluorescence intensity: ocr1+/+; 97162 ± 13111, ocr1-/-; 55094 ± 12135), however its distribution remained similar to wild-type with the majority clustered sub apically. Together, these results suggest that Ocr1 is important for endosomal function within the neuroepithelium with loss of Ocr1 activity leading to reductions in the overall levels of endocytic receptors such as megalin.
Figure 16. Enlargement of early endosomes in ocr11-/- neuroepithelial cells. A) Confocal images of coronal sections through the dorsal midbrain hindbrain boundary (MHB) of 1dpf ocr11+/+ and ocr11-/- embryos labelled with an antibody against EEA1. Images are a projection of 5 consecutive planes taken at 0.5 µm intervals from the same z-stack. Scale bars = 5 µm. B) Quantification of endosome size. Data shown is pooled from 3 independent experiments. N numbers represent total number of embryos imaged for each genotype. ** P<0.001. Error bars in (b) represent means ± s.e.m.
Figure 17. Reduced megalin abundance in ocr11 -/- neuroepithelial cells. A) Confocal images of coronal sections through the ventral forebrain of 1dpf ocr11+/+ and ocr11/-/- embryos labelled with an antibody against megalin. Images are a projection of a 20 µm tissue section imaged at 0.5 µm intervals. Scale bar = 10 µm. B) Quantification of average megalin intensity. C) Quantification of the total megalin fluorescence intensity per µm of apical membrane. D) Quantification of the total fluorescence intensity per tissue section. Data shown is pooled from 3 independent experiments. N numbers represent total number of embryos imaged for each genotype. * P <0.05, ** P <0.001, *** P<0.0001. Error bars in (b) – (d) represent means ± s.e.m.
3.4. Establishment of an endocytic assay to measure neuroepithelial endocytic uptake

Uptake assays in the zebrafish pronephros have revealed that loss of Ocrl1 leads to a reduction in endocytosis of megalin specific ligands and fluid phase markers, consistent with reduced megalin abundance at the apical brush border (Oltrabella et al., 2015). To further investigate the role of Ocrl1 in endocytosis and downstream trafficking within the neuroepithelium, a brain specific endocytic uptake assay was developed. The zebrafish ventricular system contains three cavities which begin to inflate with cerebrospinal fluid (CSF) at 18hpf, eventually giving rise to the fore, mid and hindbrain ventricles (Figure 18a-b) (Gutzman and Sive, 2009; Lowery and Sive, 2005).

Figure 18. Schematic representation of an endocytic uptake assay in the zebrafish neuroepithelium. A) Left, lateral view of the zebrafish brain at 1dpf showing the three main brain sub-divisions as labelled by F (forebrain), M (midbrain) and H (hindbrain). Right, single plane confocal micrograph of region in left hand image from 1dpf WT embryo expressing membrane-GFP mRNA. LysoTracker staining highlights the location of late endocytic compartments. B) Dorsal view of zebrafish brain at 1dpf. Right, boxed region from left hand image showing location of endocytic compartments, as labelled with the endocytic tracer RAP-Cy3. Scale bars = 10 μm
The large size of the ventricle at this stage of development allows additional ligands to be easily added to the CSF by microinjection (Lowery and Sive, 2005). As neuroepithelial cells endocytose factors found within the ventricular CSF that influence cell proliferation and survival, such as retinoic acid and FGF, exogenous ligands introduced into the CSF are also likely to be endocytosed (Chang et al., 2016; Lowery and Sive, 2009; Martin et al., 2006).

3.4.1. Characterisation of neuroepithelial endocytic tracer uptake

To test whether uptake of endocytic tracers by neuroepithelial cells could be followed by confocal microscopy, the hindbrain ventricle of transgenic embryos expressing EGFP-Rab7, a marker of late endosomes, was injected with FM4-64. FM4-64 is a lipophilic styryl dye which embeds itself in the outer leaflet of the plasma membrane before being endocytosed and distributed throughout the cells endomembrane system (Clark et al., 2011). Following injection, limited co-localisation of FM4-64 could be seen with EGFP-Rab7 at 2 minutes post injection (Figure 19a). At 10 and 20 minutes post injection however, a greater amount of FM4-64 could be seen to co-localise with EGFP-Rab7, demonstrating that FM4-64 is endocytosed by neuroepithelial cells and then enters the endocytic pathway (Figure 19b-c).

To directly assay endocytosis mediated by megalin, uptake of megalin specific ligands was also trialled. Receptor associated protein (RAP) is a chaperone that binds with high affinity to megalin and other LDL receptor family proteins within the secretory pathway. It has been used previously as a model megalin ligand in both cell culture and in vivo studies (Anzenberger et al., 2006; Christensen and Birn, 2002).

RAP follows a different itinerary to megalin following its internalisation. Whereas megalin is sorted towards Rab11 positive recycling endosomes prior to being returned to the plasma membrane (Perez Bay et al., 2016), RAP dissociates from megalin in the early endosome before being trafficked towards the lysosome for degradation (Lee et al., 2006). Following injection into the hindbrain ventricle, RAP-Cy3 predominantly co-localised with the early endosomal marker EGFP-Rab5c in
neuroepithelial cells at both 10 and 20 minutes post injection, with the amount of co-localisation remaining consistent at both time points (Figure 20a). In contrast, very little RAP-Cy3 could be seen to overlap with EGFP-Rab11a positive endosomes at all time points imaged (Figure 20b). Co-localisation of RAP-Cy3 with EGFP-Rab7 increased over time as RAP is trafficked towards and reaches degradative compartments (Figure 20c). Clathrin light chain-GFP (CLC-GFP) also co-localised with RAP-Cy3 (Figure 20d). Similar to EGFP-Rab5c, the proportion of CLC-GFP co-localisation remained consistent at all time points, in agreement with the presence of clathrin on early endosomes, as has previously been reported (Raiborg et al., 2001). Blocking endocytosis by treatment of embryos with
Dyngo, an inhibitor of the GTPase Dynamin, involved in vesicle scission from intracellular membranes, prevented uptake of RAP into neuroepithelial cells demonstrating that RAP is taken up by endocytosis (Figure 22). Thus, the megalin binding protein RAP is endocytosed by neuroepithelial cells before being trafficked through Rab5c positive endosomes towards the lysosome for degradation and is therefore suitable for use as an endocytic tracer in the neuroepithelium.
Figure 20. RAP progresses through the endocytic pathway and co-localises with endocytic markers. Confocal micrographs of the MHB region of dorsally mounted embryos showing co-localisation of RAP-Cy3 at +3, +10 and +20 minutes post injection with transiently expressed EGFP-Rab5c (a), EGFP-Rab11 (b), CLC-GFP (d) and stably expressed EGFP-Rab7 (b). 1dpf embryos were mounted dorsally and imaged by confocal microscopy. E) Quantification of co-localisation between endosomal markers and RAP-Cy3. N number indicates the number of puncta counted from a minimum of three embryos for Rab5, Rab and CLC. N number for Rab11 is from 1 embryo. Scale bars = 5 µm. Error bars in (e) represent means ± s.d.
3.4.2. Quantification of neuroepithelial endocytic uptake

To assess whether changes in neuroepithelial endocytic uptake could be detected and quantified, embryos were injected with high and low concentrations of RAP-Cy3 and fluorescence intensity measurements of RAP-Cy3 puncta performed. In order to exclude background fluorescence, images were thresholded to exclude all pixel values below a defined value. To obtain total RAP-Cy3 fluorescence intensity within the tissue, measurements were performed on all values above threshold. For measurement of average RAP-Cy3 puncta intensity, an additional segmentation step was used following thresholding to identify and outline individual puncta as shown in Figure 21a (bottom panel). The total fluorescence intensity for each puncta was then divided by its area to obtain the average.

For embryos injected with lower concentrations of RAP-Cy3, both total fluorescence intensity and average fluorescence intensity of RAP-Cy3 were lower than embryos injected with higher concentrations (Figure 21a-c). This was consistent across a number of images as applying the same method to five consecutive planes from the same z-stack produced similar results (Figure 21d-f). Additionally, in comparison to DMSO treated embryos, thresholding and segmentation of puncta from embryos treated with 40 µM Dyngo yielded no RAP-Cy3 puncta above threshold, consistent with Dynamin inhibition blocking endocytosis and entry of RAP into endosomal compartments (Figure 22a-c). The amount of RAP-Cy3 in the ventricle also appeared higher in Dyngo treated embryos at 30 minutes, indicative of reduced endocytic uptake from the ventricle. This demonstrates that quantification of RAP-Cy3 using this method can accurately detect changes in endocytic uptake.
Figure 21. Quantification of RAP endocytic tracer uptake. A) Single plane confocal micrograph from embryos injected with RAP-Cy3 showing high and low uptake 30 minutes post injection. Bottom panel; trace of puncta identified from the top panel image following thresholding to exclude all pixel values below 45. B) Quantification of total RAP-Cy3 fluorescence intensity within the tissue for each image in (a). C) Quantification of the average fluorescence intensity for each RAP-Cy3 puncta identified in (a). *P <0.05. D) Z-projection of five consecutive planes from the same embryo as imaged in (a). E) Mean total fluorescence intensity of RAP-Cy3 from the 5 consecutive z-planes shown in (d). F) Quantification of the average fluorescence intensity for each RAP-Cy3 puncta identified from images in (d). Scale bars = 5 µm. Error bars in (e) represent mean values ± s.d., bars in (c) and (f) represent the mean.
The size of RAP-Cy3 puncta could also be quantified. Endocytic compartments were labelled with RAP-Cy3 for 2 hours before embryos were treated with 100 nM Bafilomycin A. Bafilomycin A blocks lysosomal degradation through inhibition of the vacuolar ATPase (vATPase), which maintains lysosomal pH required for lysosomal hydrolase activity (Huss and Wieczorek, 2009). Bafilomycin A treatment increased the size of RAP-Cy3 labelled compartments in comparison to untreated controls as measured by thresholding and segmentation of images (Figure 23) (DMSO 0.48µm$^2$ ± 0.05, Bafilomycin A 1.8µm$^2$ ± 0.3).

