ROLE OF FIBROBLAST GROWTH FACTOR RECEPTOR IN REGULATION OF MEMBRANE TRAFFIC

Navin David Rathnakumar

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Aston University

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Abstract

Several studies show that membrane transport mechanisms are regulated by signalling molecules. Recently, genome-wide screen analyses in *C.elegans* have enabled scientists to identify novel regulators in membrane trafficking and also signalling molecules which are found to couple with this machinery. Fibroblast growth factor (FGF) via binding to fibroblast growth factor receptor (FGFR) mediate signals which are essential in the development of an organism, patterning, cell migration and tissue homeostasis. Impaired FGFR-mediated signalling has been associated with various developmental, neoplastic, metabolic and neurological diseases and cancer. In this study, the potential role of FGFR-mediated signalling pathway as a regulator of membrane trafficking was investigated. The GFP-tagged yolk protein YP170-GFP trafficking was analysed in worms where 1) FGFR signalling cascade components were depleted by RNAi and 2) in mutant animals. From these results, it was found that the disruption of the genes *egl-15* (FGFR), *egl-17*(FGF), *let-756*(FGF), *sem-5*, *let-60*, *lin-45*, *mek-2*, *mpk-1* and *plc-3* lead to abnormal localization of YP170-GFP, suggesting that signalling downstream of FGFR via activation of MAPK and PLC-γ pathway is regulating membrane transport. The route of trafficking was further investigated, to pinpoint which membrane step is regulated by worm FGFR, by analysing a number of GFP-tagged intracellular membrane markers in the intestine of Wild Type (WT) and FGFR mutant worms. FGFR mutant worms showed a significant difference in the localisation of several endosomal membrane markers, suggesting its regulatory role in early and recycling steps of endocytosis. Finally, the trafficking of transferrin in a mammalian NIH/3T3 cell line was investigated to identify the conservation of these membrane trafficking regulatory mechanisms between organisms. Results showed no significant changes in transferrin trafficking upon FGFR stimulation or inhibition.

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LIST OF ABBREVIATIONS

AP - Adaptor Protein

APS - Ammonium Persulphate

ARF - ADP Ribosylation Factors

ATP - Adenosine triphosphate

Ca^{2+} - Calcium

$CaCl_2$ -Calcium Chloride

CCP - Clathrin Coated Pits

CCV - Clathrin Coated Vesicles

CFTR - Cystic fibrosis transmembrane conductance regulator

CHC-1 - Clathrin Heavy Chain -1

CIITA - Class II major histocompatibility complex transactivator

CLR1 – Clear-1

COPI – Coat Protein I

COPII – Coat Protein II

DAB1 - Disabled -1

DAG – Diacyl Glycerol

dH_2O - Distilled water

DMEM - Dulbecco's modification of Eagle's medium

DMSO - Dimethyl Sulphoxide

DNA - Deoxyribonucleic acid

Dpy - Dumpy Phenotype

DsRNA – Double Stranded Ribonucleic Acid

ECL - Enhanced Chemiluminescence

EDTA - Ethylenediaminetetraacetic acid

EGF -Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

Egl - egg laying defective Phenotype

ENDO - Endocytosis Defect

ER - Endoplasmic Reticulum

ERK - Extracellular Signal Regulated Kinases

EtOH -Ethanol

FBS - Fetal Bovine Serum

FGF - Fibroblast Growth Factor

FGFR - Fibroblast Growth Factor Receptor

FRS2 - Fibroblast growth factor receptor substrate 2

GAP – GTPase Activating Protein

GDI - GDP dissociation inhibitor

GDP - Guanosine diphosphate

GEF - Guanine-Nucleotide Exchange Factor

GFP - Green Fluorescent Protein

GPCR - G protein coupled receptors

GRB2 - Growth factor receptor bound protein 2

GTP – Guanosine triphosphate

HTAC – α Chain of Human Interleukin 2 Receptor

HTFR - Human Transferrin Receptor

IP_3 - Inositol trisphosphate

IPTG - Isopropyl β-D-1-thiogalactopyranoside

JAK/STAT- Janus Kinase/Signal Transducer and Activator of Transcription

K_2HPO4 - Dipotassium phosphate

KCl - Pottasium Chloride

KH_2PO4 - Potassium dihydrogen phosphate

KPO_4 - Pottassium Phosphate

LB - Lysogeny broth

LDL -Low Density Lipoprotein

Let - lethal

MAPK - Mitogen-activated protein kinases

$MgSO_4$- Magnesium Sulphate

MHC – Major Histocompatibility Complex

mRNA - Messenger Ribonucleic Acid

Na_2HPO_4 - Disodium hydrogen phosphate

NaCl - Sodium Chloride

NaOH – Sodium Hydroxide

NF-Kb - Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF – Nerve Growth Factor

NGM -Nematode Growth Medium

NPC1 - Niemann-Pick C1

NSCLC – Non-Small Cell Lung Cancer

PBS - Phosphate-buffered saline

PH - Plekstrin Homology Domain

PI3K- Phosphatidylinositol-4,5-bisphosphate 3-kinase

PIP2 - Phosphotidylinositol 4,5-Bisphosphate

PKC - *Protein kinase C*

PLC-γ- Phospholipase C-gamma

PMSF - Phenylmethylsulfonyl fluoride

PTB - phosphotyrosine-binding domain

RFX - Regulatory factor X

RME -Receptor Mediated Endocytosis

RNA - Ribonucleic acid

RNAi – RNA mediated interference

RTK – Receptor Tyrosine Kinases

SDS - Sodium dodecyl sulfate

SEC – Secretory Defect

Sem - sex muscle abnormal

SH2 - Src Homology 2

SH3 - Src Homology 3

SNARE- Soluble N-ethylmaleimide attachment protein receptors

SOS – Son of sevenless guanine nucleotide exchange factor

SP12 – Signal Peptidase 12

SV40 – Simian vacuolating virus 40

TAP1 - Transporter associated with Antigen Processing 1

TEMED - Tetramethylethylenediamine

TGFβ - Transforming growth factor beta

TGN - Trans Golgi Network

TrKA - *Tropomyosin receptor kinase A*

VEGF - Vascular endothelial growth factor

VSV - Vesicular stomatitis virus

WT - Wild Type

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CHAPTER 1 INTRODUCTION

1 INTRODUCTION

A protein which is produced within a cell has to reach its correct destined location not only for proper cellular function but also to provide the impetus for cell growth and cell expansion (Barlowe & Miller 2013). Some of these proteins are transported to a particular organelle within the cell (intra-cellular) or to the cell surface for the transportation to another cell (inter-cellular) (Figure 1.1). For example, transporter proteins and many hormone receptor proteins have to be delivered to plasma membrane, water soluble enzymes such as RNA and DNA polymerases must be targeted to the nucleus and some polypeptide signalling molecules and digestive enzymes have to be directed to the cell surface for secretion from the cell. The molecular mechanisms involved in membrane trafficking have been progressively elucidated in the last few decades and the underlying machinery is complex including events like membrane bending, fission, fusion, coating and sorting (M. C. S. Lee et al. 2004; Sallese et al. 2006)

Figure 1.1: An overview of endocytic and secretory pathway (Tokarev et al. 2000) .

1.1 Overview of secretory and endocytic pathways of protein sorting

1.1.1 Secretory Pathway:

Almost all eukaryotic cells use the same secretory pathway for protein sorting which include the secreted proteins and the ones that reside inside the endoplasmic reticulum (ER), Golgi and lysosomes. These are collectively referred to as secretory proteins. Secretory pathway consists of three steps;

1. Protein synthesis and translocation across the ER membrane
2. Protein modification (folding and assembly) inside the ER.
3. Protein transport through budding and fusing of vesicles to Golgi, lysosomes or cell surface.

Thus soluble and membrane proteins slated to function at the cell surface are delivered to their final destination via secretory pathway (Figure 1.2).

The newly produced soluble and membrane proteins are translocated into the rough ER where they are modified by the addition of N-linked carbohydrates and disulphide bonds (Lodish et al. 2000). After folding they progress through the Golgi where they can still be modified for example, by the addition of O-linked oligosaccharides (Alberts et al. 2002). The secretory proteins are packaged into forward moving vesicles which fuse with each other to form a flattened membrane bound compartment known as cis-Golgi cisterna**.** The new set of cargo proteins mechanically move from the *cis* position (near to ER) to a *trans* position (farther to ER) forming a medial-Golgi cisterna and then a trans-Golgi cisterna. This whole process is called the cisternal maturation (Lodish et al. 2000). Eventually these secretory proteins reach a complex network of membranes and vesicles termed as trans-Golgi network (TGN).

The continuous flow of membranes from ER to the Golgi (anterograde transport) could result in depletion of ER membranes and extension of Golgi, therefore in order to maintain the balance of structural and functional identities of these endomembranes some protein and lipids are recycled back to the ER and this is commonly referred to as retrograde transport.

The components of the secretory pathway are universal and they are very similar from yeast to animals (J E Rothman & Orci 1992). The cargo carrying vesicles are delivered to the plasma membrane either through the trans-Golgi network or through the recycling endosomes (Brooks et al. 2009). The Golgi is assembled into three functionally distinct regions namely the cis-Golgi network (the entry face of the stack), the Golgi stack and the trans-Golgi network (the exit face of the stack). The *cis* and *trans*-Golgi are mainly involved in sorting and distribution. The proteins with different final destinations such as the lysosomes, endosomes and plasma membrane are sorted and arranged for their specific route in the TGN (James E. Rothman & Orci 1992; Griffiths & Simons 1986).

Figure 1.2: Diagrammatic representation of exocytosis; release of neurotransmitter molecules into the extracellular space (Haucke et al. 2011).

The content inside the vesicles are typically membrane proteins routed to the cell surface or luminal contents destined to be secreted into the extracellular space or organelles, like lysosomes. The other cellular functions in which exoctyosis are involved are cell-cell communications, cell polarity, immune responses and neurotransmission (Brooks et al. 2009).

After sorting at the TGN exocytosis pathway follows two different routes, the continuous delivery of membrane and cargo to the cell surface called constitutive secretion and the regulated secretion involving the redirection and of newly synthesised cargo in vesicles which are stored until they are triggered for a release by a signal (Stow et al. 2009).

Following the synthesis and modification of secretory proteins, they become fully competent to be transported forward (anterograde) with different transport vesicles to their respective destinations (Barlowe & Miller 2013). COPII coat, like other coat protein complexes help in creating a spherical vesicle from ER donor membrane and populate it with cargoes (Barlowe 1994). COPII also plays a major role in selective capture of cargoes in transport vesicles by recognizing specific sorting signals like simple acidic peptides (Malkus et al. 2002) to folded epitopes (Mancias & Goldberg 2007). The role of COPI is well established with the vesicles transported from Golgi to ER (retrograde trafficking). Similar to COPII, COPI assembly on the membrane is facilitated by small GTPase ARF1 which helps in membrane anchorage (Antonny et al. 1997).

There are two major molecular complexes conserved in most exocytic events namely the exocyst and the soluble N-ethylmaleimide sensitive factor attachment protein receptor complex (SNARE) (With the aid of various effector molecules, these complexes coordinate tethering, docking and fusion of vesicles with the plasma membrane (Brooks et al. 2009).

The attachment of secretory vesicles with target membranes is mediated by the exocyst complex (Pfeffer 1999; Guo et al. 2000). The exocyst is an evolutionary conserved octamer complex composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (Liu & Guo

2012). The exocyst complex tethers secretory vesicles to plasma membrane via a direct association with phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) (Martin 2014). RAB GTPases such as RAB11, and several other GTPase proteins such as Rho3, Cdc42 or Tc10 (orthologue of Cdc42 in mammals) mediate specific routing and tethering of vesicles to the plasma membrane through interactions with the exocyst (Brooks et al.,2009). The exocyst complex is tightly regulated by integration of key regulators of membrane trafficking such as RAB and ARF GTPases (Guo et al. 1999).

The second major complex involved in exocytosis is the SNARE complex. The SNARE complex was hypothesised in 1993 (Söllner et al. 1993). The SNARE complexes mediate membrane fusion along the secretory and endocytic pathways. There are two different SNARE groups namely the t-SNARE and the v-SNARE. All SNARE complexes contain approximately 70 amino acids comprising heptad repeats (a structural motif that consists of a repeating pattern of seven amino acids) and function on both target membranes (t-SNAREs) and transport vesicles (v-SNAREs)(Hong & Lev 2014). The t-SNAREs such as syntaxin1 and Sec9 are found on the inner plasma membrane denote the target membrane and the v-SNARE which are found on different membrane compartments denote the vesicular membrane (Stow et al. 2006; Shorer et al. 2005; Brooks et al. 2009). The t-SNARE and the v-SNARE pair with each other to form a trans-SNARE complex also called SNAREpin which consists of four helix bundle that allows membranes to get closer to each other and facilitate membrane fusion (Brooks et al. 2009). Following membrane fusion, the remaining SNARE complexes called the cis-SNARE complex undergoes disassembly initiated by the ATPase N-ethylmaleimide-senstive fusion protein (NSF) and its co-factor soluble NSF attachment protein (SNAP) to recycle SNARE for a new fusion event (Olkkonen & Ikonen 2006; Hong & Lev 2014). The other classification of SNARE complexes was based on the crystal structure of synaptic SNARE complex [(Syntaxin [Stx] 1A, synaptobrevin2 and Synaptosome associated protein (SNAP)-25B)]. The crystal structure

revealed that the central position of the four-helix bundle is comprised of an arginine contributed by synaptobrevin and three glutamine residues contributed by Stx1A and SNAP-25. Thus, these structural revelations led to the further classification of SNAREs as R-SNARE and Q-SNARE (Pieren et al. 2010). Thus a functional SNARE complex constitutes three Q-SNARE motifs and a single R-SNARE motif. R-SNAREs are generally related to v-SNAREs and Q-SNAREs related to t-SNAREs (Hong & Lev 2014).

Sec1/Munc18 (SM) proteins and several tethering factors are involved in the regulation of exocyst and SNARE complexes. SM proteins are considered to be vital components of the fusion processes and they are found to accelerate SNARE mediated fusion and contribute to the specificity of various fusion events (Südhof & Rothman 2009)(Shen et al. 2007). The four major SM proteins identified in mammals are Sly1, Vps45, Vps33 and Munc18. A recent study on Vps33 suggest that SM proteins promote the opening of a fusion pore by triggering the SNARE complexes (Pieren et al. 2010).

The tethering factors are a group of protein complexes that link the transport vesicles to their respective target membranes. Tethering factors are classified into two major categories such as homodimeric long coiled-coil proteins and multi-subunit tethering complexes (MTCs). Their function is based on the distance between two vesicles. The long coiled tethers interact with vesicles over a distance of 200nm whereas the multi-subunit tethering complexes interact with vesicles over a much shorter distance up to 30nm (Hong & Lev 2014) (Chia & Gleeson 2014). Thus these interactions between exocyst, SM proteins, tethering factors and SNARE complexes spatially and temporally organise fusion events at specific membrane compartments (Hong & Lev 2014).

1.1.2 Endocytic Pathway

Endocytosis is the process by which vesicles bud inwards at the plasma membrane thereby bringing proteins inside the cell where they are either sorted to lysosomes via late endosomes or are recycled back to the cell surface (Hancock, 2005).

Based on the material internalised, endocytosis can be broadly divided into two categories. Phagocytosis (cell eating) refers to internalisation of large molecules (>200nm) through an actin cytoskeleton based mechanism and Pinocytosis (cell drinking) is the internalisation of extracellular medium and occur via mechanisms such as clathrin-dependent endocytosis, caveolae-mediated endocytosis, macropinocytosis and dynamin–clathrin independent endocytosis (Seto et al. 2002). Cholesterol carried in LDL particles and iron atoms carried by the iron binding protein transferrin are examples of transmembrane cargo proteins that follow the endocytic pathway (Lodish et al. 2000).

By far the best characterised route of internalization of various cell surface components and solutes is the clathrin-mediated endocytosis pathway. Clathrin dependent uptake takes place at specialized sites where complex coated pits are assembled in order to internalize surface proteins. The two major proteins involved in this coated pit assembly are clathrin and AP-2 along with several other accessory proteins. The structure of clathrin (Figure 1.3), a triskelion involves three heavy chains and three light chains (Schmid 1997; Greene et al. 2000). The clathrin heavy chain has functionally distinct regions namely, an N terminal domain which interacts with a number of endocytic proteins such as AP-2, a curved region which divides the polypeptide into distal and proximal leg and the C terminal domain that forms trimerization (Liu et al. 1995). Both the distal and proximal legs are essential to form closed basket structures and the proximal leg also facilitates the binding of clathrin light chains (Schmid 1997;

Kirchhausen 2000; Ybe et al. 1998). The clathrin light chains exist in two isoforms and they are involved in regulating the assembly of clathrin triskelion (Ybe et al. 1998).

Clathrin-dependent endocytosis forms nascent vesicles using a clathrin coat (Seto, Bellen and Lloyd, 2002) and these clathrin coats (coated pits) are basket like structures around budding or invaginating membranes and vesicles (Robinson. 1994). Though these clathrin coats act as scaffolds they cannot bind directly to the membrane. This binding is mediated by clathrin adaptors which can bind directly to clathrin or lipid components of the membranes. So far six members of the clathrin adaptor complex have been identified in mammals. AP-1A, AP-2, AP-3A, and AP-4 are widely expressed and AP-5 and AP-6 are cell specific isoforms of AP-1A and AP-3A (Ohno 2006). AP-2 is a large protein complex consisting of four subunits α, β2, μ2 and δ2. α subunit is involved in driving AP-2 to the plasma membrane as well as make it

interact with other endocytic proteins. The binding sites on the β2 subunit facilitates the binding of AP-2 to clathrin (Rapoport et al. 1998) and the μ2 subunit contains a phosphoinositide binding site which helps in the recognition and sorting of cargo proteins (Rohde et al. 2002). The coated pits form at specific sites at the plasma membrane and these are called "coated pit zones" (Santini et al. 2002). The mechanisms and membrane factors that define these coated pit zones are not fully clear but phosphoinositides (PI) are found to play a major role (Vicinanza et al. 2008). PIs constitute less than 10% of total lipids yet they remain key regulators of membrane traffic and cell signalling (Haucke, 2005).

Phosphoinositides:

Phosphoinositides are phosphorylated intermediaries of phosphoinositol (PI) and is involved in several important cellular functions (Figure 1.4) including membrane trafficking (Vicinanza et al. 2008). All adaptor proteins that induce clathrin assembly are found to interact with phosphoinositides and especially PIP2 and PIP3 have been found to be important for both constitutive and regulated endocytosis (Mousavi et al. 2004). Plasma membrane $PI(4,5)P_2$ is directly involved in regulating clathrin mediated endocytosis and many of the clathin adaptors such as Epsin, AP180, Ent2p and AP-2 directly bind to $PI(4,5)P_2$. After internalization, $PI(4,5)P_2$ is rapidly dephosphorylated by lipid phosphatases (synaptojanin family) and this dephosphorylation is vital for the disassembly of clathrin coats at endocytic vesicles (Haffner et al. 2000). $PI(4,5)P_2$ is also dephosphorylated by 5-phosphatase OCRL1 that localizes to early endosomes and golgi compartment (Loi, 2006).

Figure 1.4: Phosphoinositides regulation of membrane traffic. PI(4,5)P_2 (blue) is essential for the formation of clathrin coated pits, macropninocytosis and fusion of secretory vesicles. PI(3)P (green) is localised in early endosomes and vesicles inside multivesicular bodies (MVB). PI(3)P is converted to PI(3,5)P_2 at the boundary of MVB. PI(4)P is localised to golgi and TGN complex *(Haucke, 2005).*

AP-2:

The conventional view is that AP-2 regulates the localization of clathrin assembly at plasma membrane. AP-2 consists of two binding sites for phosphoinsositides at both the N terminal region of the α subunit and the other on μ2 subunit (Musacchio et al. 1999; Kelly 1999). These two large subunits form the core domain of AP-2 and thus by associating with clathrin, accessory endocytic proteins, cargo receptors and membrane lipids they serve as the core protein recruitment hub during clathrin coated pit assembly (Praefcke et al. 2004). The

other accessory proteins such as AP180, epsins, Eps15, endophilins and amphyphysin function as adaptors (Figure 1.5) (Gallop & McMahon 2005). The current view of the coated pit formation is that the endocytic adaptors specify the location of clathrin assembly on the plasma membrane and they continue to recruit and promote clathrin polymerization (Shih et al. 1995). Targeting AP-2 to plasma membrane does not entirely rely on $PI(4,5)P_2$ binding. Although $PI(4,5)P_2$ is predominantly located at the plasma membrane, they are also found on the trans-golgi network and therefore integral membrane proteins in addition to lipids may play a role in determining nucleation sites for clathrin assembly (Martin 2001). For example, surface receptors may recruit AP-2 and initiate the nucleation of clathrin coated pits (Iacopetta et al. 1988). AP-1 localises to vesicles that bud from trans-Golgi network whereas AP-2, AP-3 and AP-4 localises to the vesicles at the plasma membrane and has the dual role of binding the clathrin coat to the membrane and also recruits cargo proteins into the pits (Smythe 2002; I S Trowbridge, J F Collawn 1993; Ohno 2006). These clathrin adaptor complexes are also found to regulate vesicular transport of cargo across different membranes (Ohno 2006).

Figure 1.5: Schematic diagram showing proteins involved in endocytosis and illustration of the recruitment role of AP-2. Amphiphysin, Epsin, Epsin15, Auxilin, and AP180 all bind to the same site on AP-2 α appendage domain in a regulatory manner at coated vesicle assembly *(Pearse et al. 2000).*

Dynamin:

Dynamin is another high molecular mass protein which plays a crucial role in endocytosis. They have an N terminal GTPase domain involved in binding and hydrolysis of GTP. The core central regions contains a pleckstrin homology domain (PH) which binds specifically to PI(4,5)P2 and the C terminal region consists of a coiled coil structure called GTPase effector domain. Following the GED domain is the PRD domain (proline-rich domain) which interacts with proteins containing SH3 domains such as amphiphysin, endophilin and actin-binding proteins. Dynamin is targeted to clathrin coated pits to form small spirals at the necks of coated pits and this achieved by SH3 binding domain within the PRD domain. Amphiphysin proteins containing SH3 domains link dynamin to the clathrin coated pits by binding both to the PRD domain of dynamin and to the adaptor protein AP-2 (Robinson. 1994).

Coat Proteins:

Coat proteins play a major role in regulation of endocytic traffic (Okamoto, 1998). Intracellular sorting of cargo, condensing cargo and their respective receptors into nascent vesicles, and targeting vesicles from donor compartment to recipient compartment are some of the functions of coat proteins (Okamoto, 1998). It is hypothesised that the tight regulation of cargos could be accomplished by specific classes of coat proteins that selectively inhibit or enhance specific vesicular trafficking steps. This hypothesis is further strengthened by the discovery of the coat protein β-NAP, a neuron specific vesicle coat protein which mediates

only apical transport in polarised cells (Lori S Newman, Matthew O McKeever, Hirotaka J Okano, 1995).

Caveolae-mediated endocytosis is another process of endocytosis which is believed to aid in receptor-mediated endocytosis in many but not all cell types. Caveoli are small flask shaped structures enriched in cholesterol and sphingolipids (Seto, Bellen and Lloyd, 2002). Caveoli are covered by a single major coat protein called caveolin (Fra et al., 1995). The dual localization of caveolae as membrane invaginations at the plasma membrane and in the trans-Golgi network suggest that they could aid in membrane trafficking both from the Golgi and from the plasma membrane (Patton, 1996).

Macropinocytosis is the third type of endocytosis similar to phagocytosis where large vesicles are formed to engulf extracellular fluid. All three above mentioned processes of endocytosis are dynamin dependent. Yet, studies suggest that there is another route of endocytosis which is dynamin- and clathrin- independent (Seto, Bellen and Lloyd, 2002) (Sandvig, 1994)

Sorting in the endosome:

The first stop of compartmentalization of internalised components is the early endosomes. From here, the destinies of the components are determined depending on their utility. If they are to be reutilized, they are then recycled back to the plasma membrane whereas molecules that are to be degraded or down regulated are transported to the late endosomes and to the lysosomes (Cavalli, Corti and Gruenberg, 2001). Endosomes are tubulovesicular structures of various shapes and sizes (Schwartz 1995; Geuze et al. 1987; Geuze et al. 1983). Immediately following endocytosis, ligands encounter endosomes. According to their distinct functions and roles, several types of compartments have been identified such as early endosomes, late endosomes, recycling endosomes and lysosomes (Huotari & Helenius 2011).

Lysosomes are organelles that store hydrolases and are considered to be the final destination where proteolytic degradation takes place (Diering & Numata 2014).

The regulation of endosomal pH is one of the important factors essential for the proper routing of molecules along the endocytic pathways. Studies suggest that early endosome maintain a pH of 6.5 followed by large endosomes with a pH of 5-5.5 and the more acidic lysosomes with a pH of 4.6 (Schwartz 1995).

The maturation model of endosomes state that they are transient and undergo defined stages as they mature along the endocytic pathway. The process of maturation is characterised by four changes, 1. An increase in number of intraluminal vesicles; 2. An increase in luminal acidification; 3. Movement in space from cell boundary to microtubule organising center (MTOC); and 4. Switching of different Rab proteins (Hu et al. 2015). Immediately after endocytosis, endocytic vesicles fuse into each other or pre-existing early endosomes with the help of GTPase Rab5 (Laifenfeld et al. 2007). Early endosomes are mostly formed around the cell periphery with a slightly acidic intraluminal pH which helps in ligand receptor dissociation. Early endosomes play an important sorting by recycling ligands back to plasma membrane for reuse or to late endosomes and to lysosomes for degradation (Li & DiFiglia 2012; Peters et al. 2001). As internal vesicles bud from the membrane of endosomes, the number of intraluminal vesicles increases and thereby maturing from early endosomes to late endosomes and thus create multivesicular bodies. The maturation from early to late endosomes is accompanied by the transition of Rab5 to Rab7 and this process is called Rab conversion (Poteryaev et al. 2010). Late endosomes or MVBs then finally fuse into lysosomes and intraluminal vesicles are degraded.

On this endo-lysosomal network, the internalized cargo can be subjected to two opposing cargo sorting systems. 1. The internalized cargo is trafficked to late endosomes and routed to lysosomes for degradation via the ESCRT system or, 2. It is transported to TGN or

cell surface for reuse via the retromer system (Hu et al. 2015). In the pathway promoting degradation, cargo proteins are transported to lysosomes and this process is initiated by cargo sorting receptor ESCRT (Endosomal sorting complexes required for transport) in a signal dependent manner. These ESCRT complexes recognise and sort ubiquitinated endosomal proteins for degradation (Raiborg & Stenmark 2009). The ESCRT machinery consists of four distinct protein complexes namely ESCRT-0, I, II and III and these are named according to the order of recruitment and function in sorting ubiquitin dependent cargo into MVBs (Stuffers et al. 2009). On the other hand, the primary function of retromer system is to conduct cargo sorting from the endosomes to TGN or to the plasma membrane via recycling pathways by various sorting receptors and SNAREs (Huotari & Helenius 2011).

Figure 1.6: Schematic representation of endosomal sorting pathways. Cargo in the endosome is sorted into their degradative pathway and eventually led into lysosomes for degradation or into a retrograde pathway for reuse. Components that are crucial in each of these pathways are listed (Burd & Cullen 2014).

Role of ubiquitin in membrane trafficking

Ligand induced activation of transmembrane receptors promotes activation of signalling cascades that control cellular processes such as cell migration, proliferation, differentiation and survival. Receptor signalling is well regulated and controlled to achieve the

best possible outcome. One such controlling mechanism is the ubiquitylation of receptors that negatively regulate receptor signalling (Haglund & Dikic 2012).

Ubiquitin is a small 8kDa protein which covalently attaches to the lysine residues of target protein through Ub ligases (E3 enzymes) (Mukhopadhyay & Riezman 2007). Ubiquitylation is more like a signal that is required for degradative receptor sorting into MVEs and lysosomal degradation (Katzmann et al. 2002; Raiborg et al. 2002; Raiborg & Stenmark 2009) and is supported by the evidence that ubiquitin fused to transferrin receptors prevented them from recycling back to the plasma membrane and instead routed them to degradative endosomal sorting (Raiborg et al. 2002).

Following internalization by either clathrin independent or clathrin dependent, receptors are directed to early endosomes (Sorkin & von Zastrow 2009) and from here these receptors are recycled back to plasma membrane for further signalling activation or targeted to lysosomes for signal termination (Raiborg and Stenmark, 2009).

Extensive studies in the last two decades have shown the importance of receptor ubiquitylation in regulating receptor endocytosis and degradative endosomal receptor sorting. Initially the role of *ub* in receptor endocytosis and endosomal receptor degradation was observed in saccharomyces cerevisiae where monoubiquitylation of the uracil permease alone induced endocytosis (Galan & Haguenauer-Tsapis 1997). Later studies on mammalian cells showed that fusion of ubiquitin to different types of receptors can promote endocytosis and endosomal sorting for degradation. Ubiquitylation in receptor tyrosine kinases have also been extensively studied and one of the most well characterised RTK is the EGFR. Immediately after the ligand induced activation of RTKs at the plasma membrane, a large number of RTKs undergo ubiquitylation. Generally, internalization is mediated by several amino acid sequences within cytoplasmic domain and the tyrosine based motif YXXO (O is an amino acid with a bulky

hydrophobic group and X is any amino acid) is involved in endocytosis of many transmembrane proteins (Owens and Evans, 1998).

The distinct role of ubiquitination in receptors in mammalian cells was first identified in platelet-derived growth factor receptor and epidermal growth factor receptor, following which ubiquitination in other tyrosine receptors were found in a ligand dependent manner by the E3 ligase Cbl (Mori et al. 1992; Galcheva-Gargova et al. 1995). Cbl is a major family of E ubiquitin ligases involved in ubiquitination of RTKs (Thien & Langdon 2001; Joazeiro et al. 1999; Levkowitz et al. 1998; Levkowitz et al. 1999). E ubiquitin ligases bind to the specific phosphorylated residues in the cytoplasmic region of RTKs (Tyr1045 in EGFR) either directly via TKB domain or indirectly through interaction with GRB2 protein (de Melker et al. 2001; Jiang et al. 2003; Schmidt et al. 2003; Thien & Langdon 2001). With thorough investigations it's found that the route of EGFR internalization and level of ubiquitylation is affected by ligand (EGF) concentration (Sigismund et al. 2005). Under low ligand doses, EGFR is more likely to undergo clathrin mediated endocytosis and are recycled back to plasma membrane whereas at higher doses, a significant level of EGFRs become ubiquitylated and undergo clathrin independent (lipid-raft dependent) intake and are targeted to lysosomes for degradation. This different intake routes was found to affect EGFR signalling as there was a prolonged EGFR signalling through the clathrin mediated endocytosis of EGFR whereas EGFR signalling was attenuated when ubiquitylated and degraded in the lysosomes (Sigismund et al. 2008). Overexpression of Cbl enhanced endocytosis whereas overexpression of dominant negative Cbl mutants or Cbl knock-down resulted in reduced endocytosis of EGFR and later studies showed that recruitment of c-Cbl resulted in endocytosis and sorting of EGFR to degradation in the lysosomes (Levkowitz et al. 1998). In case of FGFRs, outcome of ubiquitylation is slightly different. It is found that, ubiquitylation of FGFR results in intracellular sorting but not endocytosis (Cho et al. 2004). Following internalization, the FGF/FGFR complexes reach

sorting endosomes and from here, FGFRs (1-3) are routed to lysosomes for degradation whereas FGFR4 was found to be routed to recycling endosomes and from there recycled back to the plasma membrane. Subsequent studies on the levels of ubiquitylation on FGFRs showed that FGFR4 was less ubiquitinated compared to FGFRs(1-3) and these different levels of ubiquitylation was proposed to be a mechanism to regulate their sorting (Haugsten et al. 2005). FGFR ubiquitination happens by binding of c-Cbl ligase to FRS2 via Grb2. The FGF induced ternary complex formation of FRS2, Grb2 and Cbl results in ubiquitination and degradation of FRS2 and FGFR. Unlike EGFR which directly binds to Cbl via its SH2 domains, FGFRs bind to Cbl through Grb2 and FRS2. Grb2 binds to FRS2 through its SH2 domains and to Cbl via its two SH3 domains (Wong et al. 2002).

1.2 Role of RAB proteins in membrane trafficking

One of the key regulators of membrane trafficking are the RAB GTPases and they are a large family of proteins involved in secretory and endocytic pathways by coordinating several transport steps such as vesicle formation, docking, fusion and motility (Zerial & McBride 2001). RAB proteins function by cycling between a GDP (Guanosine diphosphate)-bound (OFF state) cytosolic form to a GTP (Guanosine triphosphate)-bound (ON state) membrane form and are also known to modulate the v/t-SNARE complexes. The GDP/GTP exchange is catalysed by GEFs (Guanine-Nucleotide Exchange Factors). The GTP-bound RABs then interact with their effector proteins and GTP is hydrolysed back by GAPs (GTPase Activating Proteins) (Rothman 1994). Most of the RAB proteins are ubiquitous though their expression levels may vary according to the cell type. Some RABs are tissue specific such as RAB3A (Darchen et al. 1990) (Fischer von Mollard et al. 1990) which is found only in neurons and RAB17 found only in epithelial cells (Lütcke et al. 1993). Since RABs are highly compartmentalized in organelle membranes, they remain excellent markers for studying

organelle identity and transport specificity (Zerial & McBride 2001). More detailed function of RAB proteins are described in chapter 4.

1.3 Cell Signalling

Cell signalling has become one of the most important aspects of modern biochemistry and molecular cell biology. In any multicellular organism all cells are bombarded by signals in many forms in a continuous manner and in most cases the cells have to respond to them for their survival. Cells must have the ability to detect the extracellular signalling molecules and conditions and must also be able to instigate a series of intracellular responses. The principles of signalling mechanisms are very similar across the diverse range of organisms; fungi, bacteria, animals and plants. Researchers often use their understanding of signalling mechanisms in one species and discover an analogous mechanism in a completely different species. Cell signalling not only helps in understanding the functioning of a normal cell but also to understand an aberrant cell. For example, the discovery of oncogenes has made breakthroughs in understanding cancer while the discovery of cytokines promises to be a cure for a variety of diseases (e.g. Alzheimer's disease) (Hancock., 2005).

The signalling medium could be chemical, electrical, light photons etc and in most cases signals arrive at, and are perceived at, the plasma membrane. Based on the distance and molecules involved, cell signalling can be classed into five types (Table 1.1).

Table 1.1: Types of signalling (Hancock, 2005)

Most signalling molecules are too hydrophilic and too large to penetrate through the plasma membrane and hence these bind to the cell surface receptors which are integrated in the plasma membrane. The vast majority of receptors are activated by binding of membrane-bound or secreted molecules such as hormones, growth factors, neurotransmitters and pheromones. Changes in the concentration of a metabolite (e.g. nutrients, oxygen) or by physical stimuli (e.g. light, touch, heat) can also activate receptors. The signalling molecule acts as the ligand which binds to its specific extracellular domains of the receptor. This binding causes a conformational change in the receptor which is transmitted through the cytosol causing further binding and activation of downstream signalling proteins. Thus the overall process of converting extracellular signals into intracellular responses is called as *signal transduction* (Lodish *et al.*, 2000). In all eukaryotes there are only a few dozens of signal-transduction pathways and their terminology is mostly based on the class of receptor involved (e.g. G protein-coupled receptors, receptor tyrosine kinases), class of ligand (e.g.TGFβ, Wnt, Hedgehog) or a key intracellular component such as NF-kB. The activation of cell surface

receptors results in the activation of *protein kinases*, which add phosphate groups to proteins leading to protein phosphorylation (Lodish *et al.*, 2000).

Membrane trafficking and signal transduction were once considered as unrelated disciplines of cell biology but recent advancements in research have suggested that these two are closely coupled and work in concert for a system regulation. Signalling pathways have found to be interacting and regulating membrane trafficking machinery (Seto *et al*., 2002). Endocytosis is thought to play a critical role in morphogen gradient formation. Hedgehog, Wingless and Notch signalling is found to be activated by endocytosis. Many studies have reported that membrane trafficking regulates signalling but signalling may also regulate the membrane trafficking machinery providing a mechanism through which signalling pathways modulate themselves and other pathways. One of the major signalling pathway investigated in this aspect is the Receptor Tyrosine Kinase signalling pathway (Seto *et al*., 2002).

1.4 Receptor Tyrosine Kinases

Receptor Tyrosine Kinases (RTK) is one of the major classes of cell surface receptors which regulate critical aspects of cell function such as cell proliferation, differentiation, survival and metabolism. The signalling molecules that activate RTKs are membrane bound and soluble proteins which are usually called growth factors. These RTK ligands include fibroblast growth factor (FGF), nerve growth factor (NGF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF) (Lodish *et al.*, 2000). RTKs can be split into 14 groups based on their structural patterns. RTKs possess an extracellular ligand binding domain, a single transmembrane α helix domain and a cytoplasmic domain which contains the kinase activity (Hancock., 2005). All RTKs are monomeric with the exception of insulin receptor (IR). Ligand binding induces dimerization of the receptors resulting in autophosphorylation of their cytoplasmic domains whereas the members of IR family of RTKs form disulphide linked

dimers of two polypeptide chains forming an $\alpha_2\beta_2$ heterodimer (Van Obberghen., 1994). Irrespective of the mechanism by which a ligand binds and locks an RTK into a functional dimeric state they all follow a universal phosphorylation step. In an inactive state, the kinase activity of an RTK is very low and the binding of ligands to their specific receptors make the latter into a dimeric state where the kinase in one subunit phosphorylates the tyrosine residues of the other subunit inducing a conformational change that facilitates the binding of secondary messengers such as ATP in some receptors (e.g. insulin receptor) (Lodish *et al.*, 2000). Autophosphorylation is common in RTKs and it may be intramolecular where the polypeptide chain phosphorylates itself or intermolecular where one polypeptide in a dimer phosphorylates the other. These RTKs catalyse the transfer of γ phosphate of ATP to hydroxyl groups of tyrosines on target proteins (Hunter., 1998). All RTKs induce signal transduction via the Ras (GTPase)/mitogen-activated protein kinase (MAPK) pathway (Ras/MAPK) . Activated RTKs cannot directly activate Ras and they need a link in the form of growth factor receptor bound protein 2 (GRB2) and a guanine nucleotide exchange factor (GEF) Son of Sevenless (SOS). SOS is a GEF which helps in the conversion of inactive GDP-bound Ras to active GTP-bound Ras. GRB2 is an adapter protein and it contains SH2 (Src homology 2) domains which bind to specific phophotyrosine residues of the RTK and also SH3 domains which bind and activate SOS (Grant & Sato 2006).

1.5 Fibroblast Growth Factors

The Fibroblast Growth Factors (FGFs) and their receptors (FGFRs) play a vital role in tightly regulating metazoan development, cell proliferation, survival, migration and differentiation during development and adult life. Deregulation of FGFR signalling has resulted in many human diseases especially different types of human cancer.

The human FGF family consists of 22 ligands which bind to four homologous high affinity FGFRs (FGFR1 – FGFR4). Each FGFR binds to and is activated by unique sets of FGFs. These genes are found in several multicellular organisms ranging from *C.elegans* to humans but not found in unicellular organisms. The only two FGFs found in *C.elegans* compared to 22 FGFs found in mice and humans shows the expansion of FGF gene families during the process of evolution. This diversity has also led to diverse functionalities within the system (Itoh & Ornitz 2004). The origin of FGF signalling field started in 1939 when the bovine brain extracts showed proliferation of fibroblast cells *in vitro* and characterization of this mitogenic activity was later found to be due to a 15kDa molecule named basic FGF (bFGF) based on its high isoelectric point and subsequently a second molecule possessing FGF activity was found from brain extracts and named aFGF (acidic FGF) based on its low isoelectric point. A highly sulphated glycosaminoglycan (Heparin) was later found to mediate this mitogenic activity by binding to FGFs (Mohammadi et al. 2005). Although FGFs size vary from 17-34 kDa they all share a conserved sequence of 120 amino acids with 16-65% sequence homology (Eswarakumar et al. 2005). A generic FGF protein contains a signal sequence, a core region containing the binding sites for HSPG and receptors (Figure 1.7).

1.6 Structure of Fibroblast Growth Factor Receptors

The FGFRs have a structure very similar to most RTKs and they consist of a single-pass transmembrane protein that consists of an extracellular part that binds FGF ligands, a transmembrane domain and an intracellular tyrosine kinase domain that transmits signal to the interior of the cell (Haugsten EM, Wiedlocha A, Olsnes S et al. 2010) (Figure 1.3). There are three Ig like domains (designated as D1, D2 and D3) within the extracellular ligand binding of FGFR. Between D1 and D2 is a stretch of acidic residues called the acid box and a conserved positively charged region in D2 that serves as the binding site for heparin (Eswarakumar et al.

2005) . Alternate splicing of the transcribed receptor results in a variety of different receptor isoform domains such as Ig-like I, Ig-like II, Ig-like III. The first Ig-like domain is thought to play a role in receptor autoinhibition whereas second and third Ig-like domains of the receptors are necessary for ligand binding (Haugsten *et al.*, 2010). The alternative forms that arise due to alternate splicing result in different ligand binding characteristics. For example, FGFR2b binds FGF7 and FGF10 whereas FGFR2c binds FGF2 and FGF18 (Eswarakumar et al. 2005).

Figure 1.7. Domain structure of generic FGF and FGFR proteins*: A) Main structural components of FGF. B).Main structural components of FGFR* (Böttcher & Niehrs 2005).

1.7 FGF/FGFR binding and signalling

The binding of FGF ligands to FGFRs have been widely studied. It was initially shown that each cell type expresses low and high affinity receptors for FGF. The two binding sites differed on the basis of ionic strength and buffer condition. Tyrosine phosphorylation of high affinity receptor was found to occur within 30 sec of FGF exposure. Shortly after, the first FGFR was purified from chicken embryo. The other FGFRs were soon discovered by homology based PCR (Mohammadi et al. 2005). The FGFs bind to distinct FGFRs and also to heparan sulphate

proteoglycans (HSPG) thereby forming a dimeric FGF-FGFR-HSPG ternary complex. Following FGF stimulation, signalling proteins bind to the tyrosine phosphorylation sites of the activated receptor via closely linked docking proteins.

The active FGFRs have been shown to phosphorylate multiple intracellular adaptor proteins such as factor receptor substrate 2 (FRS2), GRB2, SOS1, SHC1, SH2B1, GAB1 and PTPN11 (Ong et al. 2001; Hadari et al. 1998; Kanai et al. 1997). These complexes in turn activate different signal modules (transduction pathways that act downstream of activated FGFR) such as MAPK (RAS-RAF-MEK-MAPK) pathway, AKT (PI3K-PDK-1-PKB/AKT) pathway, and PLC (PLC-γ/PLC-ε-PIP2-IP3/DAG-PKC) pathway (Knights & Cook 2010) (Figure 1.8). The other pathways found to be associated with activated FGFR are STAT (Li et al. 1999), and NF-kB (Raju et al. 2014; Chang et al. 2014), however the mechanisms by which FGFR regulates these signal modules are still unclear (Klint & Claesson-Welsh 1999). In mammalian models, the adaptor protein FRS2 acts a hub linking several signalling pathways to the activated FGFRs. By binding to FRS2, GRB2 recruits the RAS Guanine nucleotide exchange factor SOS which in turn leads to the activation of RAS dependent signal modules (MAPK, PLC, PI3K/AKT). GRB2 also acts as link between FRS2 and GAB1 to activate PI3K/AKT in a RAS independent manner (Ong et al. 2000).

PLC pathway is regulated by FGFR by two different ways. 1. PLC-γ binds directly to FGFR (Mohammadi et al., 1991) or by the interaction of PLC-ε with RAS (Vázquez-Manrique et al. 2008), which results in hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) producing two second messengers diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (Ins(1,4,5)P3). PLC-γ activity is so crucial in regulating calcium channels present in the membranes of intracellular calcium stores (Knights & Cook 2010).