Figure 22. The dynamin inhibitor Dyngo blocks neuroepithelial endocytic uptake of RAP-Cy3. A) Left panels: Single plane confocal micrographs of embryos 30 minutes post injection of RAP-Cy3. Embryos were treated with either DMSO or 40 µM Dyngo for 2 hours prior to injection. Right panels: RAP Cy3 puncta identified following thresholding and segmentation of images. B) Total fluorescence intensity of thresholded puncta from images in (a). C) Mean fluorescence intensity of all individual puncta identified in (a) Scale bars = 5 µm. Bar in (c) represents the mean intensity of all puncta identified.
Figure 23. Measurement of RAP-Cy3 puncta area. A) Single plane confocal images of 1dpf embryos injected with RAP-Cy3 and treated with either DMSO or 100nM BafilomycinA for 1 hour 2 hours post injection. Bottom panel shows puncta identified after thresholding and segmentation. B) Quantification of segmented RAP-Cy3 puncta area. Scale bars = 5 µm. Bar in (b) represents mean size of all puncta identified in each treatment group.
3.5. Functional analysis of RAP-Cy3 endocytosis and trafficking in ocr1l1-/- embryos

To assess whether loss of Ocrl1 has any impact on endocytosis in the neuroepithelium, ocr1l1+/+ and ocr1l1-/- embryos were injected with RAP-Cy3 and imaged 30 minutes post injection. Although a small number of ocr1l1-/- embryos showed visibly reduced uptake at 30 minutes, on average, both mean fluorescence intensity of puncta within the neuroepithelium and total fluorescence intensity were comparable between ocr1l1+/+ and ocr1l1-/- embryos (Figure 24a-c)(mean fluorescence intensity of individual RAP-Cy3 puncta: ocr1l1+/+; 65.89 ± 0.67, ocr1l1-/-; 65.01 ± 0.67, total fluorescence intensity within neuroepithelial tissue: ocr1l1+/+; 274589 ± 22279, ocr1l1-/-; 261076 ± 22407). As a reduced number of vesicles may be indicative of a reduced rate of endocytosis, the number of RAP-Cy3 puncta was also counted. However, the number of RAP-Cy3 puncta at 30 minutes post injection was comparable between both genotypes (Figure 24d) (ocr1l1+/+; 697 ± 49, ocr1l1-/-; 591 ± 37). RAP-Cy3 puncta size was also unaffected in ocr1l1-/- embryos (Figure 24e) (ocr1l1+/+; 0.63 ± 0.03, ocr1l1-/-; 0.63 ±0.04). These results indicate that loss of Ocrl1 has no effect on endocytic uptake of RAP-Cy3 or its downstream trafficking.

Lack of an effect on RAP-Cy3 endocytosis in ocr1l1-/- embryos appears at odds with the reduction in megalin levels, which is likely to mediate a large proportion of RAP-Cy3 endocytosis at the apical membrane. As a positive control to test whether complete absence of megalin inhibits RAP-Cy3 endocytosis, bugeye embryos were injected with RAP-Cy3. Bugeye mutants contain a T to A conversion at amino acid position 23 which leads to a premature stop codon in place of a cysteine (Veth et al., 2011). Surprisingly, bugeye embryos showed only a very minor reduction in the average intensity of RAP-Cy3 puncta (Figure 25a-b, WT 34.8 ± 0.7, bugeye 32.7 ± 0.7) and no significant reduction in total fluorescence intensity (Figure 25c, WT 82046 ± 21707, bugeye 70102 ± 18691) 30 minutes post injection. Consistent with this, tissue sections through the brain of 1dpf bugeye embryos injected with RAP-Cy3 and fixed 30 minutes post injection show that although megalin is completely absent from the apical membrane of neuroepithelial cells, RAP-Cy3 is visible within the
Figure 24. RAP-Cy3 uptake is similar between ocr1+/+ and ocr1/- embryos. A) Representative confocal images of RAP-Cy3 uptake in the MHB region of dorsally mounted ocr1+/+ and ocr1/- embryos 30 minutes post injection. Scale bar = 5 µm B) Quantification of the mean intensity of RAP-Cy3 puncta within the neuroepithelium. C) Quantification of total RAP-Cy3 fluorescence intensity in ocr1+/+ and ocr1/- embryos. D) Quantification of the number of RAP-Cy3 puncta visible within the tissue. E) Quantification of RAP-Cy3 puncta area. Individual points on each graph represent the average values of RAP-Cy3 puncta intensity / area quantified from fifteen consecutive z-planes in individual embryos. N number represents the total number of embryos injected pooled from three independent experiments. Error bars represent mean ± s.e.m.
tissue at similar levels to WT embryos (Figure 26a-b). A similar trend was also seen with ocrl1/- embryos (Figure 26c). A reduction in megalin levels can be seen, however RAP-Cy3 uptake remains comparable to WT. This would therefore appear to explain the apparent discrepancy in results between RAP endocytosis and megalin levels, as even in the complete absence of megalin, RAP-Cy3 is taken up by neuroepithelial cells in similar amounts to WT controls. As residual levels of megalin are present in ocrl1/- embryos it is unlikely that the reduction in megalin levels caused by absence of Ocr1 would lead to a significant reduction in RAP-Cy3 endocytosis.
Figure 26. Endocytic uptake of RAP-Cy3 does not correlate with megalin abundance. Confocal images of RAP-Cy3 uptake on coronal cryosections through the MHB region of ocrl1+/+ (a), ocrl1−/− (b) and bugeye embryos. Embryos were fixed 30 minutes post injection with RAP-Cy3 before being sectioned and stained with antibodies against megalin. Scale bar = 10 µm.
3.5. Discussion

3.5.1. A second Ocr11 mutant zebrafish line displays neurodevelopment impairment

Although much is known from in vitro studies about the cellular roles of OCRL1, which of these proposed cellular functions underlies the symptoms observed in Lowe syndrome is not well understood. Zebrafish provide a good vertebrate model in which to study the physiological functions of OCRL1 as they recapitulate many of the symptoms seen in human Lowe syndrome patients (Mehta et al., 2014; Oltrabella et al., 2015; Ramirez et al., 2012). Although this model has provided additional insights into the role of OCRL1 within the kidneys (Oltrabella et al., 2015), very little is still known about OCRL1 function within the CNS. The presence of CNS abnormalities from birth suggests OCRL1 is important for neural developmental and this is supported by a previously published zebrafish model of Lowe syndrome showing increased cell death in the brain at 1dpf (Ramirez et al., 2012). The current study confirms that in a separate ocr11-/- mutant zebrafish line, Ocr11 is required for proper CNS development at 1dpf. Increased apoptosis can be observed in all regions of the brain and the dying cells are likely neuroepithelial in nature as apoptotic cells are free of markers for differentiated neurons. The absence of elevated levels of cell death in homozygous ocr11+/+ siblings suggests that this phenotype is specific to loss of Ocr11 function and not due to off target effects induced by ENU treatment. However, re-expressing WT Ocr11 to rescue increased levels of apoptosis in Tg(ubi:secAnnexinV-mVenus);ocr11-/- embryos would help to confirm that this phenotype is specific to loss of Ocr11.

3.5.2. Ocr11 is important for endosome function within neuroepithelial cells

Studies of OCRL1 in cell culture have shown that a major site of action for OCRL1 appears to be at the early endosome (Billcliff et al., 2016; Choudhury et al., 2005; Vicinanza et al., 2011). Endosomal recruitment of OCRL1, which is dependent on its interaction with the small GTPases Rab5 and Rab35 (Cauvin et al., 2016; Fukuda et al., 2008; Hyvola et al., 2006) and the presence of other endosomal OCRL1 binding partners, such as APPL1 and IPIP27A (Erdmann et al., 2007; Noakes et al., 2011; Swan...
et al., 2010), further suggest a key requirement for OCRL1 mediated PtdIns(4,5)P₂ hydrolysis on the early endosome. In support of this, numerous studies have described defects consistent with an impairment of endosomal function in the absence of OCRL1, principally enlargement of early endosomes and blockages in transport of cargo out of this compartment (Cauvin et al., 2016; van Rahden et al., 2012; Vicinanza et al., 2011). The regulation of endocytic trafficking by OCRL1 appears to be relevant to the pathology of Lowe syndrome as ocrl1⁻/⁻ mutant zebrafish have reduced levels of the endocytic scavenger receptor megalin within the pronephros (Oltrabella et al., 2015). In experiments in HK2 cells, depletion of OCRL1 leads to accumulation of megalin in enlarged PtdIns(4,5)P₂ and actin rich endosomes, providing a potential mechanistic explanation for altered megalin sorting and abundance within the pronephros (Vicinanza et al., 2011). Alternatively, OCRL1 could participate in biogenesis of megalin containing carrier intermediates to facilitate its recycling, as has been shown for formation of CIMPR trafficking intermediates cycling between endosomes and the TGN (Billcliff et al., 2016).

Whether impaired endocytic trafficking also underlies the CNS symptoms of Lowe syndrome had not previously been investigated. The data presented in this chapter provide evidence to suggest that proper endosomal function within neuroepithelial cells is dependent on Ocrl1, although a clear link to phenotype was not established. In ocrl1⁻/⁻ embryos, lack of Ocrl1 led to an enlargement of EEA1 positive endosomes and a reduction in megalin abundance. This is consistent with data from both in vitro and in vivo experiments which support of a role for OCRL1 in regulating endocytic trafficking of multiple cargos, including megalin, and suggests that dysregulated endocytic trafficking within neuronal cell types or neuronal precursors may underlie some of the CNS abnormalities seen in human Lowe syndrome patients.

In previous studies of OCRL1 function in the brain, mutant forms of Ocrl1a lacking the enhanced clathrin binding ability of isoform-a were unable to rescue neurodevelopmental phenotypes caused by loss of Ocrl1 (Ramirez et al, 2012). OCRL1 interaction with clathrin is believed to lead to its
incorporation into late stage clathrin coated pits and affect rates of endocytosis (Nandez et al, 2014; Choudhury et al, 2009). However, in this study, the predominant effect of loss of Ocr1 was enlargement of endosomes, indicative of a role for Ocr1 in endosomal trafficking rather than at the plasma membrane, consistent with no effect on endocytic uptake of RAP being observed. The ability of Ocr1 to interact with clathrin could still be relevant to endosome function in neuroepithelial cells. 

Failure to remove PtdIns(4,5)P2 from newly forming clathrin coated vesicles may carry PtdIns(4,5)P2 to endosomes and interfere with endosomal function. Alternatively, Ocr1 could affect both clathrin dependent trafficking and endosomal function separately, with a direct role for Ocr1 at the endosome in addition to regulating clathrin coated pit turnover at the plasma membrane. Ocr1 may still participate in endocytosis within the neuroepithelium. Effects of loss of OCRL1 on endocytosis in other cell types are subtle, with a delay rather than a block in endocytosis observed. Cells expressing a dominant negative form of OCRL1a show reduced endocytic uptake of transferrin at 2 and 5 mins but not at 15 and 30 mins (Choudhury et al, 2009). The endocytic uptake assay established here may not be sensitive enough to pick up small changes in rates of endocytosis in ocr1-/- mutants, or the time points examined may be too late to do so.

As Rabs are responsible for OCRL1 recruitment to the early endosome (Cauvin et al., 2016; Hou et al., 2011; Hyvola et al., 2006), the ability of Ocr1 Rab binding mutants to rescue levels of apoptosis in 1dpf ocr1-/- zebrafish brains would help to determine whether Ocr1 mediated PtdIns(4,5)P2 hydrolysis on the endosome is important for neurodevelopment. To further confirm the role of Ocr1 in neuroepithelial endocytic trafficking, future experiments could also examine levels of PtdIns(4,5)P2 and actin on endosomes by immunofluorescence microscopy. Furthermore, imaging of stably or transiently expressed GFP tagged megalin or other cargos such as EGFR or CIMPR would provide additional insights into the dynamics of endocytic trafficking in ocr1-/- embryos and how loss of Ocr1 impacts on trafficking of multiple cargos. An increase in number and length of recycling tubules in ocr1-/- mutants would support an alternative model of OCRL1 function on endosomes where OCRL1 participates directly in carrier morphogenesis (Billcliff et al., 2016). Finally, re-
expression of WT Ocrl1 and mutant forms of Ocrl1 lacking key interaction motifs would help to determine which parts of Ocrl1 structure are important for cell survival within the developing CNS.

3.5.3. RAP uptake in the neuroepithelium depends upon more than megalin

Despite a reduction in megalin abundance, no difference in endocytic uptake of the megalin ligand RAP could be detected in ocrl1-/- embryos. Interestingly however, megalin null embryos showed only a minor reduction in RAP-Cy3 endocytosis, suggesting that whilst megalin may mediate a proportion of RAP uptake in neuroepithelial cells, there is functional redundancy between megalin and another receptor.

Ligand binding by megalin is mediated by a series of cysteine rich complement type repeats arranged into four clusters along the length of its extracellular domain (Orlando et al., 1997; Saito et al., 1994). Each repeat consists of approximately 40 amino acids containing six cysteine residues spaced in a characteristic pattern (Herz et al., 1988). Found between the fifth and six cysteines is a highly conserved SDE motif involved in co-ordination of a calcium ion required for disulphide bond formation and proper folding of this module (Krieger and Herz, 1994; Saito et al., 1994). This serves to position and co-ordinate a series of asparagine residues in each repeat to form a negatively charged acidic pocket which interacts with positively charged residues exposed on the ligand (Faas et al., 1997; Fisher et al., 2006). These repeats are the defining feature of LDL receptor family members. RAP binding to megalin requires a region within the second cluster of repeats and other as yet unidentified regions of megalin structure as antibodies blocking this site do not completely abolish RAP-megalin interactions (Orlando et al., 1997).

Other LDL receptors also bind RAP with high affinity (Andersen et al., 2003; Battey et al., 1994; Kounnas et al., 1992) and could thus potentially compensate for RAP uptake in the absence of megalin. The obvious candidate for non-megalin mediated RAP uptake in neuroepithelial cells would appear to be the teleost specific megalin homologue LRP2B (Kur et al., 2011). The number and arrangement of complement type repeats is identical in LRP2B, whilst motifs within the cytoplasmic
tail important for megalin/LRP2B internalisation and trafficking are also conserved. However, LRP2B mRNA levels are extremely low during early development (1-3dpf), and are not upregulated in response to reduced megalin levels (Kur et al., 2011). Other LDL receptors known to be expressed in radial glia (a related neuroepithelial cell type), such as LRP1, ApoER2 and vLDLR, are basolateral (Auderset et al., 2016a; Donoso et al., 2009; Kang and Folsch, 2011; Luque et al., 2003; Takeda et al., 2003). A proportion of RAP endocytosis may be mediated from the basolateral membrane as RAP-Cy3 did appear to penetrate between cells following injection, but this likely represents a small percentage of total uptake. LRPS and LRP6 bind RAP with lower affinity than other members of the LDL receptor family (Li et al., 2005). These receptors interact with the Wnt receptor Frizzled, leading to formation of the LRP6-signalosome important for Wnt signal transduction (Tamai et al., 2000). The LRP6-signalosome is internalised by clathrin mediated endocytosis where signalling through this complex continues from late endosomal compartments (Hagemann et al., 2014). The localisation and expression of LRP5 and LRP6 in polarised epithelia have not been investigated directly, although there is evidence implicating the Wnt/β-catenin pathway in neuronal regeneration from radial glia (Briona et al., 2015), suggesting that LRP5/6 may be present in these cells. Thus, it is unclear which LDLR family members may mediate RAP uptake alongside megalin at the apical surface. Based on the above, LRP5/6 represent the most feasible candidates and it would be of interest to examine localisation and expression of LRP5 and 6 within the neuroepithelium.

Regardless of the identity of the receptor responsible for RAP uptake alongside megalin, any impact upon recycling and cellular abundance of this protein may be similar to that seen with megalin i.e. a partial rather than complete block in recycling. Alternatively, cell surface levels of receptors which follow the degradative pathway after internalisation, such as LRP5/6, may be unaffected by alterations in endosomal recycling. Residual levels of megalin and other LDL receptors in ocrl1-/--neuroepithelial cells may therefore be sufficient to maintain the normal rates of RAP endocytic uptake observed.
3.5.4. Linking impaired endocytic trafficking to impaired neurodevelopment in ocr1−/− embryos

Megalin is not required for development of the embryonic zebrafish brain but impairment of endosomal recycling could potentially affect other receptors important for neurodevelopment (Kur et al., 2011). Additional LDLR family members, including LRP1, vLDLR and ApoER2, have been implicated in a broad range of cellular processes critical for CNS development (Auderset et al., 2016b). As mentioned above, many of these receptors are known to reside basolaterally. Trafficking to the basolateral membrane following synthesis proceeds either directly from the trans-Golgi or indirectly through the common recycling endosome (Apodaca et al., 2012). Basolaterally localised receptors undergo recycling back to the plasma membrane following ligand binding and internalisation from basolateral Rab5 positive early endosomes, which are functionally equivalent to those at the apical membrane (Bucci et al., 1994). Through binding to Rab5, OCRL1 could therefore potentially regulate basolateral early endosome function and recycling of basolateral cargos such as LRP1 and LDLR. Alternatively, numerous other signalling pathways distinct from those modulated by LDL receptors, but dependent on recycling from apical or basolateral endosomes for correct signalling, could be impaired by altered endosome function in ocr1−/− embryos.
4. Chapter 4 - Investigating the role of Ocrl1 in lysosomal function and autophagy in the CNS
4.1. Introduction

Autophagy, the process of ‘self-eating’, is an evolutionarily conserved degradation pathway essential for maintenance of cellular homeostasis. Three main types of autophagy have so far been described, with by far the best characterised being macroautophagy (Mehrpour et al., 2010). Types of macroautophagy (herein referred to as autophagy) can be further broken down into two distinct forms that serve different purposes. Basal, constitutive levels of autophagy are involved in cellular quality control through the turnover of old and damaged organelles and misfolded or aggregated proteins (Boya et al., 2013). The second form of autophagy is induced in response to starvation in order to provide an alternate energy source to the cell (Kaur and Debnath, 2015). Depletion of nutrients leads to the deactivation of mammalian target of rapamycin complex 1 (mTORC1), which under nutrient rich conditions represses autophagy. Deactivation of mTORC1 leads to the formation of double membrane autophagosomes which non-selectively engulf portions of the cytoplasm. Sealed autophagosomes then merge with the endocytic pathway, ultimately forming an autolysosome wherein sequestered material is degraded. Material released by degradation of autophagic cargo delivered to the lysosome restores cellular nutrient levels and subsequently reactivates mTORC1 signalling, repressing autophagy (Rong et al., 2011).

Phosphoinositides have been shown to be involved in multiple steps of autophagosome maturation. Initiation of autophagosome formation is dependent on synthesis of PI(3)P, which recruits the effector proteins FYVE containing protein 1 (DFCP1) and WD repeat domain phosphoinositide interacting 2 (WIPI) to the nascent phagophore membrane (Polson et al., 2010). Similarly, PI(5)P recruits WIPI and DFCP1 independently of PI(3)P to promote autophagosome initiation during non-canonical autophagy (Vicinanza et al., 2015). Furthermore, PI(3,5)P2 and PI(4)P have been shown to modulate autophagosome lysosome fusion through functionally distinct mechanisms (Hasegawa et al., 2016; Wang et al., 2015).
Whilst PtdIns(4,5)P₂ has been implicated in the initiation of autophagy (Tan et al., 2016), the most well understood role for PI(4,5)P₂ in starvation induced autophagy occurs once degradation of material delivered to the lysosome has restored cellular energy levels to normal (Du et al., 2016; Rong et al., 2012; Yu et al., 2010). Initiation of starvation induced autophagy leads to rapid consumption of free lysosomes within the cell, which need to be replenished. Following termination of autophagy, the cellular complement of lysosomes is restored from hybrid autolysosomes through a dynamic process of autolysosome tubulation (Yu et al., 2010). Lysosomal ‘precursors’ or ‘proto’-lysosomes bud off from the end of autolysosomal tubules before maturing to become fully functional. Rong et al discovered that this process was controlled by synthesis of two distinct pools of PI(4,5)P₂ on the autolysosomal membrane (Rong et al., 2012). Following termination of autophagy, PI(4,5)P₂ synthesis by PIP5K1β on the autolysosomal body initiates tubule formation through recruitment of clathrin, AP2 and KIF5B (Du et al., 2016). A second pool of PI(4,5)P₂ is synthesised by PIP5K1α along the length and tips of newly formed autolysosome tubules where clathrin and AP2 binding mediates proto-lysosome budding from tubules. Knock down of any of these components leads to long lasting enlarged autolysosomes and failure to restore lysosome numbers.

In addition to the role of clathrin and AP2 in autophagic lysosome reformation (ALR) described above, these proteins additionally participate in recycling of lysosomal components during ongoing autophagy. PtdIns(4,5)P₂ synthesis on autolysosomes increases during starvation conditions whilst autophagy is still in progress, stimulating enhanced recruitment of clathrin and AP2 to the lysosomal membrane (De Leo et al., 2016). In cell culture, OCRL1 also associates with lysosomes under conditions of higher load, including during starvation when autophagic flux through the lysosome is high (De Leo et al., 2016; Zhang et al., 1998). The temporal profile of OCRL1 recruitment to the lysosome during starvation closely correlates with that of both clathrin and AP2, and mutant forms of OCRL1 lacking binding motifs for these proteins are unable to be recruited to the lysosome (De Leo 2016). The known functions of AP-2 and clathrin in cargo selection and membrane deformation...
at the plasma membrane hint that OCRL1 may act alongside them to regulate fission or uncoating of vesicles from the lysosome. Loss of OCRL1 consequently leads to an accumulation of autophagosomes in kidney biopsies of Lowe syndrome patients, implying an important role for OCRL1 in maintaining proper lysosomal function within the kidneys (De Leo et al., 2016).

Lysosome function is particularly important within neurons (Platt et al., 2012) as mutations in soluble or transmembrane lysosomal proteins often lead to a class of inherited diseases called lysosomal storage disorders, which rarely present without dysfunction of the CNS (Platt et al., 2012). Whether OCRL1 plays any role in lysosome function within neural tissue has not been investigated, but it may explain why some CNS symptoms of Lowe syndrome, such as generalized motor seizures, have a later onset (Schurman and Scheinman, 2009). Indeed, children born with lysosomal storage disorders often appear normal at birth before developing neurodegeneration during infancy (Platt et al., 2012). Furthermore, lysosomal storage disorders are often accompanied by neuroinflammation with a common hallmark being gliosis, a phenotype observed in human Lowe syndrome patients and in adult ocr1 mutant zebrafish (Ramirez et al., 2012; Schneider et al., 2001; Sener, 2004; Yuksel et al., 2009). This chapter therefore set out to characterise the role of OCRL1 in lysosome function within the CNS and investigate the functional consequences of loss of OCRL1 on lysosome function and autophagosome turnover.
4.2. Localisation of Ocrl1 under conditions of differing lysosomal load.