Figure 1.8. FGF/FGFR signalling cascade activation: *The complex formation of FGFs and heparin sulphate chains and FGFRs causes dimerization and trans-phosphorylation of many tyrosine residues thereby activating several downstream signalling cascades MAPK, PI3K/AKT kinase and PLC-γ/PKC. In addition to these pathways FGFR is thought to activate transcription factor STAT* (Knights & Cook 2010)

So far seven autophosphorylation sites (Y-463, Y-583, Y-585, Y-653, Y-654, Y-730 and Y766) have been mapped on FGFR1 and their roles in various FGFR1 mediated cellular responses have been studied but it is often not clear exactly which signal transduction pathways are involved. So far only PLC-γ has been shown to associate with the activated FGFR in cell mitogenesis. It has been demonstrated that autophosphorylation on Tyr766 in the carboxy terminal tail of FGFR1 creates a specific binding site for the SH2 domain of PLC-γ (Haugsten EM, Wiedlocha A, Olsnes S et al. 2010; Mohammadi et al. 1996). Finally, signal attenuation is achieved by ubiquitin ligase Cbl (Figure 1.9). Cbl binds to activated FRS2 and mediate FGFR ubiquitination that acts as a signal for receptor degradation. The signal from FGF-FGFR complex is then efficiently terminated by internalization and degradation in lysosomes by endocytosis. After endocytosis, the ubiquitinated receptors are sorted into endosomal sorting complex required for transport and further transported to lysosomes for degradation (Wesche *et al.*, 2011). A more detailed description of FGFR mediated signalling pathways are described in chapter 4.

Figure 1.9. FGF/FGFR signal attenuation: *FGFR activation results in trans-phosphorylation and activation of downstream signal transduction pathways. Active receptor signalling can be terminated through RTK dephosphorylation by protein tyrosine phosphatases. This is achieved by CLR-1 phosphatase in C.elegans. Receptor activation can also be terminated through endocytosis of active receptor complex into endosomes and then either targeted to lysosomes for degradation or recycled back to cell surface* (Knights & Cook 2010).

Thus the vesicle mediated membrane trafficking is characterised by cell signalling from the cell surface to the cytoplasm via infinite number of protein-protein interactions and second messengers and any mutations affecting RTK/Ras/MAPK signalling may cause many human syndromes and diseases including cancers (Seaman *et al.*, 1996).

1.8 Impaired FGF/FGFR signalling and associated diseases

FGF/FGFR mediated signalling has been associated with various developmental, neoplastic, metabolic and neurological diseases and also the different types of cancers. Despite advancement of technologies and disease diagnosis and treatments, lung cancer is one of the leading causes of cancer death worldwide. The dependency of exogenous growth factors is often lost or altered when there is an over production of endogenous growth factors or abnormal expression and mutation in receptor molecules leading to uncontrolled, autocrine growth stimulation. Several drugs have been designed against such complications [e.g. the antiangiogenic vascular endothelial growth factor (VEGF) antibody bevacizumab and the epidermal growth factor receptor (EGFR) small-molecule inhibitor erlotinib have both been approved for treatment of advanced non-small cell lung cancer (NSCLC)] and these drugs have been targeted against growth factors and their RTKs. However it is later found that cancer cells can still evade anticancer effects by activating alternative growth and survival pathways. Recent evidences have suggested that members of FGF family along with their four transmembrane tyrosine kinase receptors might act as autocrine as well as paracrine signalling in many solid tumour types. Fischer *et al.,* (2008) have shown that blocking FGFR1 signalling by dnFGFR1 (protein kinase truncated dominant negative FGFR1) and small molecules (SU5402) have an anti-proliferative effect on the investigated NSCLC cells. Also, mutations in the transmembrane helix of human FGFR3 causes dimerization and subsequent activation of tyrosine kinase domain affecting the extracellular part of two individual receptors to form disulphide bonds between them and this intermolecular bond forces dimerization in the absence of ligand resulting in a ligand- independent constitutive signalling. This defect is often found in bladder cancers. Mutations in kinase domain of FGFR (e.g.FGFR4) can change the conformation of the domain and cause permanent active kinases leading to diseases (e.g.

mutation at kinase domain of FGFR4 results in autophosphorylation and constitutive signalling which is often found in childhood sarcoma (Rhabdomyosarcoma) (Wesche *et al.,* 2011)

In prostate cancer, several FGFs (FGF1, FGF2, FGF6, FGF7, FGF8, FGF9) are up regulated which have a tumour promoting effect on the neighbouring epithelial cells which cause epithelial transformation and formation of differentiated prostate carcinomas (Memarzadeh *et al.,* 2007). Multiple myeloma is another example of disease caused by deregulated FGF/FGFR signalling. It is a plasma cell malignancy which is characterized by accumulation of clonal plasma cells in bone marrow and bones. Evidences suggest that there is an overexpression of FGFR3 as a consequence of chromosomal translocation t(4;14)(p16.3;q32) and it has been recognized as a potent oncogene (Wesche *et al.,* 2011)

1.9 Membrane Trafficking Deregulation and Diseases

In the last several years, different human diseases have been associated with different genes involved in intracellular sorting machinery regulating selective trafficking of secretory and endocytic pathways. Trafficking defects at each step of transport at specialized compartments such as ER, Golgi, Endosomes and Lysosomes have several disease implications. (Table 1.2) (Aridor & Hannan 2000)

The first transport step in the secretory pathway is at the ER. Properly assembled and matured proteins are exported from the ER to the Golgi. Proteins which fail to achieve functionality are identified by ER signalling receptors and degraded to prevent their accumulation.

In response to accumulation of non-matured and misfolded proteins, ER signalling pathways are activated to induce the expression of folding and transport machinery proteins to increase the transport/degradation load. In case where this up-regulation cannot eliminate the defective cargo, ER signalling responds by inducing cellular apoptosis thus eliminating the affected cell in order to preserve tissue functionality (Aridor & Hannan 2000).

The ER related diseases are classified into three categories:

1. Cargo retention and degradation- Mutated cargo that fails to achieve transport-competent conformation is eliminated by the degradation machinery. This loss of cargo function leads to diseases
2. Cargo accumulation and ER stress – Cargo is not efficiently degraded and accumulated in the ER
3. Defects in transport machinery from ER to Golgi

Similar to ER related diseases, there are diseases that affect other compartments like Golgi, TGN and endocytic systems mainly due to defects in machinery required for transport.

Defective endosomal RABs have also been associated with several diseases (Puertallano, 2004). RAB GTPases function as membrane organisers and mediate directionality and specificity of vesicle delivery. For example, RAB5 is found to mediate endocytosis and homotypic fusion between early endosomes, RAB4 and RAB11 in endocytic recycling, and RAB7 and RAB9 associated with trafficking through late endosomes (Aridor & Hannan 2000). Below is a table that shows different trafficking defects and their associated diseases.

Table 1.2. Different membrane trafficking defects and their associated diseases

Trafficking Defect	Disease Associated	Phenotype and Cellular Pathology
Cargo retention and degradation	Cystic Fibrosis	Mutations in this chloride conductance channel (CFTR) lead to the retention and degradation of the protein in the ER. The disease results from loss of chloride regulation in CFTR-expressing epithelia of secreting organs. Deletion of Phe at position 508 in CFTR accounts for more than 70% of all cases. Interestingly, this CFTRh F508 mutant retains partial activity and can be stabilized for transport and surface expression with chemical chaperones

	Diabetes Mellitus	Class 2 mutations in insulin receptor impair its transport from the ER, markedly reducing its surface expression leading to insulin resistance.
Cargo accumulation and ER stress	Congenital Hypothyroidism	Mutated thyroglobulin (Tg) accumulates in the ER leading to ER signalling and expansion of the compartment and this increase in biosynthetic activity of ER results in Goitre in some patients.
	Charcot–Marie–Tooth syndrome	Mutant protein accumulates in the endoplasmic reticulum. Signalling from the ER may contribute to abnormal growth and differentiation.
Transport machinery from ER to Golgi	Combined Factors V and VIII deficiency	This mannose-binding lectin cycles in the early secretory pathway and serves as a cargo receptor for incorporation of a specific cargo protein into COPII vesicles. Lack of ERGIC53, due to mis-sense mutations, leads to a secretion block of coagulation factors V and VIII and development of the bleeding disorder
Lysosomal Trafficking	Mucolipidosis type II	An allelic disease caused by deficiency in uridine diphospho (UDP)-N-acetylglucosamine: N-acetylglucosaminyl-1-phosphotransferase, an enzyme that mediates the addition of mannose 6 phosphate to lysosomal hydrolases within the Golgi. In patients with Mucolipidosis type II disease, the inability of addition of mannose 6 phosphate residues prevents the transport of lysosomal hydrolases to reach the lysosomes.
Diseases affecting antigen presentation	Bare lymphocyte syndrome	Lymphocytes from patients with bare lymphocyte syndrome show no surface expression of either MHC class I (defects in TAP1 and TAP2), or MHC class II (defects in CIITA and RFX-B), and therefore defective antigen presentation.

Defects in lipid transport	Niemann Pick C	NPC1 is a sterol sensing protein required for transport of cholesteryl ester (and potentially fluid phase constituents) from the late endosome/lysosome to other organelles. Defects result in an accumulation of lipid in lysosomes
Diseases affecting cytoskeleton and motor proteins	Usher's syndrome	Myosin 7 is thought to be required for transport from the Golgi to specific polarized regions of the cell. Lack of functional Myosin 7 leads to deafness and hearing loss in these patients
Defective endosomal RABs RAB27a	Griscelli Syndrome	A rare autosomal recessive disease caused by absence of RAB27a. Absence of RAB27a causes poor trafficking of melanosomes leading to accumulation of melanosomes in the central region of melanocytes resulting in defects in hair and skin pigmentation and partial albinism
Defective RAB7	Charcot-Marie-Tooth Type-2	Mutations that result in loss of RAB7 function result in severe sensory and motor neurons impairment, muscle weakness and foot ulcers
Defective RAB5 and RAB7	Thyroid autonomous adenomas (AA)	A type of benign thyroid tumour. Overexpression of RAB5 and RAB7 has been associated with (AA). An overexpression of RAB5 and RAB7 leads to an increased association with endosomes resulting in high production of thyroglobulin and thyroid hormone production
Defective RAB25	Prostate Cancer	Over expression of RAB25 is found in Prostate cancer cell lines
Defective RAB4	Cardiomyopathy	Upregulated levels of endosomal RAB4

1.10 Concept of signalling endosomes

Signal termination via degradation of activated receptor complexes was thought to be carried out by the process of endocytosis. Endocytosis was considered to be a mere 'sink' of signalling complexes. It has now become evident that the output of a signalling process depends not only on activation of a particular set of signalling molecules but also on where and how long the signal is emitted. New findings in this research have suggested that signalling machinery can be highly regulated by exploiting the functional and compartmentalisation specialisation of endocytic pathway beyond its cargo degradation. The most common view that signalling occurs only at the plasma membrane was challenged in early 90s when majority of EGFRs and their downstream signalling factors such as Shc, Grb2 and SOS were localised to the early endosomes rather than the plasma membrane shortly after ligand addition. Similar findings in nerve growth factors (NGF) bound to its activated receptor TrKA and PLC-γ1 in endocytic organelles led to the hypothesis of signalling endosomes. It was also found that signal transduction can continue even after endocytosis when EGFR was found to interact with Grb2 at endosomes (Miaczynska *et al.*, 2004).

Signalling endosomes might govern three important factors

1. Temporal regulation (varying duration of signalling result in differential biological outcomes)
2. Spatial Regulation – Non-specific cross-talk between different pathways prevented by restricting signalling cascades in space
3. Targeting signalling complexes to their site of action (Miaczynska et al. 2004)

Alternatively, recent studies suggest that at least in some RTKs signalling primarily occurs immediately following ligand binding prior to endocytosis (Auciello et al. 2013).

It is also found that many cytosolic proteins function in both membranes trafficking and signalling. E.g. β arrestin which binds activated GPCRs and terminates their interaction with

heteromeric G proteins also binds to clathrin, the clathrin adaptor protein AP-2 and membrane phosphoinositides thus functioning in the assembly of coated pits and coupling of GPCR activation to their endocytosis and then they finally β-arrestin binds Src and components of MAPK pathway and function as an activator of this MAPK pathway (Di Fiore & De Camilli., 2001). Recent investigations in many systems have implicated nearly every membrane trafficking event as a potential site for regulation by signalling pathways (Seto *et al.,* 2002). Receptor tyrosine kinase activity seems to regulate receptor internalization through phosphorylation of downstream target proteins. E.g. Eps15, which is recruited to clathrin-coated pits in response to EGFR activation and EGFR-mediated phosphorylation of Eps15 has been shown to be specifically required for ligand-induced internalization of EGFR (Confalonieri *et al.,* 2000). In addition, many downstream targets of RTK signalling directly regulate membrane trafficking. For example, Src (tyrosine kinase) phosphorylates clathrin heavy chain, stimulating clathrin coated pit formation and its SH3 domain binds and activates dynamin. Corresponding to these findings, overexpression of Src stimulates EGFR endocytosis. RTK signalling is also found to play a dual role (activation and deactivation) of RAB5 thus having both positive and negative effects on its endocytosis (Seto *et al.,* 2002). In another study, the role of all human kinases in regulating the clathrin-dependent and caveolae-dependent endocytic pathways was described by Pelkmans et al., (2005) using an RNAi-based screen. They used viruses that infect cells via endocytic uptake in this assay. Simian virus 40 (SV40) uses caveolae/raft-mediated endocytosis for host cell infection and vesicular stomatitis virus (VSV) enters cells via the clathrin-mediated pathway. By systematically knocking down each human kinase and monitoring the changes in viral infection rates in HeLa cells provided new evidences into endocytic regulation. The rate of viral infection corresponded to the level of endocytic regulation. Lower rates of viral infection suggested a reduced uptake of trafficking of viral particles whereas an increased infection rate suggested enhanced uptake of trafficking

of virus (Pelkmans et al. 2005). Thus it elucidated the significance of understanding membrane trafficking pathways via the compartmentalisation of signalling pathways (Farhan and RABouille, 2011).

Genome-wide screens in different organisms have helped scientists to identify novel regulators of membrane trafficking and cell signalling especially at FGF/FGFR signalling pathway. Even though membrane trafficking associated with cell signalling has gained immense significance in the recent years, the network of FGF signalling in mammals where FGFs interact with several FGFRs is more complicated. Hence scientists have identified other model organisms where they could analyse a similar process. The number of FGFs in invertebrates such as *Drosophila melanogaster* is only three and in *Caenorhabditis elegans*, only two (Birnbaum *et al.*, 2005).

1.11 *Caenorhabditis elegans* as a model organism

The comprehension of FGF-stimulated signalling pathways has been greatly simplified by the aid of model organisms like *Caenorhabditis elegans* and *Drosophila melanogaster*. The complexity of understanding the biology of higher organisms was a limiting step in pacing up research breakthroughs until Dr. Brenner made significant contributions using *C.elegans* as a model organism for the investigation of development biology for which he also won a Nobel Prize in 2002. *C.elegans*, a free-living soil nematode became one of the model organisms since it shares many of the essential biological characteristics with human biology. They have a conserved mode of development, neuronal function, signalling, apoptosis and aging. They are usually 1mm long and in the lab generally grown on small petri plates seeded with bacteria.

Figure 1.10: Overview of C.elegans life cycle (Altun & Hall, 2002) www.wormatlas.org

They have a simple anatomy with less than 1000 somatic cells of its transparent body all visible through a microscope. They have a quick development (egg to adult in less than three days) and an average life span of 2-3 weeks (Figure 1.10). In addition to its simple anatomy (Figure 1.11), they also serve as a simple yet powerful genetic model comprising of 5 autosomes and 1 sex chromosome. The completion of its whole genome sequencing made this organism a very suitable choice for the study of various genes homologous to humans (J. Lee et al. 2004).

Figure 1.11: Overview of C.elegans anatomy (Altun & Hall, 2002) www.wormatlas.org

1.12 RNA – Mediated Interference:

RNA-mediated interference (RNAi) is one of the most straightforward methods to inhibit the function of specific genes. This was first observed in *C.elegans* and is now used as a favourite method of knocking down gene function in various other species. The ability of double stranded RNA to block the expression of its corresponding single stranded mRNA is the principle of RNAi. The RNA endonuclease that catalyses this reaction is known as Dicer and is found in all metazoans except few simpler eukaryotes such as yeast (Lodish *et al.*, 2000). There are four ways to perform RNAi in *C.elegans.*

1. Microinjecting dsRNA into animals and observe the effects in their progeny
2. Feeding worms with bacteria expressing dsRNA of interest
3. Soaking the worms in dsRNA solution
4. shRNA (Paddison et al. 2002)

A remarkable feature of dsRNA triggered genetic interference processes in *C.elegans* is the ability of interference to spread to cells that are some distance from the initial site of dsRNA

delivery. This spreading effect was first observed when dsRNA was microinjected into the body cavity of injected animal and later it was also found in animals which were fed with food containing dsRNA or after soaking animals in dsRNA (Timmons et al., 2001). From biological and technical perspectives, the mechanism by which an organism responds to nucleic acid encountered in food has gained more interest. The paramount component of the *C.elegans* diet in the laboratory is bacteria; therefore RNAi in *C.elegans* has involved the introduction of engineered bacteria to produce dsRNA (Timmons & Fire, 1998). With the completion of *C.elegans* genome project, it became possible to use RNAi at the genome-wide level. Several labs have constructed a library of RNAi bacteria (Ex. Ahringer lab holds a library of roughly 86% of the total open reading frames (Kamath and Ahringer., 2003). According to a published data, the efficiency of RNAi is about 50% and a recent analysis of RNAi efficiency in *rrf-3* mutant background (*rrf-3* is a mutation that causes sensitivity to RNAi) showed more phenotypes for genes that did not show any phenotype in wild-type backgrounds (Simmer et al. 2002).

1.13 YP170-GFP Assay:

The most widely used assay to investigate membrane trafficking in *C.elegans* is the YP170-GFP assay and the application of RNAi together with this assay has gained valuable insights in understanding the membrane transport-signalling process *in vivo*. Yolk uptake by growing oocytes is an excellent example of the receptor mediated endocytosis. Yolk is a lipoprotein comprising of lipids and lipid binding proteins called vitellogenins. These vitellogenins are most abundantly found in developing embryos as they provide nutrients to support the rapid development of the embryo. *C.elegans* yolk is secreted from the intestine into the pseudocoelomic space (body cavity) and is taken up into vesicles within the growing oocytes by receptor mediated endocytosis following binding to the yolk receptor. Yolk transport uses

a similar route in vertebrates, from liver to bloodstream to ovum (Shen *et al.,* 1993). There are six vitellogenins in *C.elegans* genome. *vit-1, vit-2, vit-3, vit-4,* and *vit-5* all contribute to the pool of major yolk protein YP170 whereas *vit-6* encode a protein which is processed into YP88 and YP115 (DePina et al. 2011). YP170, YP115 and YP88 are homologous with vertebrate vitellogenin ApoB-100 found in mammals (Spieth *et al.*,1991). Endocytosis of yolk particles into membrane bound vesicles of the oocytes is mediated by LDL receptor RME-2 in *C.elegans*. The vitellogenin YP170 was tagged with Green Fluorescent Protein (GFP) to visualize the transport of yolk in oocytes of *C.elegans* (Grant & Hirsh 1999). The YP170-GFP fusion protein like endogenous YP170 is synthesised in the intestine and secreted into the body cavity from where it is endocytosed into oocytes by the receptor mediated endocytosis. Yolk receptor RME-2 is expressed in oocytes and endocytos yolk in an adaptor complex AP-2 and clathrin-dependent manner. The fluorescent YP170-GFP fusion is transported like endogenous yolk from intestine to oocyte, allowing *in vivo* analysis of secretion and endocytosis by fluorescence microscopy (Grant & Hirsh., 1999). The successful development of YP170-GFP assay further led Hirsh research group to identify novel membrane transport regulators and identify their homologues in vertebrates (B Grant & Hirsh, 1999).

It is found that in Wild-type (WT) worms, YP170-GFP was visible in the intestine where it is expressed, in the oocytes that take it up and in the developing embryos that are formed by fertilization of the oocytes. Blocking endocytosis by RNAi results in abnormal accumulation of YP170-GFP in the body cavity, whereas a block in secretion leads to increased accumulation of YP170-GFP in the intestine (Figure 1.12).

Figure 1.12. YP170-GFP Assay. Wild Type: *A high accumulation of YP170-GFP in oocytes and embryos and low accumulation in body cavity and intestine. Endocytosis defect: High accumulation of YP170-GFP in the body cavity and low accumulation in oocytes and embryos. Secretory defect: High accumulation of YP170-GFP in the intestine*.

This assay has helped to identify numerous genes involved in receptor mediated endocytosis (RME) and secretion (Balklava et al. 2007). From a genome-wide analysis using an RNAi-based approach on 16,300 predicted genes, 657 showed defects in YP170-GFP localization signifying their roles in endocytosis, secretion or germ line development (Balklava *et al.*, 2007). The pathways used in *C.elegans* are strikingly similar to those used in mammalian cells (Fares & Barth Grant, 2002) and this functional analysis in the worm should provide insights into human gene function (Kamath *et al.*, 2003).

1.14 FGF/FGFR Signalling pathway in *C.elegans*

The nematode worm *C.elegans* is a model system with reduced cellular and anatomical complexity compared with mammals. The complexity of FGF/FGFR network is also much reduced in this animal. The two FGFs in *C.elegans* are EGL-17 and LET-756 and only one FGFR, *EGL-15* (Popovici *et al.*, 2008). The *EGL-15* is structurally very similar to mammalian

FGFR and have the highest level of sequence conservation found within the intracellular tyrosine kinase domain and the three extracellular immunoglobin (Ig) domains (Figure 1.13) (Lo *et al.*, 2010). By sequence homology, 4 out 7 autophosphorylation sites (Y463, Y653, Y654 and Y730) were found to be well conserved between human FGFR and *EGL-15* (Borland et al. 2001). The *egl-15* gene located on chromosome X encodes two receptor isoforms *EGL-15*(5A) and *EGL-15*(5B) (Birnbaum *et al.,* 2005) and they result from the alternate splicing of exon 5 encoding a specific insert between IgL1 and IgL2 and have also reported that this insert could be the site of binding for EGL-17 and LET-756 and thus *EGL-15*(5A) acts as a receptor for EGL-17 and *EGL-15*(5B) acts as the receptor for LET-756 (Goodman *et al.*, 2003). Like its mammalian orthologues, *EGL-15* is involved in a variety of functions such as cell migration guidance, muscle protein degradation, terminal axon morphology control and fluid homeostasis. *EGL-15* is negatively regulated by a receptor tyrosine phosphatase, CLR-1 resulting in the build-up of clear fluid in the pseudocoelom (Clear phenotype) (Lo *et al.,* 2010). It is also discovered that a partial loss- of-function of LET-756/FGF could interfere with *EGL-15* activation and not allow proper sex myoblast positioning or progeny formation and also make the worms grow slowly during larval stages. Thus the functionality of these ligands may constitute alone or in cooperation with other diffusible ligands and this coordination may well have some effects on downstream signalling effectors (Popovici *et al.*, 2008).

Figure 1.13: Schematic representation of FGF ligand and receptor system in C.elegans.

(Birnbaum et al., 2005)

The intracellular signal transduction cascades activated by *EGL-15* are relatively well characterized and share a high degree of conservation with mammalian FGFRs (Polanska et al., 2009), however, it has been recently found that fibroblast growth factor receptor signalling can act independently of *rog-1* (FRS2 like gene in *C.elegans*). The Ras gene in *C.elegans* is called *let-60* which acts downstream of at least two RTKs such as LET-23 (related to EGFR) and *EGL-15* (related to FGFR) (Han & Sternberg., 1990). The Src homology 2 (SH2)/SH3 adaptor protein SEM-5 function downstream of *EGL-15* (Polanska *et al.*, 2009). The LET-60 Ras is known to stimulate the MAPK signalling cascade consisting of kinases MEK-2, LIN-45 and MPK-1 (Sundaram, 2006). In addition to MAPK pathway, *EGL-15* also regulates PLC-γ and PI3K/AKT pathways. Similar to mammalian FGFRs, the extracellular domain of *EGL-15* consists of three Ig (Immunoglobulin) repeats. Specificity and ligand binding is mediated by region between second and third Ig repeats and this region shows the highest degree of extracellular homology between human FGFRs and *EGL-15*. Out of the seven autophosphorylation sites identified in human FGFRs, *EGL-15* shares four namely Y653, Y654, Y463 and Y730. (Borland et al. 2001).

1.15 FGFR signalling system – a candidate to study membrane trafficking in *C.elegans*:

With the growing number of diseases associated with impaired vesicular trafficking and cell signalling, it is becoming increasingly critical to analyse the mechanisms that couple these two processes together. There is mounting evidence that signalling can be regulated by endocytosis, and signalling molecules also play a role in vesicular transport (Miaczynska *et al.*, 2004).

A genome-wide RNA mediated interference screen by Balklava et al., 2007 in *C.elegans* found several regulators of membrane traffic. Out of 16300 genes tested, 657 showed defects in endocytosis, secretion and germ line development including many known genes particularly associated with endocytosis and secretion. Genes that have direct link to functions such cell cycle, transcription, translation or other metabolism and indirectly associated with membrane trafficking were excluded. The final candidate transport regulators were found to be 268 of which 80% have human homologues. Among the positives from the screen, some of the FGF-FGFR signalling pathway components including *egl-15*, *sem-5*, *sos-1* and *let-60* also appeared to participate in membrane trafficking thus making the whole signalling pathway an excellent candidate to study membrane trafficking.

Through the application of reverse genetics by analysing Yolk-GFP trafficking in FGF-FGFR knocked down/ knocked out worms, it is possible to identify the combined machinery of membrane trafficking and cell signalling. Therefore, in this study the whole of FGF-FGFR signalling pathway was investigated to see how they regulate membrane traffic and also to find which transport steps are affected in case of a trafficking defect.

CHAPTER 2 MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Methods used in *C.elegans* work

All *C.elegans* strains were derived originally from the wild-type Bristol strain N2. Worm cultures, *C.elegans* husbandry and genetic crosses were performed according to standard protocols described by Brenner (1974). A complete list of strains used in this study can be found in tables 2.2 and 2.3. Temperature sensitive strains were grown at 15^{o}C and other strains were generally grown at 20^{o}C.

Table 2.1: List of all solutions and buffers used for *C.elegans* growth and maintenance

Solution/Buffer	Composition	Quantity
NGM Agar, 500ml	NaCl	1.5g
	Agar	8.5g
	Bacto peptone	1.25g
	H_2O	487.5ml
	Autoclave the above	
	1M $CaCl_2$	0.5ml
	1M $MgSO_4$	0.5ml
	1M KPO4, pH 6.0	12.5ml
	Cholesterol 5mg/ml in Ethanol	0.5ml
	Nystatin 10mg/ml in DMSO	0.5ml
NGM Agar Lite, 500ml	NaCl	0.75g
	Agar	10g
	Bacto Tryptone	2g
	KH_2PO4	1.5g
	K_2HPO4	0.25g
	Autoclave the above	
	1M $CaCl_2$	0.5ml
	1M $MgSO_4$	0.5ml
	Cholesterol 5mg/ml in EtOH	0.5ml
	1M IPTG	1ml

	Carbenicillin 50 mg/ml Nystatin 10mg/ml in DMSO	0.5ml 0.5ml
M9 Buffer, 500ml	KH_2PO_4 Na_2HPO_4 NaCl dH_2O Autoclave the above 1M $MgSO_4$	1.5g 3g 2.5g 500ml 0.5ml
1M KPO_4 (pH 6.0), 500ml	KH_2PO_4 K_2HPO_4 dH_2O Autoclave	17.8g 54.15g 500ml
1M $CaCl_2$, 500ml	$CaCl_2$ H_2O Autoclave	27.75g 500ml
1M $MgSO_4$, 500ml	$MgSO_4$ H_2O Autoclave	60.2g 500ml
Worm Freezing solution, 150ml	NaCl KH_2PO_4 Glycerol 1M NaOH Autoclave the above 1M $MgSO_4$	2.9g 3.4g 147ml 2.8ml 0.15ml
Worm Bleach Solution	Sodium hypochlorite 10N NaOH dH_2O	0.25ml 0.03ml 0.23ml

2.2 Nematode Growth Media

NGM Agar was poured into 6cm Petridishes and allowed to set. The plates were then seeded with 200 µl of overnight (O/N) culture of a uracil auxotroph strain of *E.coli* (OP50) as a source

of food for the worms. The use of uracil auxotroph strain of *E.coli* prevents the overgrowth of the bacterial lawn (Stiernagle 2006) facilitating a thin layer that helps to visualize worms distinctly. The NGM Lite medium was poured into 3cm Petri dishes.

2.3 Worm Freezing:

Worms of the desired strain were grown on two 10cm plates seeded with 1 ml of OP50. The worms were ready to be frozen when the plate had little or no food left, plenty of L1s and L2s, few eggs and no contamination. Worms were washed off the plate with 2ml of M9 buffer and transferred to a 2 ml eppendorf tube and centrifuged for 1 minute at 2000 rpm. The supernatant was removed and the pellet was washed with 2 ml of M9 buffer. 1 ml of M9 buffer was removed and an equal amount of freezing solution was added. The mix was then aliquoted into 4 cryovials labelled with the strain name and date of freezing and stored in a -80^{o}C freezer. After 24 h, one cryovial was test thawed to see if the worms recover and can be cultured.

2.4 Worm thawing:

Cryovials from the freezer were allowed to thaw at room temperature. Once all the ice had melted, they were immediately transferred to 6cm plates seeded with OP50. After 24h, live worms were transferred to fresh OP50 seeded plates.

2.5 Worm Bleaching:

To decontaminate worm strains a 10µl of freshly prepared bleach mix was spotted near the side of a 6cm plate. 20 gravid adult worms were picked and transferred into the bleach mix. The bleach solution kills all the contaminants and dissolves the worms whereas eggs are protected

by eggshell. Plates were then incubated at 20ºC for 24 hours and the newly hatched L1s were carefully transferred to fresh non-contaminated plates.

2.6 Generation of males:

Males for genetic crosses were generated by either heat shock or using *him-17* RNAi. 15-20 L4 hermaphrodites were transferred to NGM plates seeded with OP50 and incubated for 6 h at 30ºC. After 6h, the plates were moved to 25ºC and few males were obtained in their F1 progeny. Temperature sensitive strains were grown on 10 cm NGM lite plates containing *him-17* dsRNA bacteria and males were obtained in subsequent generations.

2.7 Freezing RNAi bacteria:

A single colony of RNAi clone was grown in 3 ml LB media containing 100µg/ml ampicillin and 12.5µg/ml tetracycline and incubated for 12-16 hours at 37ºC while shaking. A 920µl of this culture was transferred to a polypropylene eppendorf tube and 80µl of sterile glycerol solution was added. The culture was mixed and stored at -80ºC.

2.8 RNA-mediated interference:

RNAi by feeding is an easy, convenient yet an effective tool in determining the loss-of-function phenotype of a specific gene (Kamath 2003). A corresponding dsRNA for each of the gene was expressed in *E.coli* by inserting a segment of coding region into a plasmid vector L4440 (pPD129.36), a cloning vector which contains two convergent T7 polymerase promoters in opposite orientation. (Timmons *et al.*, 2001, Timmons & Fire 1998).

The bacterial clones were stored at -80ºC and were streaked on Luria Broth (LB)-agar plates with 100µg/ml ampicillin and 12.5µg/ml tetracycline before experiments. A 3ml LB culture

with 100µg/ml ampicillin was inoculated with a single colony of appropriate RNAi clone and grown in a shaker incubator at 37°C overnight. 100µl of the grown cultures were then seeded on 3cm NGM Lite agar medium plates and induced overnight at room temperature. Next day worms were transferred to the induced plates and incubated for indicated times. In all of the experiments, a negative control (L4440 empty vector) and a positive control (L4440-*rme-1*) were used. The *rme-1* mutants have a defective yolk uptake as they cannot efficiently recycle yolk receptor RME-2 thus producing a RME phenotype (Grant & Sato 2006).

2.9 YP170-GFP Assay:

The YP170-GFP (Yolk-GFP) assay was used to visualize yolk trafficking in *C.elegans in vivo*. Transgenic *C.elegans* strains expressing a YP170-GFP fusion protein are used to visualize the basolateral secretion of yolk by the intestine and receptor mediated endocytosis of yolk in the oocytes in adult hermaphrodites (Grant & Hirsh 1999). A typical wild type worm displays high accumulation of YP170-GFP in its oocytes and also in the developing embryos that are formed by the fertilization of oocytes and low accumulation of YP170-GFP in the intestine and body cavity. From the previous studies, it has been shown that impaired endocytosis results in increased accumulation of YP170-GFP in pseudocoelom whereas an impaired secretion leads to increased YP170-GFP accumulation in the intestine (Grant & Hirsh 1999) (Balklava *et al.*, 2007) (Figure 2.1). Hermaphrodite worms were subjected to three different exposure periods to RNAi such as short (egg to P0 adult), medium (L4 to F1 adult) and long (egg to F1 adult). P0 are the parent worms and F1 is the first generation of P0. In the short RNAi exposure experiments, the adult worms were placed onto induced dsRNA expressing bacterial lawns and allowed to lay eggs for 6h and then the adult worms were removed. After 72h, the synchronised P0 adult worms were scored for localization of YP170-GFP. In the medium RNAi exposure experiments, L4 P0 worms were placed onto induced dsRNA expressing bacterial lawns and

allowed to reach adulthood and lay eggs for 24h. The laid eggs were transferred to a new set of plates containing the same RNAi bacterial clone and allowed to grow and F1 adults were scored for YP170-GFP localisation after 72h. In the long RNAi exposure experiments, worms were grown from the egg stage to P0 adults (72h) and the eggs of these adult worms were transferred to fresh RNAi containing plates and again grown for 72h and those F1 adult hermaphrodites were scored for YP170-GFP localization. Clones that showed Secretory defect (SEC) or Receptor Mediated Endocytosis defect (RME) defects were identified and assigned as positives and clones which didn't show any abnormal YP170-GFP localisation were assigned negatives. As a general rule, a gene was found to be positive for a given phenotype if it is observed in at least 2 of every 3 (67%) worms at least in two different independent experiments (Kamath et al. 2001). Experiments were repeated thrice.

Figure 2.1: YP170-GFP assay and RNAi *a).Schematic representation of YP170-GFP trafficking b).Growth dsRNA expressing E.coli and set-up of RNAi experiment c). Phenotypes observed after RNAi. WT, ENDO and SEC representing no defects, block in endocytosis and block in secretion, respectively. Arrows point to the intestine, filled arrow heads indicate oocytes and embryos and empty arrow heads indicate accumulation of GFP signal in the body cavity indicating reduced yolk endocytosis (Balklava et al., 2007).*

2.10 RME-2-GFP Assay:

The defects in endocytosis and secretion as observed in YP170-GFP assay was further examined by the RME-2-GFP assay. The expression and localisation of the yolk receptor RME-2 can be visualised by using RME-2-GFP reporter. The RME-2-GFP transgenic strain used in RNAi experiments was the RT408, homozygous for an [*pwls116 (rme2::GFP*] transgene insertion. Endocytosis defects in oocytes results in either a steady state accumulation of RME-2-GFP at the plasma membrane and cortex of oocytes or a delayed degradation of RME-2-GFP in embryos. Secretory defects result in accumulation of RME-2-GFP in the endoplasmic reticulum or mini stacks of Golgi, or other secretory vesicles dispersed throughout the cytoplasm of the oocytes (Balklava et al., 2007). The corresponding bacterial clones for each gene of interest were fed to the RME-2-GFP animals as described previously. In all of the experiments, a negative control (L4440 empty vector) and a positive control (L4440-p115) were used. p115 is a tethering factor which facilitates Golgi biogenesis and membrane traffic in cells and it has been showed that depletion of p115 by RNAi in *C.elegans* results in retention of yolk receptor RME-2 in the ER and Golgi within oocytes (Grabski et al. 2012). Clones that showed SEC or RME defects were identified and assigned as positives and clones which didn't show any significant phenotype were assigned negatives. Experiments were repeated thrice.

2.11 Analysis of mutant worm strains for yolk trafficking defects:

The role of FGFR downstream signalling pathway components in the regulation of membrane transport was analysed by crossing a YP170-GFP transgene into mutant worms to analyse whether they phenotypically copy the results observed in RNAi experiments. The complete list mutant strains analysed is given below in table 2.2.

Table 2.2: List of all mutant and resulting transgenic strains used for analysis of YP170-GFP trafficking

Mutant Gene(Allele)	Transgene (strain RT130)	Strain Analysed
egl-15*(n1477)*	*pwIs23 (vit-2::GFP)*	*egl-15(n1477); pwIs23*
egl-17*(e1313)*	*pwIs23 (vit-2::GFP)*	*egl-17(e1313); pwIs23*
let-756 *(s2613) unc-32(e189)*	*pwIs23 (vit-2::GFP)*	*let-756 (s2613) unc-32(e189); pwIs23*
sem-5*(cs15)*	*pwIs23 (vit-2::GFP)*	*sem-5(cs15); pwIs23*
sos-1 *(cs41)*	*pwIs23 (vit-2::GFP)*	*sos-1(cs41); pwIs23*
let-60*(n1700)*	*pwIs23 (vit-2::GFP)*	*let-60(n1700); pwIs23*
lin-45*(n2018) dpy-20(e1282)*	*pwIs23 (vit-2::GFP)*	*lin-45(n2018); pwIs23*
unc-79(e1068) ***mpk-1****(n2521); pwIs23*	*pwIs23 (vit-2::GFP)*	*unc-79(e1068) mpk-1(n2521); pwIs23*
akt-1*(mg144)*	*pwIs23 (vit-2::GFP)*	*akt-1(mg144); pwIs23*
akt-2*(ok393)*	*pwIs23 (vit-2::GFP)*	*akt-2(ok393); pwIs23*
pdk-1*(sa709)*	*pwIs23 (vit-2::GFP)*	*pdk-1(sa709); pwIs23*
age-1 *(hx546)*	*pwIs23 (vit-2::GFP)*	*age-1 (hx546); pwIs23*
pkc-2*(ok328)*	*pwIs23 (vit-2::GFP)*	*pkc-2(ok328); pwIs23*
itr-1*(sa73)*	*pwIs23 (vit-2::GFP)*	*itr-1(sa73); pwIs23*

2.11.1 General procedure for setting up crosses:

A highly concentrated *E.coli* OP50 culture was obtained by centrifuging 1 ml of OP50 O/N culture for 30 sec at 12000 rpm. The supernatant was discarded and the concentrated bacterial pellet about 10µl was seeded on NGM agar medium plates and allowed to dry. The bacterial lawn is kept small to attract worms, and thus increase the chances of mating. Every mating plate was age matched and maintained in a male:hermaphrodite ratio of 5:2, respectively . For example, the reporter strain males carrying YP170-GFP transgene were crossed into a mutant

strain and a successful crossing was verified initially with a high incidence of males. The L4 F1 progeny were transferred into a new plate and allowed to grow for 24 hours. After 24 h, 6-8 gravid F1 adult hermaphrodites showing GFP signal were transferred to a fresh plate and allowed to lay eggs. 12 F2 adults displaying the mutant phenotype and GFP signal were transferred to individual seeded plates and scored for GFP signal in F3 adults. Plates where all F3 adults had GFP signal were identified and kept for further analysis by microscopy, (Figure 2.2)

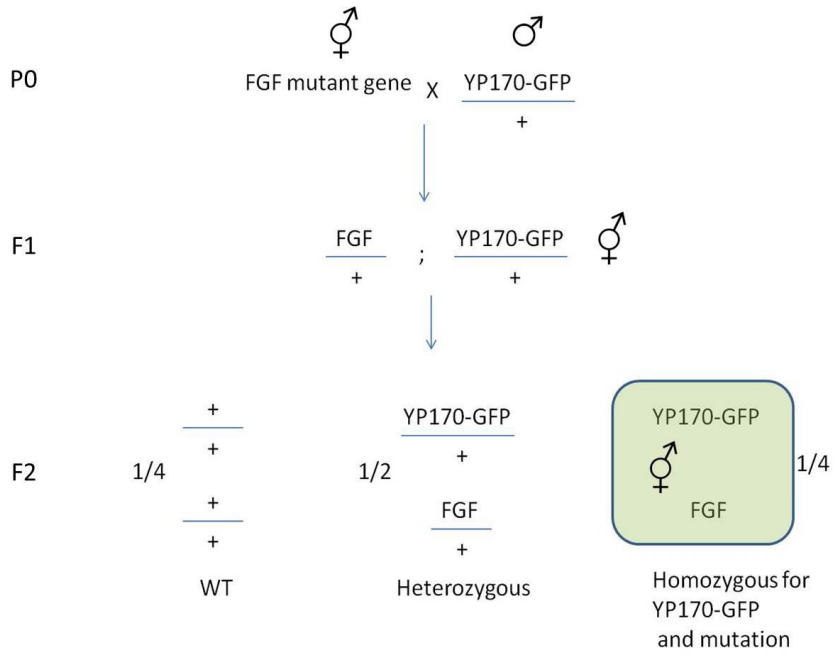


Figure 2.2: Overview of crossing YP170-GFP transgene into mutant worm strains.

Most *C.elegans* mutants with anatomic or behavioural defects are viable and can thus reproduce as homozygous hermaphrodites by self-fertilization and therefore can be maintained as stable stocks. However, some mutations that eliminate the activity of essential genes lead to a sterile or lethal phenotype. In such cases a balancer is used to maintain such detrimental mutations as heterozygotes by using a wild type allele of the affected gene. A balancer is a tightly linked marker in *trans* to the mutation (I.A.Hope, 2005). In some scenarios, especially

when studying a desired gene of interest it becomes difficult to identify a mutation if they don't have an obvious phenotype. For example, an *egl-15* mutation produces an egg laying defective (egl) phenotype and therefore it is easy to pick worms that carry *egl-15* mutation as they have difficulty in laying eggs and these eggs hatch within the worm. This is often called as "bag of worms" (Tyagi, 2009). In some cases, the mutations do not produce a distinct phenotype themselves and therefore it becomes difficult to differentiate between wild type and mutant worms. In such cases, a balancer strain which has a marker gene tightly linked to the gene of interest can be used in the crossing scheme. Below is an example that shows the crossing of GFP-CHC-1 transgene into *pkc-2* mutant worms via a *dpy-6* balancer (Figure 2.3).

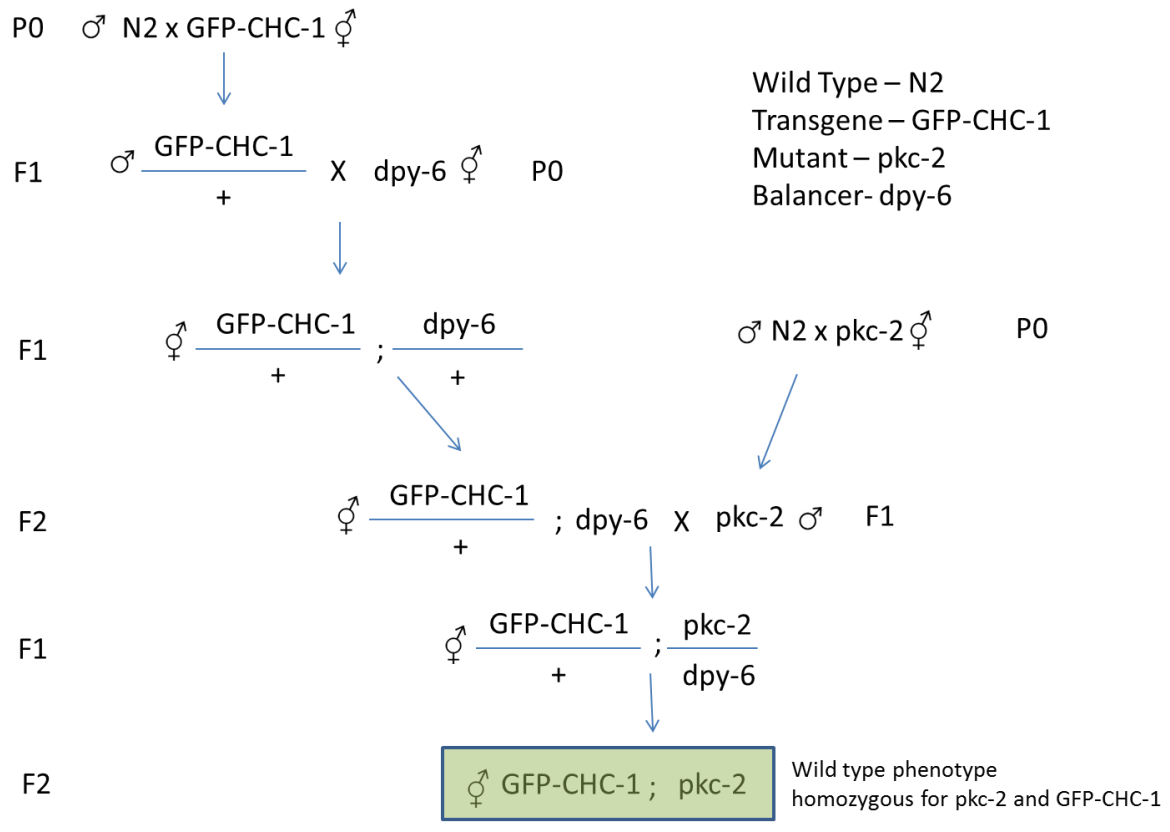


Figure 2.3: Overview of crossing GFP-CHC-1 transgene into *pkc-2* mutant worm via balancer *dpy-6*

2.12 Microscopy:

For imaging of live worms, glass slides with agarose pads were prepared. 1g of agarose was dissolved in M9 buffer by boiling. . Two layers of lab tape length wise was put on 2 glass slides. The two taped slides were put on a flat surface and a clean slide was placed between them. A drop of melted agarose was put in the centre of a clean slide and quickly covered with another clean slide placed on top of the three slides in a perpendicular fashion; Top glass slide was gently pressed until the agarose drop was flattened to a circle about 0.5 mm thick (the thickness of the tape spacers). When agarose had solidified, the two blank slides were separated by sliding one relative to the other with the agarose pad usually sticking to one of the slides. 2-4µl of 0.1M tetramisole was placed into the centre of the agarose pad and live worms were transferred into the tetramisole drop and overlaid by a coverslip. Live imaging was done using a Leica AF6000 fluorescence microscope and Leica SP5 TCSII DMI6000 confocal microscope using LAS (Leica Application Suite) AF software.