To examine whether OCRL1 plays any role at the lysosome in the CNS, the localisation of OCRL1 under conditions of differing lysosomal load was examined. Methods to label neuroepithelial lysosomes and increase lysosomal load were first tested and characterised. DQ-BSA is an endocytic tracer which fluoresces within late endosomal / lysosomal compartments once cleaved by lysosomal hydrolases. Following injection into the hindbrain ventricle and imaging 2 hrs post injection, DQ-BSA can be seen to fluoresce specifically in late endosomal compartments within the neuroepithelium (Figure 27). EGFP-Rab7 late endosomes showed strong co-localisation with DQ-BSA whilst EGFP-Rab5 positive early endosomes showed little to no overlap with DQ-BSA as expected. Thus, live imaging of lysosomes can be achieved by injection of DQ-BSA.

Figure 27. Characterisation of neuroepithelial DQ BSA uptake. Confocal micrographs of the MHB region of dorsally mounted 1 dpf EGFP-Rab5 and EGFP-Rab7 embryos injected with DQ-BSA and imaged at +2hrs post uptake. Scale bars = 5 µm.

Previous reports have described OCRL1 recruitment to the lysosome under conditions of high lysosomal load, such as following sucrose treatment or induction of autophagy (De Leo et al., 2016; Zhang et al., 1998). As described in the previous chapter, at steady state, only a small percentage of
OCRL1 co-localised with the late endocytic / lysosomal marker LAMP1 (Chapter 3, Figure 14c). To test whether OCRL1 plays a more prominent role in lysosomal function when flux through this pathway is higher, autophagy was induced in 1 dpf embryos. As the yolk sac of developing embryos prevents induction of autophagy by starvation, embryos were instead treated with the mTORC1 inhibitor Torin1, which potently inhibits mTORC1 in cell culture and induces autophagy (Thoreen et al., 2009). Confocal microscopy was used to visualise autophagosomes in transgenic zebrafish expressing GFP tagged LC3 (He et al., 2009), a canonical autophagosomal marker. Two forms of LC3 exist within cells, termed LC3I and LC3II. LC3I undergoes conversion to LC3II through cleavage of a glycine residue at the extreme C terminus of the protein to allow conjugation to phosphatidylethanolamine. LC3II is then incorporated into the inner and outer membranes of nascent autophagosomes (Tanida et al., 2008). Following fusion of autophagosomes with late endocytic compartments, LC3II is degraded within the lysosome.

By itself, treatment of embryos with Torin1 for either 3 or 6 hours led to very little increase in the number of GFP-LC3 puncta within neuroepithelial cells (Figure 28A). This is to be expected as cells upregulate lysosome biogenesis upon induction of autophagy to cope with enhanced flux through the lysosome (Settembre et al., 2011). Blocking lysosomal degradation through Bafilomycin A treatment is a common method of visualising changes in the rate of autophagosome formation masked by cellular adaptations induced by autophagy (Klionsky et al., 2016). Treatment of embryos with Bafilomycin A alongside Torin1 increased the number of GFP-LC3 positive puncta in comparison to embryos treated with either Bafilomycin A or Torin1 treatment alone, confirming that Torin1 induces autophagy within the neuroepithelium (Figure 28A). Levels of LC3II in response to these treatments were also assessed by western blot, and analysed in accordance with previously published guidelines (Klionsky et al., 2016). Combined Torin1 and Bafilomycin A treatment also enhanced LC3II levels over either Torin1 or Bafilomycin A treatment alone (Figure 28B). Furthermore, Torin1 increased the size of DQ-BSA labelled lysosomes at both 1 hour and 3 hours post treatment (Figure 28C-D). This is consistent with the increased lysosomal size observed
following induction of autophagy in cell culture (Yu et al., 2010). Increases in lysosomal load within neuroepithelial cells can therefore be induced through Torin1 treatment.

Figure 28. Torin1 induces autophagy in the neuroepithelium. A) Confocal images of the MHB region of laterally mounted 2 dpf WT GFP-LC3 embryos treated with either 1 µM Torin1, 100 nM Bafilomycin A or 1 µM Torin1 and 100 nM Bafilomycin A for 3 or 6 hrs. B) Western blot for LC3II levels in 2 dpf head lysates treated for 6 hrs with the same concentrations of drug as in (A). C) Confocal micrographs of the MHB region of dorsally mounted 1 dpf WT embryos loaded with DQ BSA for 1 hour before being treated with 1 µM Torin1 for a further 1 or 3 hours. D) Quantification of the average size of DQ-BSA puncta shown in (c). Scale bars = 5 µm. n numbers represent number of embryos imaged per treatment group. Error bars in (d) represents mean values ± s.e.m.

Live imaging of embryos expressing EGFP-Ocr1la revealed that the majority of Ocr1la is diffuse in the cytoplasm, with few visible puncta (Figure 29a). This contrasts with the distribution of Ocr1la on fixed tissue sections (Figure 14), potentially due to wash out of the cytoplasmic pool of Ocr1la during fixation and permeabilisation of tissue. However, enhanced recruitment of Ocr1la to lysosomal compartments may lead to visible foci within cells. Treatment of DQ-BSA injected embryos with
Torin1 did not lead to an increase in the number of EGFP-Ocrl1a puncta visible within neuroepithelial cells nor in the co-localisation between EGFP-Ocrl1a and DQ-BSA labelled compartments (Figure 29B). However, it is worth considering that the signal from the cytosolic Ocrl1 protein may mask its recruitment to degradative compartments.

As clathrin is responsible for the lysosomal recruitment of OCRL1 alongside AP-2, CLC-GFP was used to test whether other components of the reformation machinery which bring OCRL1 to the lysosome are recruited to autolysosomes during autophagy. In contrast to EGFP-Ocrl1a, by live imaging, clathrin puncta could be visualised on endocytic structures, likely to be early endosomes (Chapter 3, Figure 20d). However, no recruitment of CLC-GFP onto DQ-BSA positive lysosomes could be observed after 1 hour of treatment with Torin1 (Figure 29b). Thus in the neuroepithelium, neither Ocrl1 or clathrin show significant recruitment to the lysosome suggesting Ocrl1 may not play a role in lysosome function at steady state or in response to increased lysosomal load.
Figure 29. Lysosomal localisation of clathrin and OCR1La at steady state and following induction of autophagy. A) Co-localisation of clathrin with DQ-BSA pre-Torin1 treatment and post 1 hour Torin1 treatment. B) Co-localisation of OCRL1 with DQ-BSA pre-Torin1 treatment and post 1 hour Torin1 treatment. In both cases embryos were injected with DQ-BSA before being treated with 1 µM Torin1 1 hour later. Scale bar = 5 µm
4.3. Functional consequences of loss of Ocrl1 on CNS lysosomes

To test if there were any consequences to loss of Ocrl1 on lysomes during CNS development, cryosections taken from the brain of 5 dpf ocrl1+/+ and ocrl1/- embryos were stained with antibodies to LAMP1. In the forebrain of ocrl1/- mutants, both size and number of lysosomes were comparable to ocrl1+/+ control embryos suggesting that lack of Ocrl1 does not impair lysosomal function at this stage of development (Figure 30a). As previous work has shown enlargement of lysosomes in proximal tubule cells taken from Lowe syndrome patients and in HK2 cells depleted of OCRL1, lysosome size was also examined in the pronephros of ocrl1 mutants (De Leo et al., 2016). Consistent with this, and in striking contrast to the brain, lysosomes were noticeably enlarged.

Figure 30. Lysosomes are enlarged in the pronephros of OCRL1-/- embryos but not in the brain.
A) Confocal micrograph of transverse cryosection through the forebrain of ocrl1+/+ and ocrl1/- embryos stained with an antibody to LAMP1. Images represent a single plane from a 12 µm tissue section. B) Confocal micrograph of transverse cryosection through the pronephros of 5dpf ocrl1+/+ and ocrl1/- embryos stained with an antibody to LAMP1 and the apical brush border marker 3G8. Images are a z projection of a 12 µm tissue section imaged at 0.5 µm intervals. Insets show enlarged lysosomes stained with LAMP1. Dashed line indicates outline of pronephros. Scale bars = 5 µm
In the pronephros of 5 dpf ocrl1\textsuperscript{1/-} embryos. This suggests that Ocrl1 is important for lysosomal function in the pronephros and highlights a differential requirement for Ocrl1 in regulating lysosomal function within the CNS and kidneys (Figure 30b).

4.4. Functional consequences of loss of Ocrl1 on CNS autophagy

The lack of an effect on lysosome morphology in the CNS of ocrl1\textsuperscript{1/-} embryos suggests that in contrast to the kidneys, Ocrl1 is dispensable for lysosomal function within the brain at this stage of development. As turnover of autophagosomes is dependent on efficient lysosomal clearance, it should follow that autophagic flux would also show differential sensitivity to loss of Ocrl1 in the CNS and pronephros. To investigate autophagosome levels in ocrl1 knockout embryos, ocrl1 mutants were crossed with transgenic GFP-LC3 zebrafish (He et al., 2009) in order to visualise autophagosomes by fluorescence microscopy.

Transgenic GFP-LC3 embryos from both genotypes showed strong expression in the lens, as has previously been reported (Figure 31a, left panel) (He et al., 2009). Basal autophagy occurs at a constitutive rate in neurons (Maday and Holzbaur, 2014), however within the forebrain of ocrl1\textsuperscript{1/+} embryos, GFP-LC3 was predominantly diffuse (Figure 31a), with few visible LC3 puncta. This suggests that neural tissue has low levels of autophagosome formation at this stage of development or that autophagosomes are quickly cleared. Consistent with the above results showing normal lysosome function in the CNS of ocrl1\textsuperscript{1/-} mutants, ocrl1\textsuperscript{1/-} embryos showed no increase in GFP-LC3 fluorescence compared to ocrl1\textsuperscript{1/+} controls at 5 dpf (Figure 31a). To confirm this result, cryosections through the forebrain of ocrl1\textsuperscript{1/+} and ocrl1\textsuperscript{1/-} embryos were stained with an antibody to Gabarap, which plays a similar role to LC3II in autophagosome biogenesis and labels mature autophagosomes (Kabeya et al., 2004). Discernible Gabarap puncta could be observed on both control and mutant cryosections, which contrasts with the lack of GFP-LC3 puncta visible at the same developmental timepoints (Figure 31b). These puncta may be autophagosomes which have fused with the lysosome that are not detectable by GFP-LC3 due to the acidic environment of the lysosome quenching GFP
fluorescence (Kimura et al., 2007). However, no increase in the number of Gabarap puncta could be seen in ocr1/- mutant embryos (Figure 31b).

In the pronephros, no autophagosomes were visible in the proximal tubule of ocr1+/+ with either GFP-LC3 or Gabarap, again indicating efficient clearance of autophagosomes (Figure 32a-b). In ocr1/- embryos, autophagosome clearance was impaired, as both GFP-LC3 and Gabarap puncta accumulated in cells of the proximal tubule, In line with the impairment of lysosomal function observed above. This indicates that in the pronephros, loss of Ocr1 impacts upon lysosome function, consequently leading to an inability to efficiently maintain rates of autophagosome clearance, which accumulate in the absence of Ocr1.
Higher rates of flux through the lysosome induced by starvation-induced autophagy have been shown to stimulate greater lysosomal recruitment of OCRL1 to the lysosome, indicating a greater need for OCRL1 function under conditions of higher lysosomal load. Impaired clearance of lysosomal substrates, such as autophagosomes, may only become apparent when rates of flux through the lysosome are high. This may be why the pronephros displays an accumulation of autophagosomes, as the high rate of endocytosis and delivery of material to the lysosome may place degradative compartments under sustained pressure. To test whether \textit{ocrl1/-} could cope with higher lysosomal loads within the CNS, \textit{ocrl1+/-} and \textit{ocrl1/-} embryos, sustained high rates of autophagy were induced by treatment of embryos with 1 \mu M Torin1 for 16 hours. Western blotting for LC3II levels in head lysates from untreated 2 dpf \textit{ocrl1+/-} and \textit{ocrl1/-} embryos revealed no difference in
autophagosome levels (Figure 33a, lanes 1-2). LC3II was comparable in untreated embryos, consistent with staining for GFP-LC3 and Gabarap in Figure 31 A-B. Induction of autophagy with Torin1 led to a slight increase in LC3II in both genotypes, however levels were comparable between control and ocr11-/- embryos (Figure 33a, lanes 3-4). This indicates that lack of Ocr11 function does not impede autophagosome clearance in the CNS even when the lysosome is placed under greater pressure by inducing sustained high rates of autophagic flux.

Finally, as pathological changes in neurons caused by impairment of lysosomal function can often take long periods of time to manifest (Platt et al., 2012), the long term effects of loss of OCRL1 on autophagosome turnover were assessed in 8 month old adult ocr11+/+ and ocr11-/- brain lysates. Western blotting for LC3II in whole brain lysates showed no difference in between ocr11+/+ and ocr11-/- fish suggesting that Ocr11 is not required for efficient autophagosome turnover in mature neurons (Figure 33b). Collectively, these results argue against a significant role for Ocr11 in lysosome function with the CNS at any stage of development. This contrasts with the pronephros where loss of
Ocrl1 had a clear impact on both lysosome function, as indicated by an increase in lysosome size, and autophagosomes clearance, which accumulated in the absence of Ocrl1.
4.5. Discussion

Previous work investigating the function of OCRL1 has revealed that in addition to its known role in regulating trafficking at the early stages of the endocytic pathway (Mehta et al., 2014), OCRL1 also participates in regulating lysosome function (De Leo et al., 2016; Zhang et al., 1998). Initial studies showed that OCRL1 associates with lysosomes following treatment of cells with the non-digestible lysosomal substrate sucrose (Zhang et al., 1998), which consumes free dense core lysosomes into late endosomal / lysosomal hybrid compartments (Bright et al., 1997; DeCourcy and Storrie, 1991). Further work has revealed that OCRL1 is recruited to the lysosome on demand in response to higher rates of cargo delivery to the lysosome, such as during autophagy (De Leo et al., 2016). This is dependent on interaction with clathrin and AP2, two proteins critical for reformation of lysosomes upon completion of autophagy (De Leo et al., 2016). The importance of the lysosomal function of OCRL1 in relation to the symptoms of Lowe syndrome are not known, particularly within the CNS, although autophagosomes do accumulate in proximal tubule cells taken from human Lowe syndrome patients (De Leo et al., 2016). As long lived post mitotic cells, neurons are particularly sensitive to perturbations in lysosomal function, as evidenced by neuronal dysfunction being a hallmark of many lysosomal storage disorders (Platt et al., 2012). An attractive possibility therefore is that OCRL1 mediated PtdIns(4,5)P₂ hydrolysis on the lysosome may be relevant to the CNS symptoms of Lowe syndrome, but this had not previously been investigated. In this chapter, the role of OCRL1 in lysosome function and autophagosome turnover in both the developing neuroepithelium and in mature adult tissue was investigated.

4.5.1. Loss of Ocr11 does not impact on lysosome function or autophagosome turnover in the CNS.

Testing the functional consequences of loss of Ocr11 on lysosomes at early stages of development revealed no difference in lysosome size or autophagosome turnover at any stage of larval development or in adult fish. In contrast, the pronephros of ocr11/⁻ mutant embryos was very
sensitive to loss of OCRL1 as both an increase in autophagosome numbers and enlargement of lysosomes could be observed.

The different requirements for OCRL1 in lysosome function within the CNS and kidneys is potentially linked to the rates of endocytosis and autophagy in these tissues. The human kidney produces 180 litres of ultrafiltrate per day containing 10-30 grams per litre of low molecular weight proteins (LMWP). These proteins are reabsorbed by cells of the proximal tubule and catabolised within the lysosome into small peptides and individual amino acids. Traffic to the lysosome within cells of the proximal tubule is therefore consistently high and a high degradative capacity is likely to be required. The proximal tubule epithelium is equipped to deal with the high load imparted by the ultrafiltrate through higher expression of lysosomal hydrolases and a greater number of free dense core lysosomes housing these enzymes (Haga et al., 1988).

The need to actively maintain the number of free lysosomes and the optimum concentration of lysosomal hydrolases within degradative compartments is also important. Repeated rounds of endosomal fusion can quickly deplete free lysosomes, dilute hydrolase concentration within active compartments and slow degradation efficiency. Maintaining a store of lysosomes enriched in lysosomal hydrolases allows the cell to hold the upper hand in dealing with steady state degradative load and also provides a buffer against changes in endocytic or autophagic flux. To achieve this, soluble and transmembrane lysosomal proteins are condensed from hybrid late endosomal / lysosomal compartments back into dense core lysosomes (Bright et al., 2005; Bright et al., 1997; Pryor et al., 2000). Lysosome reformation is in part thought to be mediated through a process similar to that of endocytic vesicle formation at the plasma membrane involving AP-2, clathrin and OCRL1 (De Leo et al., 2016; Rong et al., 2012; Traub et al., 1996). If the machinery required to reform lysosomes is impaired, the balance between consumption and reformation can become skewed, with potential consequences for degradation of material trafficked through the endocytic and autophagic pathways.
The function of OCRL1 in other cellular processes may provide some insight into the presence and absence of lysosomal and autophagic abnormalities in distinct tissues of *ocrl1*-/- fish. At the plasma membrane, absence of OCRL1 delays but does not block transferrin endocytosis (Choudhury et al., 2009; Nandez et al., 2014) and leads to an increase in the number of free clathrin coated vesicles within the cortical region of the cell, indicative of impaired vesicle uncoating (Nandez et al., 2014). Similarly, altered clathrin dynamics at the lysosome through loss of OCRL1 may delay rates of lysosomal recycling, but not enough to profoundly affect lysosomal homeostasis in certain cell types. In the absence of OCRL1 *in vivo*, tissue specific demands placed upon the lysosome may only lead to defects in lysosomal homeostasis within certain tissues. For instance, the high rates of endocytosis in the kidney presumably dictate that a high rate of reformation is required. If reformation rates are slowed, as may be the case with loss of OCRL1, the continued high volume of material delivered to lysosomes from the ultrafiltrate could overwhelm degradative compartments, leading to cargo accumulation and a situation that quickly becomes irretrievable. This could be compounded by reduced levels of lysosomal hydrolases, which OCRL1 regulates through trafficking of the cation independent mannose phosphate receptor (CIMPR) (Billcliff et al., 2016; Cauvin et al., 2016; Choudhury et al., 2005; van Rahden et al., 2012; Vicinanza et al., 2011), responsible for delivery of newly synthesised lysosomal enzymes to the lysosome. In contrast, in cell types where rates of endocytosis or autophagy are lower than in the kidneys, such as in neurons or neuronal precursors, loss of OCRL1 and slowing of reformation rates may be more manageable. Lower rates of delivery to the lysosome would provide some ‘breathing space’ to allow reestablishment of the equilibrium between lysosomal fission and fusion. This could occur either through compensatory PtdIns(4,5)P$_2$ dependent or even PtdIns(4,5)P$_2$ independent mechanisms. Indeed, In addition to PtdIns(4,5)P$_2$ dependent lysosome reformation, both PI(3)P and PI(4)P have been shown to be involved in regulating the dynamics of lysosomal fission (Chang et al., 2014; Munson et al., 2015; Sridhar et al., 2013).
4.3.2. Ocrl1 is not recruited to lysosomes on demand in the neuroepithelium

Autophagosome fusion with the lysosome was shown to be one of the primary drivers of clathrin, AP2 and OCRL1 recruitment to lysosomal compartments in a process termed the ‘lysosomal cargo response’ (De Leo et al., 2016). Arrival of autophagic cargo at the lysosome is thought to lead to PtdIns(4,5)P₂ synthesis on lysosomal compartments through TLR9 mediated recognition of methylated CpG motifs found on mitochondria. Damaged or old mitochondria are sequestered in autophagosomes and delivered to the lysosome to be degraded and are thus lysosomal substrates (Oka et al., 2012). TLR9 signalling subsequently stimulates PtdIns(4,5)P₂ synthesis through recruitment of the PtdIns(4,5)P₂ kinases, PIP5K1α and PIP5K1β. PtdIns(4,5)P₂ synthesis on lysosomes in turn recruits AP2, clathrin and OCRL1 to mediate recycling of lysosomal proteins (De Leo et al., 2016). In contrast to the findings reported by De Leo et al, Torin1 mediated induction of autophagy within the neuroepithelium did not appear to induce any kind of lysosomal cargo response. Torin1 robustly induced autophagy as evidenced by an increase in LC3 II levels and lysosome size, however neither OCRL1 or clathrin could be observed to increase their association with DQ-BSA positive compartments.

Using alternative methods to confirm this result will be important as live imaging of Ocrl1 may have limitations. Contrasting with the punctate pattern of Ocrl1 observed on fixed cryosections, EGFP-Ocrla appears predominantly diffuse by live imaging with few visible puncta. This difference in distribution is likely due to the cytosolic pool of Ocrl1 visible by live imaging masking sites of Ocrl1 membrane association. Performing the same experiments using fixed tissue sections and antibodies against LAMP1 would help to confirm whether or not Ocrl1 is recruited to the lysosome under conditions of higher lysosomal load. However, by live imaging clathrin could be observed to co-localise with endocytic structures labelled by RAP, but was not recruited to DQ-BSA labelled lysosomes following Torin1 treatment. As clathrin is responsible for Ocrl1 recruitment to the lysosome this would argue against a lysosomal cargo response being mounted in neuroepithelial cells in response to arrival of autophagic cargo.
Lack of OCRL1 and clathrin association with lysosomes may be due expression levels of TLR9 within the neuroepithelium. Precise levels of TLR9 in neuroepithelial cells have not been determined, however in the developing mouse brain immunofluorescence experiments have revealed that the majority appears to be found in neurons rather than precursor cells (Kaul et al., 2012). Low levels of a key node in this signalling pathway suggest that the arrival of autophagic cargo within lysosomes may not stimulate strong recruitment of PIP5K and in turn PtdIns(4,5)P$_2$ synthesis and enhanced association of clathrin, AP2 and OCRL1 with the lysosome.