2.13 Analysis and Quantification of Membrane Markers:

Membrane markers labelling specific membrane compartments such as the endosomes, lysosomes, plasma membrane, endoplasmic reticulum (ER), Golgi, and intestine were analysed to identify the role of *egl-15* in membrane trafficking. The list of transgenic lines with membrane markers used in this study is given in table 2.3.

Table 2.3: List of all transgenic strains used to study localization of membrane markers

Membrane Marker /Transgene	Mutant Gene/Allele	Strain Analysed
*dkIs8(vha-6::**chc-1**::GFP)*	*egl-15(n1477)*	*egl-15(n1477); dkIs8*
*pwIs481[vha-6::**mans**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs481*
*pwIs446[vha-6::**ph**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs446*
*pwIs506[vha-6::**sp12**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs506*
*pwIs601[vha-6::**arf-6**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs601*
*pwIs87[vha-6::**rme-1**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs87*
*pwIs717[vha-::GFPC65T**HTFR**short]*	*egl-15(n1477)*	*egl-15(n1477); pwIs717*
*pwIs112[vha-6::**hTAC**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs112*
*pwIs72[vha6::GFP::**rab-5**]*	*egl-15(n1477)*	*egl-15(n1477); pwIs72*
*pwIs170[vha-6::**rab-7**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs170*
*pwIs206[vha6-p::**rab-10**::GFP].*	*egl-15(n1477)*	*egl-15(n1477); pwIs206*
*pwIs69[vha-6::**rab-11**::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs69*
*pwIs355[vha-6::**rab-35**minigene::GFP]*	*egl-15(n1477)*	*egl-15(n1477); pwIs355*
*dkIs8(vha-6::**chc-1**::GFP)*	*sem-5(cs15)*	*sem-5(cs15);dkls8*
*pwIs72[vha6::**rab-5**::GFP]*	*sem-5(cs15)*	*sem-5(cs15);pwls72*
*pwIs170[vha-6::**rab-7**::GFP]*	*sem-5(cs15)*	*sem-5(cs15);pwls170*
*dkIs8(vha-6::**chc-1**::GFP)*	*let-60(n1700)*	*let-60(n1700);dkls8*
*pwIs72[vha6::**rab-5**::GFP]*	*let-60(n1700)*	*let-60(n1700);pwls72*
*pwIs170[vha-6::**rab-7**::GFP]*	*let-60(n1700)*	*let-60(n1700);pwls170*

Intracellular localization of GFP-CHC-1, MANS-GFP, GFP-hTFR, GFP-hTAC was quantified by counting the number of puncta within a unit area of 400μm^2 in the intestine. GFP-ARF-6, RME-1-GFP, GFP-RAB-5, GFP-RAB-7, GFP-RAB-10, GFP-RAB-11, and GFP-RAB-35 were quantified by determining the number of labelled punctate and tubular structures within a unit area of 400μm^2 in the intestine. Five different areas were chosen in each animal at adult stage and 8 animals were scored for each strain. For PH-GFP, the size of the labelled vacuoles was measured in each animal at adult stage within a unit area of 500μm^2. For GFP-

SP12, the average total fluorescence intensity per unit area of 1μm^2 was quantified in 10 different areas in each animal at adult stage and 6 animals were scored. All of the above quantification was done using ImageJ software. Statistical analysis was carried out by Student's two-tailed unpaired t-test using the software Graph Pad.

2.14 Antibodies:

Table 2.4: The antibodies used in *C.elegans* study

Primary Antibodies – Western Blotting			
Name of Antibody	**Host**	**Source**	**Dilution**
GFP	Rabbit	Cell Signalling	1 in 2000
Actin	Mouse	Developmental Studies Hybridoma Bank	1 in 500
Secondary Antibodies – Western Blotting			
Anti-Rabbit HRP	Goat	Cell Signalling	1 in 3000
Anti-Mouse HRP	Horse	Cell Signalling	1 in 3000

2.15 SDS-polyacrylamideGel Electrophoresis:

Polyacrylamide gels are prepared by the free radical polymerization of acrylamide and the cross linking agent N N' methylene bis acrylamide. The reaction was initiated by 10% Ammonium persulfate (APS) and catalysed by Tetramethylethylenediamine (TEMED).

Both 8% and 15% running gels were used for western blotting depending on the size of the protein that is being detected and prepared with chemicals as shown. Higher the protein size,

lower the resolving gel percentage and vice versa. A 3% stacking gel was used in all experiments (Table 2.5).

Table 2.5: Composition of Gels used in SDS-PAGE Analysis:

Resolving gel	8%	15%
H_2O (ml)	4.94	3.05
Tris (ml)	1.7	1.7
60% (w/v) Sucrose (ml)	1.1	1.1
10% (w/v) SDS (µl)	67	67
40% (w/v) Acrylamide (ml)	1.35	2.7
10% (w/v) APS (µl)	50	50
TEMED (µl)	6	6

Stacking Gel	3%	6%
H_2O (ml)	1.725	1.6
Tris (ml)	0.8	0.8
10% (w/v)SDS (µl)	33	33
40% (w/v)Acrylamide (ml)	0.375	0.5
10% (w/v) APS (µl)	20	20
TEMED (µl)	3.3	3.3

Table 2.6: List of all solutions and buffers used in SDS-PAGE:

Name of Solution/Buffer	Composition	Quantity
10X Laemmli Buffer (1L)	Tris Glycine SDS dH_2O	30g 144g 10g 1L
5X Laemmli loading dye	Glycerol 10% (v/v) SDS 2% Tris HCl 1.5M (pH 6.8) β-mercaptoethanol 5%(v/v) Bromophenol blue 1% (v/v)	10ml 2g 4 ml 5 ml 1 ml
10X Running Buffer, 1L (pH 8.3)	Tris Glycine SDS dH_2O	30g 144g 10g 1L

10% (w/v) APS	Ammonium Persulfate dH_2O	1g 10ml
10% (w/v) SDS, 100 ml	Sodium Dodecyl Sulfate distilled H_2O	10g 100ml
10X Electroblot Transfer Buffer (pH 8.3), 1L	Tris Base Glycine dH_2O	30.25g 144g 1L
1X Electroblot Transfer Buffer, 1L	10x Electroblot Transfer Buffer Methanol dH_2O	100ml 200ml 700ml
10X PBS, 500ml	NaCl KCl Na_2HPO_4-$7H_2O$ H_2O	40g 1g 13.4g 500ml
PBS-Tween20, 1L	10X TBS Tween 20 dH_2O	100ml 0.5ml 1L
10X TBS, 1L pH 8.0	Tris NaCl 1M HCl dH_2O	60.6g 87.6g 1L
ECL- Electrochemiluminescence Detection Mix	1M Tris pH 8.5 90mM Coumaric acid 250mM Luminol 37% H_2O_2 dH_2O	1ml 0.02ml 0.05ml 0.003ml 9ml

2.16 Worm lysis for SDS PAGE:

Exactly 30 worms of each strain of interest were transferred to separate microfuge tubes containing 15µl of M9 buffer. The worms were homogenised in 4X laemmli buffer by boiling them for 5min and stored at -20^{o}C until use.

2.16.1 Gel Preparation:

The Biorad mini gel apparatus was assembled and the resolving gel solution was added carefully to avoid creating air bubbles. The stacking gel was poured immediately and the comb was inserted ensuring that there are no air bubbles. The gel was allowed to solidify for 45 minutes.

2.16.2 Electrophoresis:

The gel tank was set up as per the manufacturer instructions.. The running buffer was poured into the chamber submerging the gel completely and the comb was removed. The samples (15µl) and protein ladder (5µl) were then loaded into the wells and the power supply was connected. The gel was initially run on low voltage (60V) for 20 minutes following by higher voltage (140V) for 60 minutes.

2.16.3 Electrotransfer:

The gel was carefully retrieved after the run from the glass plates and assembled in a sandwich containing equal sized whatman filter sheets, nitrocellulose membrane, and sponges. The sponge, filter sheets, and nitrocellulose were soaked in transfer buffer. A sandwich was made as follows; sponge, 2 filter sheets, gel, nitrocellulose membrane, 2 filter sheets and sponge. The sandwich was then placed in the transfer apparatus and an icepack was placed on the sides of the chamber. The transfer buffer was added to the apparatus and ensured that the sandwich was fully covered with buffer. The whole apparatus was then placed in thermofoam box covered with ice. The transfer was carried out for 90 minutes at 200mAmp.

2.16.4 Blocking and Antibody incubation:

The nitrocellulose membrane was carefully removed from the transfer apparatus and blocked with 5% (w/v) skimmed milk in 1X PBS for 1 hour. The membrane was then incubated with

the primary antibody overnight at 4^{o}C on a shaker. The membrane was then washed with 1X PBS for 10 minutes thrice and was incubated with the secondary antibody for 1 hour at room temperature. The membrane was washed again with 1X PBS for 10 minutes thrice. The ECL mix was then added to the blot and the results were visualized in the dark room.

2.17 Methods used in Mammalian Cell Model

2.17.1 Materials

DMEM (Dulbecco's Modified Eagle Complete Medium, high glucose), PBS (Phosphate Buffered Saline, pH 7.2), and Alexa Fluor488 transferrin conjugate from human serum were from Life Technologies, Invitrogen, UK. Tryspin-EDTA, Fibronectin (from bovine plasma), SU5402 (FGFR inhibitor), PD157890 (EGFR inhibitor), and Phosphatase inhibitor cocktail II were from Sigma-Aldrich, UK. Recombinant mouse FGF-1 was from CellGS, Cambridge, UK. FBS (Fetal Bovine Serum, SA Origin) and Penicillin-Streptomycin were from Lonza, UK. Primary antibodies - FGFR1 (rabbit), Phospho-FGFR1 (mouse), α-Tubulin(mouse) and Secondary antibodies – HRP-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were from Cell Signalling Technology, USA.

2.18 Cell Culture

NIH/3T3 cells were propagated in DMEM supplemented with 10% (v/v) foetal bovine serum, 100 U/ml pencillin and 100 U/ml streptomycin in a humidified atmosphere with 5% CO_2 and 95% air at 37^{o}C.

2.19 Passaging cells:

In order to maintain a healthy stock of cells, cells were grown in above mentioned conditions until they reached approximately 80% confluency. The media was then removed from the flask and the cells were rinsed with 1X PBS prior to incubation at 37^{o}C with 1X trypsin EDTA solution for few minutes. Following cell detachment, trypsin action was stopped by adding serum complemented DMEM. Cells were then collected into a 15 ml centrifuge tube and centrifuged at 4000 rpm for 5 minutes at room temperature. The supernatant was removed and

fresh media was added to the tube depending on the size of the pellet. If necessary, cells were counted using a haemocytometer. A 20ul aliquot of cell suspension was applied to a haemocytometer chamber and cells were counted in 4 individual square fields using a phase contrast microscope. Desired number of cells was then seeded into flasks or dishes containing fresh media.

2.20 Freezing cells:

Freshly centrifuged cells after passage were suspended in a freezing mixture. About 1-1.5ml of cells in freezing mixture consisting of 90%(v/v) Fetal Bovine Serum (FBS) and 10%(v/v) Dimethyl Sulphoxide (DMSO) were taken into cryovials. The cryovials were initially placed into -20^{o}C freezer for an hour before being transferred to -80^{o}C freezer. For long term storage cells were transferred to liquid nitrogen.

2.21 Thawing cells:

The vials containing cells were removed from -80^{o}C and were quickly thawed by dipping into warm water bath and very gently agitated until fully thawed. The cell suspension was then transferred into T25 cm flasks containing serum complemented DMEM and incubated at 37^{o}C in a humidified atmosphere with 5% CO_2 and 95% air for 12 hours before changing the medium when cells had attached.

2.22 Cell Homogenisation for SDS-PAGE Analysis

50,000 to 100,000 cells were seeded into 6cm or 10cm tissue culture dishes and allowed to grow until 70 – 80% confluent. Cells were then washed with 1X PBS twice. After aspirating the PBS, 100 µl of homogenising buffer consisting of 0.25M Sucrose, 2mM EDTA, 5mM tris-

HCl pH 7.4 and 200mM Phenylmethylsulfonyl Fluoride (PMSF) was added to the dishes and the cells were quickly scraped using sterile cell scrapers and collected in a 1.5ml eppendorf tube. The cells were then sonicated on ice at 6-9 amps for 10 sec twice. Of the 100 µl of protein sample, 10µl of sample was collected for protein quantification (using the nanodrop) and the remaining 90µl aliquot was stored in -20^{o}C for later use. On the day of running the gel protein samples were mixed with 5X protein loading dye and loaded onto the gel.

2.23 FGFR1 Expression Assay

To analyse the expression of FGFR1 in NIH/3T3 cells, cells were harvested 48 hours after seeding on 10 cm plates with $5x10^5$ cells/plate. Whole cell lysates were prepared by scraping the cells using the homogenising buffer. The obtained cells were sonicated on ice at 6-9 amps for 10 sec twice and protein concentrations were determined using the Nanodrop as per manufacturer's instructions. 50 µg of protein of each sample was separated on 8% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. FGFR expression levels were detected using anti-FGFR1 antibody as described in methods section 2.15.

2.24 FGFR1 Phosphorylation Assay

NIH/3T3 cells were harvested 48 hours after seeding on 10 cm plates with $5x10^5$ cells/plate. On the day of the experiment, cells were serum starved for 30 minutes and further induced with FGF1 or inhibited with increasing amounts of SU5402 (2µM, 20µM and 40µM) (FGFR inhibitor) for another 30 minutes. PD157890 (EGFR inhibitor) at 20µM was used as a negative control. Whole cell lysates were prepared by scraping the cells using the homogenising buffer with a phosphatase inhibitor cocktail. The obtained cells were sonicated on ice at 6-9 amps for 10 sec twice and protein concentrations were determined using the nanodrop. 50 µg protein sample of each condition was separated in 8% SDS polyacrylamide gels and transferred onto

nitrocellulose membranes. Phospho-FGFR1 levels were detected by western blot as described in methods section 2.15.

Table 2.7: The antibody dilutions used in mammalian model study

Primary Antibodies – Western Blotting			
Name of Antibody	Host	Source	Dilution
FGFR1	Rabbit	Cell Signalling	1 in 2000
Phospho-FGFR(Tyr653/654)	Mouse	Cell Signalling	1 in 2000
α-Tubulin	Mouse	Sigma	1 in 2000
Secondary Antibodies – Western Blotting			
Anti-Rabbit HRP	Goat	Cell Signalling	1 in 3000
Anti-Mouse HRP	Horse	Cell Signalling	1 in 3000

2.25 Measurement of Transferrin Uptake and Recycling using Fluorescence Microscopy

NIH/3T3 cells were seeded on fibronectin coated cover slips at a density of 20,000 cells/well in a 24 well plate and incubated in a 5% CO_2 atmosphere at 37^{o}C for 24 hours prior to the experiment. On the day of experiment, the cells were serum starved for 30 minutes to deplete endogenous transferrin and growth factors and further induced with FGF1 (100ng/ml) or inhibited with SU5402 (20µM) (FGFR inhibitor) for another 30 minutes. To measure the transferrin uptake, cells were incubated with 5 µg/ml Alexa Fluor488-conjugated transferrin for 30 min in a 5% CO_2 atmosphere at 37^{o}C. Cells were then washed twice with ice- cold PBS followed by an acid wash (0.5% acetic acid, 0.5M NaCl, pH 3.0) and another three washes with ice-cold PBS to remove unbound transferrin. Cells were then fixed using 3.7% (v/v)

paraformaldehyde in PBS for 30 minutes at room temperature. After fixation, the cover slips were washed once with PBS and mounted on slides and the total cell fluorescence was imaged using a fluorescence microscope.

To measure transferrin recycling, cells were seeded and grown as above. On the day of experiment, the cells were serum starved for 30 minutes to deplete endogenous transferrin and growth factors and further induced with FGF1 or inhibited with SU5402 (FGFR inhibitor) for another 30 minutes. Cells were then incubated with 5µg/ml of Alexa Fluor488 transferrin for 30 minutes in a 5% CO_2 atmosphere at 37ºC. At the end of internalization, surface bound and unbound transferrin was removed as above and the cells were incubated in a complete medium for 30 minutes. The cells were then washed once in ice-cold PBS and fixed in 3.7% (v/v) paraformaldehyde in PBS for 30 minutes at room temperature. The coverslips were mounted and imaged using a fluorescence microscope. Images of at least 50 cells per condition from random different fields of the cover slip were imaged and the total cell fluorescence was quantified using the Image J software.

2.26 Quantification Analysis:

In order to measure the Tf uptake and recycling, random cells located on the coverslips were imaged at fixed intensity settings and conditions. Alexa Fluor488 transferrin intensity in the cells was calculated for each condition (Control, FGF-1 induced and FGFR inhibited) at specific time point (at 30 min uptake and at 30 min recycling) using Image J software. Cells to be analysed were selected and a free shape was drawn around the membrane of the cell. The image was then first converted to a 32 bit and further converted to an 8 bit. A histogram was then obtained for the cell with individual pixel intensity. The minimum pixel intensity of the background was also obtained. The sum of all pixel intensities of the selected cell was

calculated based on the background value. 50 cells were analysed for each condition and thus the total cell fluorescence were compared between conditions and at varying time points.

2.27 Measurement of Transferrin Uptake and Recycling using Flow Cytometry

Transferrin uptake and recycling was also measured using the flow cytometry. NIH/3T3 cells were seeded at a density of $1X10^5$ cells in 6 cm tissue culture plates and incubated in a 5% CO2 atmosphere at 37ºC for 24 hours prior to the experiment. On the day of experiment, the cells were serum starved for 30 minutes to deplete endogenous transferrin and growth factors and further induced with FGF1 (100ng/ml) or inhibited with SU5402 (20µM) for another 30 minutes. To measure the transferrin uptake, cells were incubated with 5 µg/ml Alexa Fluor488 transferrin at varying intervals of time such as 10, 20 and 30 min in a 5% CO_2 atmosphere at 37ºC. At the end of internalization cells were then washed twice with ice- cold PBS followed by an acid wash (stripping buffer 0.5% acetic acid, 0.5M NaCl, pH 3.0) and another three washes with ice-cold PBS to remove unbound transferrin. The cells were then trypsinised,pelleted by centrifugation, and resuspended in how much? PBS. The samples were then analysed using the flow cytometer (10,000 events). The mean fluorescence for each time point was calculated.

To measure transferrin recycling using the flow cytometer, after internalisation of Alexa488-transferrin for 30 minutes, cells were washed with ice-cold PBS and surface bound transferrin was removed by an acid wash. The cells were then incubated in complete medium at varying time points 10, 20 and 30 minutes. After incubation the cells were trypsinized and resuspended in PBS and analysed using the flow cytometer. The mean fluorescence for each time point was calculated.

2.28 Statistical Analysis:

Statistical Analyses were performed using statistical software package GraphPad Prism 5. The total fluorescence intensity data are expressed as the mean ± s.e.m of 50 cells per condition. One way analysis of variance (ANOVA) and the Dunnet test were used to compare data between 3 or more groups. P value ≤ 0.05 were considered statistically significant.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

To investigate how membrane trafficking is regulated by Fibroblast Growth Factor Receptor (FGFR) mediated cell signalling using *C.elegans* as a model organism:

- By establishing gene depletion by RNAi and analyse the trafficking of YP170-GFP
- By analysing YP170-GFP trafficking in FGFR mutant worm strains

To analyse the localization and morphology of various membrane markers in worm intestine and to pinpoint which membrane transport step is regulated by worm FGFR

- By crossing GFP-tagged intracellular membrane marker into FGFR mutant strain and analyse the resulting phenotype by confocal microsopy

To analyze whether regulation of membrane traffic by FGFR is conserved in mammalian cells by

- By using of FGFR inhibitors and FGF1 stimulation and analyse transferrin uptake and recycling using Flow Cytometry and Confocal Microscopy

CHAPTER 3: WORM FGFR (*egl-15*) REGULATES MEMBRANE TRAFFICKING VIA MAPK AND PLC-γKINASE PATHWAYS

3 WORM FGFR (*egl-15*) REGULATES MEMBRANE TRAFFICKING VIA MAPK AND PLC-γ PATHWAYS

3.1 Introduction

C.elegans was the first multicellular organism whose genome was completely sequenced. Since sequencing, the concept of reverse genetics has greatly helped scientists to identify and characterise different genes on a large scale (Anon 1998). The network of FGF-FGFR signalling in mammals is difficult to define as each FGF binds to several FGFRs and vice versa. Whereas in invertebrates such as *Drosophila melanogaster* and *C.elegans* only three FGFs and two FGFs have been identified so far, respectively (Birnbaum et al. 2005). With just two FGFs *egl-17* and *let-756* and the lone FGFR *egl-15*, the complexity of FGF-FGFR network is reduced in *C.elegans*. One of the reasons hypothesised for such complexity in mammals is due to the large scale duplication in a chordate ancestor that led to the large scale increase in the number of genes (Ohno, 1970). The *C.elegans* FGFR is found to be the orthologue of vertebrate FGFRs 1-4. The FGFRs show a high degree of sequence conservation between vertebrates and invertebrates (Figure 3.1 & Table 3.1) and FGFRs in worms and flies appear to regulate cellular responses such as proliferation, differentiation, and migration similar to those FGFRs regulated in mammalian cell culture systems (Popovici et al. 1999).

Table 3.1: FGF-FGFR signalling components in *C.elegans* and mammals

Component	***C.elegans* genes**	**Mammalian genes**
FGF	*egl-17, let-756*	FGFs (1-22)
FGFR	*egl-15*	FGFRs (1-4)

Ras activating complex	*sem-5*	Grb2
	sos-1	*Sos*
MAPK Pathway	*let-60*	*Ras*
	lin-45	*Raf*
	mek-2	*Mek*
	mpk-1	*Mpk*
AKT Kinase Pathway	*age-1*	*PI3K*
	pdk-1	*PDPK-1*
	akt-1	*Akt*
	akt-2	*Akt*
PLC-γ Pathway	*plc-3*	*PLC-γ*
	itr-1	*Itpr-1*

	pkc-2	*PKC-α*

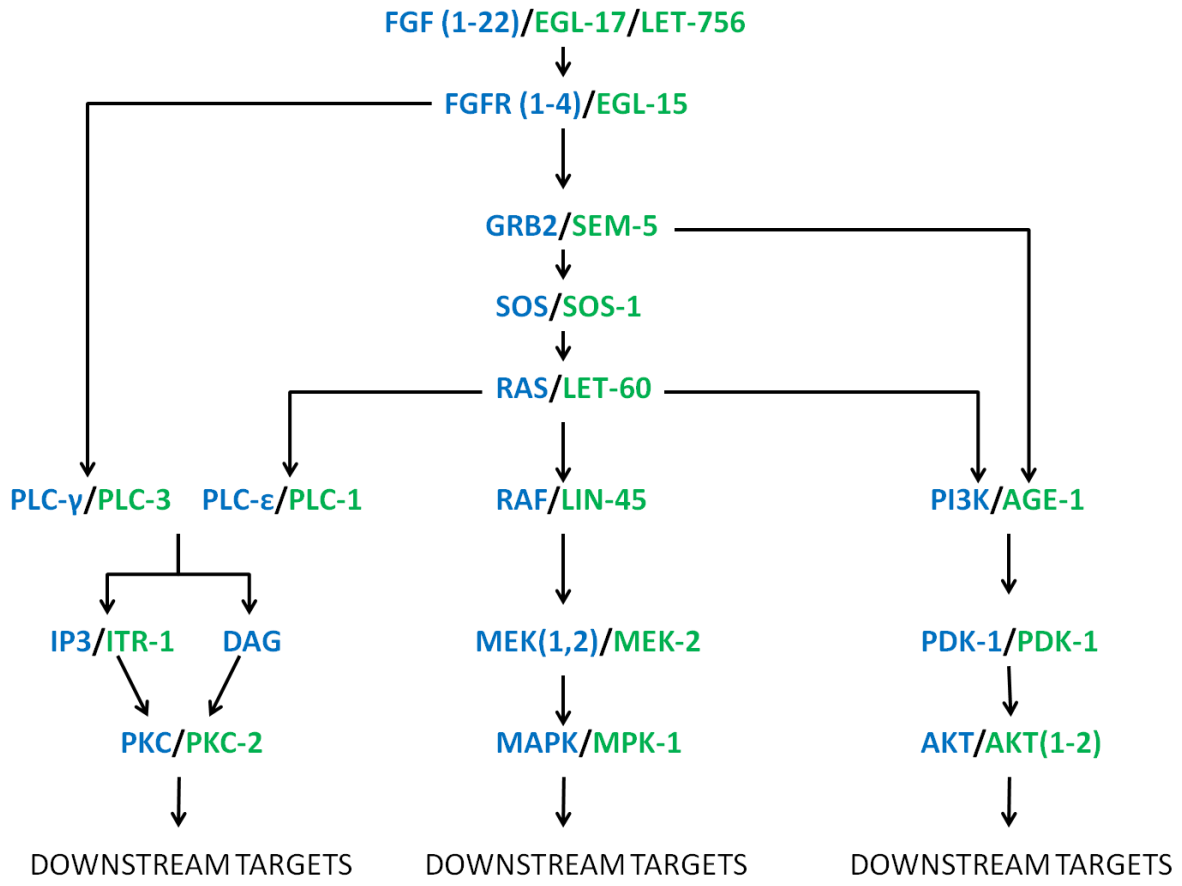


Figure 3.1: FGF-FGFR signalling pathways between mammals and *C.elegans. The signalling components of C.elegans coded in green and mammalians in blue.*

Reverse genetics has been widely used in *C.elegans* since 1998 when it was found that the introduction of double stranded RNA resulted in the inactivation of endogenous genes (Fire et al. 1998). This technique called the RNA-mediated interference or RNAi has become the common tool to study genes in *C.elegans* because it is rapid and simple in identifying gene function on a large scale. The early use of this technique involved the injection of dsRNA into

the head or tail of the adult hermaphrodites as it produced robust interference even across cellular boundaries but other alternate methods that were found in subsequent studies showed that RNAi could be performed by simply soaking worms in dsRNA solution (Tabara et al. 1998) or by feeding the worms with *E.coli* expressing target gene dsRNA (Timmons & Fire 1998). Of all the three RNAi methods (injecting, soaking and feeding), RNAi by feeding is found to have more advantages than the others namely, it's less complicated and less laborious and it is comparatively cheaper than the other methods. There are bacterial libraries available for scientists that contains almost all of the predicted genes in *C.elegans* (Fraser et al. 2000) (Kamath et al. 2003).

Thus initially in this study, bacterial clones containing the dsRNA were used to target the desired *C.elegans* FGFR signalling pathway components and the RNAi by feeding approach was combined with the YP170-GFP and RME-2-GFP assays as described in the Materials and Methods. The transgenic *C.elegans* strain RT130 *[unc-119(ed3); pwls23 [vit-2::GFP; unc-119]* that expresses major yolk protein YP170 fused to GFP was used to analyse the basolateral secretion of yolk by the intestine and receptor mediated endocytosis of yolk by oocytes in adult hermaphrodites thereby elucidating the role of worm FGFR in membrane transport. The Yolk-GFP assay combined with RNAi was carried out by knocking down the expression of target genes such as FGFR *egl-15*, both FGFs *egl-17* and *let-756*, downstream RAS activating complex components *sem-5,sos-1, and let-60,* MAPK pathway components *lin-45, mek-2, and mpk-1,* AKT kinase pathway components *akt-1, akt-2, pdk-1, and age-1* and PLC-γ pathway component *plc-3* by feeding worms with their respective dsRNA expressing bacterial clones. The other two components of *plc-3* pathway, *pkc-2 and itr-1* couldn't be analysed by RNAi since they weren't available from the library during the time of analysis. The *rme-1* RNAi was used as positive control, as it gives a strong RME phenotype and the negative control L4440 (an empty vector) were used in all experiments.

For every gene of FGFR pathway, at least 80% of the worms showed a strong yolk trafficking defect. All experiments were repeated thrice and below results show a representative of observed phenotypes.

3.2 RESULTS

3.3 Analysis of YP170-GFP trafficking in FGF-FGFR signalling pathway components by RNAi.

Depletion of FGF-FGFR upstream signalling pathway components by RNAi

From the initial analysis of FGF-FGFR signalling components by RNAi, an impaired localization of Yolk-GFP was observed in animals where *egl-15* and *sem-5* were depleted and a WT phenotype was observed in *egl-17, let-756, sos-1* and *let-60* depleted animals suggesting the significance of *egl-15* and *sem-5* as positive regulators of membrane transport (Figure 3.2). At first, worms were exposed to dsRNA at their L4 stage and their progeny at adult hermaphrodite stage were analysed. Worms that were depleted of *egl-15* and *sem-5* including the positive control *rme-1* showed a very strong Yolk-GFP trafficking defect whereas worms that were depleted of *egl-17, let-756, sos-1* and *let-60* showed a wild type phenotype. Though *egl-15* showed a high accumulation of Yolk-GFP in the intestine suggesting a secretory defect, more Yolk-GFP were also found in the body cavity. Those worms that didn't show a phenotype were exposed to dsRNA for a longer time from egg to P0 adult hermaphrodites and those hermaphrodites were allowed to lay eggs again and their progeny were analysed. *sos-1* and *let-60* showed a weak Yolk-GFP trafficking defect on long dsRNA exposures whereas *egl-17* and *let-756* still showed a wild type phenotype. The worms subjected to RNAi displayed specific phenotypes which served as positives to analyse gene knock down. For example, *egl-15, egl-*

17, *sem-5* knock-down worms by RNAi show an egg laying defective phenotype. *sos-1* shows a weak egl and clumpy phenotype. *let-756* and *let-60* show a weak and scrawny phenotype.

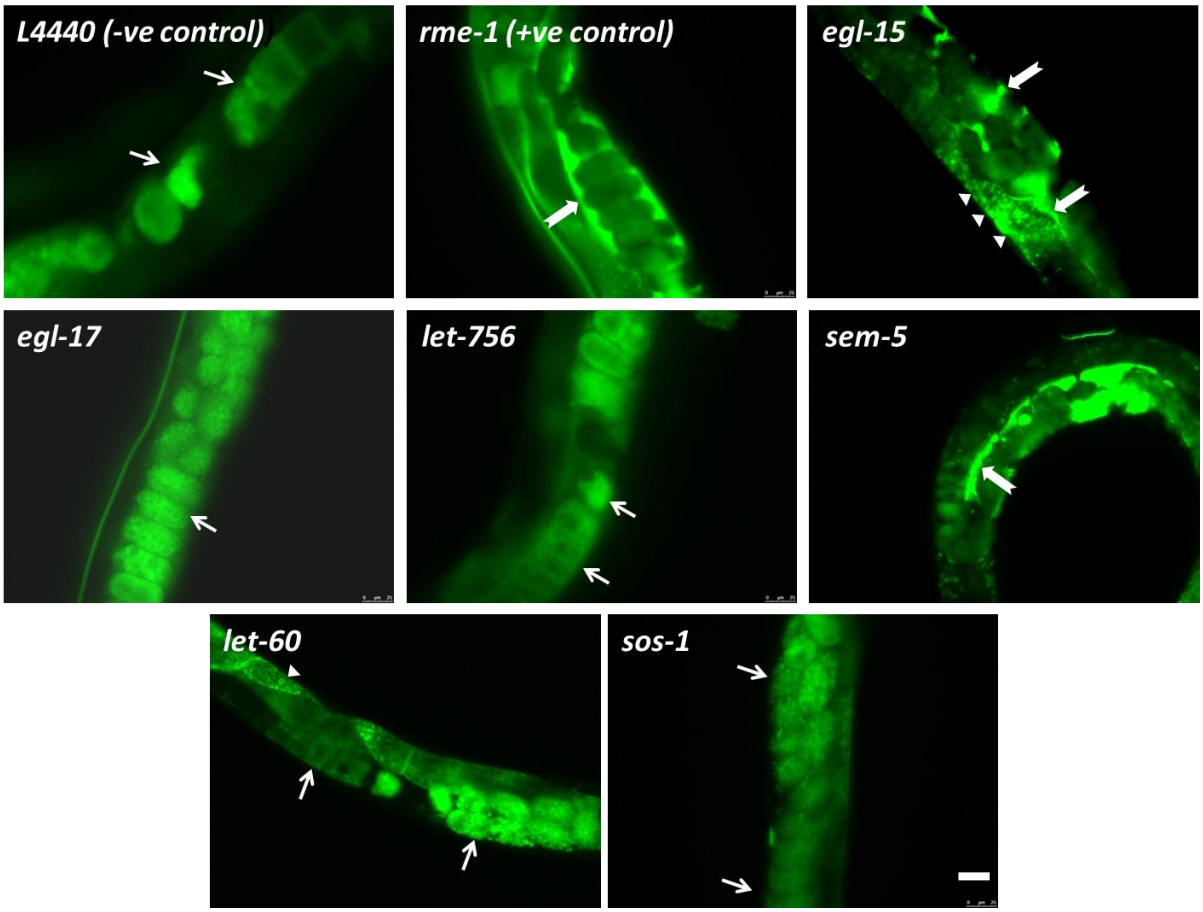


Figure 3.2: Analysis of FGF-FGFR upstream signalling pathway components for defects in membrane trafficking by RNAi. *Yolk-GFP in oocytes and embryos indicated by normal arrows. Notched arrows indicate body cavity and arrow heads indicate intestine. Localization of Yolk-GFP in control RNAi L4440 (empty vector) showing a WT phenotype with high Yolk-GFP in oocytes and embryos and low in intestine and body cavity. rme-1 RNAi was used as a positive control showing an endocytosis defect with a very high accumulation of Yolk-GFP in body cavity and less accumulation in intestine, oocytes and embryos as indicated by notched arrows. egl-15 knock-down worms showing an increased Yolk-GFP accumulation in the intestine (indicated by arrow heads) and pseudocoelom and less accumulation in oocytes and*

embryos. sem-5 knock-down worms showing an endocytosis defect. let-756, egl-17, let-60, sos-1 showing a WT phenotype. N=3. Scale bar, 25µm.

Depletion of MAPK signalling pathway components by RNAi

Yolk-GFP trafficking in signalling components of MAPK (*lin-45, mek-2 and mpk-1*) signalling pathway were analysed by RNAi. MAPK components are essential for the development of the worm and hence these worms were exposed to dsRNA starting at L3-L4 stage and were analysed at their adult stage. *lin-45, mek-2* and *mpk-1* animals show a scrawny weak phenotype when compared to wild type. It was found that the animals treated with dsRNA for *lin-45, mek-2* and *mpk-1* showed a strong endocytosis defect demonstrated by the increased presence of Yolk-GFP in pseudocoelom and low in oocytes, embryos and intestine suggesting the role of MAPK pathway in regulation of membrane trafficking (Figure 3.3).

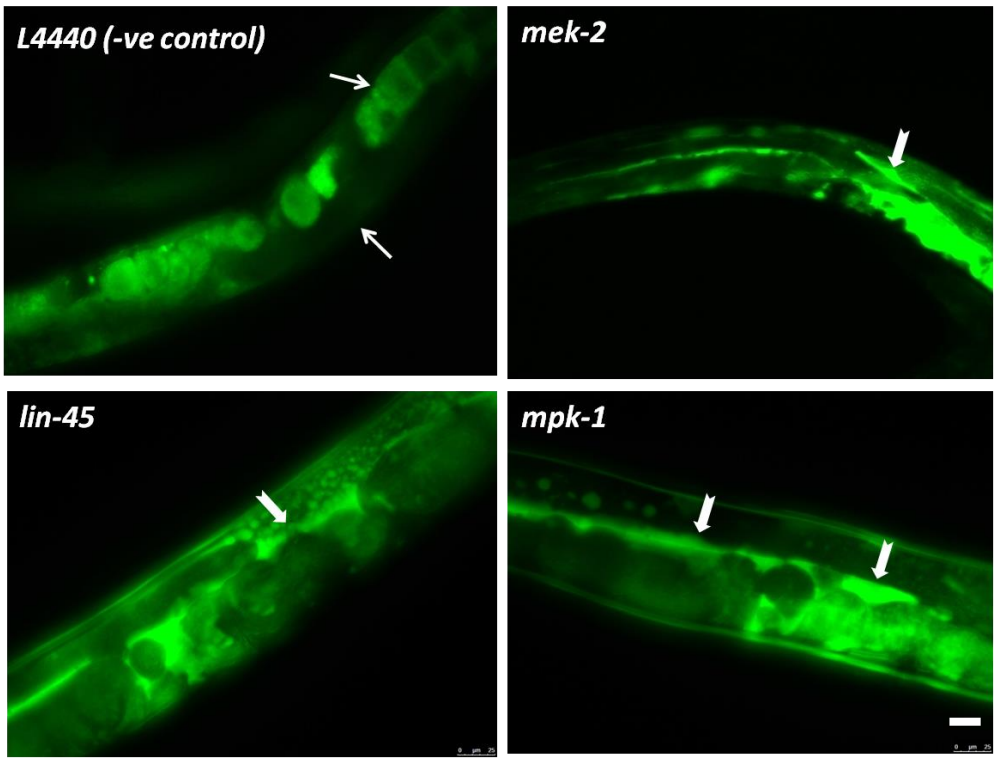


Figure 3.3: Analysis of MAPK signalling pathway components for defects in membrane trafficking by RNAi: *Yolk-GFP in oocytes and embryos indicated by normal arrows. Notched*

arrows indicate body cavity and arrow heads indicate intestine. Localization of Yolk-GFP in control RNAi L4440 (empty vector) showing a WT phenotype with high Yolk-GFP in oocytes and embryos and low in intestine and body cavity. mek-2, lin-45 and mpk-1 knock-down worms showing an endocytosis defect with a very high accumulation of Yolk-GFP in pseudocoelom and reduced/little accumulation in intestine, oocytes and embryos. N=3, Scale bar, 25µm.

Depletion of phosphatidyinositol-3-kinase/AKT kinase signalling pathway components by RNAi

The downstream signalling events in *C.elegans* mediated through the AKT kinase pathway and their candidate genes *akt-1, akt-2, pdk-1* and *age-1* were investigated. Animals subjected to *akt-1, akt-2* and *age-1* RNAi bacteria show a weak egl phenotype and *pdk-1* show an egl and often display a bag of eggs phenotype. The RNAi results from this study showed that all these animals produced a WT phenotype (Figure 3.4). Worms were exposed to dsRNA at their L4 stage and their progeny at adult hermaphrodite stage were analysed. All the worms showed a complete wild type phenotype. Next worms were exposed to dsRNA for a longer time (egg to adult P0 hermaphrodites to egg to adult F1 hermaphrodite), but even with an extended exposure to RNAi worms did not display any yolk trafficking defects, suggesting that PI3K/AKT signalling pathway might not be involved in membrane trafficking.

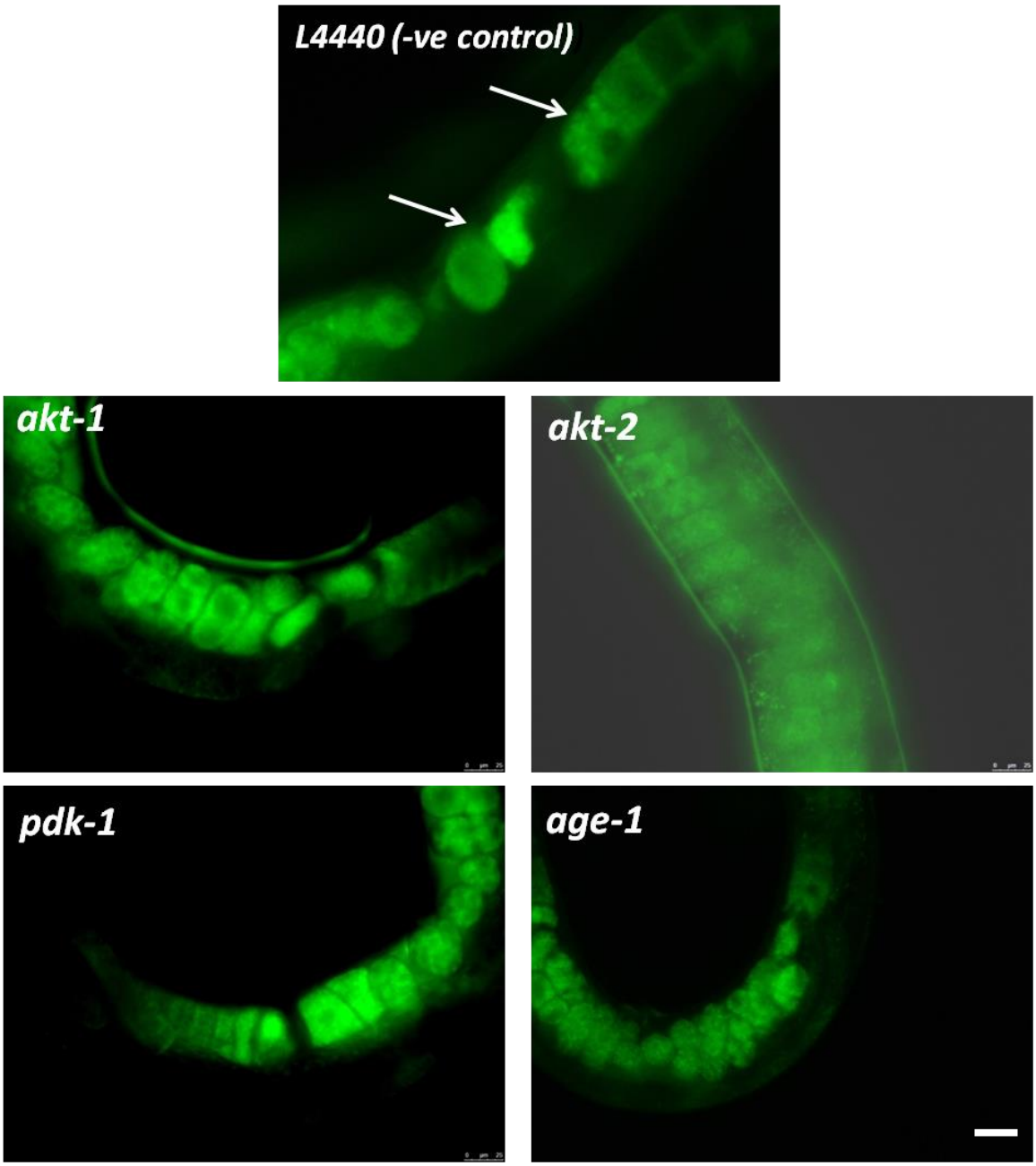


Figure 3.4: Analysis of phosphatidyinositol -3-kinase/AKT kinase pathway components for defects in membrane trafficking by RNAi: *Yolk-GFP in oocytes and embryos indicated by normal arrows. Localization of Yolk-GFP in control RNAi L4440 (empty vector) showing a WT phenotype with high Yolk-GFP in oocytes and embryos and low in intestine and body cavity. akt-1, akt-2, pdk-1, age-1 animals showing a WT phenotype. N=3. Scale bar, 25µm*

Depletion of PLC signalling pathway components by RNAi

The downstream signalling events in *C.elegans* mediated through the PLC-3 were analysed by RNAi. A high accumulation of Yolk-GFP was found in the body cavity and to some extent in intestine compared to Yolk-GFP in oocytes and embryos. The other components of PLC-3 signalling pathway such as *itr-1* and *pkc-2* couldn't be analysed by RNAi due to unavailability of dsRNA clones from the library during the time of experiments. This RNAi result suggested that *plc-3* (PLC-γ) could have a role in membrane transport however; it couldn't be concluded without the analysis of other genes in the pathway at this stage. (Figure 3.5)

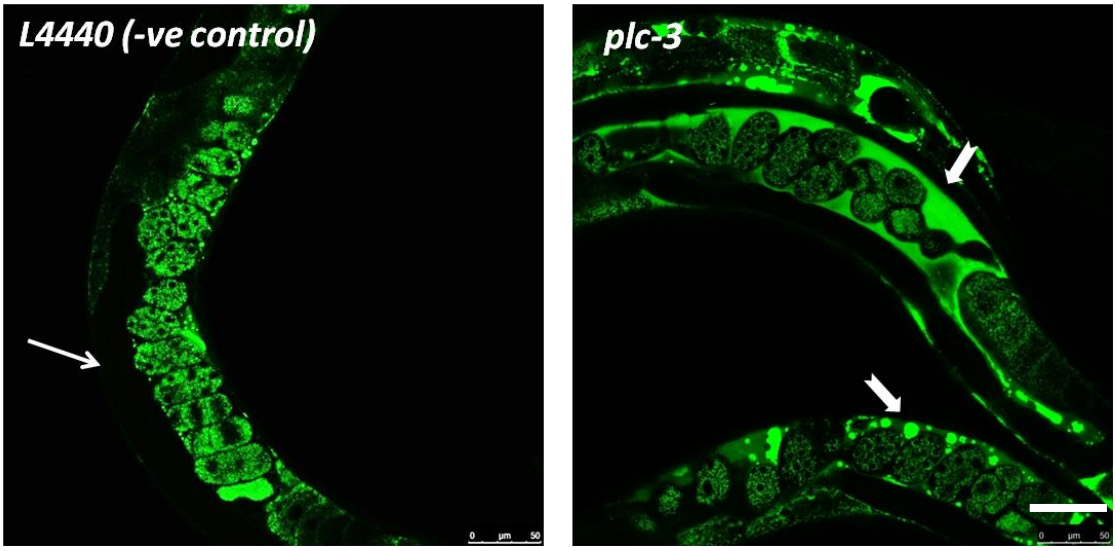


Figure 3.5: Analysis of PLC-3 for defects in membrane trafficking by RNAi: *Yolk-GFP in oocytes and embryos indicated by normal arrows and notched arrows indicate body cavity. Localization of Yolk-GFP in control RNAi L4440 (empty vector) showing a WT phenotype with high Yolk-GFP in oocytes and embryos and low in intestine and body cavity. plc-3 knock--down worms showing a strong endocytosis defective phenotype with high accumulation of Yolk-GFP in body cavity. Scale bar, 50µm.*

Hence from the initial RNAi experiments, it was found that the worm FGF-FGFR pathway regulates membrane transport via the MAPK and PLC-3 pathway and not through the AKT

Kinase pathway. Further to these experiments, it was necessary to investigate yolk trafficking in mutant (knock-out) worms to see if they phenocopy the same as observed in RNAi (knock-down) worms.