The presence of TLR9 in neurons would appear to suggest that this signalling mechanism is also in place within neurons however. It is therefore somewhat surprising that no defect in autophagosome turnover could be observed in adult brain tissue taken from ocr11/- fish. Perhaps as mentioned above, this could be due to tissue specific differences in flux through the degradative pathway or alternative routes of reformation predominating over others within neurons.

Overall, the minimal impact on lysosomal function within the brains of ocr11/- zebrafish would imply that lysosomal abnormalities may not significantly contribute to CNS pathology in Lowe syndrome.
5. Chapter 5 - Investigation of neuroepithelial lysosome dynamics
5.1. Introduction

Variations in lysosome morphology have been observed in a number of cell types and under a number of different conditions. Whilst in the majority of cell types lysosomes adopt a vacuolar morphology, lysosomes can also tubulate to serve a number of different purposes. For instance, rather than having a collection of discrete lysosomal units, *Drosophila* muscle tissue *in vivo* contains an interconnected network of tubular lysosomes that extends throughout the cytoplasm, potentially to help maintain sarcoplasmic proteostasis (Johnson et al., 2015). Lysosomes can also shift between globular and tubular morphologies in response to differing stimuli. Stimulation of macrophages with LPS leads to striking morphological changes in lysosomes as they take on a pronounced tubular morpholgy, although the functional importance of this switch remains unclear (Swanson et al., 1987; Swanson et al., 1985). As discussed in the previous chapter, tubulation is also critical following termination of autophagy as a homeostatic mechanism to enhance restoration of cellular lysosome numbers (Yu et al., 2010), and also in the maintenance of steady state homeostasis between lysosomes and end lysosomes (Bright et al., 2016; Bright et al., 2005).

Insight into the mechanisms controlling lysosomal tubulation have been provided by the finding that mTORC1 plays a key role regulating tubulation of lysosomes (Mrakovic et al., 2012; Saric et al., 2016; Yu et al., 2010). Torin1 treatment of macrophages blocks LPS induced tubulation, whilst inhibition of mTORC1 signalling also blocks tubule formation during ALR, leading to long lasting enlarged autolysosomes (Saric et al., 2016; Yu et al., 2010). In macrophages, extracellular danger signals such as bacterial cell wall components are detected by cell surface pattern recognition receptors that include the TLR family (Akira and Takeda, 2004). Upon challenge with LPS, signalling through TLR4 converges on mTORC1 to stimulate lysosomal tubulation. Torin1 treatment prevents LPS induced lysosomal tubulation, indicating that mTORC1 serves to co-ordinate lysosome positioning and motility under these conditions (Saric et al., 2016). Tubulation of autolysosomes during ALR is also mTORC1 dependent. During starvation induced autophagy, the release of free amino acids broken down in the lysosomal lumen reactivates mTORC1, triggering biogenesis of reformation tubules on
autolysosomes (Yu et al., 2010). Inhibiting reactivation of mTORC1 with rapamycin blocks autolysosome tubulation demonstrating that ALR is dependent on active mTORC1 signalling.

mTORC1 may regulate tubulation through altering recruitment of molecular motors to lysosomes. The small GTPases Rab7 and Arl8b interact indirectly with dynein and kinesin family members through RILP and SKIP respectively (Jordens et al., 2001; Rosa-Ferreira and Munro, 2011). Rab7 and Arl8b and their effectors have all been shown to be involved in lysosomal tubulation in response to LPS stimulation as dominant negative forms of these proteins prevent tubulation (Mrakovic et al., 2012). Furthermore, LPS stimulation increases the association of Arl8b with lysosomes, stimulating anterograde movement towards the cell periphery and lysosomal tubulation (Saric et al., 2016).

Tubulation during ALR has also been shown to involve Rab7 and Kinesins, although a direct link between mTORC1 signalling and changes in motor protein association with autolysosomes has not been established. The kinesin family member KIF5B drives autolysosome tubulation through association with clathrin and PtdIns(4,5)P₂, with KIF5B knockdown inhibiting tubule formation (Du et al., 2016). This may be co-ordinated with Rab7 dissociation from autolysosomes following reactivation of mTORC1 signalling (Yu et al., 2010). Loss of Rab7 mediated recruitment of the minus end directed motor dynein through RILP may allow plus end directed transport of lysosomes to predominate, promoting lysosomal tubulation. KIF5B dependent lysosomal tubulation has been shown to be size dependent with vesicles of a size greater than 500nm more frequently undergoing tubulation in contrast to those less than 200nm, which instead underwent KIF5B dependent long range movements (Su et al., 2016).

Changes in cytosolic pH have also been shown to alter lysosomal motility and positioning and may therefore also influence lysosomal tubulation (Heuser, 1989; Korolchuk et al., 2011). Cytosolic pH increases during starvation conditions, reducing lysosomal association of Arl8b and Kinesin. This leads to movement of lysosomes towards the cell centre and perinuclear clustering (Korolchuk et al., 2011). Conversely, restoration of normal cytosolic pH following termination of autophagy drives
movement of lysosomes back to the cell periphery. Thus, a picture of lysosomal tubulation emerges where motor protein association, lysosome size and cytosolic pH to contribute to determining lysosome morphology.

Although the phenomenon of autophagic lysosome tubulation is widely conserved across a number of cell lines derived from various species and tissues (Yu et al., 2010), this behaviour has yet to be observed in vivo. Additionally, whether ALR and lysosomal tubulation occur in the brain is unknown as in SH-SY5Y cells, lysosomes do not undergo tubulation following starvation (Yu et al., 2010). These factors and the proposed role for OCRL1 alongside clathrin and AP-2 in lysosomal function during periods of high lysosomal load made it desirable to attempt to establish an assay to monitor lysosome dynamics in vivo within the CNS. The experiments performed in this chapter set out to establish markers to perform live imaging of lysosomes in vivo within the neuroepithelium and from here examine whether recycling events could be observed at steady state. Conditions likely to lead to enhanced recycling were also trialled to increase the likelihood of observing such events.
5.2. Establishment of methods to study lysosomal dynamics in neuroepithelial cells

The unique characteristics of zebrafish as a model system and the amenability of the neuroepithelium to live imaging makes them ideally suited to investigate lysosome dynamics \textit{in vivo}.

To visualise lysosomes by live imaging, embryos were injected with mRNA encoding either LAMP1-GFP or LAMP1-RFP. LAMP1-GFP labelled hydrolytically active compartments as marked by the lysosomal substrate DQ-BSA (Figure 34a).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure34.png}
\caption{Neuroepithelial lysosome dynamics. A) Transiently expressed LAMP1-GFP labels degradative compartments. Embryos injected with LAMP1-GFP mRNA at the one cell stage were loaded with DQ-BSA for 2 hrs before being imaged by confocal microscopy. Region shown is from the midbrain of a dorsally mounted 1dpf embryo. Scale bar = 5µm B) Example of a fission event from a LAMP1-GFP positive compartment. Embryos were injected with RAP-Cy3 and imaged after 1 hour. Arrowheads highlight bud emerging from parent LAMP1-GFP positive compartment over a ten second period. Scale bar = 5 µm.}
\end{figure}
Fusion and fission events could be observed within neuroepithelial cells, albeit at a very low rate. Vesicles containing the cargo RAP-Cy3 could be seen to extend from LAMP1-GFP labelled lysosomes before undergoing scission and moving away from the parent compartment (Figure 34b).

Approaches were taken to enhance the likelihood of observing recycling events, as there occurrence at steady state was relatively rare. Earlier studies using a cell free assay to investigate lysosome biology in vitro have shown that the vATPase inhibitor Bafilomycin A blocks reformation of lysosomes from hybrid late endosomal / lysosomal compartments (Pryor et al., 2000). An approach was therefore taken to block lysosome reformation through treatment of embryos with Bafilomycin A followed by drug wash out and subsequent visualisation of lysosomal dynamics by confocal microscopy. To visualise late endosomal / lysosomal compartments, LAMP1-RFP was transiently expressed in transgenic EGFP-Rab7 zebrafish and 1 dpf embryos treated with 100 nM Bafilomycin A for 2 hours. Surprisingly, prior to any drug wash off, Bafilomycin A treated embryos displayed elongated tubular LAMP1-RFP positive structures which often spanned the whole length of neuroepithelial cells (Figure 35a-b). This was unexpected as in the context of lysosomal tubulation during autophagy, tubulation has only been shown to occur at the end of degradation cycles.

To confirm that the tubulation phenotype observed after Bafilomycin A treatment was not due to overexpression of LAMP1, lysosomes in WT embryos were labelled with RAP-Cy3 and imaged live by confocal microscopy following Bafilomycin A treatment. Tubular structures could occasionally be observed at later timepoints following RAP-Cy3 injection (Figure 36a, +180 mins). Compared to untreated controls, Bafilomycin A treatment led to an increase in the number of tubules visible within the neuroepithelium (Figure 36b-c). These tubules co-localised with transiently expressed LAMP1-GFP confirming that they were of lysosomal origin (Figure 36d). Thus, inhibition of vATPase activity following Bafilomycin A treatment leads to tubulation of lysosomes labelled with RAP-Cy3 as well as in embryos overexpressing LAMP1. This suggests that tubulation is not an artefact of LAMP1
overexpression and may represent a physiological response to changes in lysosomal pH or degradation efficiency in neuroepithelial cells.

Figure 35. Bafilomycin A treatment leads to tubulation of LAMP1 RFP labelled compartments. A) Images taken from the hindbrain region adjacent to the MHB of laterally mounted EGFP-Rab7 embryos injected with LAMP1 RFP mRNA and imaged by confocal microscopy 2hrs post treatment with 100nM Bafilomycin A. Scale bar = 10 µm. B) Quantification of the number of tubules visible in the MHB region. Data are pooled from three independent experiments with each data point representing one individual embryo. Statistical analysis was performed using students t-test *** P<0.0001. Error bar in (b) represents mean ± s.e.m.
Figure 36. Baflomycin induces tubulation of RAP-Cy3 labelled lysosomes. A) Confocal micrograph of the MHB region of dorsally mounted WT embryos injected with RAP Cy3 and imaged at 5, 30 and 180 minutes post injection. Scale bar in main image = 10 µm, inset = 5 µm B) Confocal micrograph of MHB region in DMSO and Baflomycin A treated embryos. Embryos were injected with RAP-Cy3, incubated at 28°C for 2 hours and then treated with 100nM Baflomycin A for 1 hour before being imaged. C) Quantification of number of tubules in the MHB region of DMSO and Baflomycin A treated embryos. N number indicates number of embryo quantified in each treatmnt group from 1 experiment. D) RAP-Cy3 tubules are lysosomal. Embryos transiently expressing LAMP1-GFP were treated as in B and imaged by confocal microscopy. Scale bar = 5 µm. Error bars in represent mean ± s.e.m.
5.3. Mechanisms governing Bafilomycin induced neuroepithelial lysosome tubulation

Figure 37. Bafilomycin induced lysosomal tubulation is mTOR dependent. A) Confocal micrograph of laterally mounted EGFP-Rab7 embryos transiently expressing LAMP1-RFP treated with either DMSO, 100 nM Bafilomycin A or 100 nM Bafilomycin A and 1 uM Torin1. Embryos were imaged 2 hours post treatment. B) Western blot for mTOR activity in embryos treated with either 100 nM Bafilomycin A or 1 uM Torin1 for 2 hours.

Lysosomal tubulation during ALR and in LPS stimulated macrophages has been shown to be dependent on active mTORC1 signalling (Saric et al., 2016; Yu et al., 2010). To examine whether tubulation in Bafilomycin A treated embryos was also controlled by mTOR, transgenic EGFP-Rab7 embryos expressing LAMP1-RFP were treated with either Bafilomycin A alone or Bafilomycin A plus Torin1. Tubules were again visible in Bafilomycin A treated embryos however co-treatment of embryos with Bafilomycin A and Torin1 prevented tubule formation and caused enlargement of late endosomes / lysosomes indicating that following inhibition of mTOR activity lysosomal tubulation is blocked (Figure 37a).
In vitro, Bafilomycin A and the related compound concanamycin have been shown to block mTORC1 signalling (Zoncu et al., 2011). The vATPase interacts with mTORC1 on the lysosomal membrane in an amino acid dependent fashion to control mTORC1 activation state (Zoncu et al., 2011). This presents a potential paradox, as treatment with Bafilomycin A and inhibition of vATPase activity could potentially block mTORC1 signalling. To examine whether Bafilomycin A treatment of embryos interfered with mTORC1 signalling, lysates from 1 dpf embryos treated with either DMSO or 100 nM Bafilomycin A were probed with antibodies to phosphorylated p70-s6k, an mTORC1 substrate that is phosphorylated when mTORC1 is active. Bafilomycin A treatment did not block mTORC1 signalling, as phosphorylation of p70 s6k was unaffected by Bafilomycin A treatment (Figure 37b, top panel, lane1 vs lane 2). In contrast, treatment of embryos with Torin1 completely abolished p70 s6k phosphorylation (Figure 37b, top panel, lane 1 vs lane 3). These results support the view that the Bafilomycin A induced tubules are mTOR dependent. In addition, and as expected, Bafilomycin A blocked lysosomal degradation, as evidenced by an increase in the lysosomal cargo LC3II (Figure 37b, panel 3, lane1 vs lane 2).

To determine whether tubules were in any way related to clathrin dependent reformation tubules, observed during ALR (Rong et al., 2012), embryos were injected with CLC-GFP to visualise cellular pools of clathrin. It is known that clathrin increases its association with vacuolar lysosomes in response to autophagosome-lysosome fusion, and associates along the length of reformation tubules to enhance recycling of lysosomal components (De Leo et al., 2016; Rong et al., 2012). Increased lysosomal localisation of clathrin in response to Bafilomycin A treatment would hint that these tubules could be clathrin dependent reformation tubules.

At steady state, labelling of fixed cryosections of CLC-GFP expressing embryos with antibodies to Golgin84 and EEA1 revealed that the majority of intracellular neuroepithelial clathrin was adjacent to the cis-Golgi marker Golgin84, with clathrin being found at the trans-Golgi, and a smaller amount of CLC-GFP showing co-localisation with early endosomes (Figure 38a). Tubulation of lysosomes was
not visible on cryosections from Bafilomycin A treated embryos owing to the labile nature of these structures following fixation, as has been described previously (Heuser, 1989). Bafilomycin A treatment enlarged the size of compartments labelled with LAMP1, indicating that lysosomal degradation had been blocked. Although tubules could not be visualised on fixed cryosections, little

Figure 38. Clathrin association with lysosomes does not increase following Bafilomycin treatment. A) Coronal cryosections through the hindbrain region of WT embryos injected with CLC-GFP mRNA at the one cell stage and fixed at 1 dpf. Sections were stained with antibodies to EEA1 or Golgin84. Arrowheads point to co-localisation between CLC-GFP and EEA1. B) Embryos transiently expressing CLC-GFP as in (A) were treated with 100 nM BafilomycinA1 for 2 hours then fixed, sectioned and stained with an antibody to LAMP1. Scale bars = 10 µm.
to no clathrin could be detected on the main body of LAMP1 labelled lysosomes, suggesting that clathrin is not associated and by inference that the tubules are unlikely to be reformation tubules (Figure 38b).

5.4. Dependence of tubule formation on vATPase activity

In the previous sections, Bafilomycin A induced tubulation was observed in embryos overexpressing LAMP1 and following Bafilomycin A treatment of RAP-Cy3 labelled lysosomes. Tubulation of lysosomes following Bafilomycin A treatment could be due to a number of possibilities. Higher lysosomal loads induced by blocking lysosomal degradation with Bafilomycin A may lead to the observed change in lysosome morphology. Alternatively, as the vATPase has been shown to interact with a number of proteins and have effects independent of its ability to maintain the lysosomal pH gradient, tubulation could be independent of the effect of Bafilomycin A treatment on lysosomal pH and degradation (De Luca et al., 2014; Zoncu et al., 2011).

To determine whether tubulation of lysosomes was specific to inhibition of the vATPase or occurred in response to other treatments that block lysosomal degradation, embryos were treated with the ionophore Nigericin. Nigericin is a bacterial toxin which transports K+ across cellular membranes in exchange for H+, thereby dissipating the lysosomal proton gradient and neutralising lysosomal pH (Grinde, 1983; Steinrauf et al., 1971). To confirm that Nigericin alters lysosomal pH in vivo, embryos were stained with Lysotracker. Lysotracker is a weak base only partially protonated at neutral pH and freely permeable to cell membranes whilst in this state (Pierzynska-Mach et al., 2014). The high pH of the lysosome leads to Lysotracker protonation and its accumulation within acidic compartments. Treatment of embryos with agents increasing lysosomal pH would therefore be expected to decrease the amount of Lysotracker accumulating within the lysosome.
Treatment of embryos with 100 nM Bafilomycin A led to a reduction in Lysotracker staining of acidic organelles as anticipated (Figure 39a-b). Nigericin treatment reduced the amount of Lysotracker staining to a similar extent (Total fluorescence intensity: DMSO; 31614 ± 4516, Bafilomycin A; 6084 ± 2689, Nigericin; 4738 ± 1145). Consistent with an increase in lysosomal pH caused by Bafilomycin A and Nigericin, degradation of lysosomal cargo was also blocked by both treatments. Pre-treatment of embryos with Bafilomycin A or Nigericin 30 minutes prior to hindbrain ventricle injection of an equal mixture of DQ-BSA and Alexa 488-BSA reduced DQ-BSA intensity in both Bafilomycin A and Nigericin treated embryos (Figure 39c). The effect of Nigericin on DQ-BSA cleavage appeared
marginally stronger however, as whilst a small amount of DQ-BSA was visible in embryos treated with Bafilomycin A, DQ-BSA signal was completely absent in Nigericin treated embryos. The reduction in DQ-BSA signal intensity was not due to impaired endocytosis following Bafilomycin A or Nigericin treatment (Kozik et al., 2013) as Alexa 488-BSA accumulated within the neuroepithelium in all treatment groups, albeit at reduced levels in Nigericin and Bafilomycin A treated embryos (Figure 39c). Thus, Nigericin mimicks the effects of BafilomycinA on lysosomal pH and degradation.

In contrast to Bafilomycin A however, Nigericin treatment of LAMP1-RFP embryos did not lead to lysosomal tubulation. Although a slight increase in the number of tubular lysosomes could occasionally be observed, Nigericin treatment typically led to enlargement of vacuolar lysosomes reminiscent of Bafilomycin A and Torin1 treatment (Figure 40a-b). These results would suggest that tubulation is specific to inhibition of the vATPase and is not a general response to increased lysosomal load or an increase in luminal pH.
Figure 40. Effect of Nigericin treatment on lysosome morphology. A) Confocal micrographs of the MHB region of laterally mounted EGFP-Rab7 embryos transiently expressing LAMP1-RFP and treated with either DMSO, 100 nM Bafilomycin A or 10 µM Nigericin for 2 hours. Scale bar = 5 µm. B) Quantification of the number of tubules in each treatment group. Data is from one experiment with each data point representing one embryo. *P < 0.05. Error bars in (b) represent mean ± s.e.m. * P<0.05.
5.5. Discussion

This chapter set out to study lysosome dynamics in the zebrafish neuroepithelium, with the initial aim of looking at the effects of loss of OCRL1 on lysosome dynamics. Unexpectedly, in attempting to study lysosome reformation it was found that following Bafilomycin A treatment, neuroepithelial lysosomes marked with LAMP1-RFP underwent a change in morphology from a vacuolar shape to elongated tubular structures that often spanned most of the length of the cell. As very little work has been published on neuroepithelial lysosomes, this phenotype was deemed to be worthy of further investigation and formed the basis for the set of experiments described above.

Bafilomycin A is widely used to study many processes relating to lysosome biology. It is thus surprising that this behaviour has not been reported in the literature before. This may be due to the methods used when studying lysosomes by microscopy as many experiments are performed in fixed cells where tubules are not well preserved (Heuser, 1989). This response also be could be linked to the biology of neuroepithelial cells which exhibit many unique cellular adaptations linked to their function and bipolar spindle like morphology (Taverna et al., 2016; Willardsen and Link, 2011).

Both of the methods used to visualise lysosomes here rely on conditions which either overexpress key lysosomal proteins or induce higher lysosomal loads which may influence organelle behaviour. It would thus be of importance to examine the effects of Bafilomycin treatment on lysosomes not overexpressing LAMP1 or injected with RAP. In frame knockin of a fluorescent tag into the endogenous LAMP1 locus would allow visualisation of LAMP1 dynamics at physiological levels.

5.5.1. Signalling pathways and trafficking machinery involved in neuroepithelial lysosome tublation

The appearance of tubules following vATPase inhibition led to an investigation of whether these tubules are reformation tubules similar to those observed extending from autolysosomes and endolysosomes (Bright 2005, Yu 2010, Bright 2016). No recruitment of clathrin to lysosomes could be seen following Bafilomycin A treatment however, arguing against tubules being related to
PtdIns(4,5)P₂ dependent reformation tubules. Tubules did require active mTORC1 signalling, as treatment with the mTOR inhibitor Torin1 blocked tubule formation. Tubulation in response to Bafilomycin A treatment in neuroepithelial cells is therefore subject to the same mTORC1 dependent regulatory mechanisms as in other situations where lysosomal tubulation has been described (Saric et al., 2016; Yu et al., 2010).