3.4 Analysis of Yolk-GFP trafficking in mutant worms of the FGF-FGFR signalling pathway.

3.4.1 FGFs and FGFR are involved in regulation of membrane trafficking in *C.elegans*

In order to analyse Yolk-GFP trafficking in the mutant worms of FGF-FGFR signalling pathway, the Yolk-GFP transgene *[unc-119(ed3); pwls23 [vit-2::GFP; unc-119]* was introduced into the mutant animals (as shown in table 2.3 in methods) by crossing. Firstly, the Yolk-GFP trafficking in FGF mutants (*egl-17* and *let-756*) and the FGFR mutant (*egl-15*) worms was analysed.

egl-15 (FGFR)

The *egl-15* mutant strain MT3456 – [*egl-15(n1477)*] was crossed with the Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit-2::GFP; unc-119]*. The resulting F2 progeny that were homozygous for Yolk-GFP and *egl-15* mutation (with egg laying defective phenotype) were picked and analysed. It was found that a strong accumulation of Yolk-GFP fusion protein was observed in the body cavity and prominent vacuolated structures in the intestine and less in the oocytes and embryos of *egl-15* mutant worms suggesting a combination of both a secretory and an endocytosis defects (Figure 3.6). This defective Yolk-GFP trafficking in *egl-15* mutants was similar to that observed in *egl-15* RNAi experiment supporting the role of *egl-15* in regulation of membrane trafficking.

egl-17 (FGF):

The *egl-17* mutant strain, CB1313- *[egl-17(e1313)]* was used for the analysis of Yolk-GFP trafficking and was crossed with RT130 *[unc-119(ed3); pwls23 [vit-2::GFP; unc-119]* strain. The resulting F2 progeny that contained the GFP and were homozygous for *egl-17* (with egg laying defective phenotype) were picked and analysed (Figure 3.6). *egl-17* mutants showed a strong accumulation of Yolk-GFP both in the intestine and also in the body cavity suggesting that the ligand *egl-17* is important for efficient yolk trafficking.

let-756 (FGF):

The *let-756* mutant strain used in this experiment was FF628 [*let-756(s2613) unc-32(e189)]III* displaying an Unc (uncoordinated) phenotype and was crossed with RT130 *[unc-119(ed3); pwls23 [vit-2::GFP;unc-119]* strain. The gene variation of *let-756(s2613)* is located in *cis*- to *unc-32(e189)* which serves as a marker and hence the F2 progeny that displayed an Unc phenotype were picked and analysed (Figure 3.6). A high accumulation of Yolk-GFP was found in the body cavity and intestine and less in the oocytes and embryos suggesting that *let-756* is important in regulation of membrane trafficking.

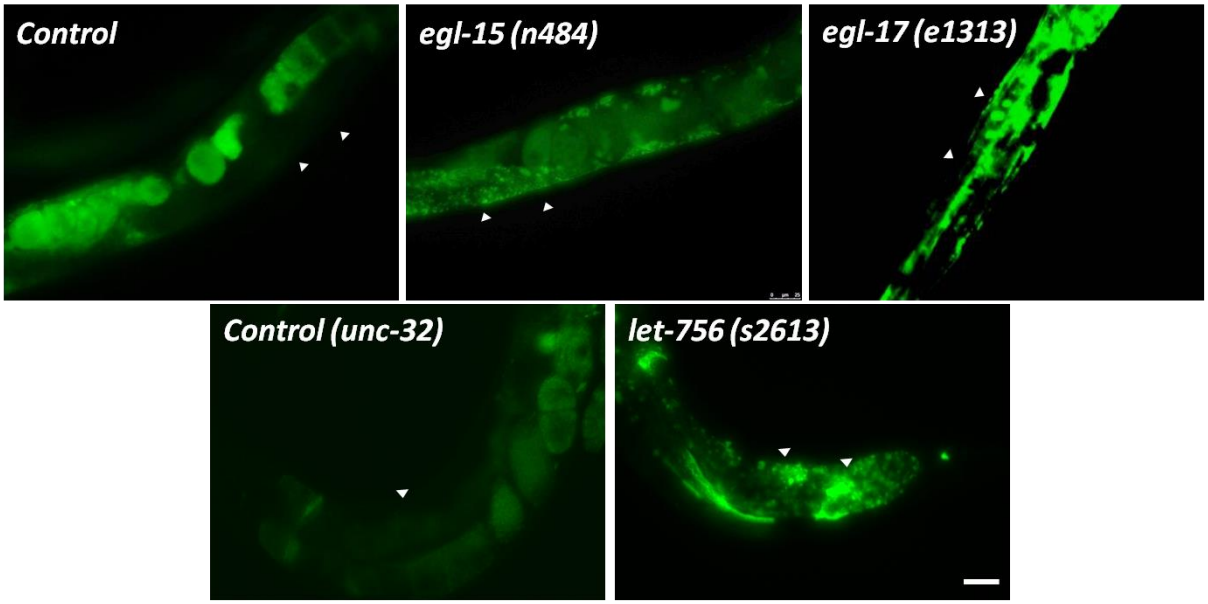


Figure 3.6: Yolk-GFP trafficking in FGF-FGFR mutant worms. *The worm FGFs egl-17 and let-756 and the worm FGFR egl-15 are required for efficient trafficking of Yolk-GFP in C.elegans. Arrowheads indicate the worm intestine. The N2 and unc-32 control worms showing a WT phenotype with high accumulation of Yolk-GFP in embryos and oocytes and little accumulation of Yolk-GFP in intestine. egl-15 and let-756 mutants showing an abnormal accumulation of Yolk-GFP in the intestine (arrowheads) and reduced accumulation in oocytes and embryos. egl-17 mutants showing abnormal accumulation of Yolk-GFP in the intestine (arrowheads) and body cavity and reduced accumulation in oocytes and embryos. Scale bar, 25µm*

3.4.2 RAS activating complex is essential for proper Yolk-GFP trafficking

sem-5:

The Ras activating complex consisting of *sem-5* and *sos-1* was analysed. The *sem-5* mutant strain used in for analysis was UP148 – *[sem-5(cs15)]*. Yolk-GFP was introduced into *sem-5* mutant worms by crossing the Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit-2::GFP; unc-119]* with UP148 – *[sem-5(cs15)* strain. The worms that were homozygous for Yolk-GFP and Egl phenotype were picked and analysed. *sem-5* mutants showed an increased accumulation and prominent vacuolated structures positive for Yolk-GFP in intestine and body cavity and reduced accumulation in oocytes and embryos suggesting the role of *sem-5* in regulation of yolk trafficking (Figure 3.7).

sos-1:

The mutant strain of *sos-1* used for analysis was a temperature sensitive strain UP604 [*sos-1(cs41)*]. Yolk-GFP was introduced into *sos-1* mutant worms by crossing the Yolk-GFP strain

RT130 *[unc-119(ed3); pwls23 [vit-2::GFP; unc-119]* with [*sos-1(cs41)*] strain. The resulting transgenic mutant strain ZIB24 - *[let-60(n1700); pwls23)]* was grown at 25°C to induce mutation and was imaged (Figure 3.7). *sos-1* mutant worms displayed a normal accumulation in oocytes and embryos but there was still an increased accumulation in body cavity.

let-60:

The *let-60* mutant strain used in this experiment was MT4698- [*let-60(n1700)*] and was crossed with RT130 *[unc-119(ed3); pwls23 [vit-2::GFP; unc-119]* strain. *let-60* loss of function mutants are not viable or get arrested at larval stage, therefore *let-60* gain of function mutation strains were used in this analysis. Gain of function mutation in *let-60* produces a multivulva (Muv) phenotype which serves as a marker and hence the F2 progeny that displayed a Muv phenotype and positive for Yolk-GFP were picked and analysed. *let-60* mutant worms displayed an endocytosis defect with a high accumulation of Yolk-GFP in the pseudocoelom and reduced in oocytes and embryos (Figure 3.7).

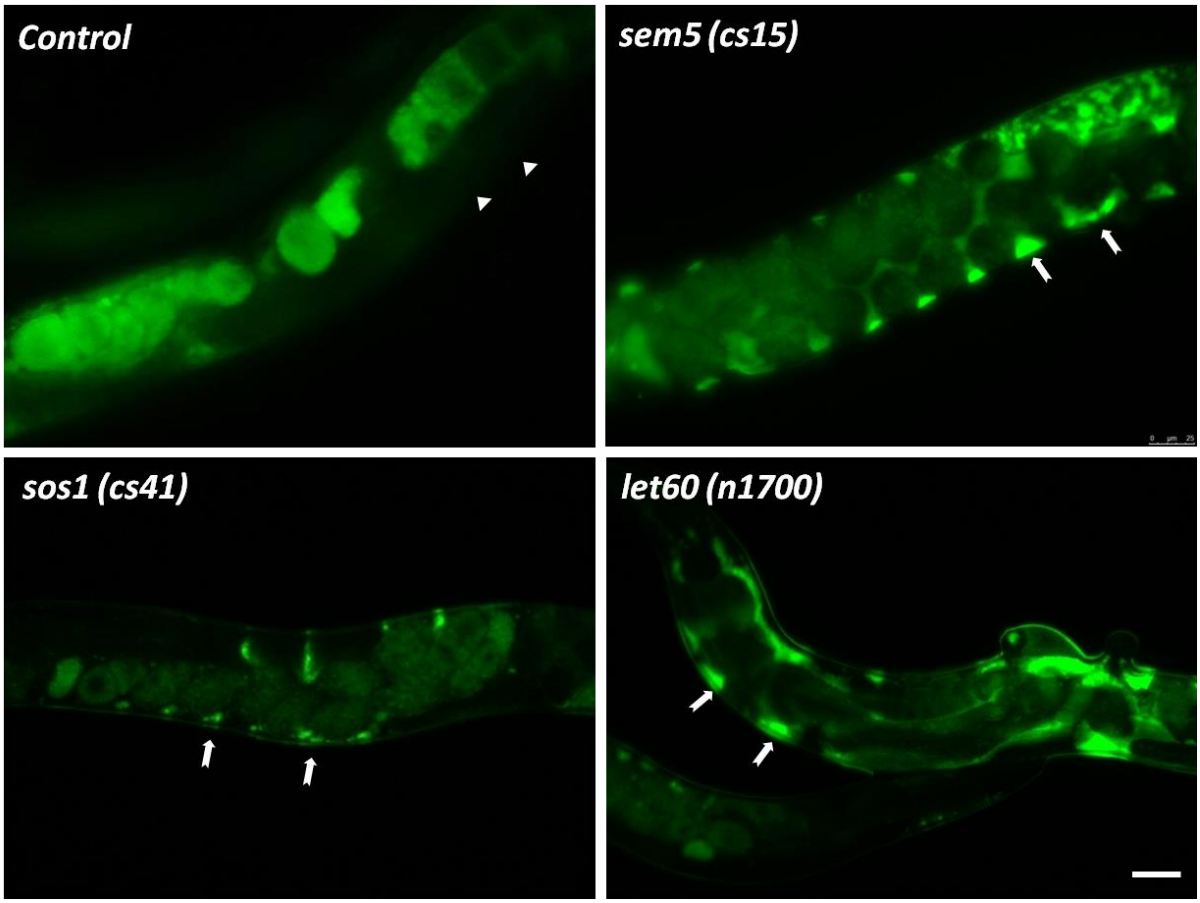


Figure 3.7. Analysis of Yolk-GFP trafficking in RAS activating complex: *The control N2 worms showing a WT phenotype with high accumulation of Yolk-GFP in embryos and oocytes and little accumulation of Yolk-GFP in intestine. sem-5 mutants showing abnormal accumulation of Yolk-GFP signal in the body cavity and intestine and reduced accumulation in oocytes and embryos. sos-1 mutants displaying a weak endocytosis phenotype with slightly increased accumulation of Yolk-GFP in body cavity (indicated by notched arrows). let-60 mutant worm displaying a strong endocytosis defective phenotype with high accumulation of Yolk-GFP in body cavity. Scale bar, 25µm.*

3.4.3 MAPK signalling cascade affects membrane trafficking

lin-45:

The *lin-45* mutant strain used in this experiment was WU48 [*lin-45(n2018) dpy-20(e1282)*]. The strain was crossed with RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* strain. The allele of *lin-45(n2018)* is positioned in *cis* to *dpy-20(e1282)* which acts as a marker mutation to precisely identify worms that are homozygous to *lin-45.* The F2 progeny that displayed a 'dumpy' phenotype and were positive for Yolk-GFP were picked and analysed. *lin-45* mutants showed a strong accumulation of Yolk-GFP fusion protein in the body cavity suggesting that they are required for proper membrane trafficking (Figure 3.8).

mpk-1:

The *mpk-1* mutant strain analysed was SD184- *[unc-79(e1068) mpk-1(n2521]*. The strain was crossed with RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* strain. The allele *mpk-1(n2521)* is positioned in *cis* to *unc-79(e1068)* which acts as a marker mutation to precisely identify worms that are homozygous to *mpk-1.* The F2 progeny that displayed an Unc (uncoordinated) phenotype and were positive for Yolk-GFP were picked and analysed. The *mpk-1* mutant worm displayed a high accumulation of Yolk-GFP in the body cavity suggesting a strong endocytosis defect (Figure 3.8).

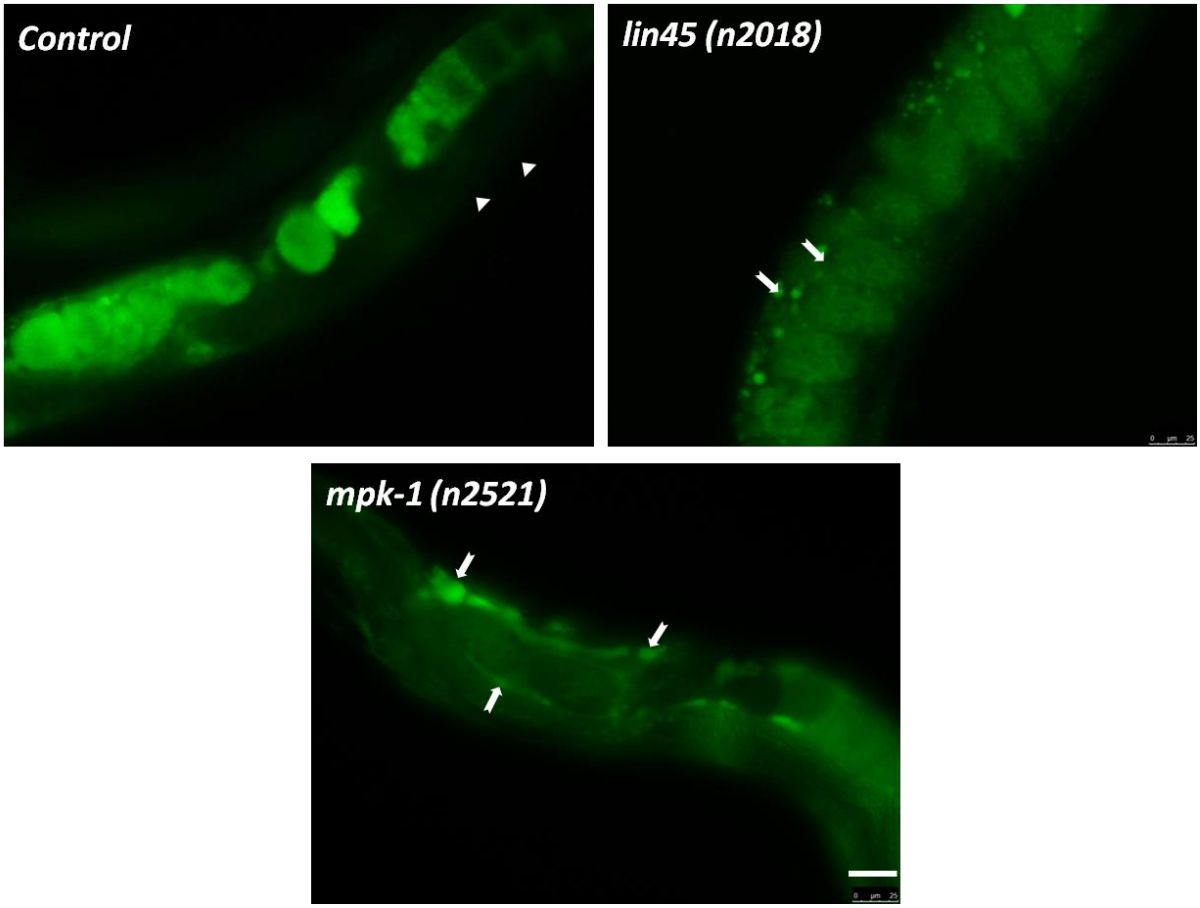


Figure 3.8: Analysis of YP170-GFP trafficking in MAPK signalling pathway mutant worms. *The control N2 worms showing a WT phenotype with high accumulation of Yolk-GFP in embryos and oocytes and little accumulation in intestine (indicated by arrowheads). Both, lin-45 and mpk-1 mutant worms displaying a strong endocytosis defective phenotype with high accumulation of Yolk-GFP in body cavity (indicated by notched arrows). Scale bar, 25µm*

3.4.4 PI3K/AKTsignalling cascade is not involved in regulation of membrane trafficking

age-1:

The *age-1* mutant strain used in this analysis was TJ1052-*[age-1 (hx546)]*. *age-1* mutants are difficult to differentiate from wild type animals and therefore, a balancer strain dpy-10 was used. The dpy-10 balancer strain has GFP in its pharynx. The Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* was first crossed into the balancer *dpy-10* and the resulting F2 progeny homozygous for Yolk-GFP and dpy-10 (dumpy phenotype with GFP in its pharynx) were crossed with male *age-1* mutants. The resulting F1 progeny has *dpy-10* positioned *trans* to *age-1* and therefore from self fertilization the worms that are homozygous for Yolk-GFP and *age-1* with a wild type phenotype lacking GFP in the pharynx are picked and analysed. Thus *age-1* mutant worms positive for Yolk-GFP were picked and analysed. From the analyses, it was found that *age-1* mutants showed a WT phenotype with low Yolk-GFP in intestine and body cavity and normal Yolk-GFP presence in oocytes and embryos suggesting that they might not be required as a signalling component for regulation/control of membrane trafficking (Figure 3.9).

pdk-1:

The *pdk-1* mutant strain used in the analysis was GR1318-*[pdk-1(mg142); pwls23]*. Since *pdk-1* mutants don't have a distinct marker phenotype themselves, they had to be identified by using a balancer. The Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* was first crossed into a balancer *dpy-3* and the resulting F2 progeny homozygous for Yolk-GFP and *dpy-3* (dumpy phenotype) were crossed into *pdk-1* mutants. *Dpy-3* is positioned *trans* to *pdk-1* and therefore from self fertilization they produce worms homozygous for Yolk-GFP and *pdk-1* with a wild type phenotype. Thus pdk-1 mutant worms positive for Yolk-GFP were picked

and analysed. From the analyses, it was found that *pdk-1* mutant worms do not show any defects in Yolk-GFP localization and were found to display a WT phenotype suggesting that they might not have a role in regulation of membrane trafficking. (Figure 3.9)

akt-1 and akt-2:

The *akt-1* and *akt-2* mutant strains used in this analysis were JT573-[*akt-1(sa573)*] and VC204 – *[akt-2(ok393)]* respectively. In order to isolate *akt-1* and *akt-2* mutants from wild type worms, genetic balancers *unc-42* and *unc-3* respectively were used. The Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* was first crossed into the balancer strain carrying *unc-42* and the resulting F2 progeny homozygous for Yolk-GFP and *unc-42* (unc phenotype) were crossed with akt-1 mutant (males). The resulting F1 progeny gets unc-42 positioned *trans* to *akt-1* and therefore from self fertilization the worms that are homozygous for Yolk-GFP and *akt-1* with a wild type phenotype were carefully picked and analysed. A similar approach was taken in identifying *akt-2* mutants using *unc-3* balancer strain. From the analysis, both, *akt-1* and *akt-2* mutants showed a WT phenotype with low levels of Yolk-GFP in intestine and body cavity and normal Yolk-GFP presence in oocytes and embryos suggesting that they might not be required as a signalling component for regulation of membrane trafficking (Figure 3.9). Thus from this analysis of the AKT Kinase signalling pathway for Yolk-GFP trafficking, it is evident that AKT kinase pathway might not have any significant role in regulation of membrane trafficking.

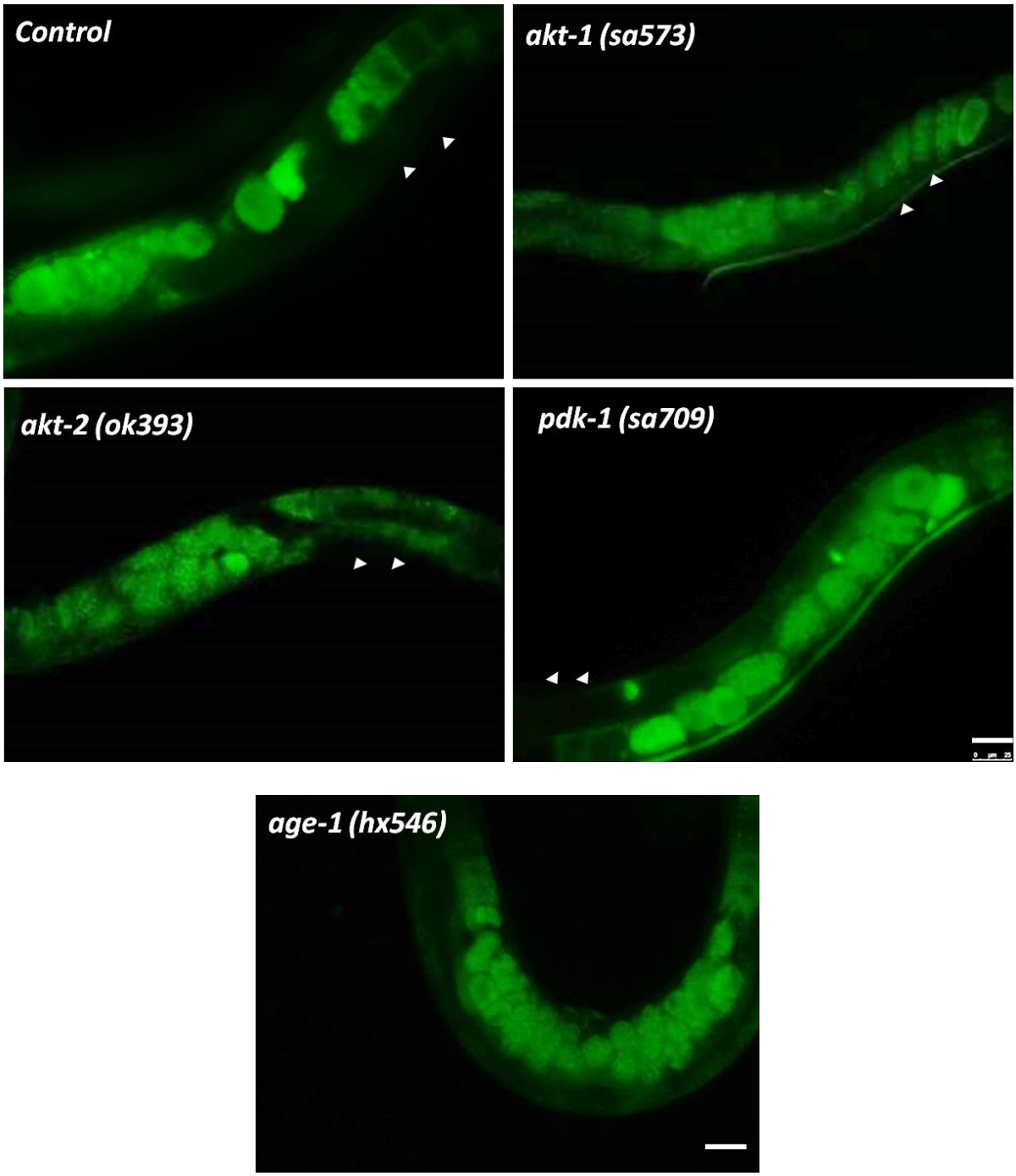


Figure 3.9: Analysis of YP170-GFP trafficking in PI3K/AKT signalling pathway mutant worms. *Control N2 worms and age-1, pdk-1, akt-1 and akt-2 mutant worms showed a WT phenotype with low levels of Yolk-GFP signal in intestine and body cavity and normal presence in oocytes and embryos. Scale bar, 25µm.*

3.4.5 PLCγ (PLC-3) signalling cascade is involved in regulation of membrane trafficking

itr-1

The *itr-1* mutant strains used in this analysis were JT73-[*itr-1(sa73)*]-loss of function (lf) and PS2582-[*itr-1(sy290) unc-24(e138)*]-gain of function (gf). The *itr-1* gain of function strain carries unc-24 which is positioned in *cis* to *itr-1* and therefore shows an unc phenotype. This unc phenotype serves as a marker when identifying *itr-1* gf mutants from wild type animals. The Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* was directly crossed into itr-1 gf mutants and the resulting F2 progeny homozygous for [*itr-1(sy290) unc-24(e138)*] and Yolk-GFP showing an unc phenotype were picked and analysed.

The itr-1 loss of function mutant strain doesn't carry a marker phenotype and therefore they were identified by using unc-24 strain as a genetic balancer. The Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* was first crossed into the balancer strain carrying *unc-24* and the resulting F2 progeny homozygous for Yolk-GFP and *unc-24* (unc phenotype) were crossed with *itr-1* (lf) mutant (males). The resulting F1 progeny gets *unc-24* positioned *trans* to *itr-1* and therefore from self fertilization the worms that are homozygous for Yolk-GFP and *itr-1* with a wild type phenotype were carefully picked and analysed

itr-1(sa73) is a loss of function mutant is viable at 20^{o}C but behaves as a near null at 25^{o}C (H. a. Baylis & Vázquez-Manrique 2012). It was found that *itr-1(sa73)* mutant showed a strong yolk-GFP trafficking defect with high accumulation of YP170-GFP in body cavity suggesting their role in regulation of membrane trafficking whereas *itr-1(sy290)* gain of function mutant showed a wild type phenotype (Figure 3.10).

pkc-2

The *pkc-2* mutant strain analysed was VC127 *[pkc-2(ok328)]*. *Pkc-2* mutants are difficult to differentiate from wild type animals and therefore, a balancer strain dpy-6 was used.

The Yolk-GFP strain RT130 *[unc-119(ed3); pwls23 [vit2::GFP; unc-119]* was first crossed into the balancer dpy-6 and the resulting F2 progeny homozygous for Yolk-GFP and dpy-6 (dumpy phenotype) were crossed with male *pkc-2* mutants. The resulting F1 progeny has dpy-6 positioned *trans* to *pkc-2* and therefore from self fertilization the worms that are homozygous for Yolk-GFP and *pkc-2* with a wild type phenotype were picked and analysed.

From the analysis it was found that *pkc-2* mutants showed a high accumulation of Yolk-GFP in the pseudocoelom and reduced accumulation in intestine and embryos indicating a strong endocytosis defect (Figure 3.10). Thus from this analysis of the PLC/PKC-2 signalling pathway for Yolk-GFP trafficking, it is evident that the signalling components of PLC-γ pathway are essential for proper regulation of membrane transport.

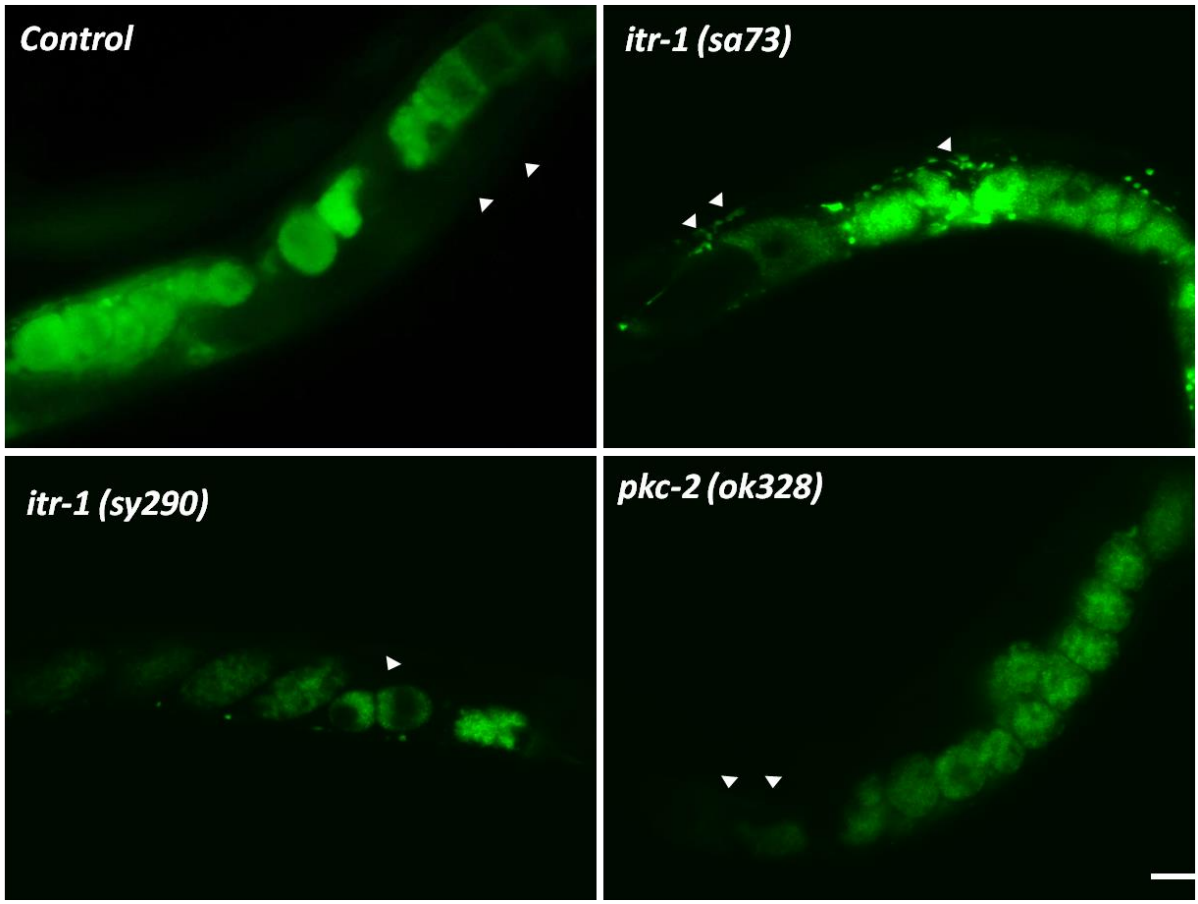


Figure 3.10: Analysis of Yolk-GFP trafficking in PLC-3 signalling pathway mutant worms. *The control N2 worms showing a WT phenotype with high accumulation of Yolk-GFP in embryos and oocytes and little accumulation of Yolk-GFP in intestine. itr-1(sa73) mutant worms showing a high accumulation of Yolk-GFP in the body cavity. itr-1(sy290) and pkc-2 (ok328) showing a WT phenotype. Scale bar, 25µm.*

3.5 Discussion

From previous studies it was found that the worm FGFR *egl-15* and some of the downstream components of FGF-FGFR signalling pathway such as *sem-5*, *sos-1* and *let-60* could possibly regulate membrane trafficking (Balklava et al. 2007). Here in this study, the role of FGF-FGFR signalling pathway in membrane traffic was analysed. YP170-GFP assay is one of the widely used method to analyse membrane trafficking as it shows the common membrane trafficking defects in endocytosis and secretion. The components of FGF-FGFR signalling pathway were knocked-down (using RNAi) or knocked-out (using mutants) and trafficking of Yolk-GFP was analysed. From the initial RNAi experiments it was found that the worm FGFR *egl-15*, component of Ras activating complex *sem-5*, MAPK components including *lin-45*, *mek-2*, *mpk-1* and PLC-3 pathway component *plc-3* all showed a strong Yolk-GFP trafficking defects. Surprisingly the ligands of *egl-15* such as *egl-17* and *let-756* showed a WT phenotype whereas the key activators of downstream transduction pathways such as *sos-1* and *let-60* showed a weak Yolk-GFP trafficking defect. It was also found that the members of AKT kinase pathway showed a WT phenotype. These positives obtained from Yolk-GFP assay were also tested with the RME-2-GFP assay. The FGF-FGFR signalling components which showed a Yolk-GFP trafficking defect also showed a poor trafficking of RME-2-GFP (results not shown). Though RNAi is one of the robust tools in silencing genes, it has its drawbacks and one needs to be careful when interpreting results. The possible interpretations from this initial RNAi results where that some of the genes that showed a WT phenotype either didn't respond to RNAi cnock-down or alternatively they don't regulate membrane trafficking. The reasons known to affect RNAi efficiency are 1. Endogeneous genes that inhibit RNAi silencing 2. Tissue specificity (Timmons 2004). In some cases, genes are thought to possess intrinsic RNAi resistance but the reasons are not yet fully understood (Cutter et al. 2003). Genes such as *eri-1, lin-15* and *rrf-3* are known to strongly reduce RNAi effectiveness and this is the reason why

some RNAi experiments are carried out in either *eri-1* or *rrf-3* mutant backgrounds. In one of the studies, RNAi against GFP in *C.elegans* showed decreased levels of GFP in all tissues except the neurons and it is believed that neuronal cells might express a protein that interferes with RNAi mechanism. RNAi efficiency also varies depending on temperature and protein half-life. Therefore in order to verify the yolk trafficking defective phenotypes, FGF-FGFR signalling pathway mutants were used. Most of the mutants displayed a similar phenotype as observed in RNAi experiments however this time the genes such as *egl-17, let-756, sos-1* and *let-60* showed a Yolk-GFP trafficking defect. The loss of function mutation in let-60 would result in lethality and are not viable and hence gain-of-function let-60 mutational strain was used in this study. The gain-of-function mutation produced an endocytosis defect.

The loss of let-60 functionality by RNAi produced no distinct phenotype unlike the previous study by Balklava et al., 2007 of let-60 RNAi on a sensitive background (rrf-3) which produced an endocytosis defect. From this it can be inferred that the background sensitivity could be a factor inducing trafficking defects in case of RNAi and either the loss or gain of function mutation affects trafficking since a balanced and optimum level of signalling could be required to regulate trafficking. Underactivating or overactivating the FGFR signalling might result in trafficking defects. One of the ways to confirm this hypothesis would be to use gain of function mutation strains for all FGFR signalling components.

The signalling components from the AKT kinase still showed a WT phenotype in mutant worms suggesting that they may not be involved in membrane trafficking. PLC-γ pathway components including *itr-1* and *pkc-2* which were not analysed in RNAi experiments were investigated as mutants. *itr-1* (loss of function) mutation showed a strong Yolk-GFP trafficking defect whereas *pkc-2* showed a WT phenotype.

Previous studies have proved that *plc-3* and *itr-1* act through IP3 pathway to regulate defecation in *C.elegans*. A similar assumption can be made here that *plc-3* and *itr-1* to function through IP3 to regulate membrane trafficking. Though it is interesting to find *pkc-2* showing a wild type phenotype, it is possible that it could be due to redundancy. There are four genes that encode PKC namely *tpa-1*, *pkc-1*, *pkc-2* and *pkc-3* in which *pkc-2* alone encodes six PKC isoforms (H. A. Baylis & Vázquez-Manrique 2012). Previous studies have shown that PKC-2 executes an isoform specific physiological function (Tabuse 2002). Therefore one can say that a loss of function mutation could have been compensated by other components associated with the IP3 signalling pathway. Taking all these into consideration, *egl-15*, the worm FGFR is found to regulate membrane trafficking via MAPK and PLC-γ signalling pathways.

CHAPTER 4: WORM FGFR (*egl-15)* REGUALTES SEVERAL STEPS OF ENDOCYTOSIS

4 WORM FGFR *egl-15* REGULATES SEVERAL STEPS OF ENDOCYTOSIS.

4.1 Introduction

The precise steps at which membrane trafficking is affected can be identified by studying specific membrane markers that are enriched on different compartments. Different compartments are used in trafficking of different cargo proteins. The internalised cargo is transported to early endosomes where they are sorted to be either delivered to trans-Golgi network or recycled to plasma membrane or degraded by the lysosomes (Chen et al. 2010).

The *C.elegans* intestine is a one cell layer thick polarized epithelial tube (Leung et al. 1999). Epithelial cells are polarized and have an apical and basolateral plasma membrane (Altschuler et al. 1999). The apical membrane faces the lumen and helps in the uptake of nutrients from the environment and the basolateral membrane faces the body cavity and helps in exchange of molecules between the intestine and body cavity (Shi et al. 2012). They are both different in composition and functions. The clathrin-mediated endocytosis at the basolateral membrane is similar to that of constitutive endocytosis at the plasma membrane of non-polarised cells whereas, the clathrin-mediated endocytosis at the apical membrane is too slow about one fifth compared to that of endocytosis at the basolateral membrane (Altschuler et al. 1999).

Different membrane markers and cargo proteins are used in studying different trafficking defects based on their function, localization, distribution and their own trafficking.

The RAB protein markers tagged to GFP are commonly used to study the membrane trafficking. RABs selectively regulate the transport of vesicles and help in the fusion of these vesicles with appropriate donor membranes. They have been primarily associated with vesicle docking and it is assumed that RABs initiate transport vesicle budding from the donor compartment and fuses it with the acceptor? compartment by docking and is finally recycled

back to the donor compartment (P Novick & Zerial 1997). The RAB proteins are generally maintained in their GDP bound inactive form by the RAB GDP dissociation inhibitor (RAB GDI) (Garrett et al. 1994). It is found that the association of RAB GDI with RAB proteins are disrupted by factors like RAB effectors and thus facilitate the binding of RAB to the membrane. Following this, a GDP/GTP nucleotide exchange occurs which results in the activation of RAB proteins which renders RAB resistant to removal by RAB GDI (Ullrich et al., 1994; Soldati et al., 1994). The active RABs now come in contact with effector proteins that facilitate trafficking in the destined pathway. The GTPase-activating proteins (GAPs) then hydrolyse RABs (GTP bound-ON form) which reverts them back to inactive GDP-bound OFF form (Pfeffer 2001). This exchange reaction between GDP/GTP is catalysed by GEF (Guanine Exchange Factors). The inactive RAB is then extracted by RAB GDI from the membrane. The RAB which is now bound to GDI is reattached from a membrane and continue the cycle again (Collins 2003). The role of GEFs and GAPs are also important as they determine which nucleotide is bound to RAB proteins on the membrane and regulate the association of RAB proteins to RAB GDI (Fukui et al. 1997). In order to bind to membranes, RAB proteins must be prenylated which is facilitated by the catalysing enzyme RAB geranylgeranyltransferase (GGTase). Thus, all these factors maintain RAB proteins in an equilibrium between the membrane and the cytosol (Peter Novick & Zerial 1997). Different RAB proteins are involved in each step of vesicular transport and this finding was initially seen from studies in yeast. For example, the yeast RAB GTPase Sec4p, a key regulator of exocytosis in yeast helps in the transport of Golgi-derived secretory vesicles to the plasma membrane (Salminen & Novick 1987). The association of RABs with specific effector proteins play a major role in vesicle formation, tethering, cargo selection, vesicle movement and fusion.

The recruitment of effectors by RABs is essential for vesicle movement along actin or microtubule-based cytoskeletal structures. For example, in mammalian cells, RAB11 regulates

plasma membrane recycling by interacting with myosin Vb through its effector protein RAB11 family interacting protein 2 (RAB11-FIP2) (Hales et al. 2002). RABs have also been found to interact with microtubule based motor proteins such as kinesin (plus end motors) and dynein (minus end motors) to carry out vesicle movement. Some RABs can also function in both the endocytic (retrograde) and exocytic (anterograde) pathways. For example, RAB6 which has been found to be localised with Golgi regulates a retrograde traffic between endosomes, Golgi and ER but recently studies also show that they regulate an exocytic traffic to the plasma membrane (Dejgaard et al. 2008; Girod et al. 1999; Grigoriev et al. 2007; White et al. 1999). Thus the association of RAB proteins to different membranes and their role in formation, docking and fusion between different compartments make them excellent markers to precisely study exactly which transport step is affected. Below is a table showing some of the RAB markers that are used to identify the defects in transport steps.

Table 4.1. RAB proteins, their localisation and function

Protein	**Localisation**	**Function**
RAB1A, RAB1B RAB2	Golgi intermediate compartment *cis* Golgi network (CGN)	ER to Golgi transport (Saraste et al. 1995) (Tisdale et al. 1992)
RAB4A, RAB4B	Early endosomes	Recycling from early endosome to plasma membrane (van der Sluijs et al. 1992)
RAB5	Plasma membrane, Clathrin coated vesicles Early endosomes	Plasma membrane to early endosome transport (Gorvel et al. 1991)
RAB7	Late endosomes	Early to late endosome transport (Papini et al. 1997)

RAB9	TGN, late endosomes	Late endosome to TGN transport (Lombardi et al. 1993)
RAB10	Early endosomes Golgi and post Golgi vesicles	Endocytic recycling (Shi et al. 2012)
RAB11	TGN, recycling endosome, Post-Golgi complexes	Transport through recycling Endosomes (Ullrich et al. 1996)
RAB35	Plasma membrane, early Endosomes, clathrin coated pits (CCP)	Controls fast recycling to Plasma membrane (Kouranti et al. 2006)

Along with RABs, another small GTPases which have been implicated in membrane trafficking are the ADP ribosylation factors (ARFs) (Chavrier & Goud 1999). Arf proteins have been found to be involved in regulation of both endocytic and exocytic pathways by maintaining the organelle integrity, assembly of coat proteins (Boman & Kahn 1995), vesicle formation, tethering and docking (Chavrier & Goud 1999). Arf proteins were first identified as cofactors for cholera toxin and are grouped under the members of Ras superfamily of GTP proteins (Kahn & Gilman 1984) (Kahn & Gilman 1986). So far, six mammalian ARF proteins have been found and they are classified into three classes based on their primary structure. ARFs 1, 2, and 3 are in Class I, ARFs 4, and 5 are in Class II and ARF6 is in Class III.