To date, the machinery involved downstream of mTORC1 signalling in lysosome tubulation has predominantly been shown to involve the small GTPases Rab7 and Arl8b and various plus end and minus end directed molecular motors (Mrakovic et al., 2012; Saric et al., 2016). Inhibition of mTORC1 signalling in macrophages alters the balance of motor protein association with lysosomes to potentially control lysosomal positioning and motility. Arl8b association with lysosomes increases upon mTORC1 inhibition favoring anterograde transport of lysosomes and tubulation (Saric et al., 2016). As tubules in Bafilomycin treated are mTORC1 sensitive (Table 8) (Figure 41), this raises the possibility that tubulation in neuroepithelial cells is also due to changes in motor protein association with lysosomes and requires further investigation.

5.5.2. Potential mechanisms of lysosomal tubulation following Bafilomycin A treatment

Using Nigericin to investigate whether tubulation is a general response to impaired lysosomal pH or specific to inhibition of the vacuolar ATPase revealed that tubulation appeared to be specific to Bafilomycin A treatment. Tubule numbers were comparable to controls in Nigericin treated embryos. This suggests that induction of tubulation by Bafilomycin A is independent of its effect on lysosomal pH as Nigericin increased lysosomal pH to a similar extent as Bafilomycin A, as visualised by Lysotracker. Therefore, in neuroepithelial cells, the active vATPase complex would appear to suppress lysosomal tubulation, with vATPase inhibition leading to a change from a uniquely vacuolar morphology to the tubular structures observed here.

How might inhibition of the vATPase lead to lysosomal tubulation? The vATPase is composed of two multisubunit V0 and V1 domains. ATP hydrolsis by the V1 domain facilitates rotation of the V0
membrane domain, with this action driving movement of proteins into the lysosomal lumen to maintain lysosomal pH (Forgac, 2007). The V1 domain of the vATPase interacts directly with components of the mTOR signalling pathway in an amino acid dependent fashion (Zoncu et al., 2011). Inhibiting ATP hydrolysis blocks rotation of the V1 domain and interaction with mTOR, whilst dissipating the lysosomal proton gradient independent of vATPase activity does not, demonstrating that vATPase regulation of mTOR is independent of its function in establishing the lysosomal proton gradient (Zoncu et al., 2011). Thus, the vATPase can influence additional cellular processes through protein-protein interactions. Potentially, inhibition of vATPase activity with Bafilomycin A could inhibit vATPase interactions that affect lysosome positioning and motility separable from establishment of the pH gradient. The Rab7 effector RILP, involved in interactions with Dynein, has recently been shown to bind the V1G1 subunit of the vATPase to regulate V1G1 stability and localisation to the lysosomal surface (De Luca et al., 2014). Bafilomycin A treatment could alter vATPase interactions with proteins involved in controlling motor protein association with lysosomes leading to the tubulation observed here (Figure 41).

A pH independent effect of the vATPase on lysosomal tubulation is difficult to reconcile with the mechanism of action of Bafilomycin A however. Interactions of the vATPase with mTORC1 and RILP are through the V1 domain responsible for ATP hydrolysis. In contrast, Bafilomycin A binds to the V0 complex and inhibits movements of V0 complex subunits required for proton transport into the lysosomal lumen. The mechanism of action of Bafilomycin A is therefore seemingly inextricable from establishment of lysosomal pH. One possibility is that blocking movements of the vATPase V0 domain induces conformation changes which inhibit interaction of the vATPase with proteins required for lysosome motility and positioning, but this is highly speculative.

Other treatments which interfere with lysosomal pH or degradation through mechanisms distinct from those of Bafilomycin and Nigericin would help to shed further light on the mechanism of lysosomal tubulation in neuroepithelial cells. As mTORC1 activity requires free amino acids within
the lysosomal lumen, treatment of embryos with Nigericin could inhibit lysosomal pH and degradation to a greater degree than Bafilomycin A, preventing breakdown and release of amino acids and inhibiting mTORC1 activity. Consistent with this idea, higher concentrations of Bafilomycin (>2uM) than those used here block mTORC1 activity (Zoncu et al., 2011). As tubules are mTOR dependent, Nigericin treatment could therefore block lysosomal tubulation. Western blotting for mTORC1 substrates would determine whether treatment with Nigericin inhibits mTORC1 signalling and provide additional evidence that Bafilomycin A induced lysosomal tubulation is due to specific inhibiton of the vATPase.

**Table 8- Summary of conditions under which lysosomal tubulation occurred in the neuroepithelium**

<table>
<thead>
<tr>
<th>Condition</th>
<th>vATPase / mTOR activity</th>
<th>Tubulation?</th>
<th>Potential GTPase and motor protein association based on mTOR activation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>mTOR active vATPase active</td>
<td>-</td>
<td>Arl8b - SKIP - Kinesin, Rab7 - RILP-dynein</td>
</tr>
<tr>
<td>+ Bafilomycin A</td>
<td>mTOR active vATPase inactive</td>
<td>Yes</td>
<td>Arl8b - SKIP - Kinesin, Rab7-RILP-dynein</td>
</tr>
<tr>
<td>+ Bafilomycin A, Torin1</td>
<td>mTOR inactive vATPase inactive</td>
<td>No</td>
<td>Rab7-RILP-dynein</td>
</tr>
</tbody>
</table>

**Figure 41. Schematic representation summarising conditions under which lysosomal tubulation occurred in the neuroepithelium.**

A) In untreated embryos, lysosomes adopt a vacuolar morphology. As mTOR is active, both Arl8b and Rab7 can associate with lysosomes. B) Bafilomycin A treatment leads to lysosomal tubulation. Changes in motor protein association with lysosomes which may induce tubulation cannot be due to changes in mTOR activity which is still active in the presence of Bafilomycin A. C) Co-treatment of Bafilomycin treated embryos with the mTORC1 inhibitor Torin1 blocks lysosomal tubulation. As mTORC1 has been shown to alter Arl8b association with lysosomes in immune cell types, Torin1 treatment may prevent the lysosomal association of Arl8b and anterograde (+ end) directed movement of lysosomes required for tubulation.
6. Chapter 6


6.1 Final Conclusions

Since the discovery of OCRL1 as the causative gene for Lowe syndrome, much progress has been made in uncovering the cellular functions of OCRL1. Knowledge of Lowe syndrome pathology has been expanded upon by the use of animal models to investigate the physiological roles of OCRL1 in tissues relevant to the disease (Inoue et al., 2017; Oltrabella et al., 2015; Ramirez et al., 2012). In particular, insights into Lowe syndrome pathology in the kidney have come from a zebrafish model of Lowe syndrome, which shows impaired endocytic trafficking of megalin with the pronephros (Oltrabella et al., 2015). However in the CNS, symptoms of Lowe syndrome have yet to be attributed to particular cellular processes affected by OCRL1.

Zebrafish Ocrl1 also appears to play a key role in endocytic trafficking within the neuroepithelium. The localisation of Ocrl1 to endosomal compartments containing megalin, and the reduced megalin abundance and enlarged endosomes observed in ocrl1-/- mutant embryos point to an endocytic function in this tissue. Lowe syndrome patients display symptoms affecting the CNS shortly after birth, including hypotonia, consistent with impaired neurodevelopment (Loi, 2006). Neurogenesis requires that neuronal progenitors respond to appropriate cues that influence multiple cellular behaviours including cell fate decisions and pro and anti-survival signalling (Gotz and Huttner, 2005). Sensing and interpretation of these signals depends on cell surface receptors and downstream trafficking, which Ocrl1 could affect through perturbation of receptor recycling through endosomes or impaired endocytosis. The work presented here is consistent with impaired receptor recycling potentially underlying impaired neurodevelopment in zebrafish, although an effect on endocytosis cannot fully be ruled out. It will be important in any future experiments to link impaired receptor recycling to increased levels of cell death at 1dpf to definitively prove that neurodevelopmental phenotypes are due to dysregulation of endocytic trafficking rather than other proposed functions of OCRL1. Approaches to identify which receptors and signalling pathways may be affected by loss of Ocrl1 would help to achieve this. RNA sequencing in embryonic brain tissue may provide clues as to
which pathways are altered in the absence of Ocr1, with alterations in mRNA levels potentially indicating the identity of receptors and recycling pathways affected.

In addition to regulating endocytic trafficking, OCRL1 is also important for regulating lysosomal function with depletion of OCRL1 affecting clearance of lysosomal cargos including autophagosomes (De Leo et al., 2016). This is clearly important in the kidneys as lack of OCRL1 function in proximal tubule cells of humans and zebrafish disrupts lysosomal function (De Leo et al., 2016). Additionally, autophagosome clearance is impaired in proximal tubule cell biopsies taken from Lowe syndrome patients (De Leo et al., 2016). In agreement with De Leo et al, in this study ocr1-/- embryos accumulated autophagosomes and had enlarged lysosomes within proximal tubule cells of the pronephros, further supporting an important role for OCRL1 in maintaining lysosomal function in the kidneys. It has been hypothesised that accumulation of autophagosomes and failure to clear autophagosomal cargo such as damaged or old mitochondria leads to prolonged TLR9 signalling, which regulates inflammatory responses (De Leo et al., 2016). Prolonged TLR9 signalling could lead to inappropriate inflammatory responses in the kidneys, causing kidney damage. Additionally, blockages in lysosomal degradation can interfere with the endocytic pathway upstream of the lysosome which may contribute to disease progression.

In contrast to the pronephros, the lack of an effect of loss of Ocr1 on autophagosome turnover and lysosome function in the CNS of ocr1 mutant zebrafish suggest that Ocr1 does not significantly contribute to maintaining lysosomal homeostasis in the CNS. This would also indicate that loss of Ocr1 function at the lysosome may not be relevant to the CNS pathology of Lowe syndrome. Potentially, changes may take longer to accumulate within neuronal cells than at the timepoints looked at here (8 months). LC3II levels in the ocr1-/- mutant could therefore be examined at later timepoints during adult life. However, the later the lysosomal system is affected, the less relevance to Lowe syndrome pathology OCRL1 lysosomal function would appear to have as many symptoms develop during childhood and adolescence (Schurman and Scheinman, 2009).
Investigating lysosomal dynamics in neuroepithelial cells revealed that Bafilomycin A treatment leads to tubulation of lysosomes. This wasn’t seen with Nigericin, which neutralises lysosomal pH and inhibits degradation to a similar degree as with Bafilomycin A. This would indicate a direct role for the vATPase in suppressing lysosomal tubulation, independently of maintaining the lysosomal proton gradient. No data exists in the literature describing similar effects on lysosomes in other cell types, suggesting this may be specific to neuroepithelial cells. Further experiments could serve to identify additional vATPase interaction partners that may regulate this response or examine motor protein association with lysosomes in the presence and absence of Bafilomycin A.
7. References


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