Among these ARF proteins, ARF1 and ARF6 are the most extensively studied and are often used as representative members of the family (Moss & Vaughan 1998) (Randazzo et al. 2000). ARF1 has been associated with trafficking from ER to Golgi and transport from the trans Golgi network, whereas ARF6 has been associated with endocytosis, receptor recycling, and

phagocytosis. ARFs have also been found to activate Phospholipase D and also involved in lipid metabolism which helps in the maintaining the integrity of organelle structure and their transport. The molecular basis underlying ARF regulation of membrane trafficking has been well established in ARF1 compared to that of ARF6 and vesicle coat proteins such as COPI and the AP1-clathrin complex play a major role in this regulation. They help in formation of vesicles and sorting and transport of cargoes between membrane bound organelles. Similar to RABs, ARFs also cycle between GTP-bound (active) and GDP-bound (inactive) states. The GTP/GDP cycle and the activity of GEFs and GAPs are essential to complete the sequence of events comprising of sorting, transporting and formation of vesicles.

In this study, we used ARF6 as the representative in *C.elegans*. ARF6 is localised to the cell periphery and based on their nucleotide status they cycle between the plasma membrane and the intracellular endosomal compartment (D'Souza-Schorey et al. 1995). Peters et al(1995) have shown one of the roles of ARF6 in membrane trafficking. They took two mutant forms of ARF6, ARF6 which is defective in GTP hydrolysis and hence remains active in GTP bound conformation (constitutively active) and the other mutant which is in the GDP bound conformation (dominant negative or inactive). They found that the GTP-bound ARF6 was localised exclusively to the plasma membrane which resulted in a decreased rate of transferrin uptake whereas, the GDP-bound ARF6 was localised in intracellular compartments and resulted in an intracellular accumulation of transferrin receptors and inhibition of recycling of ligands back to the cell surface suggesting that ARF6 could be involved in recycling rather than uptake. Later studies showed that ARF6 is localised to a recycling compartment and could regulate the outward flow of recycling membrane (D'Souza-Schorey et al. 1998).

Alpha mannosidase II-GFP, a specific Golgi marker (Rolls et al. 2002) along the secretory pathway was used in this study to analyse the distribution and localisation of GFP-MANS in the *egl-15* mutant worms. The *C.elegans* contain many small Golgi stacks unlike the

vertebrates that contain one large Golgi stack near the nucleus (Shi et al. 2012). Shi et al (2012) have also reported recently that GFP-MANS colocalise with RAB-10 significantly in the trans-Golgi network and apical recycling endosomes which shows that MANS along with RAB-10 performs its functions in the secretory pathway.Similar to GFP-MANS, SP12 (Signal peptidase 12), a 12 kDa endoplasmic reticulum marker was used to identify if transport steps along the ER was affected. The distribution and localisation of GFP-SP12 in the *egl-15* mutant worms was analysed.

The amount of information available on the sorting and recycling of molecules from the endosomes to the plasma membrane is less compared to the information available on the transport events of internalisation. Recent studies have shown that RME-1 is a novel protein which is found to be involved in endocytic recycling. In *C.elegans*, the amount of yolk uptake is dependent on the efficiency of yolk receptor recycling and it has been shown that RME-1 is essential in yolk receptor recycling (Grant et al. 2001). Loss of RME-1 function results in endocytic defects in most cell types and it has been found that RME-1 is expressed predominantly in the plasma membrane and recycling endosomes and particularly in basolateral recycling endosomes in the intestine of *C.elegans*. RME-1 mutants also show enlarged endosomes filled with endocytic fluid in the intestine thereby showing its role in endocytic recycling (Grant & Caplan 2008). Thus, the marker GFP-RME-1 has been used in this study to see if the loss of *egl-15* function would have any impact on endocytic recycling.

In this study we also investigated some of the transmembrane cargo proteins to see their trafficking with the loss of *egl-15* function. The human transferrin receptor (hTFR)-) and the α-chain of the human interleukin 2 receptor (hTAC) were expressed as GFP fusions in the *C.elegans* intestine and the localisation and distribution of these were compared between wild type and *egl-15* mutant animals. The hTFR undergoes a clathrin-dependent endocytosis to the early endosomal system and gets recycled back to plasma membrane through the Endosomal

Recycling Compartment (ERC) whereas hTAC undergo a clathrin-independent endocytosis via non-coated invaginations like caveolae (Kurzchalia & Parton 1999) and are recycled back to the plasma membrane through a distinct tubular endosomal compartment (Radhakrishna & Donaldson 1997; Caplan et al. 2002).

The initial study to understand the role of FGFR signalling pathway components in membrane trafficking using YP170-GFP and RME-2-GFP assays suggested that FGFR affects membrane transport via the MAPK and PLC-γ pathways. We observed defects of both endocytosis and secretion with the disruption of FGFR pathway components. The next focus of study was to pinpoint exactly which transport step was affected. The different membrane compartments such as endosomes (early/late/recycling), lysosomes, Golgi, ER and their respective marker proteins (Figure 4.1) were analysed in the intestine of worms. The expression of these membrane markers in the intestine was achieved from the intestine- specific *vha-6* promoter. The distribution, localization, morphology and expression levels of these membrane markers were compared and analysed between wild type and *egl-15* mutant worms.

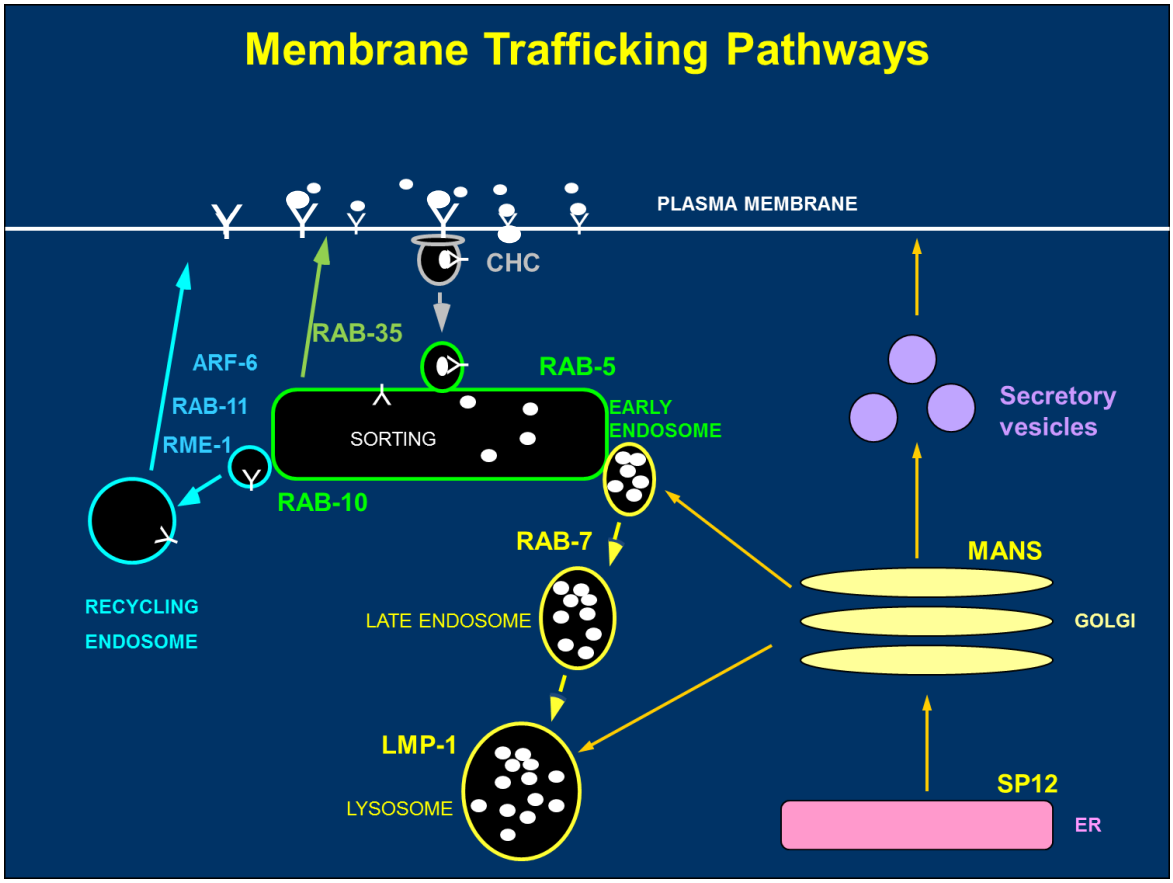


Figure 4.1: Overview of membrane trafficking pathways, compartments and membrane markers.

Table 4.2: Membrane compartments and their respective membrane markers

Membrane Marker	Compartment
RAB-5	Early/late endosomes
RAB-7	Early/late endosomes and early lysosomes
RAB-10	Basolateral early endosomes, Golgi and apical recycling endosomes
RAB-11	Basolateral early endosomes, Golgi and apical recycling endosomes
RAB-35	Early recycling endosomes
MANS	Golgi

SP-12	Endoplasmic Reticulum
RME-1	Basolateral recycling endosomes
CHC-1	Clathrin Coated Pits (Plasma membrane)
hTAC	Clathrin-independent cargo
hTFR	Clathrin-dependent cargo
PH domain of PLCδ	Plasma membrane, recycling endosomes

4.2 RESULTS

4.3 *egl-15* mutation affects clathrin-mediated endocytosis

Analysis of GFP-CHC-1 distribution

CHC-1, a marker for clathrin-coated pits and vesicles was analysed. The CHC-1 strain GK35 [*unc-119(ed3); pwls72 [vha-6p::GFP::chc-1;unc-119]* and the *egl-15* mutant strain MT3456 *[egl-15(n1477)]* were used in this analysis. The GK35 strain was crossed into *egl-15* mutant animals and were analysed. Clathrin-coated vesicles are formed at the plasma membrane and are actively involved in endocytosis. They are also found at the trans-Golgi network but their precise function at this organelle is still unclear (Robinson & Pimpl 2014). In this study, GFP-CHC-1 labelled a very large number of punctate structures throughout the cytoplasm likely representing Clathrin Coated Pits (CCPs) and Clathrin Coated Vesicles (CCVs) at the PM and TGN in the wild-type intestine. However, in *egl-15* mutants, a significantly reduced number of these punctate structures were observed (Figure 4.1).

A reduced accumulation of GFP-CHC-1 could indicate a decreased number of clathrin- coated pits or decreased clathrin expression. Either way, this phenotype could indicate impairment in clathrin-mediated uptake from the plasma membrane. Previous studies in mammalian cells have shown that FGFR activation increases clathrin spots on the plasma membrane and promotes clathrin-mediated endocytosis and FGFR itself is internalised through a clathrin-dependent endocytosis (Auciello et al. 2013). The decreased accumulation of CHC-1 in *C.elegans* intestine from this study could also suggest that the worm FGFR *egl-15* could be associated with the clathrin-dependent pathway. Clathrin is also found to localise at the TGN. Taken together, this result suggests that the functions of the clathrin along the endocytic/sercretory pathway and may be even the recycling could be affected in worms lacking FGFR function.

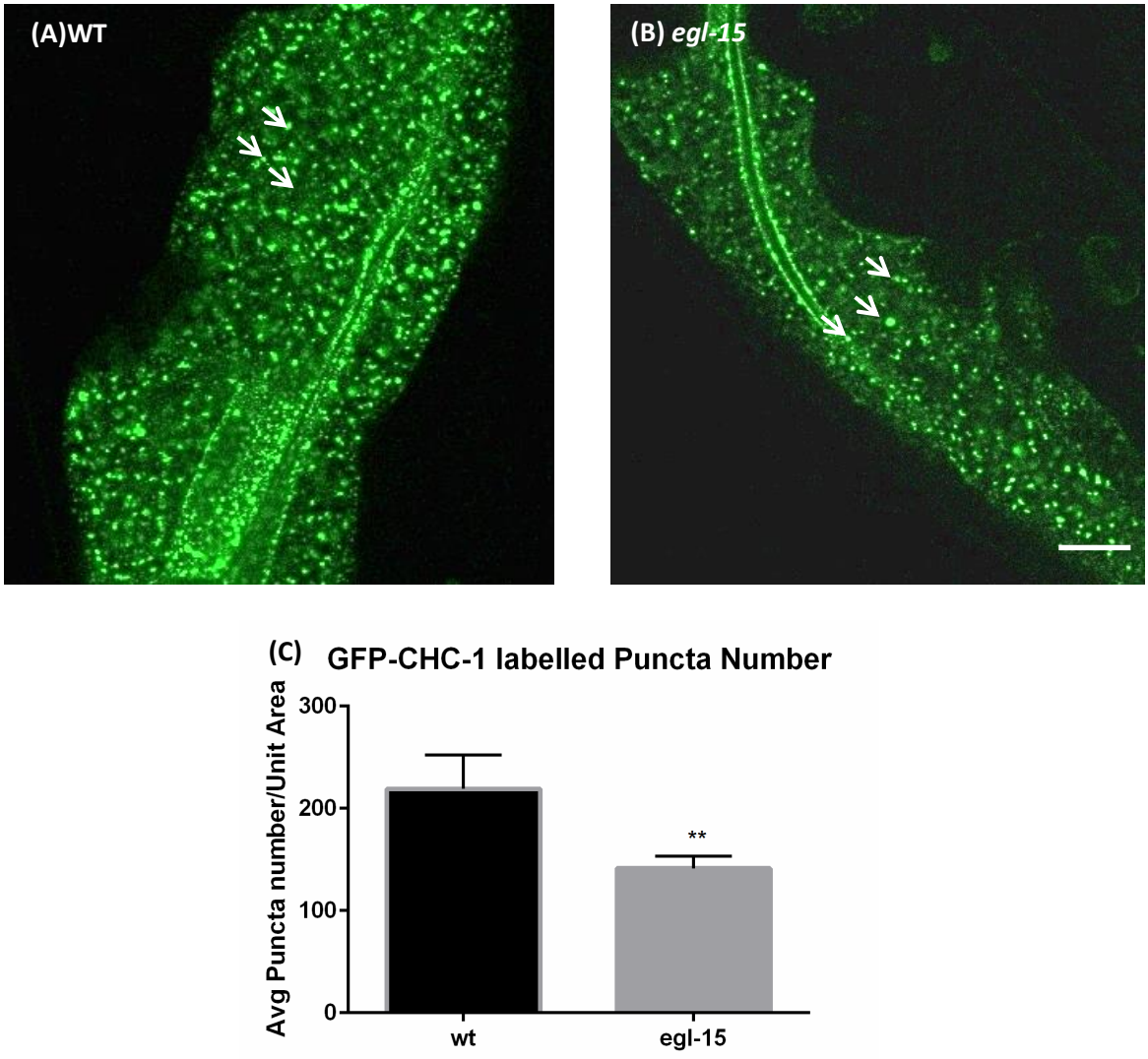
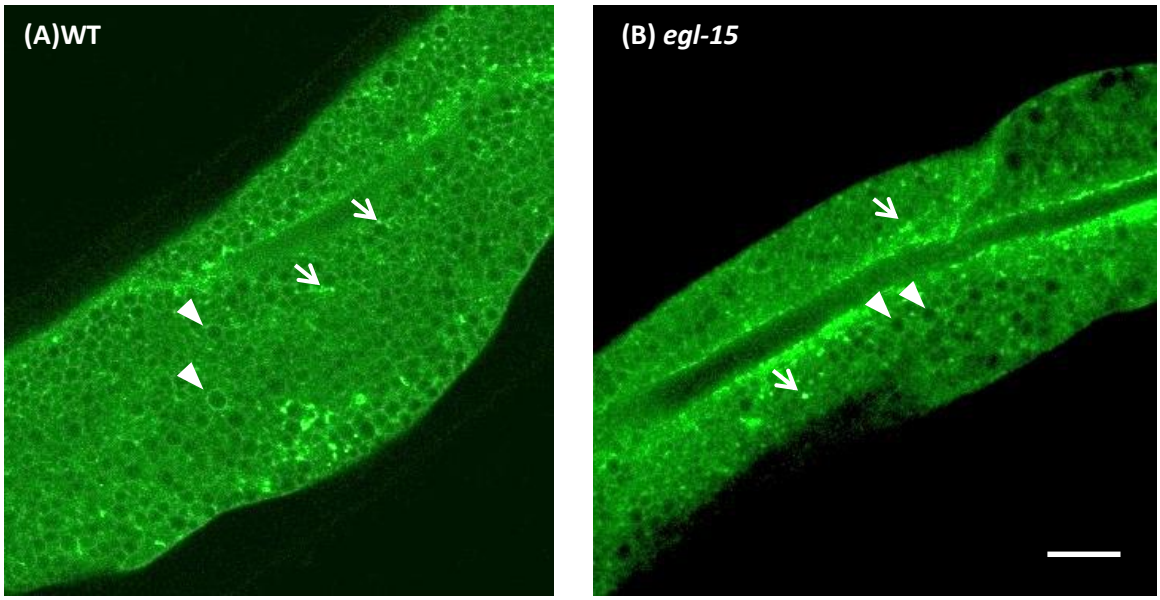


Figure 4.2: Confocal images displaying GFP-CHC-1 in the intestine of WT and egl-15 mutants. *A high number of punctate structures observed in the intestine of wild-type worms (A) and a significantly reduced number of puncta observed in the intestine of egl-15 mutants (B).Arrows indicate puncta (endosomes). Quantification of endosome number as visualised shown in (C). Error bars represent standard deviation of the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and egl-15 mutant animals. ** P<0.05. Scale bar=10 µm*

4.4 *egl-15* mutants show early to late endosome trafficking defect

Analysis of GFP-RAB-5 distribution

The distribution of various endolysosomal proteins in *egl-15* mutants was examined. GFP-RAB-5 is associated with the early endosomes (Grant *et al.*, 2006). The GFP-RAB-5 strain RT327 [*unc-119(ed3); pwls72 [vha-6p::GFP::RAB-5;unc-119]* and the *egl-15* mutant strain MT3456 *[egl-15(n1477)]* was used in this analysis. The RT327 strain was crossed into *egl-15* mutant animals and the resulting mutant transgenic worms were analysed. GFP-RAB-5 labelled a very small number of punctate structures and many ring like vesicles in wild- type intestine which likely represent early and late endosomes respectively. In *egl-15* mutants, however, GFP-RAB-5 labelled more of punctate structures and significantly fewer ring-like vesicles suggesting that the early to late endosome step transport step is affected (Figure 4.3).



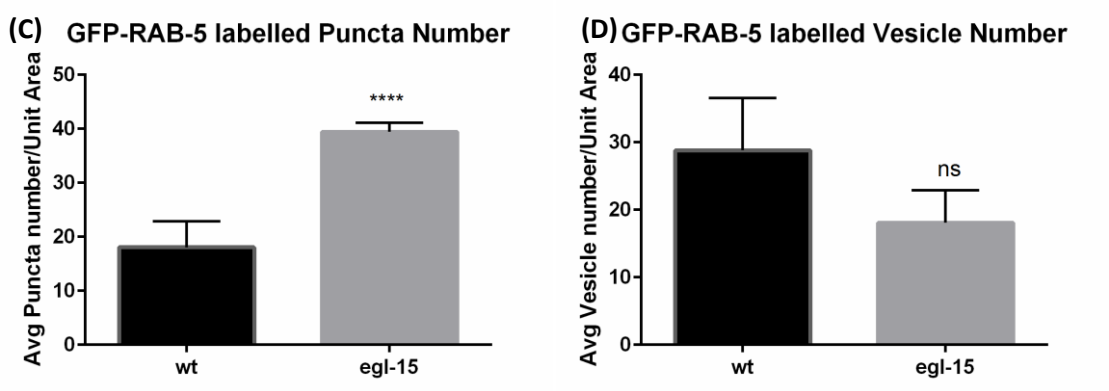


Figure 4.3: Confocal images displaying GFP-RAB-5 in the intestine of WT and egl-15 mutants*. A high number of ring like vesicles (late endosomes) and less number of puncta (early endosomes) observed in the intestine of wild-type worms (A) and on the contrary a reduced number of vesicles and high number of puncta observed in the intestine of egl-15 mutants (B). Arrows indicate puncta (early endosomes) and arrow heads indicate vesicles (late endosomes). Quantification of endosome number as visualised shown in C and D. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and egl-15 mutant animals. ** P<0.05. Scale bar=10 µm*

Analysis of GFP-RAB-7 distribution

RAB-7, a marker for early/late endosomes and early lysosomes was analysed (Grant *et al.*, 2006). The GFP-RAB-7 strain RT476 [*unc-119(ed3); pwls170 [vha-6p::GFP::RAB-7;unc-119]* and the *egl-15* mutant strain MT3456 *[egl-15(n1477)]* was used in this analysis. The RT476 strain was crossed into *egl-15* mutant animals and the resulting mutant transgenic worms were analysed. It was found that, GFP-RAB-7 animals labelled distinct ring like vesicles which represent the late endosomes in wild-type intestine. However, in *egl-15* mutant animals, there was a significant reduction in these ring-like vesicles but with few aggregates of

punctate structures (rarely seen in wild-type animals) likely to represent early endosomes (Figure 4.4). This result suggests that the transport step from early to late endosomes is affected in *egl-15* mutants.

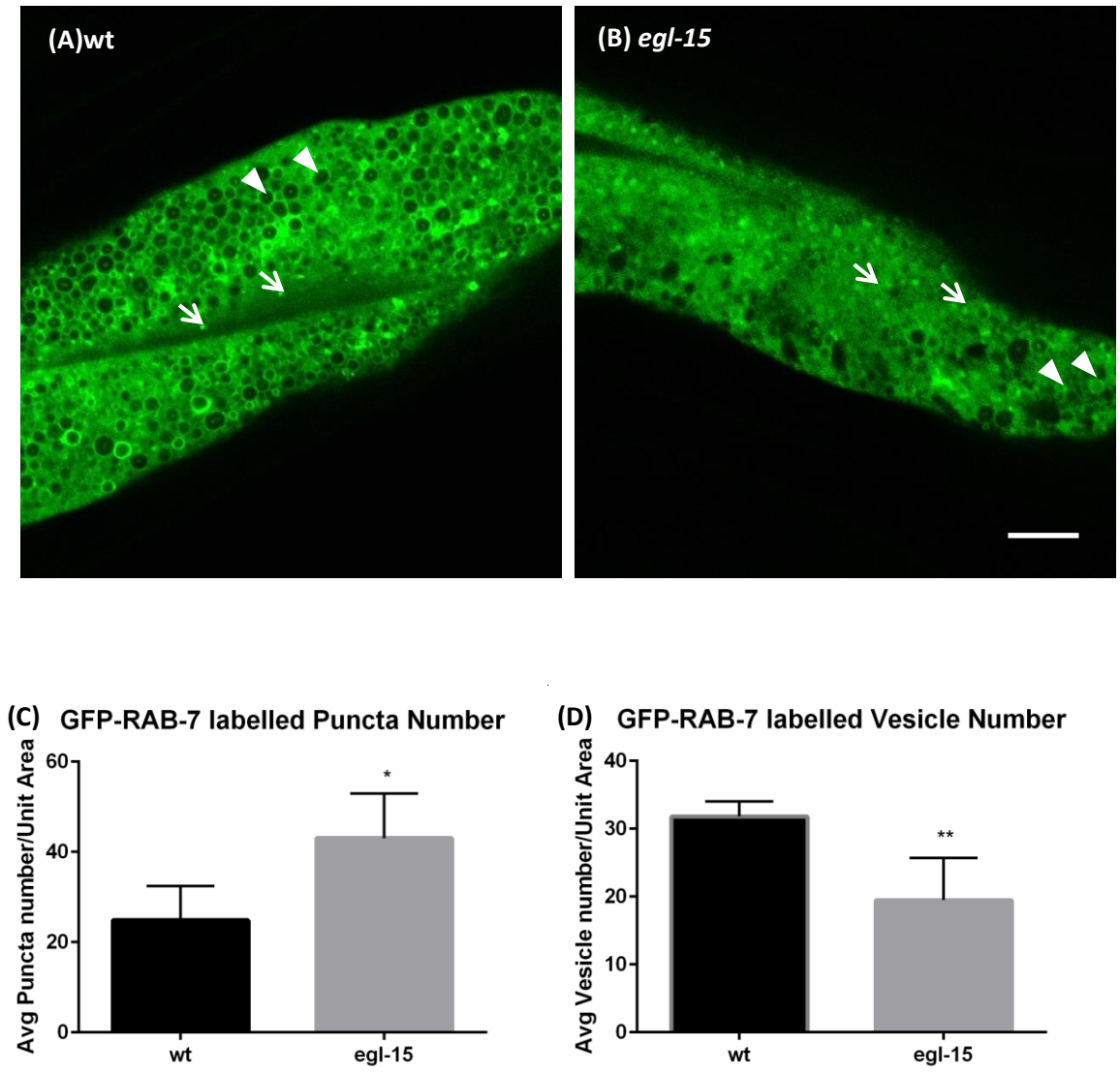
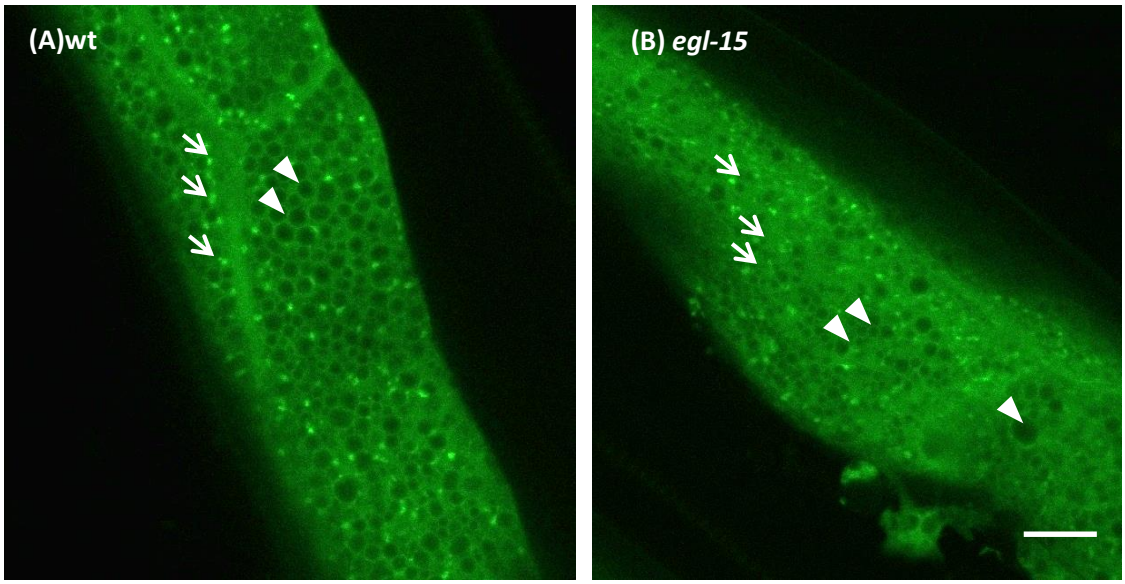


Figure 4.4: Confocal microscopy images displaying GFP-RAB-7 in the intestine of WT and egl-15 mutants. *A high number of ring like vesicles (late endosomes) and small number of puncta (early endosomes) observed in the intestine of WT worms (A) and on the contrary a reduced number of vesicles and high number of puncta observed in the intestine of egl-15 mutants (B). Arrows indicate puncta (early endosomes) and arrow heads indicate vesicles (late endosomes). Quantification of endosome number as visualised shown in C and D. Error bars*

*represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and egl-15 mutant animals. ** P<0.05. Scale bar=10 µm*

Analysis of GFP-RAB-10 distribution

RAB-10 associates predominantly with early endosomes, some apical recycling endosomes and the Golgi (Grant *et al.*, 2006). The GFP-RAB-10 strain RT525 [*unc-119(ed3); pwls206 [vha6p::GFP::RAB-10;unc-119]* and the *egl-15* mutant strain MT3456 *[egl-15(n1477)]* was used in this analysis. The RT525 strains were crossed into *egl-15* mutant animals and the resulting mutant transgenic worms were analysed. It was observed that GFP-RAB-10 displayed both a punctate staining pattern and ring like vesicles in the wild-type intestine. Although not significantly different, a slightly higher number of GFP-RAB-10 punctate structures and vesicles were observed in *egl-15* mutants (Figure 4.5).



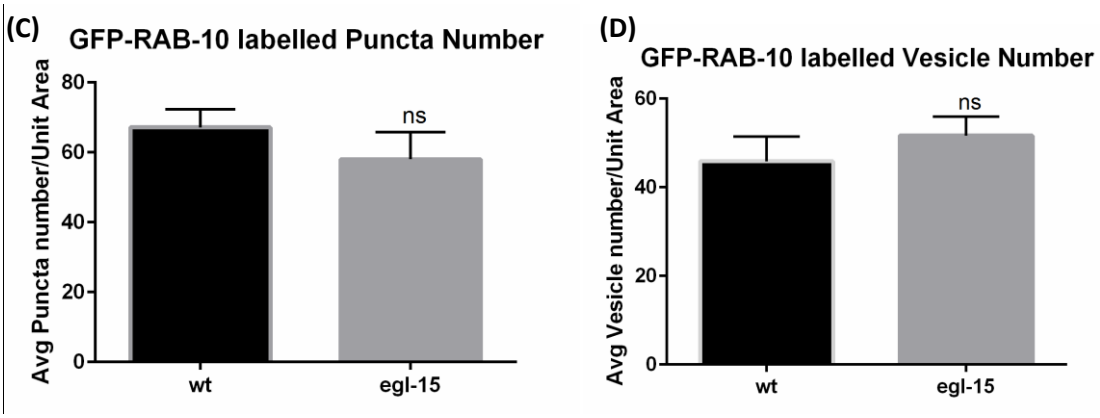


Figure 4.5: Confocal microscopy images displaying GFP-RAB-10 in the intestine of WT and egl-15 mutants. *Arrows indicate puncta (early endosomes) and arrow heads indicate vesicles (endosomes). No significant difference was observed in the puncta or the vesicle number between the wild-type (A) and egl-15 mutants (B). Quantification of endosome number as visualised shown in C and D. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Scale bar=10 µm*

4.5 *egl-15* mutations affect recycling endosomes

Analysis of GFP-RAB-35 distribution

RAB-35 is associated with early recycling endosomes (Sato *et al.*, 2008). The GFP-RAB-35 strain RT910 [*unc-119(ed3); pwls355 [vha6p::GFP::RAB-35]* and the *egl-15* mutant strain MT3456 *[egl-15(n1477)]* were used in this analysis. The RT910 strains were crossed into *egl-15* mutant background animals and the resulting mutant transgenic worms were analysed. GFP-RAB-35 labelled distinct small ring-like vesicles which represent the recycling endosomes in wild-type intestine. However, in *egl-15* mutant animals, there was a significant reduction in these ring-like vesicles (Figure 4.6) suggesting that RAB-35-positive recycling endosomes are affected.

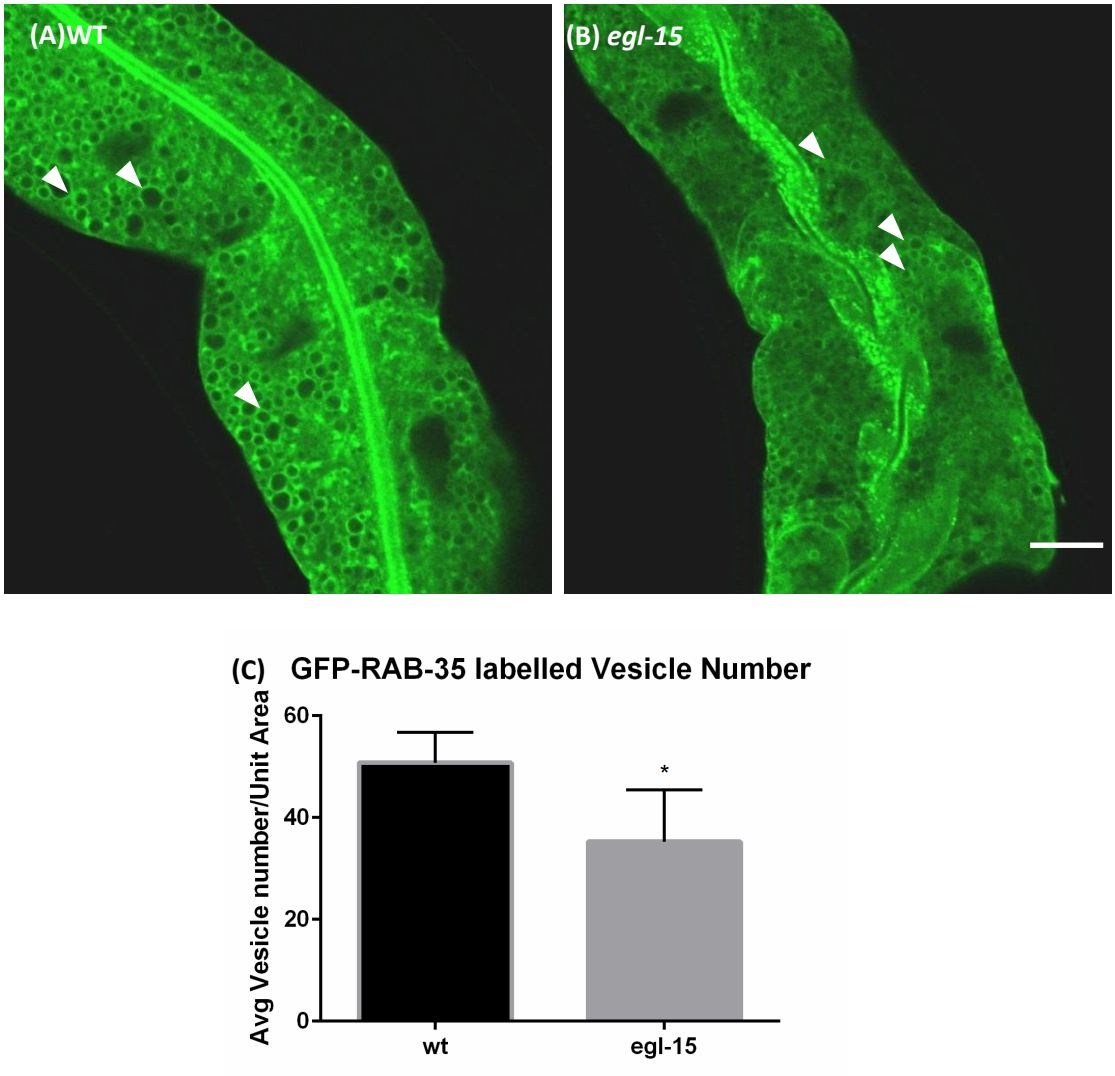
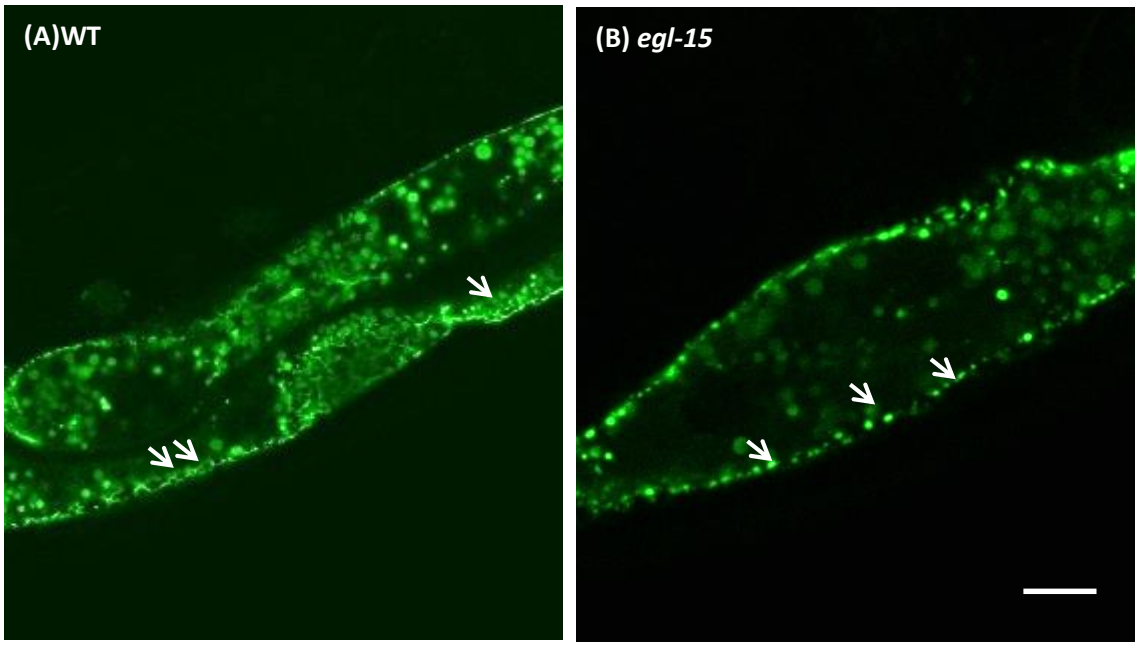


Figure 4.6: Confocal microscopy images displaying GFP-RAB-35 in the intestine of wild-type and *egl-15* mutants. A significant difference was observed in the vesicle size and number between the WT (A) and *egl-15* mutants (B). Arrowheads indicate ring like vesicles (recycling endosomes). Quantification of endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and *egl-15* mutant animals. ** P<0.05. Scale bar=10 µm

Analysis of RME1-GFP distribution

RME-1 is associated with basolateral recycling endosomes in worm intestine (Grant et al., 2006). The GFP-RME-1 strain RT348 [*unc-119(ed3); pwls87 [vha-6p::GFP::rme-1]* and the *egl-15* mutant strain MT3456 *[egl-15(n1477)]* was used in this analysis. The RT348 strain was crossed into *egl-15* mutant animals and the resulting mutant transgenic worms were analysed. GFP-RME-1 labelled a mesh-like tubular-vesicular staining pattern, recycling endosomes (Grant *et al.*, 2006) along the basolateral membranes in the wild-type intestine However, in *egl-15* mutants, there was significantly reduced labelling of GFP-RME-1 puncta at both the apical and basolateral membranes suggesting a trafficking defect of endosomes (Figure 4.7)



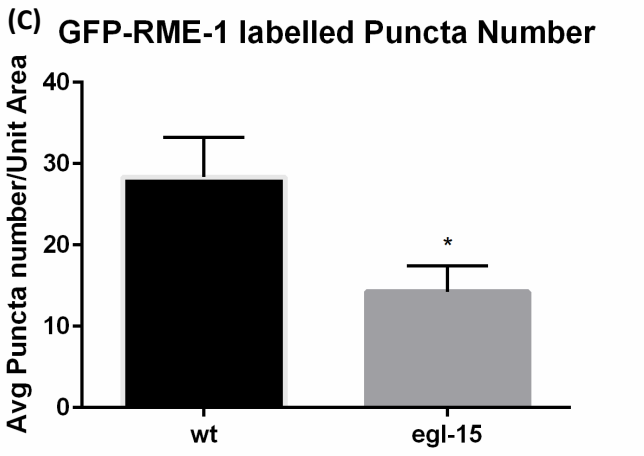


***Figure 4.7: Confocal images displaying** GFP-RME-1 **in the intestine of WT and egl-15 mutants**. A significant difference was observed in the vesicle number between the WT (A) and egl-15 mutants (B). Arrows indicate punctate structures (recycling endosomes) in the basolateral compartments. Quantification of recycling endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=20 each, 4 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and egl-15 mutant animals. ** P<0.05. Scale bar=10 µm*

Thus, from these investigations to it was found that endocytic trafficking through early endosomes is affected in *egl-15* mutants leading to disruption of recycling and late endosomes. The next focus was to study some of the cargo proteins such as hTfR and hTAC to see if their trafficking was affected in the mutant worm intestines.

4.6 Loss of *egl-15* function affects affect cargo protein transport

Analysis of GFP-hTFR distribution

hTFR, the human transferrin receptor is a cargo protein that is clathrin-dependent for its uptake and is normally transported to recycling endosomes or directly to plasma membrane from early endosomes following internalization (Lin *et al.*, 2001). GFP-hTFR has been previously shown to label basolateral membranes in wild-type worm intestine with localization to plasma membrane and proximal small endosomal vesicles and tubules (Chen et al., 2006). The GFP-hTFR strain RT1970 [*unc-119(ed3); pwls717 [vha-6p::GFP::c65ThTFRshort]* was crossed into *egl-15* mutant worms and the resulting mutant transgenic worms were used for analysis. GFP-hTFR labelled several punctate structures likely representing early/recycling endosomes in the worm intestine. Quantification results showed a significantly reduced number of GFP-

hTFR positive punctuate in *egl-15* mutants compared to wild type worms suggesting a cargo trafficking defect (Figure 4.8).

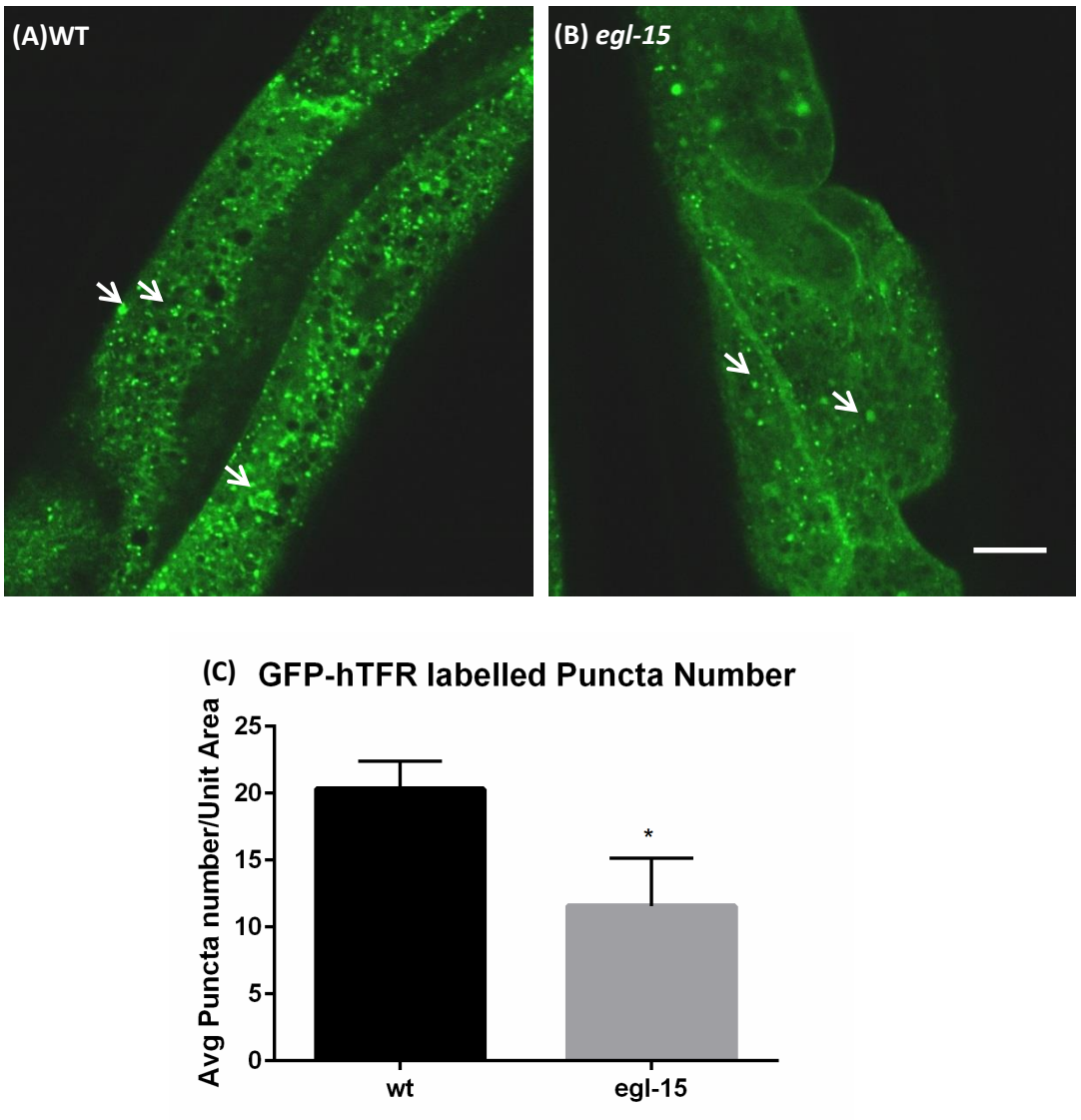


Figure 4.8: Confocal images displaying GFP-hTFR in the intestine of WT and egl-15 mutants. *A significant difference was observed in the puncta number between the wild-type (A) and egl-15 mutants (B). Arrows indicate punctate structures (endosomes). Quantification of endosome number (C) Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and egl-15 mutant animals. ** P<0.05. Scale bar=10 µm*

Analysis of GFP-hTAC

hTAC, the α chain of the human interleukin-2 receptor is a transmembrane cargo protein similar to hTFR but is internalised via clathrin-independent route (Caplan *et al.*, 2002). Like hTFR, hTAC has been primarily known to label basolateral membranes in wild-type worm intestine with localization to plasma membrane and proximal small endosomal vesicles and tubules (Grant *et al.*, 2006). The GFP-hTAC strain RT393 [*unc-119(ed3); pwls112 [vha6p::GFP::hTAC;unc-119]* was crossed into *egl-15* mutant worms and were used for analysis. It was found that GFP-hTAC labelled the apical and basolateral membranes in the wild-type worms however, they labelled weakly them in *egl-15* mutants. Also, the GFP-hTAC labelled puncta was not so visible and appeared quite diffused in *egl-15* mutants which made it difficult to quantify. Also, GFP-hTAC labelled abnormal big vacuoles in few worms. These results suggest that hTAC trafficking is compromised by loss of *egl-15* function (Figure 4.9).

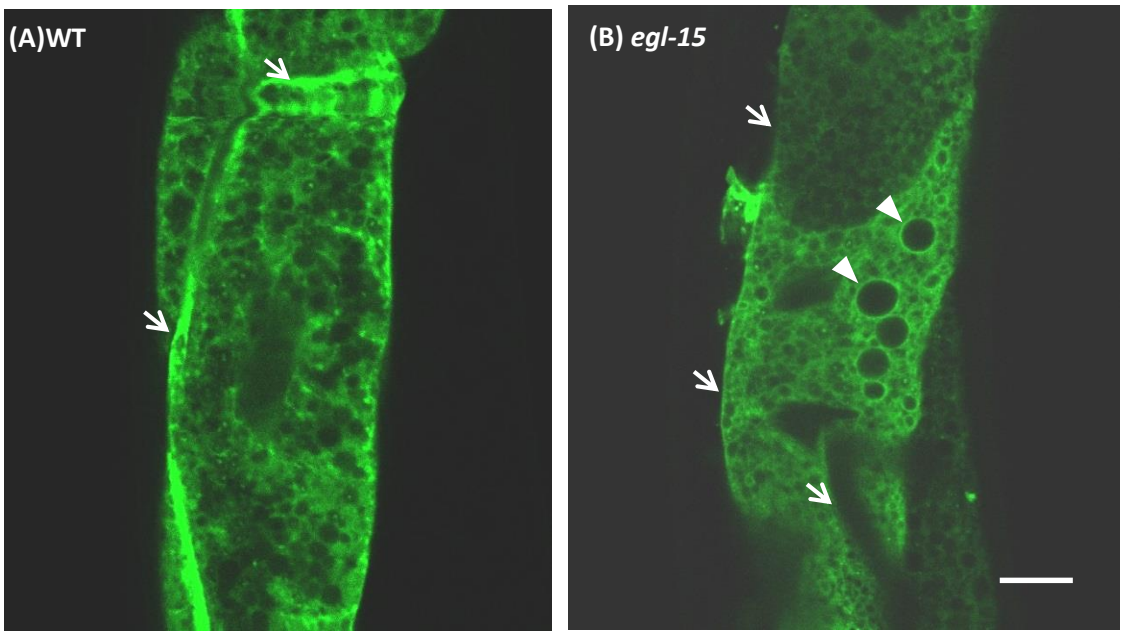


Figure 4.9: Confocal images displaying GFP-hTAC in the intestine of wild-type and egl-15 mutants. *Arrows indicate GFP-hTAC-puncta labelled more significantly along the membranes*

in the intestine of wild-type animals (A) to that of egl-15 mutants (B). Arrow heads indicate large abnormal vacuoles observed in egl-15 mutants (B). Scale bar=10 µm.

Analysis of ARF-6-GFP:

ARFs are a group of GTPases that are involved in membrane traffic. For example, in polarised cells MDCK (Madin-Darby Canine Kidney) cells, ARF-6 is thought to regulate clathrin-mediated endocytosis at the apical plasma membrane (Altschuler et al. 1999). In CHO cells ARF6 is found to be involved in endosomal recycling and in Hela cells ARF6 was found to be affecting the clathrin-independent cargo and not clathrin-dependent (Shi et al. 2012). Thus ARF6s function varies depending on the cell type.

For a long time, ARF-6 was known to localize only at the plasma membrane and intracellular endosomal compartments (D'Souza-Schorey et al. 1998) (Shi *et al.*, 2013) but studies in the last decade have shown that ARF-6 is localised to a recycling compartment and hence could also be involved in regulating the outward flow of recycling membrane (D'Souza-Schorey et al. 1998). For this study, the GFP-ARF-6 strain RT1579 *[unc-119(ed3); pwls601 [vha-6p::GFP::arf-6;unc-119]* was crossed into *egl-15* mutant animals and the resulting mutant transgenic worms were used for analysis. GFP-ARF-6 labelled the apical membrane to a higher degree than the basolateral membrane and a slightly diffused mesh like vesicles and punctate structures throughout the cytoplasm in the intestine of wild-type animals (Figure 4.10). In *egl-15* mutants, GFP-ARF-6 marked the apical membrane to the same extent as observed in wild-type however in the cytoplasm, enlarged vesicles positive for ARF-6-GFP were found. Moreover there was a significant reduction in number of GFP-ARF-6 labelled vesicles in *egl-15* mutants compared to wild type worms.

These vesicles likely represent enlarged early endosomes suggesting a transport defect from the early to recycling or late endosomes.

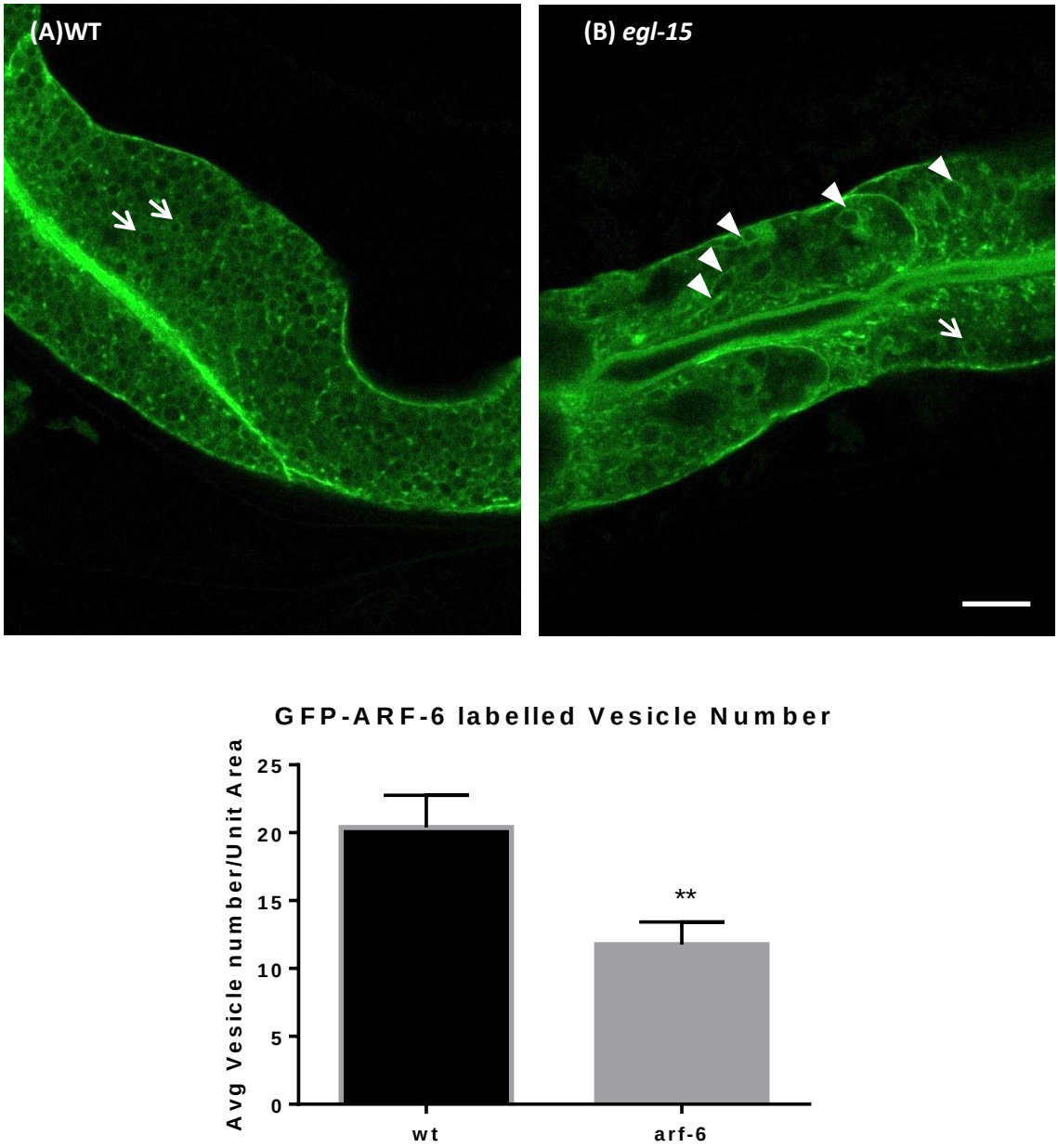
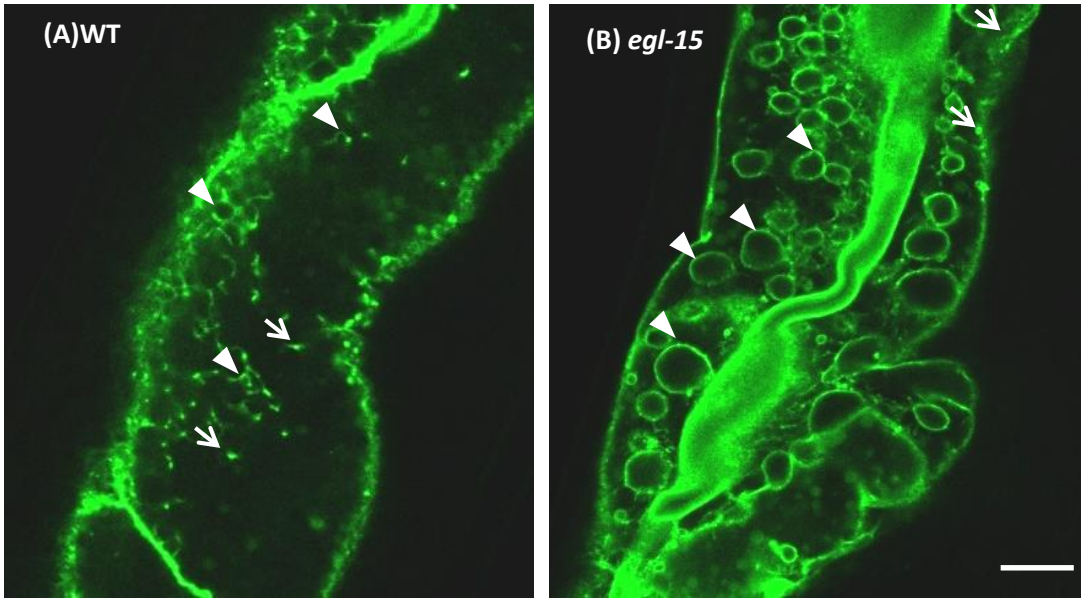


Figure 4.10: Confocal images displaying GFP-ARF-6 in the intestine of WT and egl-15 mutants. *Arrows indicate ring-like vesicles (early/recycling endosomes) in the wild-type (A) and egl-15 mutant animals (B). Arrowheads indicate enlarged abnormal endosomes in the intestine of egl-15 mutants only (B). Asterisks indicate a significant difference in the two-tailed Student's t test between control and egl-15 mutant animals. ** P<0.05. Scale bar=10 µm*

Analysis of PI(4,5)P_2 (PH domain of PLCδ):

Plekstrin Homology domain of PLCδ is a marker for the PI(4,5)P_2 which is mainly localized at the plasma membrane. The PH-GFP strain RT1120 *[unc-119(ed3); pwls446 [vha-*

6p::GFP::PH;Cbunc-119] was crossed into *egl-15* mutant animals and the resulting mutant transgenic worms were used for analysis. It was found that PH-GFP labelled the basolateral and apical membranes in the wild-type intestine. Strikingly in *egl-15* mutants, GFP-PH labelled abnormally big vacuoles in the cytoplasm likely representing enlarged endosomes (Figure 4.11)



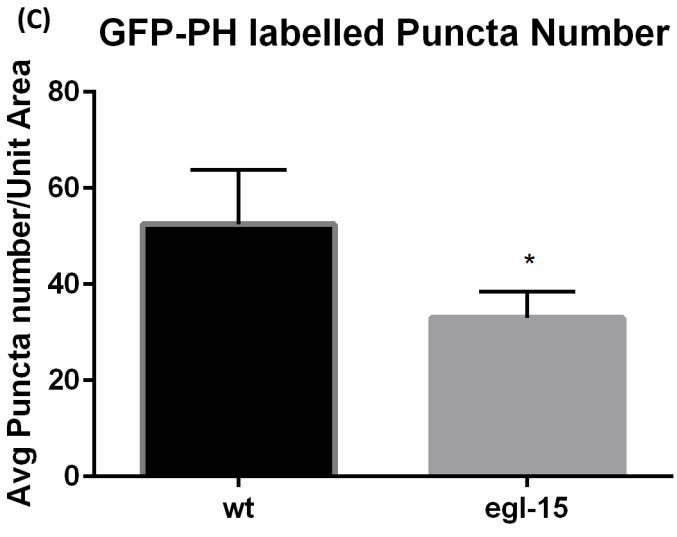


Figure 4.11: Confocal images displaying PH-GFP in the intestine of WT and egl-15 mutants. *Arrows indicate punctate structures (endosomes) in both wild-type (A) and egl-15 (B) mutant animals and arrowheads indicate large abnormal endosomes only in egl-15 mutants (B). A significant difference was observed in the puncta number between the wild-type and egl-15*

*mutants. Quantification of endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and egl-15 mutant animals. ** P<0.05. Scale bar=10 µm*

Thus, from these analyses of distribution of endolysosomal marker proteins and trafficking of transmembrane cargo proteins, it was evident that *egl-15* affects endocytic trafficking through the early endosomes leading to disruption to recycling and late endo/lysosomes. The next focus was to examine the exocytic membrane markers to see if the loss-of-function of *egl-15* affects exocytosis.

4.7 Loss of *egl-15* function doesn't impair exocytic trafficking

Analysis of GFP-SP12:

To understand the role of *egl-15* in exocytosis, it was important to study the membrane compartments such as ER and Golgi along the secretory pathway. The GFP fusion to the ER-resident signal peptidase SP12 has been a widely used marker to study the ER in *C.elegans* oocytes, embryos and intestine (GRABski *et al.,* 2011). The GFP-SP12 strain RT1369 *[unc-119(ed3); pwls526 [vha6p::GFP::sp-12;unc-119]* was crossed into *egl-15* mutant animals and the resulting mutant transgenic worms were used for analysis. The GFP-SP12 was found to be much diffused in the intestine of both the wild-type and *egl-15* mutant animals. The quantification of fluorescence intensity showed no difference between the wild type and *egl-15* mutant animals (Figure 4.12) suggesting that the loss of *egl-15* function might not impact secretion.

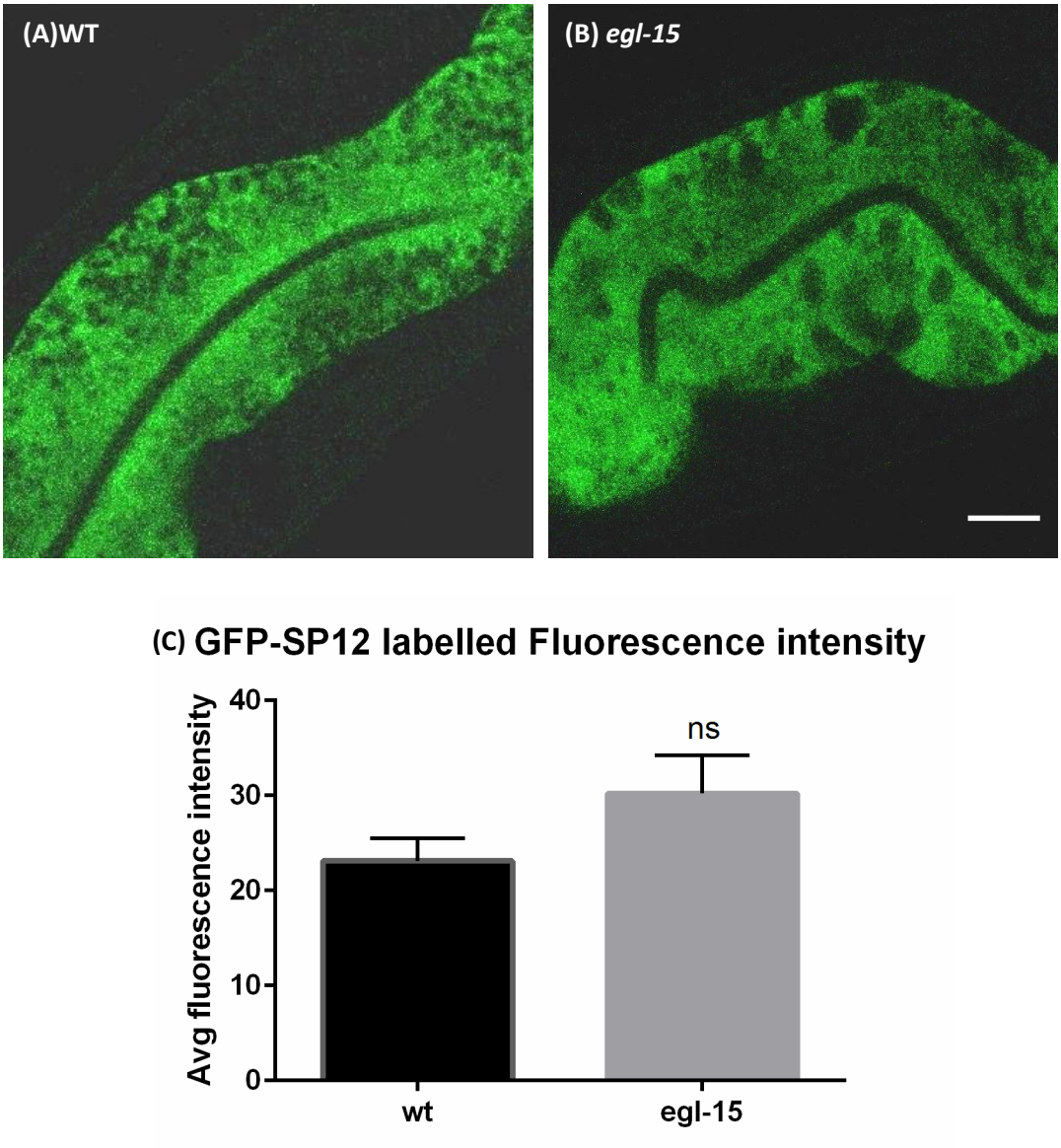
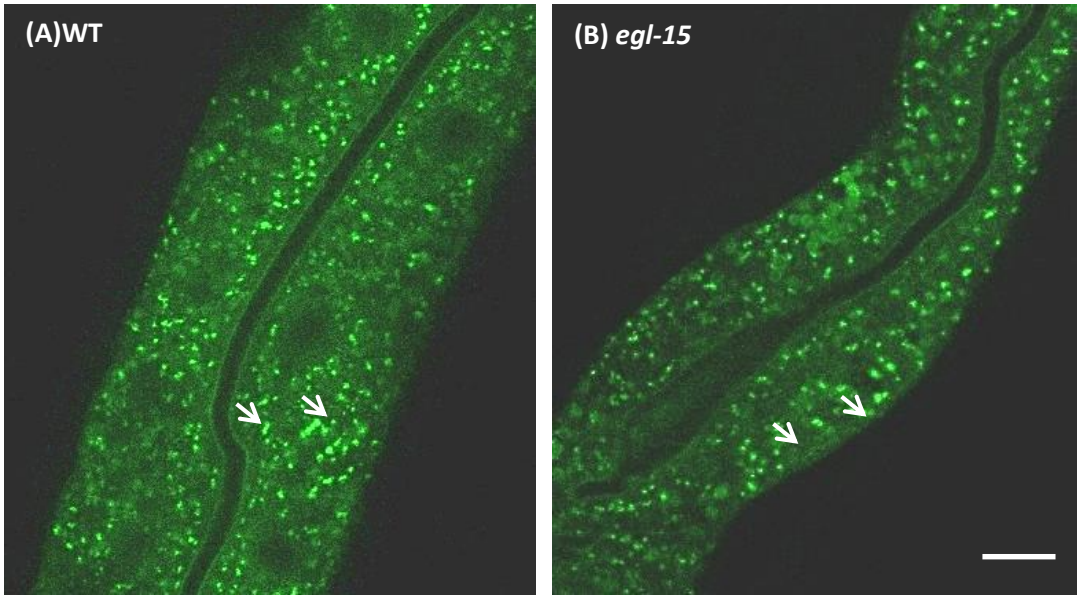


Figure 4.12: Confocal images displaying GFP-SP-12 labelling in the intestine of WT and egl-15 mutants. *A diffused pattern of GFP-SP-12 staining was observed in both wild-type (A) and egl-15 (B) mutant animals and no difference was found in the fluorescence intensity. Quantification of fluorescence intensity as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Scale bar=10 µm.*

Analysis of MANS-GFP:

MANS-GFP (α mannosidase II-GFP) is a marker used to study the Golgi compartment in *C.elegans*. Unlike the mammalian cells that contain only one large juxtanuclear Golgi stack, *C.elegans* have several 'mini-stacks' dispersed throughout the cytoplasm (Grant *et al.*, 2012). The MANS-GFP strain RT1242 *[unc-119(ed3); pwls481 [vha-6p::GFP::MANS;unc-119]* was crossed into *egl-15* mutants and the resulting mutant transgenic worms were used for analysis. Consistent with previous studies it was found that GFP-MANS labelled several 'Golgi mini-stacks' in the wild-type intestine and *egl-15* mutants displayed a similar number of Golgi mini stacks throughout the cytoplasm (Figure 4.13) suggesting that *egl-15* might not be affecting the transport steps along the secretory pathway.



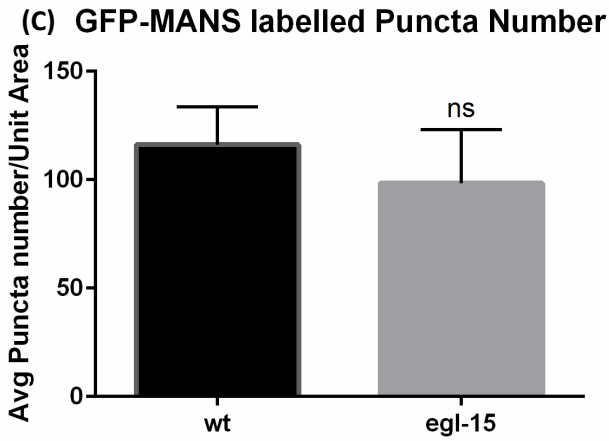


Figure 4.13: Confocal images displaying GFP-MANS labelling in the intestine of WT and egl-15 mutants. *Arrows indicate puncta (Golgi mini-stacks). A high number of Golgi-positive punctate structures were observed in the intestines of wild-type worms (A) and egl-15 mutants animals (B). Quantification of puncta number as visualised shown in (C). Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Scale bar=10 µm.*

4.8 Protein expression levels of membrane markers unaffected in *egl-15* mutants

In identifying the precise membrane transport steps affected by the loss of *egl-15* function by analysing the localisation and distribution of membrane markers in *C.elegans* intestine, it was found that *egl-15* mutants often showed a less or decreased accumulation or distribution of these markers. Hence it was important to know if *egl-15* regulates only the localisation or does it affect even the protein expression of these markers. Of all membrane markers which were significantly different in *egl-15* mutants to that of wild type worms, GFP-CHC-1 and GFP-RAB-35 showed the strongest phenotypical difference. Hence the protein expression levels of these two membrane markers were investigated using western blot analysis. It was found that there is no difference in protein expression level in both these markers between the wild type and *egl-15* mutants (Figure 4.14).

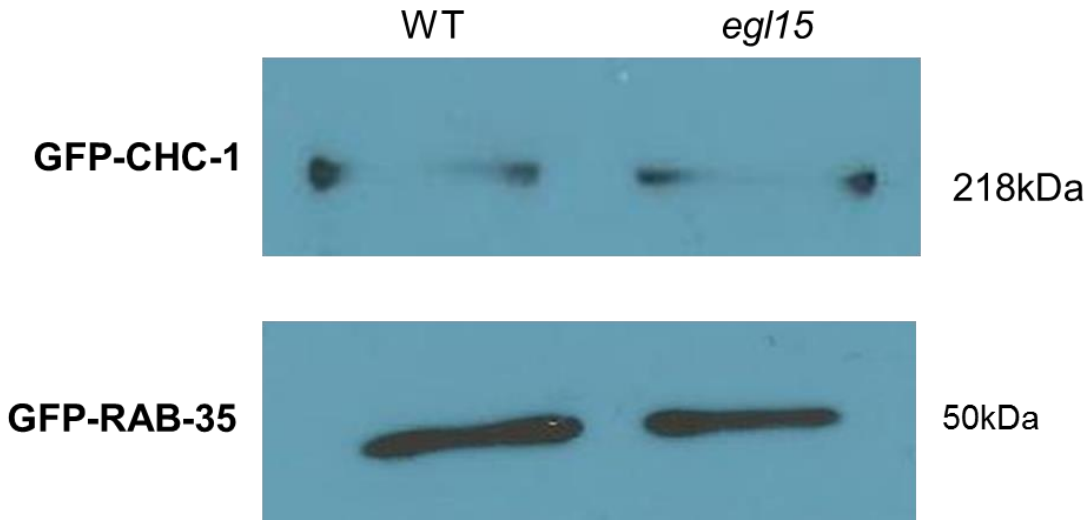


Figure 4.14: Protein expression levels of membrane markers in WT and egl-15 mutants*. Worm lysates were subjected to SDS-PAGE and Immunoblotting as described in materials and methods. Equal amount of protein (30µg of each sample) was loaded onto each well. GFP-CHC-1 (218kDa) and GFP-RAB-35 (50kDa) showed no difference in expression levels between wild type and egl-15 mutants.*

4.9 Discussion

Protein transport between compartments is coordinated by a complex system involving vesicles, intracellular organelles like Golgi, ER, lysosomes, endosomes and autophagosomes. This transport is mediated by various events such as transport of vesicles, organelle fusion or fission, maturation which changes these organelle identities based on the requirements of the cell, combined work of coat proteins, cytoskeletal proteins, motor proteins etc. Amidst of such a complex network, it becomes important to identify exactly which transport steps are affected in case of a failure in membrane trafficking. In this study, the distribution and localisation of well characterised membrane markers enriched in specific compartments and transport routes of cargo proteins were used to identify the transport steps affected by loss of *egl-15* function. Clathrin is an important protein whose structure is well characterised.

Clathrin coated vesicles are either plasma membrane/golgi derived or endosome derived. Endosome derived CCVs are differentiated from plasma membrane derived by size, lack of the adapter protein complex AP-2 and continuity with endosomes. Proteins carried by plasma membrane derived CCVs are first delivered to sorting endosomes from where they are transported to TGN, lysosomes or recycling endosomes. The two major structural units of clathrin in *C.elegans* are CHC-1 (Clathrin Heavy Chain) and CLIC-1 (Clathrin light chain). Several studies have shown that CHC-1 is essential for endocytosis and viability in *C.elegans* and serves as a marker for CCVs. From the analysis of distribution and localisation of CHC-1 between wild type and *egl-15* mutants, results suggests that *egl-15* might affect early endocytic as well as retrograde steps from the TGN within the cell.

Clathrin as such do not bind directly to cargo or membranes but mediated by special clathrin adaptors like the heterotetrameric AP-2 adaptor complex, APM-2, Epsin, DAB-1 (a PTB domain of disabled protein family) and dynamin. Many such clathrin adaptors bind to lipid domains like PI(4,5)P2 for plasma membrane recruitment. This structural binding is achieved

by special domain architectures depending on the binding component, for example plekstrin homology (PH) domain in case of dynamin. In this study, the localisation and distribution of PI(4,5)P_2 marker GFP-PH(PLCδ) was analysed in *C.elegans* intestine. Similar to previous reports, wild type worms labelled GFP-PH(PLCδ) along the apical and basolateral plasma membrane as well as internal puncta and tubules (Shi et al. 2012) however, in *egl-15* mutants, very large vacuolated structures positive for GFP-PH(PLCδ) were found throughout the intestine. It was also found that GFP-ARF-6 labelled similar enlarged structures in the intestine of *egl-15* mutants. Shi et al (2012) have recently reported that these internal puncta and tubules positive for GFP-PH(PLCδ) are actually endosomes and they colocalise and extensively overlap with ARF-6 along the basolateral recycling pathway and ARF-6 regulates endosomal PI(4,5)P2. Though colocalisation of GFP-PH(PLCδ) and ARF-6 weren't carried out in this study, the previous findings of association of ARF-6 with PI(4,5)P_2, and display of similar defective phenotype (enlarged endosomes) positive for ARF-6 and PI(4,5)P_2 in the intestine of *egl-15* mutants only suggests that that loss of *egl-15* function could affect membrane trafficking along the basolateral recycling pathway. This assumption is further strengthened from the analysis of distribution and localisation of GFP-RME-1 in *C.elegans* intestine. In wild type worms, GFP-RME-1 strongly labels tubule-vesicular endosomes along the basolateral membrane and also weakly labels structures near the apical membrane (Chen et al. 2006) however in *egl-15* mutants, there was a significantly weaker labelling of GFP-RME-1 positive recycling endosomes along basolateral membranes.

RAB proteins are known to be key regulators of membrane traffic. Different RABs are required for different trafficking events including endocytosis, recycling and exocytosis. The regulation, localisation and distribution of some of the RAB GTPases were analysed in this study with the loss of *egl-15* function. From the analysis of GFP-RAB-5 and GFP-RAB-7, it can be said that

the transport step from early to late endosomes is affected by the loss of *egl-15* function. RAB-5 and RAB-7 are key components of early and late endosomes respectively. Rink et al (2005) have shown the association of RAB-5 in smaller early endosomes which progresses into larger late endosomes where RAB-5 is replaced with RAB-7. The high accumulation of puncta (early endosomes) and less of the ring like vesicles (late endosomes) positive for GFP-RAB-5 and also less number of ring like vesicles (late endosomes) positive for GFP-RAB-7 in *egl-15* mutants compared to wild type worms suggest that early endosomes have failed to mature into late endosomes and transport step from early to late endosomes is affected. A similar interpretation can be made from the analysis of recycling endosome marker GFP-RAB-35 where the number of recycling endosomes is significantly reduced in *egl-15* mutants compared to wild type worms suggesting that transport steps via endocytic recycling could be affected.

Given these results, RAB-10, which is known to regulate endocytic recycling in *C.elegans* intestine (Chen et al. 2006) was analysed. Previous studies have shown that GFP-RAB-10 mark extensively at the early endosomes especially near the basal plasma membrane in the top focal plane and also localise Golgi associated structures at the medial plane. They are also known to weakly label late endosomes or apical recycling endosomes (Chen et al. 2006). Thus, RAB-10 functions in multiple compartments in *C.elegans* intestine and is thought to be involved in basolateral recycling, apical recycling, secretion (Chen et al. 2006) and is even thought to promote maturation of early endosomes to recycling endosomes (Shi et al. 2012). Surprisingly, in this study no significant difference was found in GFP-RAB-10 positive puncta between wild type and *egl-15* mutants. One could assume two possibilities that either *egl-15* doesn't involve RAB-10 in endocytic recycling or due to its association with multiple compartments there could be a RAB-10 functional redundancy.

Since early and recycling endosomes are affected in *egl-15* mutants, cargo proteins such as hTFR and hTAC were investigated to see if their transport was affected by the loss of *egl-15* function. As hypothesised, the trafficking of both these cargo proteins was compromised in *egl-15* mutants. There was a significant reduction in GFP-hTFR puncta in *egl-15* mutants compared to wild type worms. This reduction in GFP-hTFR could be due to an early endocytic/uptake defect where intestinal cells are unable to uptake hTFR. The recyling of hTFR, hTAC are RAB-10 and RME-1 dependent in *C.elegans* intestine. Loss of *egl-15* function was found to affect several RABs (RAB-5, RAB-7 and RAB-35) including the regulation of ARF-6 and RME-1. These proteins are key in trafficking of different cargo proteins; therefore it is not surprising to see the trafficking of cargo proteins such as hTFR and hTAC compromised in *egl-15* mutants.

The role of *egl-15* in the secretory pathway was also investigated by analysing the Golgi and ER membrane markers such as GFP-MANS and GFP-SP-12. No significant difference was found in the localisation or distribution of these markers between wild type and *egl-15* mutants suggesting that *egl-15* might not affect secretory pathway.

From all these analyses, though the reduced accumulation or distribution of membrane markers suggest particular transport steps being defective, it was important to investigate whether *egl-15* was affecting the protein expression levels of any of these markers. Therefore, the protein expression levels of membrane markers such as GFP-CHC-1 and GFP-RAB-35 that showed the strongest phenotypical difference between wild type and *egl-15* mutants were analysed by western blot. Results showed no difference in protein expression levels between the wild type and *egl-15* mutant animals.

Taken together, these results suggest that *egl-15* could affect membrane trafficking at different endocytosis events such as early uptake, early to late endosomes or recycling. The results also indicate that *egl-15* might not be affecting the secretory pathway atleast in *C.elegans* intestine.

Also *egl-15* doesn't seem to affect the protein expression of the two membrane markers analysed (CHC-1 and RAB-35). Thus from these results it was evident that *egl-15* affects membrane trafficking in *C.elegans* intestine. The next step of investigation was to identify how *egl-15* regulates membrane trafficking in the intestine. In other words, experiments were conducted to identify which signalling modules were involved in membrane trafficking in *C.elegans* intestine.

CHAPTER 5: *egl-15* AFFECTS MEMBRANE TRAFFICKING VIA PLC-γ PATHWAY IN *C.elegans* INTESTINE

5 *egl-15* AFFECTS MEMBRANE TRAFFICKING VIA PLC-γ PATHWAY IN *C.ELEGANS* INTESTINE

5.1 Introduction

As described in previous two chapters, *egl-15* affected Yolk-GFP trafficking via MAPK and PLC-γ signalling pathway in *C.elegans.* From the analysis of membrane markers in the *C.elegans* intestine, it was found that loss of *egl-15* function affects different endocytosis steps like uptake and endocytic recycling. In this chapter, some of the membrane markers found to be regulated by *egl-15* in the worm intestine were investigated if they were regulated via MAPK or PLC-γ pathway.

The small GTPase *let-60/*Ras is one of the key players in transducing signalling through many downstream pathways, such as MAPK, PI3K/AKT and PLC-γ. One of the most conserved pathways via the *let-60* is the MAPK kinase pathway. In *C.elegans,* EGF and FGF are the two main signalling pathways which are found to signal clearly through *let-60*. They account to most biological functions involving *let-60* and studies in *C.elegans* were the first to show that *sem-5/GRB2* (Clark et al. 1992) acts upstream of *let-60* and *lin-45*/Raf (Han et al. 1993) acts downstream of *let-60.* The association of *egl-15* with MAPK via *let-60* is known to regulate essential biological functions in *C.elegans* such as germline developments, hypodermal fluid homeostasis, oocyte growth and differentiation, aging, sex muscle differentiation and body muscle maintenance (Château et al. 2010; Lee et al. 2007; Okuyama et al. 2010).

On the other hand, PLC-γ signalling pathway is essential for the regulation of intracellular calcium signals which in turn regulate several other cellular responses (Foskett et al. 2007). PLC-γ signalling is initiated by hydrolysis of $PI(4,5)P_2$ which is catalysed by PLC-γ.

So far, six isoforms of PLCs have been identified such as PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ, and PLC-η. In *C.elegans*, five PLC genes encoding four of the six families have been identified. *plc-2* and *egl-8* (PLC-β), *plc-3* (PLC-γ), *plc-4* (PLC- δ) and *plc-1*(PLC-ε) (Gower et al. 2005). Although these isoforms share common motifs, they initiate varied cellular responses based on family specific regulatory domains (Vázquez-Manrique et al. 2008).

Therefore in this chapter, these two signalling pathways (MAPK and PLC-γ) were put to test to see how they regulated membrane trafficking in *C.elegans* intestine. Hence the regulation of membrane markers that showed the strongest phenotypical difference between wild type and *egl-15* mutant worms from earlier findings was chosen in this analysis. The regulation of these membrane markers via the RAS-MAPK activating complex including *sem-5* and *let-60* and the components of PLC-γ pathway including *plc-3, itr-1* and *pkc-2* were analysed.

5.2 RESULTS

5.3 *let-60*/Ras does not affect membrane trafficking in *C.elegans* intestine

Analysis of GFP-CHC-1 localization in sem-5 and let-60 mutants

CHC-1, a marker for clathrin-coated pit was analysed. The GFP-CHC-1 strain GK35 *[unc-119(ed3); pwls72 [vha-6p::GFP::chc-1;unc-119]* was crossed into *sem-5 and let-60* mutant strains such as [UP148- *sem-5(cs14)*] and [MT4698-*let60(n1700)*] respectively and were analysed. It was found that *let-60* showed no difference in puncta number however, *sem-5* mutants showed a significant reduction in the number of puncta compared to wild type worms (Figure 5.1). It was interesting to observe that *let-60*, a key player of MAPK signalling pathway showing no difference in GFP-CHC-1 phenotype especially when they showed a Yolk-GFP trafficking defect in previous studies. In case of *sem-5* mutants, the significant reduction in puncta number can be associated to its role in receptor-mediated endocytosis. In the analysis of Yolk-GFP trafficking by FGF-FGFR signalling pathway components, *sem-5* was found to be one of the positive regulators which showed a strong endocytosis defect and therefore it's not surprising to see *sem-5* mutants affecting the localisation of GFP-CHC-1. In addition to GFP-CHC-1, endosomal markers GFP-RAB-5 and GFP-RAB-7 were also analysed in *sem-5* and *let-60* mutants (Images not shown) (Figure 5.2). No difference was found between wild type and *sem-5* and *let-60* mutants. Here, it is important to note that *let-60*, a major component to transduce signalling via MAPK showed no difference in morphology, localisation and distribution of the above analysed membrane markers suggesting that MAPK signalling pathway does not regulate membrane trafficking at least in intestine.

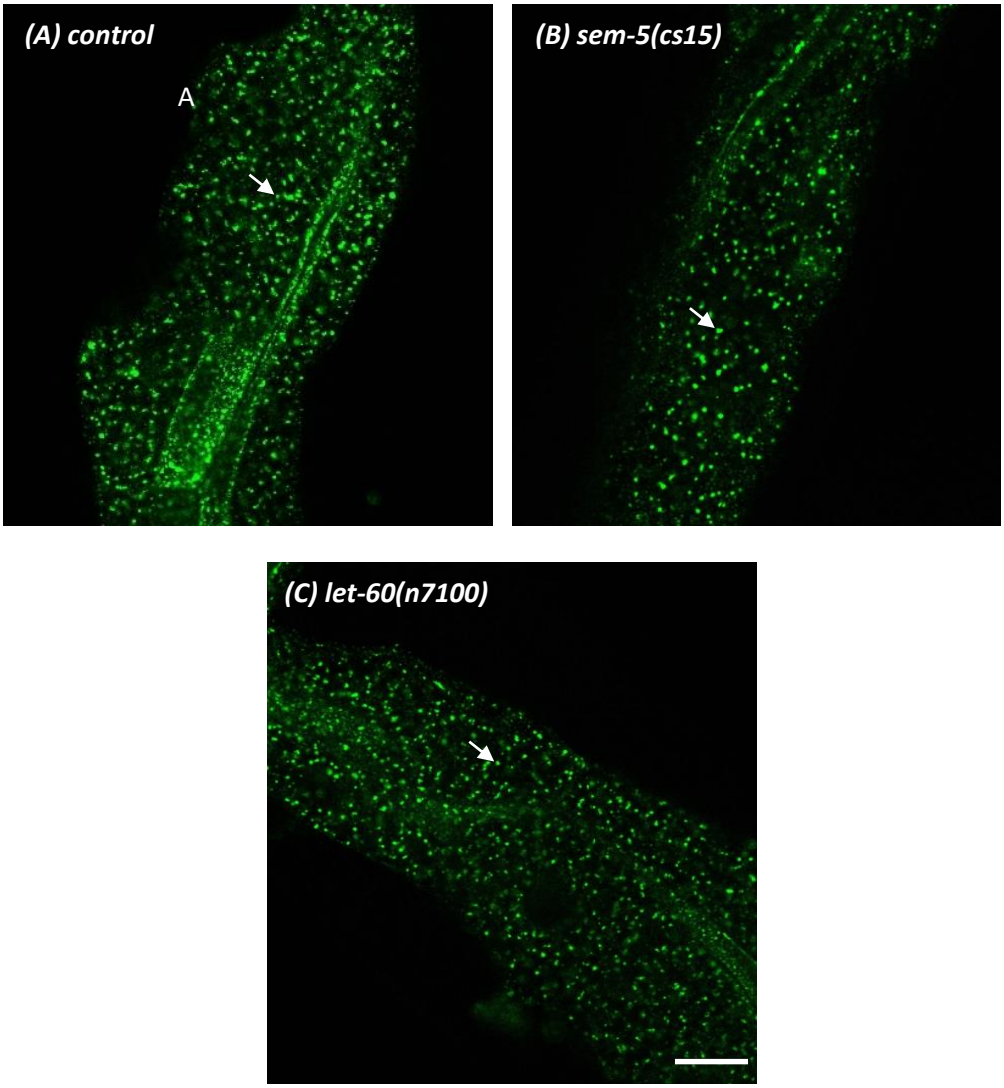

(A) control
A
(B) sem-5(cs15)
(C) let-60(n7100)
(D)
GFP-CHC-1 labelled Puncta Number
Avg Puncta number/Unit Area
300
200
100
0
*
wt
sem-5
let-60

Figure 5.1: Confocal images displaying GFP-CHC-1 in the intestine of WT, sem-5 and let-60 mutants. *A high number of punctate structures observed in the intestine of wild-type (A) and let-60 mutants (C) and a significantly reduced number of puncta observed in the intestine of sem-5 mutants (B).Arrows indicate puncta (endosomes). Quantification of endosome number as visualised shown in (D). Error bars represent standard deviation of the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and sem-5 mutant animals. * P<0.05. Scale bar=10 µm*

Analysis of GFP-RAB-5 and GFP-RAB-7 distribution in sem-5 and let-60 mutants

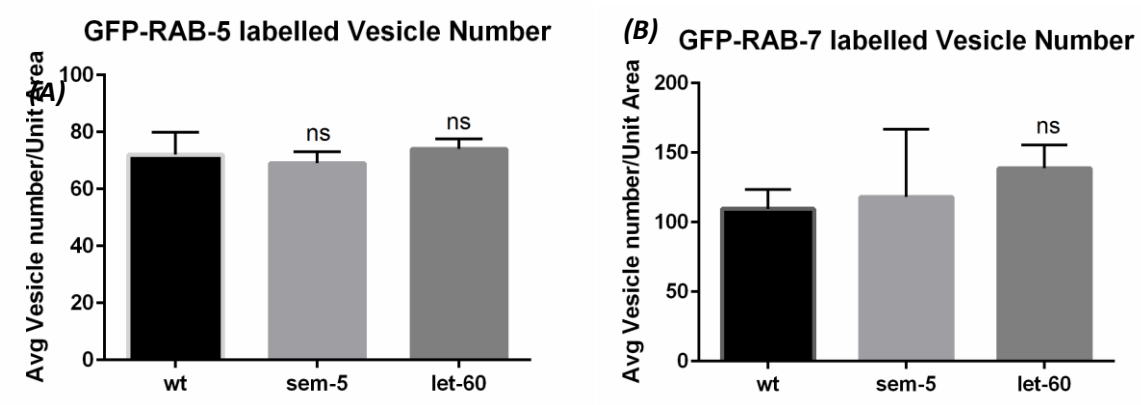


Figure 5.2: Comparison of GFP-RAB-5 and GFP-RAB-7 between WT, sem-5 and let-60 mutants in C.elegans intestine. *No difference in localisation and distribution of GFP-RAB-5 and GFP-RAB-7 between wild type and sem-5, let-60 mutants. Quantification of endosome number of GFP-RAB-5 (A) and GFP-RAB-7 (B). Error bars represent standard deviation of the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine).*

The other study which involved the knock down of *plc-3* by RNAi showed a strong Yolk-GFP trafficking defect (chapter 4). *plc-3* which encodes PLCγ in *C.elegans* is a key component of IP3 signalling pathway. Also the analysis of membrane marker GFP-PH (domain of PLCδ)

showed enlarged endosomes likely indicating a recycling defect in *egl-15* mutants. These results suggested a strong case for the role of IP3 signalling pathway in membrane traffic. Therefore, it was important to investigate how PLC-γ signalling pathway regulates different steps of membrane transport in *C.elegans* intestine.

The two key components of PLC-γ signalling pathway are the IP3 receptor (IP_3R) ITR-1 and protein kinase C (PKC). ITR-1 is the only known IP3 receptor in *C.elegans*. IP_3R is located within the ER and is involved in calcium release in response to IP3 production. The IP3 signalling is also found to be essential for functions like spermatheca dilation, ovulation (Clandinin et al. 1998; Bui & Sternberg 2002), embryonic development and morphogenesis (Yin et al. 2004). In this study, the role PLC-γ signalling pathway affecting the different membrane transport steps in *C.elegans* intestine was analysed.

5.4 Loss of PLC-γ signalling pathway components affect early endocytosis and recycling

In order to see how PLC-γ signalling pathway affects different transport steps in *C.elegans* intestine, membrane markers such as CHC-1, ARF-6, Plekstrin homology domain PH and RAB-35 were analysed. These membrane markers were chosen based on their significant difference in localisation and distribution between wild type and *egl-15* mutants.

Analysis of GFP-CHC-1 localization in plc-3 RNAi-depleted animals

The distribution of CHC-1, a marker for clathrin-coated pits and vesicles was analysed in *plc-3* RNAi-depleted animals. The GFP-CHC-1 strain GK35 *[unc-119(ed3); pwls72 [vha-6p::GFP::chc-1;unc-119]* was used in this analysis. CHC-1-GFP labelled a very large number of punctate structures throughout the cytoplasm in the intestine of wild-type animals. However,

in *plc-3* depleted animals, a significantly smaller number of these punctate structures were observed (Figure 5.3). As previous described, a reduced accumulation of GFP-CHC-1 could indicate a decreased number of clathrin coated pits or decreased clathrin expression. Either way, this phenotype could indicate impairment in clathrin-mediated uptake, secretion or recycling.

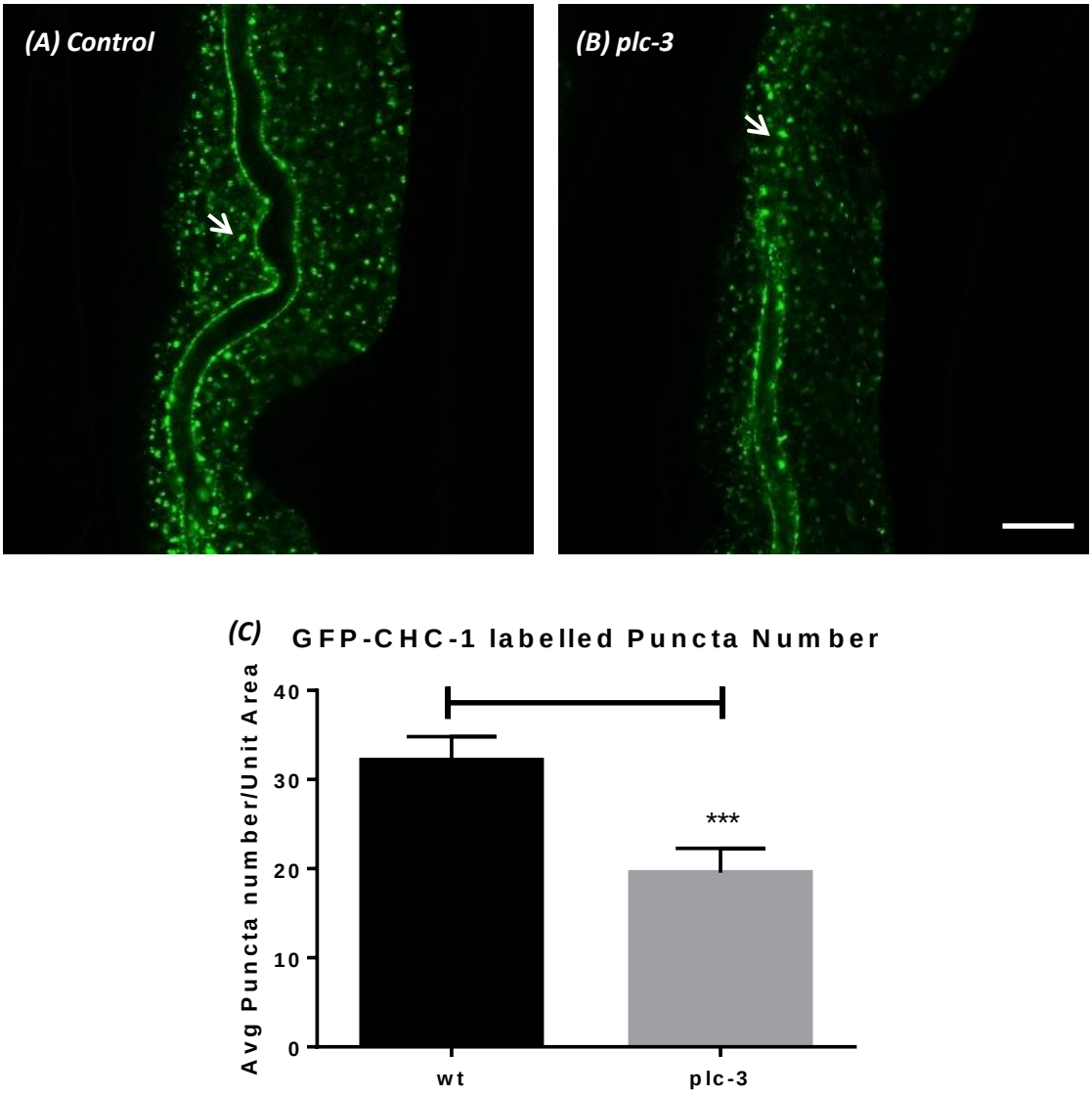
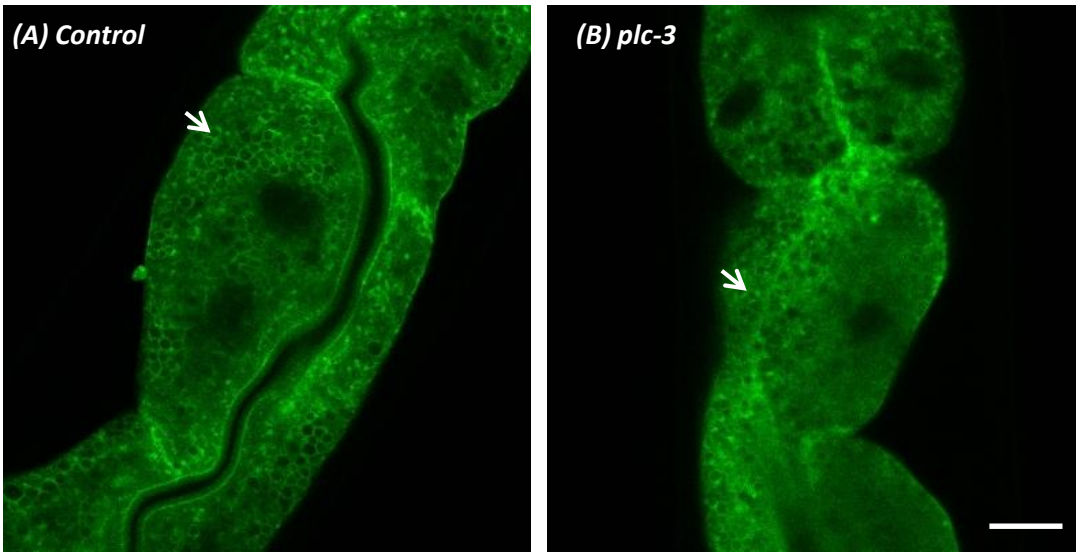


Figure 5.3: Confocal images displaying GFP-CHC-1- in the intestine of WT and plc-3 knock--down animals. *A high number of punctate structures observed in the intestine of wild-type worms (A) and a significantly reduced number of puncta observed in the intestine of plc-3 knocked-down animals (B). Arrows indicate puncta (endosomes). Quantification of*

*endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and plc-3 mutant animals. * P<0.05. Scale bar=10 µm*

Analysis of GFP-RAB-35 localization in plc-3 knock-down animals

GFP-RAB-35 is associated with recycling endosomes (Sato *et al.*, 2008). The GFP-RAB-35 strain RT910 [*unc-119(ed3); pwls355 [vha-6p::GFP::rab-35minigene]* was used in this analysis. GFP-RAB-35 animals labelled distinct small ring-like vesicles which represent the recycling endosomes in wild-type intestine. However, in *plc-3* knock- down animals, there was a significant reduction in these ring-like vesicles (Figure 5.4) suggesting that RAB-35 positive recycling endosomes are affected.



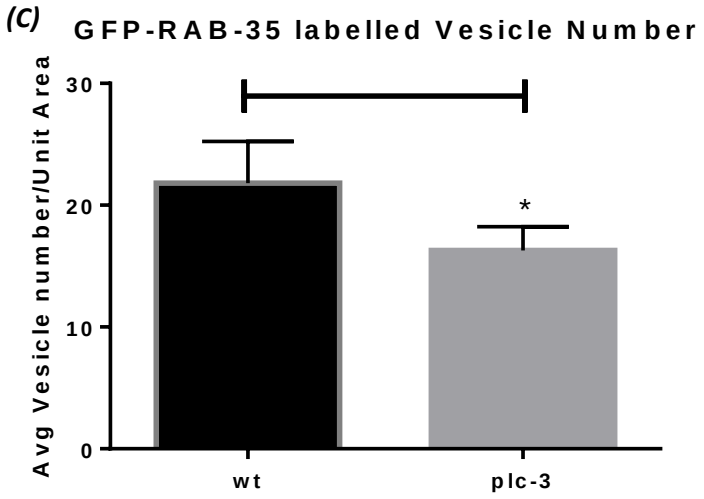


Figure 5.4: Confocal images displaying GFP-RAB35-GFP in the intestine of WT and plc-3 knock-down animals. *A significantly higher number of ring-like vesicle structures observed in the intestine of wild-type worms (A) compared to the intestine of plc-3 knock-down animals (B). Arrows indicate ring-like vesicles (recycling endosomes). Quantification of endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control and plc-3 mutant animals. * P<0.05. Scale bar=10 µm*

Analysis of GFP-ARF-6 localization in plc-3 knock-down animals

In polarized cells such as the worm intestine, it is proposed that ARF-6 mediates apical uptake of clathrin-dependent cargo (Altschuler *et al.*, 1999). ARF-6 is known to localize along the cytoplasmic face of the plasma membrane (Peters et al. 1995). The GFP-ARF-6 strain RT1579 *[unc-119(ed3); pwls601 [vha6p::GFP::arf-6;unc-119]* was used in the analysis. It was found that, GFP-ARF-6 labelled the apical more than the basolateral membrane and appeared slightly diffused with mesh-like vesicles and punctate structures throughout the cytoplasm in the

intestine of wild-type animals (Figure 5.5). Quantification of GFP-ARF-6 labelled vesciles showed no significant difference between control and *plc-3* depleted worms.

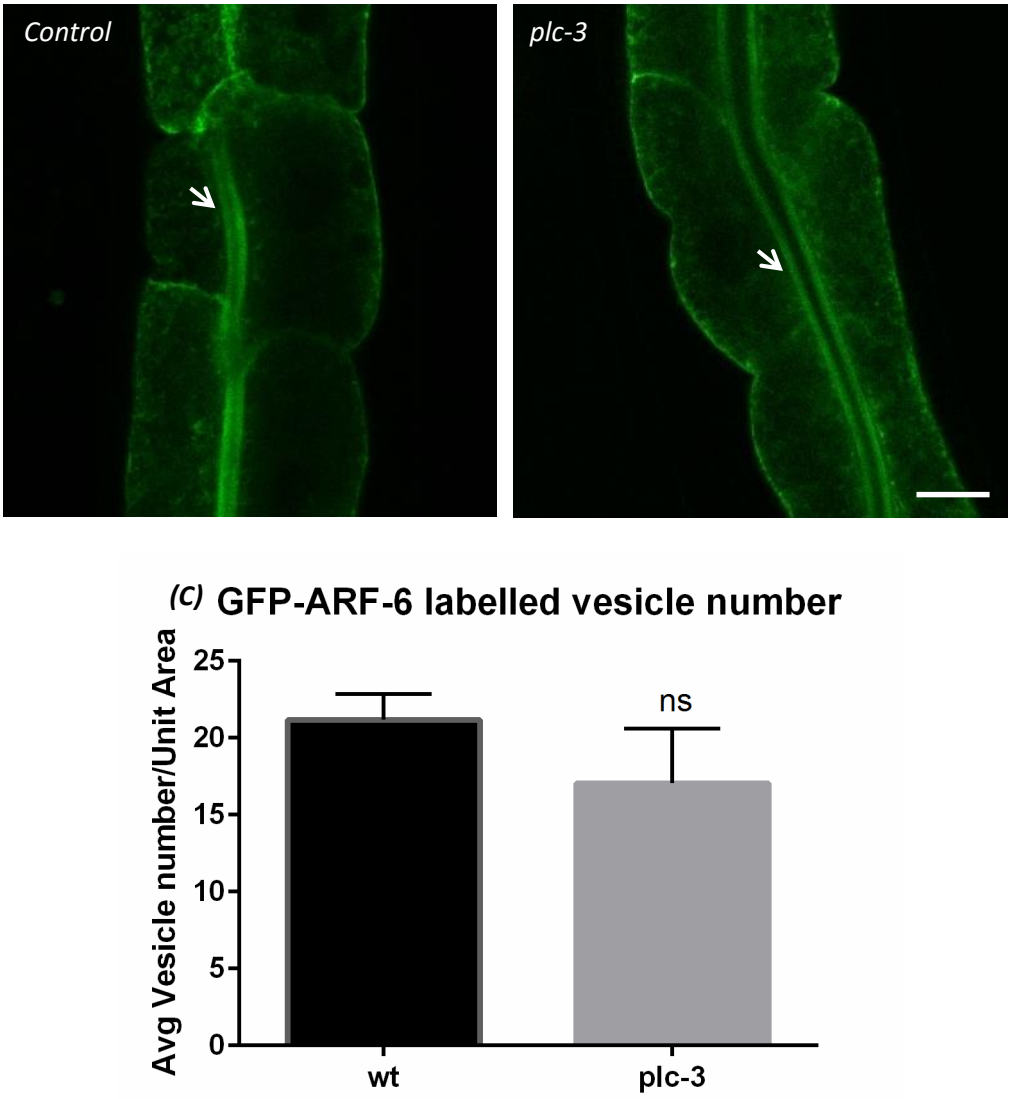
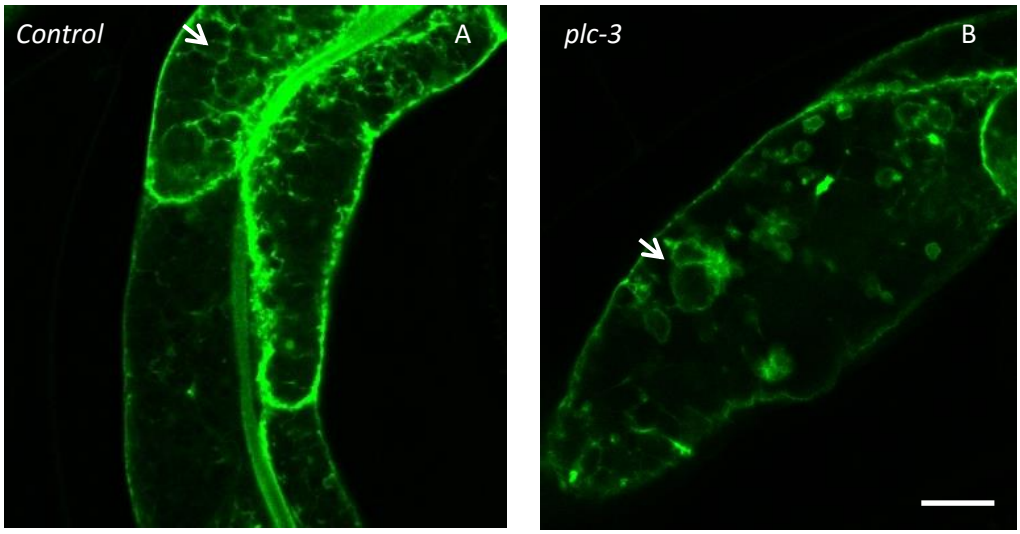


Figure 5.5: Confocal images displaying GFP-ARF-6 in the intestine of WT and plc-3 knock-down animals. *No difference was observed in GFP-ARF-6 labelled structures between wild-type worms (A) and plc-3 knock-down worms (B). Quantification of vesicle number as visualised shown in C. Arrows indicate diffused mesh-like vesicles (endosomes). Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). N=3 Scale bar, 10µm*

Analysis of GFP-PH distribution and localization in plc-3 knock-down animals:

Plekstrin Homology domain of PLC-δ (GFP-PH) strain RT1120 *[unc-119(ed3); pwls446 [vha-6p::GFP::PH;Cbunc-119]* was used in the analysis. It was found that GFP-PH labelled the basolateral and apical membranes in the wild-type intestine (Figure 5.6). Strikingly, in some of the *plc-3* knocked-down animals, PH-GFP labelled several abnormally big vesicles in the cytoplasm likely representing enlarged endosomes. The number of vesicles per unit area was also found to be reduced in *plc-3* knock-down animals compared to wild type. This was a similar phenotype observed in *egl-15* mutants suggesting that an impaired *plc-3* might affect membrane trafficking. .



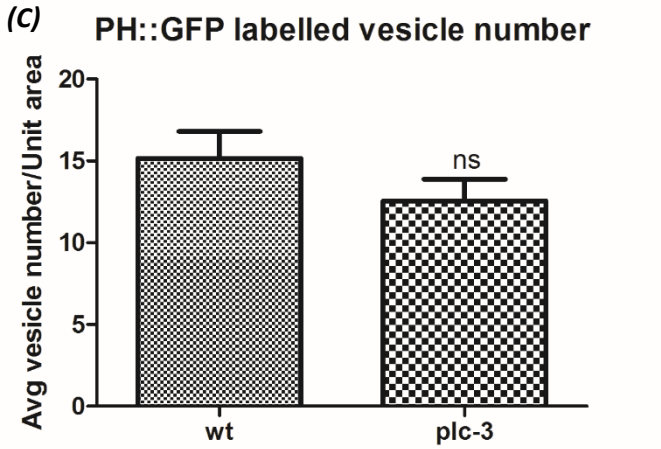


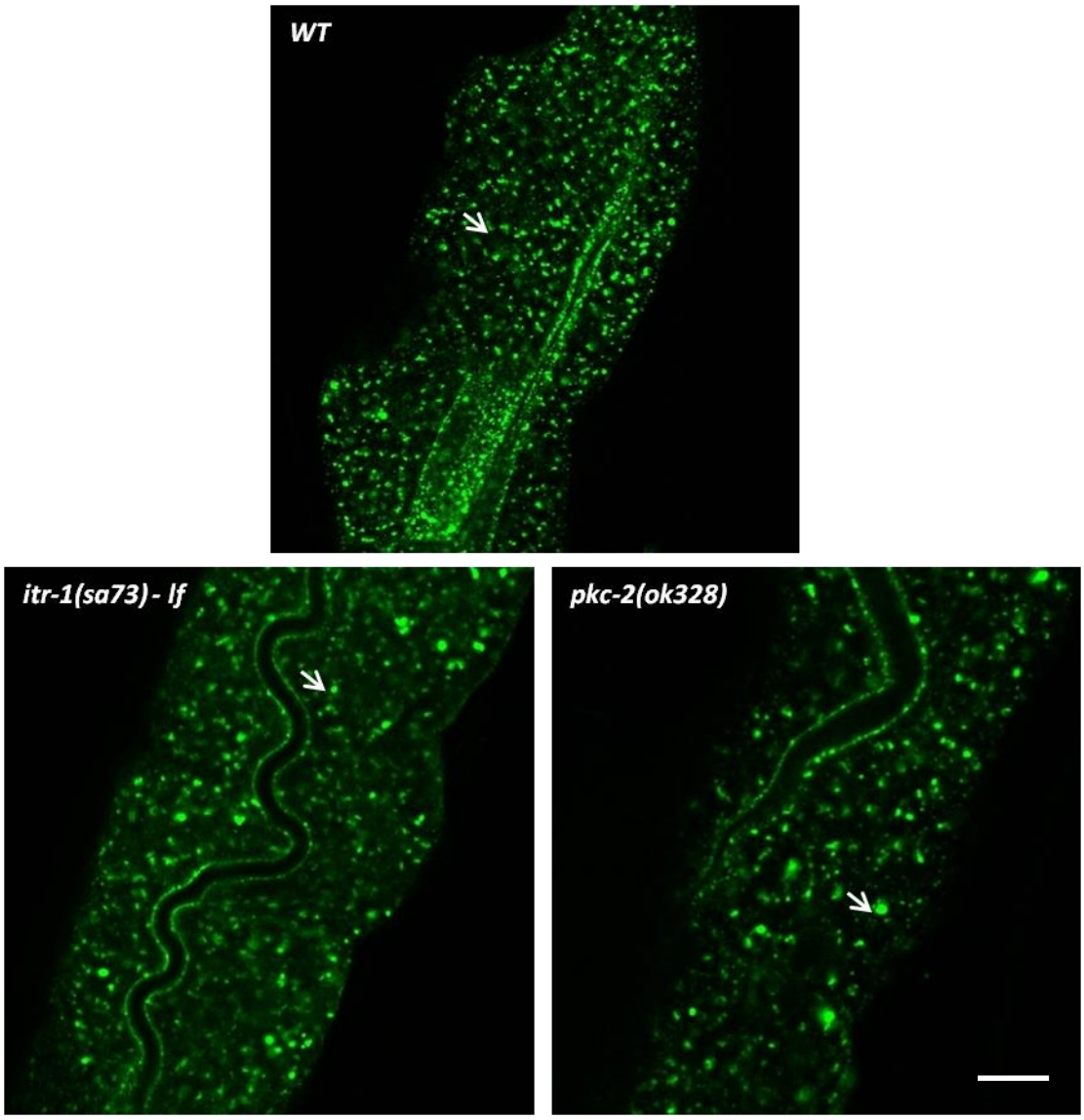
Figure 5.6: Confocal images displaying GFP-PH localization in the intestine of WT and plc-3 knock-down animals*. No significant difference was observed in PH-GFP labelled structures between wild-type worms (A) and plc-3 knocked-down worms (B). Arrows indicate enlarged vesicles in plc-3 depleted animals. Quantification of endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). N=3, Scale bar, 10µm*

5.5 PLC-γ signalling pathway mutants show similar localisation, distribution and morphology of membrane markers as observed in *plc-3* RNAi-depleted animals

Following the analysis of PLC-γ signalling pathway components by RNAi, mutants were analysed to see if they phenocopy the same.

Analysis of GFP-CHC-1 distribution in PLC signalling pathway mutants:

CHC-1, a marker for clathrin-coated pits and vesicles was analysed. The GFP-CHC-1 strain GK35 [*unc-119(ed3); pwls72 [vha-6p::GFP::chc-1;unc-119], VC127[pkc-2(ok328)],*and *JT73[itr-1(sa73)]* were used in this analysis. GFP-CHC-1 strain was crossed into *itr-1, and pkc-2* mutant animals and the resulting mutant transgenic animals were analysed. It was found that the GFP-CHC-1 labelled a very large number of punctate structures throughout the cytoplasm in the wild-type intestine. However, in *itr-1 and pkc-2* mutants, a significantly reduced number of these punctate structures were observed (Figure 5.7). This was a similar phenotype observed in *plc-3* knock-down animals by RNAi. Also, reduced number of GFP-CHC-1 puncta in *itr-1* and *pkc-2* mutants suggest that these downstream components of PLC-γ pathways do play a significant role in regulation of membrane traffic.


WT
itr-1(sa73) - lf
pkc-2(ok328)

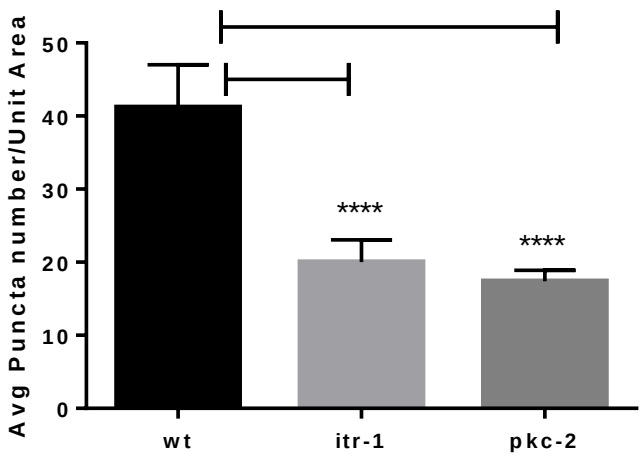
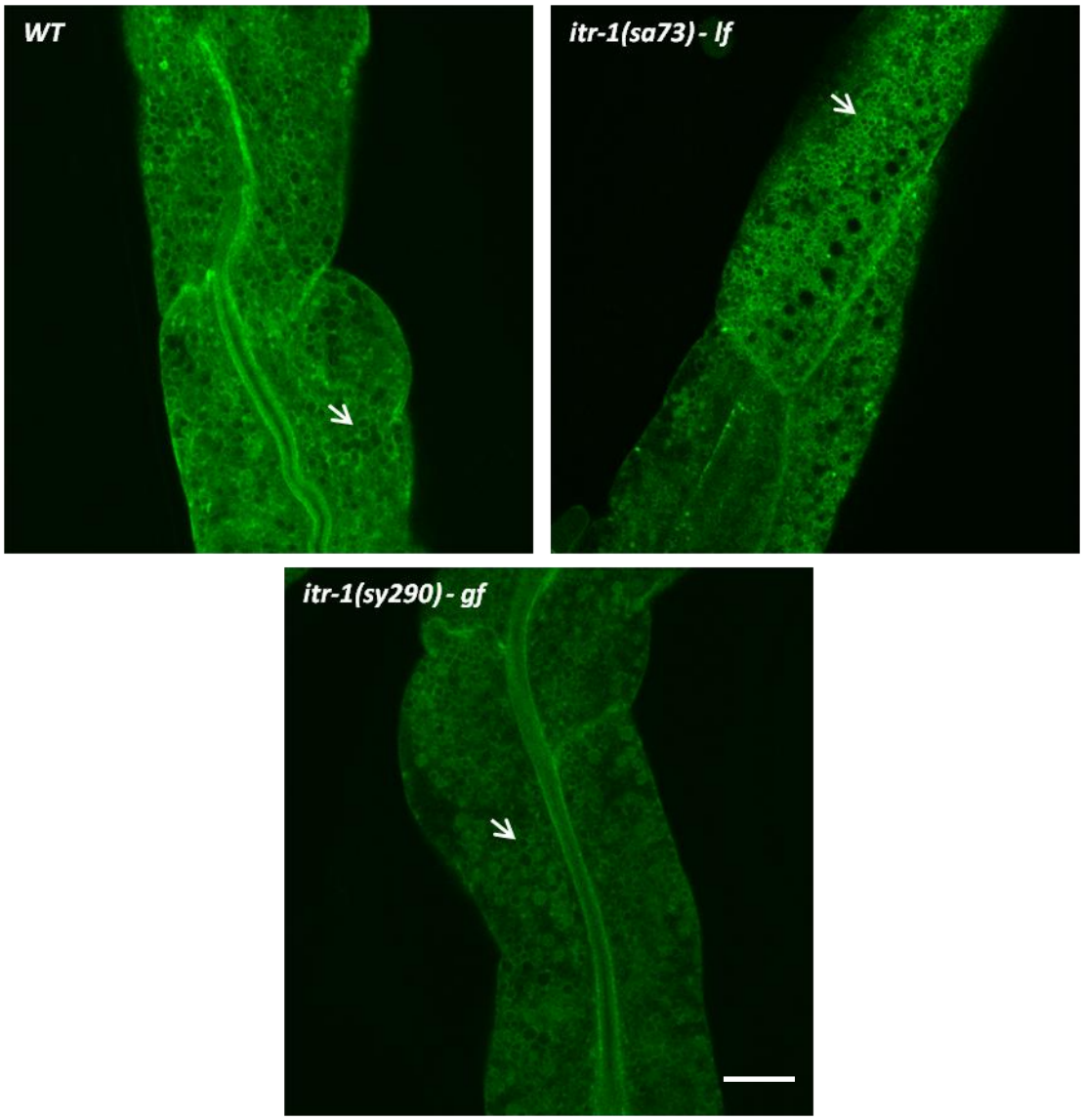

GFP-CHC-1 labelled Puncta Number
Avg Puncta number/Unit Area
50
40
30
20
10
0
****
****
wt
itr-1
pkc-2

Figure 5.7: Confocal images displaying GFP-CHC-1 in the intestine of wt, itr-1, and pkc-2 mutants. *A high number of punctate structures observed in the intestine of wild-type worms (A) whereas a significantly reduced number of puncta observed in the intestine of itr-1(B), and pkc-2 (C). Arrows indicate puncta (endosomes).Quantification of endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine). Asterisks indicate a significant difference in the two-tailed Student's t test between control, sem-5 and pkc-2 mutant animals. **** P<0.0001. Scale bar=10 µm.*

Analysis of GFP-RAB-35 distribution in PLCγ signalling pathway mutants:

GFP-RAB-35 is associated with recycling endosomes (Sato *et al.*, 2008). The GFP-RAB-35 strain RT910 [*unc-119(ed3); pwls355 [vha-6p::GFP::rab-35minigene], JT73[itr-1(sa73)](lf), , and PS2582 [itr-1 (sy290) unc-24(e138)](gf)* were used in this analysis. The RT910 strains were crossed into *itr-1 (lf),and itr-1 (gf)* mutant animals and the resulting mutant transgenic animals were analysed. GFP-RAB-35 labelled distinct small ring-like vesicles which represent the recycling endosomes in wild-type intestine. However, in *itr-1 (lf)* mutant animals, there was a significant reduction in these ring-like vesicles whereas *itr-1*(gf) mutant animals showed a wild type phenotype with no difference with the control. (Figure 5.8) This result suggests that RAB-35-positive recycling endosomes could be affected with loss of *itr-1* function in PLC-γ pathway.


WT
itr-1(sa73) - lf
itr-1(sy290) - gf

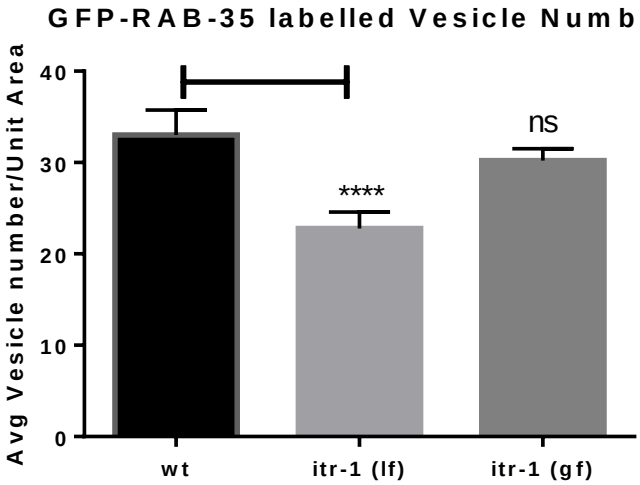

GFP-RAB-35 labelled Vesicle Number
Avg Vesicle number/Unit Area
40
30
20
10
0
****
ns
wt
itr-1 (lf)
itr-1 (gf)

Figure 5.8: Confocal images displaying GFP-RAB-35 in the intestine of WT, itr-1 (loss of function), and itr-1 (gain of function) mutants. *A high number of ring-like vesicles observed in the intestine of wild-type worms (A) and itr-1 (gf) (C) mutants whereas a significantly reduced number of vesicles observed in the intestine of itr-1(B). Arrows indicate ring-like vesicles (recycling endosomes). Quantification of endosome number as visualised shown in C. Error bars represent standard deviation from the mean (n=30 each, 6 animals sampled in 5 different regions of each intestine. Asterisks indicate a significant difference in the two-tailed Student's t test between control and itr-1 (lf) mutant animals. **** P<0.0001. Scale bar=10 µm.*

5.6 Discussion

From the analysis of membrane markers in the *C.elegans*, results indicate that membrane trafficking, at least in the intestine is regulated via PLC-γ signalling pathway rather than MAPK kinase pathway. From earlier investigations it was found that Yolk-GFP trafficking was affected by loss of *egl-15* function both via MAPK and PLC signalling pathways. With this understanding, studies were carried out to understand the different membrane transport steps that could be affected via this pathway. *let-60*/Ras which regulates Yolk-GFP trafficking in the germline does not seem to affect membrane trafficking in the intestine. There could be many factors behind this differential regulation of MAPK in *C.elegans*. One of the reasons could be that, the *C.elegans* intestine is a polarised epithelium. Polarised cells normally maintain distinct basolateral and apical membrane domains with different lipid and compositions (Grant & Sato 2006). Much of our understanding so far about endocytosis and exocytosis dynamics are from studies of non-polarized cells. What makes polarized cells different from non-polarized cells is the difference in distribution of endosomal compartments. Polarized cells have two types of endosomes such as apical endosomes and basolateral endosomes. Some studies suggest that basolateral endosomes are thought to regulate uptake and recycling of receptors and ligands which are normally involved in cell maintenance (Kelly 1993) while apical endosomes are thought to be involved in cell type specific processes such as trancytosis (Barroso & Sztul 1994) however, other studies have shown that no specialised apical endosomal compartments exists (Apodaca et al. 1994). Recent studies on transferrin internalisation have shown that some of the endosomes near the apical membrane failed to label internalised transferrin whereas basolateral endosomes were enriched (Knight et al. 1995; Hughson & Hopkins 1990). Also, studies on neurons (polarized cells) show the existence of different endosomal populations where basolateral endosomes regulate housekeeping functions whereas apical endosomes located near the axons are involved in recycling of synaptic vesicle proteins (Parton

et al. 1989; Mundigl et al. 1993; Bonzelius et al. 1994). All these studies make it clear that polarized cells contain distinct endosomal compartments that differ functionally and biochemically (Wilson & Colton 1997). With these two different plasma membranes in polarized cells, there is a need for proteins to be transported to the appropriate target membrane. Proteins can be targeted to from TGN to either of the membranes or can be first sent to one surface and later transcytosed to the other (Mostov & Cardone 1995). For a long time, it was thought that basolateral transport was spontaneous and would occur by default. But studies have shown that specialized signals are required to target apical and basolateral proteins to appropriate domains (Lipschutz et al. 2001). Thus there is a possibility that MAPK signal transduction could be target specific in polarized cells such as intestine and non-polarized cells such as oocytes.

The other factor to consider is the role of *let-60*/Ras and how it regulates MAPK signalling pathway. In general, it has been proposed that Ras acts upstream of Raf and activation of Ras results in phosphorylation of Raf, which in turn phosphorylates and activates MEK and MEK activates MAPK. MAPK finally carries the signal into the nucleus where they phosphorylate transcription factors and yield the appropriate cellular function. MAPK pathway is not always Ras dependent. Studies show that MAPK signal transduction can be induced even via a Ras independent mechanism (Büscher et al. 1995). Also, in another study, despite the loss of Ras and Raf, High density lipoprotein (HDL) induced MAPK phosphorylation by a possible involvement of PKC (Rentero et al. 2006).

Therefore, the membrane markers such as CHC-1, RAB-5 and RAB-7 that failed to show a significant difference in localization and distribution between wild type and *let-60* mutants suggest three possibilities. 1) MAPK pathway may not be involved in regulation of membrane trafficking in *C.elegans* intestine. 2) MAPK signal transduction might be Ras independent in

C.elegans intestine and 3) Loss of Ras and Raf activity could be compensated by PKC as found in other studies.

From the analysis of PLC-γ sigalling pathway components, it was found that loss of *itr-1* or *pkc-2* resulted in a significant difference in localization and distribution of membrane markers such as CHC-1 and RAB-35. Taking all these into consideration, it is very likely (not conclusive) that *egl-15* might regulate membrane trafficking via PLC-γ signalling pathway in the intestine and there is a possibility that PKC might compensate an impaired Ras activating complex thereby still phosphorylating the MAPK pathway.

CHAPTER 6: FGF-FGFR SIGNALLING AND MEMBRANE TRAFFICKING IN MAMMALIAN SYSTEMS

6 FGF-FGFR SIGNALLING AND MEMBRANE TRAFFICKING IN MAMMALIAN SYSTEMS

6.1 Introduction

FGFR signalling pathways and membrane trafficking in mammalian cells are complicated compared to that of invertebrates like *C.elegans* and *D.melanogaster*. The results obtained from the investigations conducted on *C.elegans* membrane trafficking and FGFR signalling were to be tested on mammalian cells in order to compare the underlying molecular machinery. In this chapter, the role of FGFR signalling pathway in membrane trafficking is analysed in mammalian cells. The FGF signalling events are complex by the sheer number of ligands and receptors. The mammals have four FGFRs (FGFR1, FGFR2, FGFR3 and FGFR3) and each receptor has its own alternative spliced isoforms (Burke et al. 1998). This broad range of ligands and receptors with their diverse functions and genetic redundancy makes it difficult to characterise their signalling properties and their impact on cells (Klint & Claesson-Welsh 1999). The major downstream signal transduction pathways associated with activated FGFRs are the well-established Ras-MAPK cascade, PI3 Kinase/PDK/Akt pathways and PLC-γ pathways (Boilly et al. 2000) (Coleman et al. 2014).

Activation of FGFRs can trigger a diverse cellular responses including proliferation, migration, differentiation and membrane trafficking (Auciello et al. 2013) whereas a deregulation could lead to several developmental defects and other pathological conditions (Aridor & Hannan 2000). Many different pathways have been associated with RTK endocytosis but recently it has been found that FGFR internalisation as such is dynamin-dependent and clathrin-mediated (Auciello et al. 2013).

The FGF-FGFR interactions are quite complicated as each receptor can bind to multiple different ligands with the same affinity and this binding of FGFs to FGFRs is stabilised by

heparin sulphate proteoglycans (Haugsten et al. 2005). In terms of tissue distribution in normal adult tissues, it is found that the ligands FGF1 and FGF2 and the receptors FGFR1 and FGFR2 are most widely expressed (Hughes & Hall 1993) (Hughes 1997).

From phylogenetic tree analysis, it was found that the most conserved human homologue to *C.elegans* FGFR *egl-15* was the FGFR1. Therefore, in this study, NIH/3T3 swiss mouse fibroblast cell line was chosen to study FGFR signalling and membrane trafficking as they express detectable levels of FGFR1 (Zhen et al. 2007). The FGFR inhibitor SU5402 was used to inhibit the function of FGFR and the trafficking of transmembrane cargo protein transferrin tagged to a flurophore was analysed. In previous studies it has been found that SU5402 a potent FGFR inhibitor can specifically inhibit the autophosphorylation of FGFR1 kinase in NIH/3T3 cells thereby blocking the receptor tyrosine kinase signalling properties (Mohammadi 1997).

6.1.1 Transport routes of different cargo proteins (Tf, LDL)

Transferrin (Tf) is a widely studied cargo protein used in the analysis of membrane trafficking. It is an 80kDa clathrin-dependent glycoprotein distributes iron to tissues. Tf helps in the uptake of iron in cells by binding to iron molecules. Such iron binding Tf is also called as holo transferrin and the iron unbound called apo-transferrin. At neutral pH, transferrin binds ferric ions with high affinity whereas in acidic conditions transferrin binds with a low affinity (Bleil & Bretscher 1982). Tf binds to its receptor (TfR) and enters cells through clathrin-mediated endocytosis (Pearse & Robinson 1990). Once endocytosed, Tf is sorted along the trafficking pathway into three different endosomal systems such as early endosomes, late endosomes and recycling endosomes. Recent studies have shown that there are two distinct early endosomes with different maturation kinetics; dynamic endosomes and static endosomes and the sorting of Tf ligands begins at the cell surface into any of these endosomes. The early endosomes that mature faster into late endosomes are classified as dynamic endosomes and the slower maturing

endosomes are classified as static endosomes (Lakadamyali et al. 2006). Cargo proteins such as LDL (low density lipoprotein) are found to be preferentially taken into dynamic endosomes and further transported to lysosomes for degradation whereas, Tf follows the recycling pathway and remains enriched in the static population of early endosomes (Figure 6.1). There is eventually a second sorting process where the tubular formations in early endosomes sort the cargo either directly back to the cell surface (fast recycling) or to the perinuclear endocytic recycling compartment (slow recycling) (Schweitzer et al. 2011). The Tf trafficking pathway is extensively studied and well established. The core components involved in the internalisation of Tf are clathrin, adaptor proteins, and dynamin (Marsh & McMahon 1999) (Grant & Donaldson 2009) (Conner & Schmid 2003). The clathrin complex (three heavy chains and three light chains) create the initial invaginations at the plasma membrane followed by the adaptor proteins such as AP-2 that recruit cargo and clathrin to ligands and finally dynamin which completes the vesicle formation and scission from the cell surface.

The other major regulators of Tf trafficking are the RAB GTPases. In one study, it was found that Tf became increasingly transported to recycling endosomes when Rab4 was over expressed (van der Sluijs et al. 1992). There is also evidence that Rab35 plays a major role in trafficking Tf via the fast recycling pathway (Kouranti et al. 2006) and Rab8 via the perinuclear endocytic recycling compartment (slow recycling pathway) (Hattula et al. 2006).

Figure 6.1: An overview of Transferrin and LDL trafficking. *Both transferrin and LDL are internalised via clathrin-mediated endocytosis. After uncoating, LDL is preferentially taken into dynamic endosomes where it follows the degradation pathway into lysosomes whereas Tf is taken into both dynamic and static population of endosomes. A faster recycling route takes transferrin directly to the plasma membrane and a slow recycling route takes Tf first to the endocytic recycling compartment before it is trafficked back to the cell surface* (Mayle et al. 2012).

Therefore in order to understand the role of FGFR in membrane trafficking in mammalian cells, the FGFR in fibroblast cell line NIH/3T3 was stimulated with FGF or inhibited using a potent chemical FGFR inhibitor SU5402 and trafficking of fluorophore-tagged Transferrin was analysed and compared to that of a control using confocal microscopy and flow cytometry.

6.2 RESULTS

6.3 Endogenous FGFR highly expressed in NIH/3T3 cells

In order to study FGFR signalling in mammalian cells, the choice of cell line was important. It was vital to study FGFR signalling in cells that are known to express FGFRs. The NIH/3T3 cells are mouse embryonic fibroblast cells. 3T3 refers to 3 day transfer inoculum of $3x10^5$ cells. The expression of FGFR1 in NIH/3T3 cells was first confirmed by western blot analysis. NIH/3T3 cells showed detectable levels of FGFR1. As shown in Figure 6.2, FGFR1 was expressed in the NIH/3T3 cells as proteins with molecular mass of approximately 120 kDa. This result confirmed the expression of endogenous FGFR1 in NIH/3T3 cells consistent with previous reports from (Zhen et al. 2007)

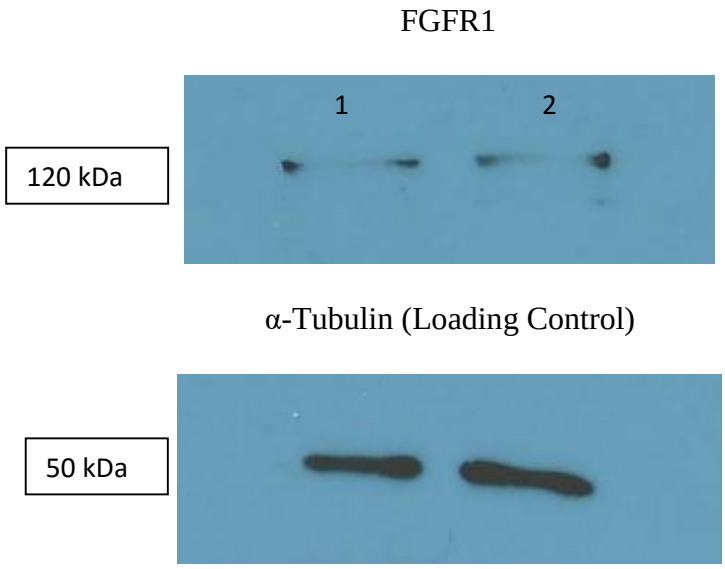


Figure 6.2: Expression of FGFR1 protein in NIH/3T3 cells. *NIH/3T3 cell lysates were prepared and subjected to SDS-PAGE and Immunoblotting as described in materials and methods. Lanes 1 and 2 represent duplicates. α-Tubulin was used as the loading control.*

6.4 SU5402, an FGFR antagonist, inhibits the phosphorylation of FGFR

The ability of SU5402 to inhibit the protein tyrosine kinase activity of FGFR1 was investigated in NIH/3T3 cells. The cells were serum starved for 30 minutes and treated with SU5402 for 30 minutes at 37^{o}C. Cells were then lysed and the protein expression levels of FGFR1 were identified using FGFR1 and phospho-FGFR1 antibodies as described in Materials and Methods. It was found that the phosphorylation of FGFR1 was inhibited between 10-20µM of SU5402 which is consistent with a previous study by Mohammadi et al., 1997. It was also found that SU5402 completely inhibited FGFR1 phosphorylation at 40µM.

As a control, EGFR inhibitor was also used to test whether EGFR inhibitors affect FGFR phosphorylation. It was found that the drug didn't have any noticeable inhibitory effect on the tyrosine kinase phosphorylation of FGFR (Mohammadi 1997). Also, the stimulation of FGFR1 with FGF-1 didn't show any significantly increase in Phospho-FGFR band (Figure 6.3). From these results it was evident that SU5402 inhibits the phosphorylation of FGFR1 in NIH/3T3 cells and a 20µM concentration of SU5402 was used in all subsequent experiments.

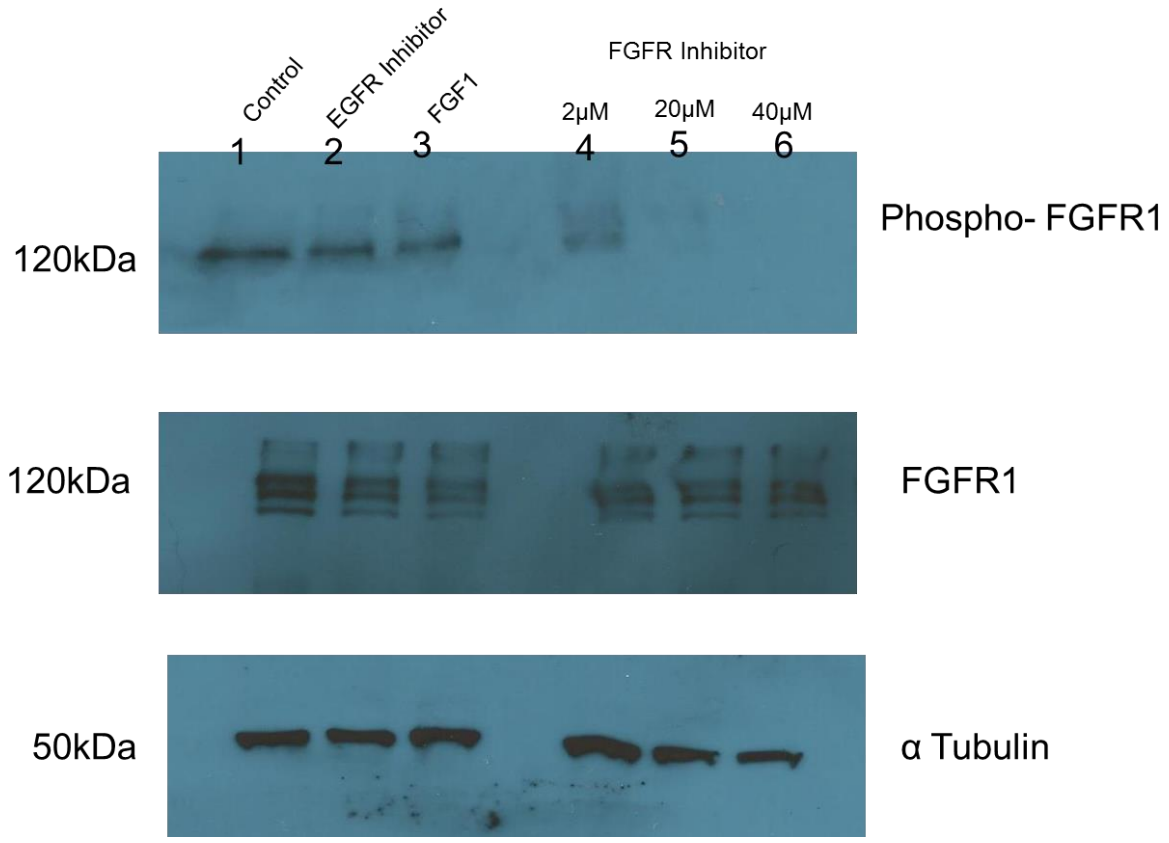
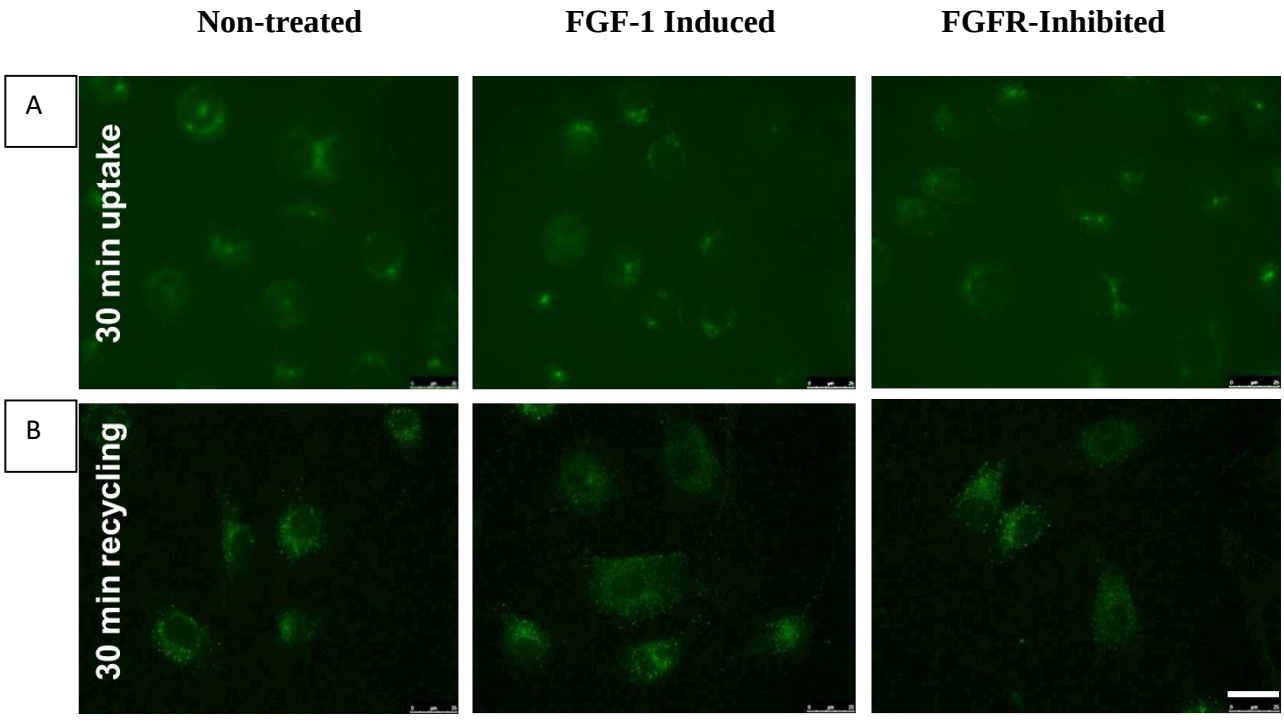


Figure 6.3: SU5402 inhibits phosphorylation of FGFR in NIH/3T3 cells: *NIH/3T3 cells were serum starved for 30 minutes and incubated further with increasing amounts of SU5402 or with FGF-1 or EGFR inhibitor for 30 minutes. After washing with ice-cold PBS, cells were lysed and subjected to SDS-PAGE and immunoblotting as described in Materials and Methods. Lane 1 (non-treated), lane 2 (EGFR inhibited 20μM, -ve control), lane 3 (FGF-1 induced) and lanes 4,5, and 6 represent increasing amounts of SU5402 2μM, 20μM and 40μM* respectively.

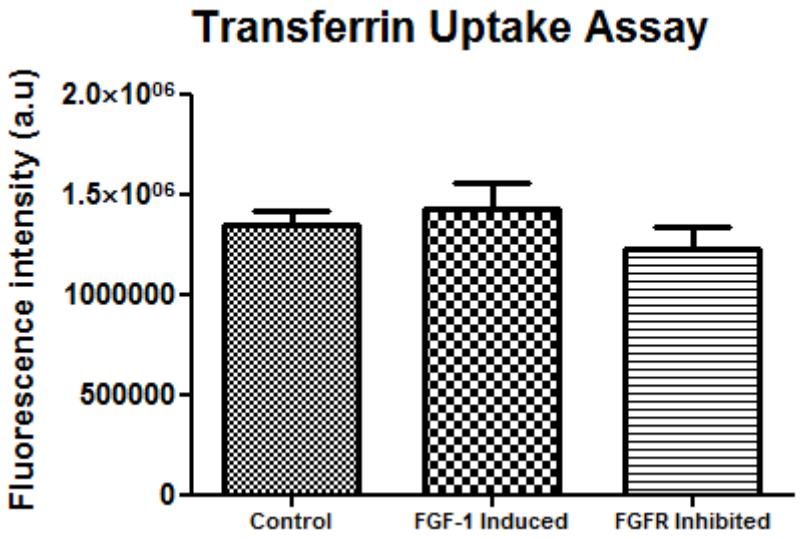
6.5 Knock-down of FGFR signalling does not affect transferrin trafficking

To test the relevance of FGFR inhibition in the process of membrane trafficking, the uptake and recycling of Alexa Fluor488-conjugated transferrin was investigated using both the confocal microscopy and flow cytometry. The NIH/3T3 cells were either induced or inhibited using FGF-1 and SU5402 respectively and the trafficking of Alexa Fluor488-conjugated transferrin was analysed. The uptake of transferrin was analysed at 30 minutes after incubation with Alexa488-transferrin. It was found that there was no difference in the transferrin uptake or recycling with either FGF1 induction or FGFR inhibition (Figure 6.4a and 6.4b).

Figure 6.4: Transferrin uptake and recycling in NIH/3T3 fibroblasts.



C



D

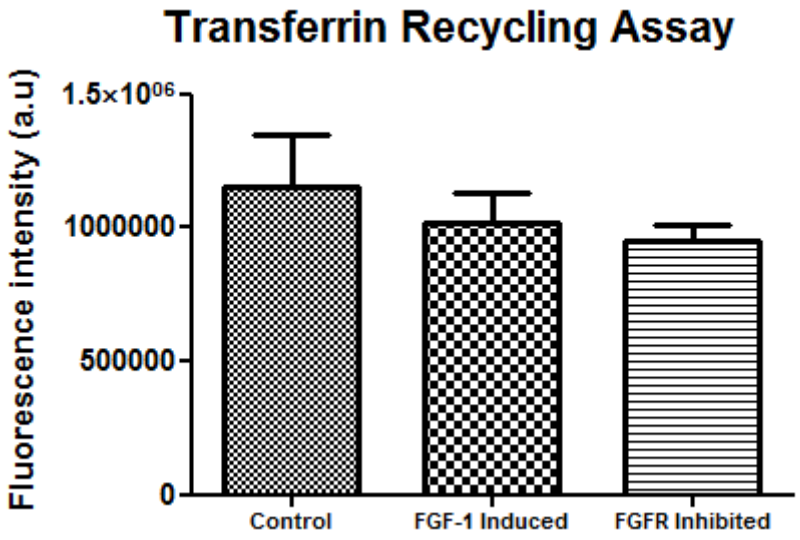
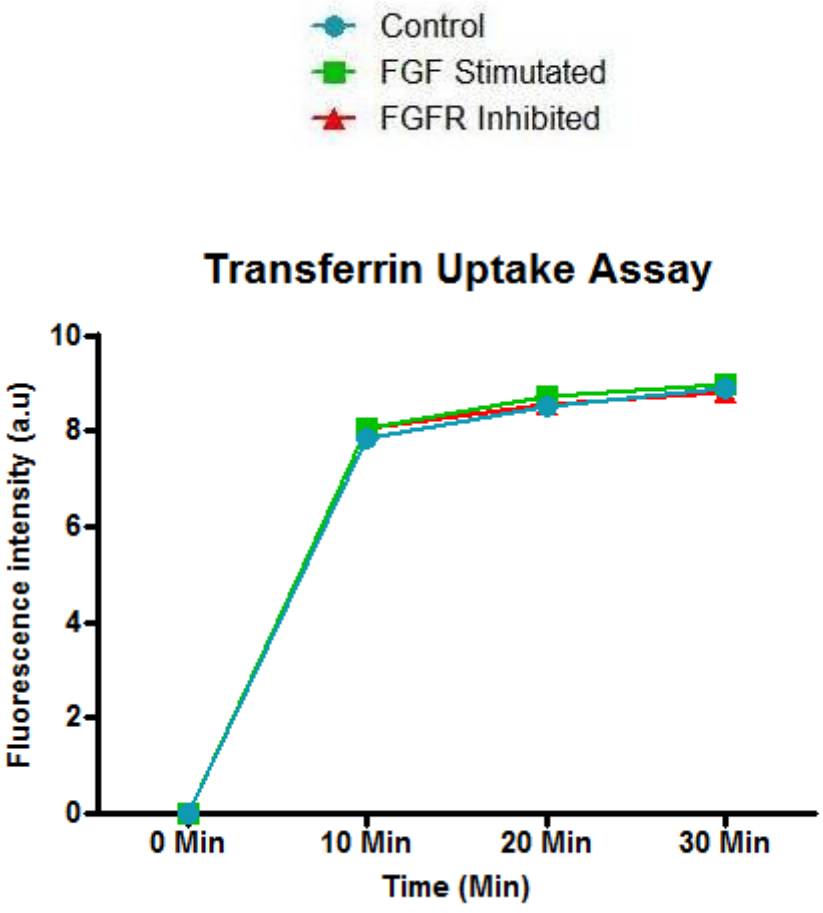


Figure 6.4: Analysis of Transferrin uptake and recycling by confocal microscopy in NIH/3T3 fibroblasts:

NIH/3T3 cells expressing endogenous FGFR1 were either induced with FGF-1 or inhibited with FGFR1 inhibitor SU5402 for 30 minutes and incubated with Alexa Fluor488- transferrin for 30 minutes (uptake). Following transferrin internalization, cells were incubated in complete medium for a further 30 minutes (recycling) at 37°C and cells were rinsed, fixed and

analysed by fluorescence microscopy. (A). Transferrin localisation and distribution after 30 min uptake and 30 min recycling (B).

Quantification of *Tf fluorescence intensity* as visualised shown in (C) and (D). Error bars represent standard deviation of the mean (n=50 cells per condition). 'NS' indicate no significant difference in the two-tailed Student's t test between control and FGF-1 stimulated/FGFR inhibited cells. P>0.05. Scale bar=25 µm



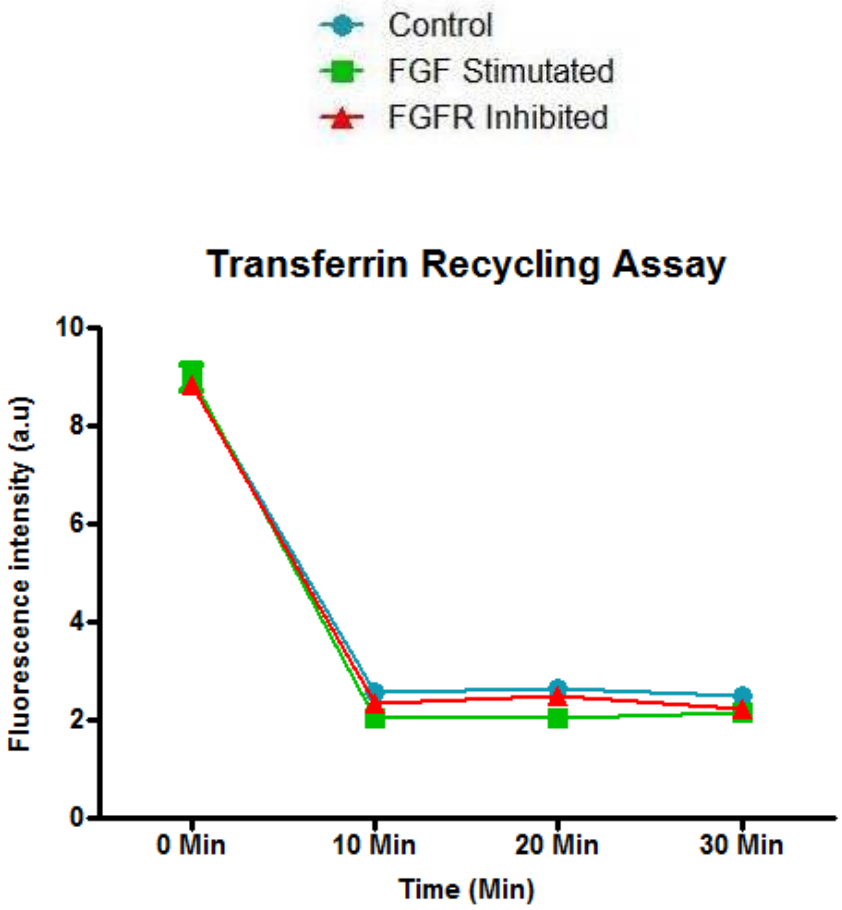


Figure 6.5: Analysis of Transferrin uptake and recycling by flow cytometry in NIH/3T3 fibroblasts: *Tf uptake and recycling unaffected by FGF-1 stimulation or FGFR1 inhibition. NIH/3T3 cells expressing endogenous FGFR1 were either induced with FGF-1 or inhibited with FGFR1 inhibitor SU5402 for 30 minutes and incubated with Alexa Fluor488 transferrin at varying time intervals 10 min, 20 min and 30 min. Mean Tf fluorescence intensity at each point was measured using flow cytometry. For recycling assays after transferrin internalization, cells were washed with ice-cold PBS and stripping buffer and incubated in complete medium at varying time intervals 10 min, 20 min and 30 min at 37°C . Mean Tf fluorescence intensity at each point was measured using flow cytometry*

6 Discussion

Transferrin is a widely used cargo protein to study membrane trafficking. The transport routes are very well characterised which makes it conducive to the researcher to identify any impairment in trafficking. As mentioned earlier, transferrin is a key component in bringing iron into the cells. Transferrin binds to transferrin receptors at the cell surface which then forms a dimer and internalises two iron molecules. Ligand sorting happens at cell surface and transported along the early endosomes, late endosomes and recycling endosomes thus making it an excellent candidate to study both uptake and recycling membrane trafficking pathways. Studies have also shown how GTPases such as RABs, ARFs and proteins such as clathrin, dynamin, actin and phosphoinositides (PIP2) (Shi & Grant 2013) affect membrane trafficking . Thus analysis of transferrin trafficking also helps to identify some of its key regulators. Therefore, in this study it was hypothesised that inhibiting or enhancing the function of FGFR would affect transferrin trafficking in mammalian cells. Similar to previous studies, this study also shows that NIH/3T3 cells expressed detectable levels of FGFR and inhibition of FGFR by SU5402 (FGFR inhibitor) reduces the phosphorylation of FGFR. However, from the FGFR phosphorylation assay it was found that the addition of FGF did not trigger a strong FGFR phosphorylation compared to that of control thereby making it inconclusive whether transferrin trafficking was actually affected by modulating FGFR signalling in this study.

One possibility could be that the serum deprivation of cells for 30 minutes might not have been sufficient and subjecting them to a longer overnight serum starvation might induce a stronger FGFR phosphorylation with addition of FGF ligands.

Though the results from this study are inconclusive, similar studies on transferrin trafficking and FGFR signalling in Hela cells have shown that transferrin trafficking is unaffected by modulating FGFR signalling.

It was found that in Hela cells, FGFR inhibition or stimulation didn't affect transferrin uptake though it affected clathrin spots (Auciello et al. 2013). Regulation of membrane traffic by FGFR signalling is found to be clathrin mediated and dynamin dependent (Auciello et al. 2013). Transferrin internalisation is also a clathrin-mediated endosytosis. Therefore, the increased number of clathrin spots however with no difference in transferrin uptake suggests that FGFR signalling could be cargo specific (Auciello et al. 2013). The other possibility is the role of adaptor proteins which could yield differential trafficking of transferrin. For example, DAB2 (Disabled Protein-2), an adaptor protein involved in erythroid differentiation in hematopoietic cells regulates transferrin transport differently. While DAF2 inhibition affects transferrin internalization in human K562 erythroleukemic cells, a similar transferrin defect is not found in Hela cells. More importantly, it's found that transferrin trafficking could also vary with different culture conditions (Chu et al. 2014).

Taking all these into considerations, it could be said that though FGFR signalling may show cargo specificity, other factors such as adaptor proteins, cell specificity and varying cellular conditions could also affect transferrin trafficking, but from this study it is still inconclusive how FGFR signalling affect transferrin trafficking. Though inhibition of FGFR signalling showed no change in transferrin transport, inducing FGFR signalling with ligands such as FGF-1, FGF-2 also did not produce any strong FGFR phosphorylation response and hence with no ligand dependent activation of FGFR it's still not conclusive that FGFR signalling does not impact on Tfr uptake and recycling.

CHAPTER 7: GENERAL DISCUSION

7 GENERAL DISCUSSION

The FGFR pathway is known to regulate cell function via three known signalling pathways MAPK, AKT and PLC-γ (Eswarakumar et al. 2005). Recently, from a genome-wide analysis, Balklava et al., 2007 found several candidate genes in the FGFR downstream signalling pathway that could be involved in regulation of membrane trafficking. This study was focused on how these FGFR signalling components regulate membrane trafficking, the key transport steps affected and finally to compare the FGFR signalling and membrane trafficking events between *C.elegans* and mammalian cells.

The role of *egl-15* as a signalling component has been associated with different functions within the *C.elegans*. One of the well-established functions is the role *egl-15* in sex myoblast migration. The loss of *egl-15* functionality leads to animals with missing or mispositioned sex muscles causing Egl (egg laying defect) phenotype (DeVore et al. 1995). An increased *egl-15* signalling leads to fluid accumulation resulting in a clear (Clr) phenotype while a decreased signalling leads to Scr (Scrawny) or Let (Lethal) phenotypes (Huang & Stern 2004), but how FGFR signalling affects membrane trafficking is not well established yet and this work shows some insights on how FGFR signalling could co-ordinate membrane trafficking. The YP170-GFP assay combined with RNAi used in this study initially showed that FGFR signalling regulates membrane trafficking via the MAPK and PLC-γ pathways but not the AKT Kinase pathway. The efficiency of gene knock-down in *C.elegans* using RNAi is known to be usually high. Previous studies have reported that even small amounts of dsRNA are enough to silence genes. This was observed when just 2 molecules of dsRNA inactivated abundant UNC-22 mRNAs in *C.elegans* (Timmons & Fire 1998; Alder 2003). One possible explanation is that the RNAi signal is replicated and amplified into new dsRNA and these amplified dsRNAs can move between cells (Alder 2003). Analysing the upstream and downstream components of the whole FGFR system, it was found that genes such as *egl-15, sem-5, let-60, mpk-1, mek-2, plc-*

3, itr-1 all showed a strong yolk-GFP trafficking defect whereas genes such as *egl-17, let-756, lin-45, and sos-1* showed somewhat a milder phenotype and showed a stronger defective yolk trafficking phenotype only after extended time on RNAi. This could be due to number of factors like endogenous inhibitors of RNAi like *eri-1* and *rrf-3*, tissue specificity, half-life of protein and even external factors such as temperature. It should be noted that much of the genome-wide analysis have been done in *rrf-3* mutant worms, as the *rrf-3* mutation enhances sensitivity to RNAi. This was well established in *C.elegans* neurons where there was a clear loss of GFP expression in *rrf-3* background however not in wild-type worms (Simmer et al. 2002). The RNAi in *C.elegans* is heritable and can be maintained for three or more generations (Grishok 2005) but RNAi in progeny spread less efficiently unless genes expressed in the germline are targeted (Grishok et al. 2000). Therefore, a longer exposure to dsRNA increases RNAi efficiency even in subsequent progenies. But even with longer exposure for more generations, the signalling components of AKT pathway did not show any phenotype. Thus from these initial results, it was concluded that the AKT pathway might not have any role in regulation of membrane trafficking compared to the MAPK and PLCγ pathways. Though RNAi is revolutionary and easy, they do have some practical limitations such as intrinsic factors within the gene itself which make silence RNAi mechanism, RNAi antagonizing pathways, the lack of heritability beyond F1 generation of RNAi targeting somatic genes and chromatin factors (Grishok 2005). To circumvent these issues and to see if the same phenotypes are mimicked in mutant strains, mutant worms were used in the analysis. As predicted, the yolk trafficking phenotypes observed in RNAi experiments was observed in mutants, too. This time, the genes that showed milder phenotypes with RNAi such as *egl-17, let-756, lin-45, sos-1* showed a strong yolk trafficking defective phenotype in mutants. Thus, this display of variation in phenotype strength between RNAi (knock-down) and mutant (knock-out) could only be attributed to RNAi insensitivity, specificity or factors such as temperature and protein half life.

Thus these results show the significance of the full functionality of the FGFR signalling components and how an impairment in signalling would affect membrane trafficking.

RTKs are an intricate network of signalling pathways comprising various multi protein complexes that assemble at different intracellular locations for a timely and specific biological response. MAPK is one of the most widely studied pathways associated with RTKs. Generally, dimerization of RTKs leads to receptor activation and autophosphorylation. The RTK intracellular domains have specific binding sites for proteins that contain Src-homology 2 (SH2) and PTB domains. The activated RTKs then recruit various proteins to its sites such as Src, PLCγ, Shp-2, PI3K and adaptor proteins such as Grb2, SHC and docking proteins such as IRS, FRS. (McKay & Morrison 2007).

This study shows the signal transduction pathways downstream of *egl-15* is required for proper membrane trafficking. From this study, it is found that the FGF-FGFR signalling pathway regulating membrane trafficking follows two signal transduction pathways. One is the well-established Ras/MAPK cascade via the *sem-5*/Grb2 adaptor protein (Lo et al. 2010) and the other is the PLC-γ signalling pathway. The trafficking defects observed with the complete loss of either *egl-17* or *let-756* suggests the common understanding that the ligand is crucial for receptor activation. Interestingly, with RNAi experiments on *egl-17* and *let-756*, no yolk-trafficking defect was observed however in *egl-17* and *let-756* mutants there was strong Yolk-GFP trafficking defect. This result from RNAi experiments suggests that *egl-17* and *let-756* could be RNAi insensitive.

The Ras/MAPK cascade has been previously well studied in *C.elegans* and been shown to be essential for fluid homeostasis chemoattraction (Lo et al. 2010) and protein degradation (Szewczyk & Jacobson 2003) and once again from this study, it is evident that this *egl-15* is

essential for membrane trafficking. The subsequent signalling effector genes such as *sem-5, sos-1* and *let-60* showed differential trafficking defects.

Of all the yolk trafficking defects observed, the defects observed in *egl-15* mutants showed accumulation of Yolk-GFP in the intestine as well as the body cavity whereas, the *sem-5* mutants showed a severe endocytosis defect with high accumulation of yolk in the body cavity. This difference in Yolk-GFP trafficking showing both an endocytosis as well as a secretory defect via the same signal transduction pathway could be due to many reasons. The different isoforms of *egl-15* could possibly explain such a scenario. Different isoforms of *egl-15* and their functionalities change based on differences in their structure. It was found that the two isoforms of *EGL-15*, *EGL-15*(5A) and *EGL-15*(5B) mediate opposing mechanisms when regulating sex myoblasts where *EGL-15* (A) is required for SM chemoattraction while *EGL-15* (5B) is required for SM chemorepulsion to maintain SM migration in balance (Lo et al. 2008). A similar mechanism could be applied by *egl-15* in maintaining membrane trafficking.

The strength and duration of signalling can also have contrasting biological responses. For example, the activation of MAPK yields to different biological outcomes. In PC12 cells, EGF stimulates a transient MAPK activation which leads to cell proliferation however, when EGF receptors were overexpressed the MAPK activation became sustained and this resulted in cell differentiation (Gotoh et al. 1990; Nguyen et al. 1993; Traverse et al. 1994). Thus a transient and sustained MAPK activation can trigger different cellular responses (Ebisuya et al. 2005). The magnitude of signalling can also produce contrasting cellular responses. For example, during cell cycle, moderate levels of MAPK activity result in accumulation of CDK (Cyclin Dependent Kinase) complexes whereas high levels of MAPK activity stimulate CDK inhibitor p21 which reduces CDK accumulation causing G1 arrest (Sewing et al. 1997; Woods et al.

1997; Roovers & Assoian 2000) . Therefore, loss of *egl-15* function might relate to regulate endocytosis defect and also an opposite secretory defect.

Tyrosine autophosphorylation of RTKs is crucial for recruiting several other signalling proteins. The auto phosphorylation sites are located in the non-catalytic sides of the FGFR and signalling proteins with SH2 and PTB domains binding to these sites. The PLCγ pathway was the other pathway that was found to be regulating membrane trafficking. Upon the activation of FGFR, PLCγ is rapidly recruited to the activated FGFR through the binding of its SH2 domains to pTyr sites in receptor molecule. An activated PLCγ hydrolyses its substrate $PI(4,5)P_2$ to DAG and IP3. IP3 stimulates the release of Calcium which in turn activates a family of calmodulin-dependent protein kinases.

From this study, it was found that *plc-3, itr-1* and *pkc-2* showed trafficking defects similar to the ones observed in *egl-15* mutants. However, the key MAPK signalling pathway components *sem-5* and *let-60* which was found to regulate Yolk-GFP trafficking didn't show any differences in the localisation and distribution of some of the membrane markers in intestine. As mentioned previously, this could be due to the fact that the *C.elegans* intestine is a polarized epithelium having different plasma membranes (apical and basolateral) and different endosomal compartments. Protein transport via these different endosomal compartments require specific signals and hence this could probably be a reason that MAPK having a differential regulation. The other possibility could be that PKC might compensate an impaired function of Ras and Raf and therefore membrane trafficking in *C.elegans* intestine could be PKC dependent MAPK phosphorylation.

The difference in vesicle number, size and localisation in *egl-15* mutants suggest that *egl-15* might play a major role in endocytic sorting. Previously, (Wilde et al. 1999) have demonstrated that EGFR receptor signalling upon ligand activation stimulates modification and recruitment

of clathrin, and also activation of other RTKs could cause an increase in the number of clathrin-coated pits. Very recently, (Auciello et al. 2013) showed that FGFR activation in Hela cells increased both the number of CCPs and the events of clathrin-mediated endocytosis. In this current study, it was found that *egl-15* mutants showed a reduced number of CHC-1-GFP positive puncta compared to the wild type worms. This shows that *egl-15* could affect number of endocytosis events in a cell.

The distribution of various endolysosomal proteins were examined in *egl-15* mutants. The GFP-RAB-5, a marker for early endosomes and GFP-RAB-7, marker for late endosomes (Chen et al. 2010) showed a similar pattern of distribution and localization with a high number of ring like vesicles (late endosomes) and less number of puncta (early endosomes) observed in the intestine of wild-type worms and on the contrary a reduced number of vesicles and high number of puncta observed in the intestine of *egl-15* mutants. These results suggest that early to late endosome transport is affected.

GFP-RAB-35, a recycling endosome marker (Sato et al. 2008), demonstrated a reduction in the ring-like vesicles representing recycling endosomes in *egl-15* mutants when compared to wild type worms suggesting that *egl-15* was involved in regulation of the recycling step of membrane trafficking. . It is also found that RAB-35 accumulates on early endosomes and functions in a direct recycling route from early endosomes to the plasma membrane in *C.elegans* (Kouranti et al. 2006). It is also reported that from the kinetic studies in mammalian cells RAB35 is involved in rapid recycling of transferrin from early endosomes.

With the Golgi, apical and basolateral recycling endosome marker, GFP-RAB-10 (Shi et al. 2012), although not significantly different, a slightly higher number of GFP-RAB-10 punctate structures and vesicles observed in *egl-15* mutants compared to wild type worms supporting the role of *egl-15* in regulation of endocytic recycling. With GFP-RAB-35 showing a

significant difference in endosome number compared to GFP-RAB10, this result might point that *egl-15* could work preferentially on a faster recycling route.

The reduced number of GFP-hTFR labelled puncta and enlarged endosomes positive for hTAC-GFP in *egl-15* mutants also suggest a cargo trafficking defect. Enlarged intestinal endosome phenotypes have been previously described for *rme-1* and *rab-10* mutants (Zhang et al. 2001). But the RME-1-GFP localization and distribution in *egl-15* mutants in this study didn't show abnormal enlarged vesicles but rather poorly labelled RME-1-GFP along the apical and basolateral membranes in *egl-15* mutants compared to the wild type animals. The RME-1-GFP labelling in *egl-15* mutants in this study is similar to that of RME-1-GFP labelling in *tat-1* and *chat-1* mutants where the punctate staining pattern of GFP-RME-1 along the basolateral membranes was significantly reduced in *egl-15* mutants and GFP-RME-1 either formed large aggregates in the cytoplasm or became diffusive (Chen et al. 2010) indicating that RME-1-positive recycling endosomes are disrupted with loss of *egl-15* function. These results indicate that *egl-15* might likely be involved in transport of cargo via recycling endosomes in an *rme-1* dependent manner. Thus from the analysis of all these membrane markers, it can be said that *egl-15* affects endosomes along the endocytic uptake and recycling pathways and the loss of *egl-15* function could affect trafficking of cargo proteins.

To find out how well membrane trafficking via FGFR signalling is conserved between mammals and *C.elegans*, a mouse fibroblast cell line (NIH/3T3) was used and transferrin trafficking was analysed after stimulating or inhibiting FGFR.

Transferrin is transported in most cells through a well characterised uptake and recycling pathways. Diferric transferrin binds to transferrin receptor at the cell surface and is internalised and delivered to sorting endosomes. The mild acidic pH of the sorting endosome dissociates iron from the protein and the transferrin receptor together with apo-transferrin is recycled directly to the plasma membrane or routed to the recycling endosomes, from which they are

delivered to the cell surface (Maxfield & McGraw, 2004). In this study, the uptake and recycling of transferrin (Alexa Fluor488 transferrin) in NIH/3T3 was analysed. The transferrin trafficking was compared between control (normal conditions), FGF-1 stimulated and FGFR inhibited cells. The tyrosine kinase activity of FGFR was blocked by using an FGFR inhibitor SU5402. Analysis was carried out using two different methods - a flow cytometry and fluorescence microscopy.

The results from this analysis are inconclusive. Addition of FGF ligands to enhance FGFR signalling did not show any significant difference in FGFR phosphorylation compared to that of control. There was no significant difference in both transferrin uptake and recycling between control, FGFR inhibited and FGFR enhanced conditions. More than the transferrin trafficking itself, it's not clear why addition of FGF ligands failed to produce a stronger FGFR phosphorylation. One possibility could be that the cells serum deprivation of 30 minutes might not have been sufficient and subjecting them to a longer overnight serum starvation might induce a stronger FGFR phosphorylation with addition of FGF ligands.

Recently, Auciello *et al.*, 2013 reported that neither the FGF treatment nor the SU5402 inhibition had any impact on the transferrin entry in Hela cells suggesting that FGFR signalling could be cargo specific. In addition to cargo specificity, cell specificity, adaptor proteins, and varying cellular conditions could also affect transferrin trafficking (Chu et al. 2014).

SU5402, the FGFR inhibitor directly interacts with the catalytic domain of FGFRs (Mohammadi 1997). SU5402 is also known to block the FGF-1 induced tyrosine autophosphorylation of MAP kinases (ERK1 and ERK2) (Mohammadi 1997). MAP kinase activation is dependent on the intracellular kinase activity of FGFR1 and to find that transferrin uptake or recycling unaffected by SU5402 further suggests that the FGFR and its well

established downstream signalling cascade MAP kinase might not be involved in transferring trafficking is NIH/3T3 cells.

Another possible explanation would be that specific phosphorylation sites in FGFR would be key in regulation of membrane traffic. Though SU5402 blocks the autophosphorylation of major tyrosine residues at tyr-653 and tyr-654 which is essential for major biological responses including MAP kinase activation, the other phosphorylation residues go unblocked. For example, as discussed in the results, membrane trafficking is found to be regulated by FGFR activation of MAP kinase or PLCγ, but it has been found that PLC-γ activation of FGFR requires autophosphorylation at tyr-766 which then leads to influx of Ca^{2+} and activation of protein kinase C. It has been found that Tyr 653 and 654 are conserved in all known members of FGF receptor family and tyrosines 463, 583 and 585 are conserved among human, mouse, chicken Drosophila, *Xenopus* and *C.elegans*. Therefore, it would be interesting to study all the possible autophoshorylation sites in *egl-15* and to find the residues which are inhibited upon *egl-15* knock-down.

Thus using *C.elegans* as a model organism to analyse how FGF-FGFR signalling pathway regulates membrane trafficking, it was found that

1. *egl-15* (worm FGFR) affects Yolk-GFP trafficking via MAPK and PLC-γ signalling pathway and not via AKT pathway
2. *egl-15* regulates membrane trafficking via PLC-γ pathway at least in the intestine.
3. *egl-15* might affect different endocytic/recycling transport steps by impairing early, late and recycling endosomes in the *C.elegans* intestine.
4. *egl-15* might not be involved in secretory pathway at least in *C.elegans* intestine

These results open the window to further characterise the relation between FGF-FGFR signalling pathway and membrane trafficking. Future studies could involve the analysis of more membrane markers to precisely conclude the exact transport steps that could be affected. Also, by immunostaining the dissected intestines of *egl-15* mutant worms expressing YP170-GFP with antibodies against molecular markers of secretory/endocytosis compartments would help us understand how Yolk-GFP is regulated in *C.elegans*. Co-localisation studies on membrane markers such as RABs could be done by staining them with anti-RAB antibodies to see how they overlap between wild type and *egl-15* mutants. This could help us understand probably FGFR mediated defects in RAB maturation and how it impairs membrane trafficking. It would also be interesting to study the FGFR phosphorylation sites that are activated or inactivated during membrane trafficking using mass spectrometry. Like transferrin, other cargo proteins such as MHC-1 can be analysed in mammalian cell models to better understand how well FGFR signalling and membrane trafficking are conserved in mammalian models. It would also be interesting to identify the downstream transcription factors activated by FGF-FGFR signalling pathway during membrane trafficking. Thus, this study once again shows how cell signalling and membrane trafficking are intertwined and how the former regulates the latter. Understanding this relationship further would help us to identify several membrane trafficking diseases caused by deregulated signalling pathways and thus would help to serve as a therapeutic target.

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APENNDIX

List of all strains used in this work

Strain Name	Genotype
RT130	*unc-119 (ed3);pwIs23 [VIT-2::GFP]*
MT3456	*egl-15(n1477)X.*
MT1079	*egl-15(n484)X.*
ZIB107	*egl-15(n484); pwIs23*
ZIB20	*egl-15(n1477); pwIs23*
FF628	*let-756 (s2613) unc-32(e189) III.*
CB1313	*egl-17(e1313)X.*
MT3188	*egl-17(n1377).*
NH2192	*egl-17(ay8) X.*
NH2103	*egl-17(ay6) X.*
ZIB35	*egl-17(e1313); pwIs23*
ZIB109	*egl-17(ay6); pwIs23*
ZIB108	*egl-17(n1377); pwIs23*
ZIB110	*egl-17(ay8); pwIs23*
MT4698	*let-60(n1700)*
MT2124	*let-60(n1046)*
ZIB112	*let-60(n1046); pwIs23*
ZIB24	*let-60(n1700); pwIs23*
UP604	*sos-1(cs41)*
RT1222	*sos-1(cs41); bIs1*
ZIB78	*sos-1(cs41); pwIs23*
MT4185	*sem-5(n1779)*
UP148	*sem-5(cs15)*

ZIB2	*sem-5(cs15); pwls23*
ZIB118	*sem-5(n1779); pwls23*
MT7052	*lin-45(sy96) unc-24(e138)*
WU48	*lin-45(n2018) dpy-20(e1282)*
WU49	*lin-45(n2506) unc-24(e138)*
ZIB115	*lin-45(sy96) unc-24(e138); pwls23*
ZIB116	*lin-45(n2506) unc-24(e138); pwls23*
ZIB111	*lin-45(n2018) dpy-20(e1282); pwls23*
CB1068	*unc-79(e1068) mpk-1(n2521)*
SD939	*mpk-1(ga111)unc-79(e1068)*
MH37	*mpk-1(ku1)unc-(e189)*
ZIB100	*unc-79(e1068) mpk-1(n2521); pwls23*
ZIB113	*mpk-1(ku1)unc-(e189); pwls23*
ZIB114	*mpk-1(ga111)unc-79(e1068); pwls23*
BQ1	*akt-1(mg306)*
RB759	*akt-1(RB759);*
GR1310	*akt-1(mg144)*
JT573	*akt-1(sa573)*
ZIB80	*akt-1(mg306); pwls23*
ZIB81	*akt-1(mg144); pwls23*
ZIB73	*akt-1(ok525);pwls23*
ZIB119	*akt-1(sa573); pwls23*
VC204	*akt-2(ok393)*
ZIB31	*akt-2(ok393); pwls23*
JT709	*pdk-1(sa709)*
GR1318	*pdk-1(mg142)*

ZIB79	*pdk-1(sa709); pwIs23*
ZIB117	*pdk-1(mg142); pwIs23*
VC127	*pkc-2(ok328)*
JT73	*itr-1(sa73)*
PS2582	*itr-1(sy290) unc-24(e138) IV*
ZIB217	*itr-1(sa73); pwIs23*
ZIB211	*itr-1(sy290) unc-24(e138) IV; pwIs23*

List of strains used in the analysis of membrane markers

Strain Name	**Genotype**
GK35	*dkIs8(vha-6-GFP-CHC)*
ZIB16	*egl-15(n1477); dkIs8(vha-6-GFP-CHC)*
RT1242	*pwIs481[vha-6-MANS-GFP-E]*
ZIB9	*egl-15(n1477) X; pwIs481[vha-6-MANS-GFP-E]*
RT1120	*unc119(ed3); pwIs446[vha6-PH-GFP Cbunc119]F*
ZIB17	*egl-15(n1477); unc119(ed3); pwIs446[vha6-PH-GFP Cbunc119]F*
RT1323	*unc-119 (ed3); pwIs506[vha-6-SP-12-GFP]*
ZIB25	*egl-15(n1477); unc-119 (ed3); pwIs506[vha-6-SP-12-GFP]*
RT1579	*pwIs601[vha-6-ARF-6-GFP]*
	egl-15(n1477); pwIs601[vha-6-ARF-6-GFP]
RT348	*unc-119(ed3); pwIs87[vha6-GFP RME-1]*
ZIB54	*egl-15(n1477); unc-119(ed3); pwIs87[vha6-GFP RME-1]*
RT1970	*pwIs717[vha6-gfp(Asp718)c65THTFRshort]*
ZIB11	*egl-15(n1477); pwIs717[vha6-gfp(Asp718)c65THTFRshort]*
RT393	*unc-119(ed3); pwIs112[vha-6 GFP Cb unc-119 hTAC]*
ZIB12	*egl-15(n1477); unc-119(ed3); pwIs112[vha-6 GFP Cb unc-119 hTAC]*

RT327	*unc-119(ed3); pIs72[vha6-GFP::RAB5; unc-119(+) D1]*
ZIB18	*egl-15(n1477); unc-119(ed3); pIs72[vha-6::GFP::rab-5; unc-119(+) D1]*
RT476	*pwIs170[vha6 GFP CE RAB-7 Cb unc-119(+)]*
ZIB52	*egl-15(n1477); pwIs170[vha6 GFP CE RAB-7 Cb unc-119(+)]*
RT525	*unc-119(ed3)III; pwIs206[vha6p::GFP::RAB-10 + Cb unc-119(+)].*
ZIB130	*egl-15(n1477); unc-119(ed3)III; pwIs206[vha6p::GFP::RAB-10 + Cb unc-119(+)].*
RT311	*unc-119(ed3); pwIs69[vha6-GFP::RAB-11; unc-119(+)]*
RT910	*unc119; pwIs355[vha6::GFP::RAB35minigene]*
ZIB138	*egl-15(n1477); unc119; pwIs355[vha6::GFP::RAB35minigene]*