Chapter 1

Introduction
1.1 **Optineurin**

Optineurin (Optic neuropathy inducing protein) is a multifunctional protein, mutations in which have been implicated in glaucoma and amyotrophic lateral sclerosis (ALS), both being neurodegenerative disorders (Rezaie et al. 2002; Maruyama et al. 2010). It is a 577 amino acid protein of 67 kilodaltons (KD), in humans. It was first identified in 1998 by Li et al. in a yeast two-hybrid screening using an adenoviral protein E3-14.7K (group C early transcription region protein 3 of 14.7 KD) as bait (Li et al. 1998). It has been variously named as FIP2 (fourteen KD interacting protein 2, as it interacts with adenoviral protein E3 14.7K), NRP (NEMO related protein, because of its similarity to NEMO (NF-κB Essential MOdifier), HYP-L (huntingtin interacting protein), transcription factor IIIA interacting protein (TFIIIA-intP) (Faber et al. 1998; Li et al. 1998; Moreland et al. 2000; Schwamborn et al. 2000).

1.1.1 **Expression profile of optineurin**

Though mutations in optineurin have been linked to diseases associated with retinal ganglion cells and motor neurons, it is expressed in almost all the tissues. It is present in all parts of the body with particularly high levels in tissues taken from human brain, liver, heart, pancreas and skeletal muscle, as seen by mRNA expression levels (Li et al. 1998). In mouse, high expression levels were seen in heart, brain, liver, kidney and testis compared to skeletal muscle, spleen and lungs (Rezaie and Sarfarazi 2005). Its presence in non ocular tissues in significant amount indicates that optineurin has a common role to play in other cell types of the body, apart from its specific functions in retinal ganglion cells.

Optineurin gene is present on the short arm of human chromosome 10 (10p14-15) in a glaucoma-associated locus GLC1E spanning 37 kb of genomic region. **OPTN** gene consists of 16 exons, three of which are in 5’ UTR (UnTranslated Region). Alternative splicing gives four splice variants of transcripts, which give rise to the same protein (Rezaie et al. 2002). Human **OPTN** gene shows strong homology to mouse and monkey optineurin both at gene as well as protein level. Mouse optineurin shares 78% identity whereas monkey optineurin shows 96%
1.1.2 Domains in optineurin protein

Secondary structure prediction of optineurin has revealed the presence of several coiled coil (CC) domains, one leucine zipper (LZ) domain, one ubiquitin binding UBAN (Ubiquitin binding in ABIN and NEMO) domain (UBD) towards its C-terminus and a C₂H₂ type zinc finger domain which also binds ubiquitin, at its C-terminus (Li et al. 1998; Schwamborn et al. 2000; Zhu et al. 2007; Laplantine et al. 2009). Recently, an LC3-binding domain, LIR (LC3 interacting region) has also been shown to be present in optineurin (Wild et al. 2011) (Figure 1.1). LZ domain of optineurin spans from 144-170 aa, UBD spans from 424-509 aa, LIR from 169-209 aa and Zn finger domain from 556-577 aa. Coiled coil and leucine zipper domains have been implicated in its dimerization while the presence of zinc finger domain suggests its involvement in DNA binding (Wolfe et al. 2000; Burkhard et al. 2001). However, this is yet to be demonstrated experimentally. UBD of optineurin has been shown to bind to Lysine-63 linked polyubiquitin chains (Zhu et al. 2007).

Figure 1.1. Schematic of optineurin showing its domain organisation and its interacting proteins. CC- coiled coil, UBD- ubiquitin binding domain, LZ- leucine zipper, LIR- LC3 interacting region, ZF- zinc finger. The region of optineurin interacting with various proteins are also indicated. Htt- Huntingtin, mGluR- metabotropic glutamate receptor; MYPT1- myosin phosphatase targeting subunit 1; TBK1- TANK binding kinase 1; RIP1- receptor interacting protein 1.
1.1.3 Interacting proteins of optineurin

As optineurin does not have any enzymatic or catalytic property, it is expected to execute its functions by its interaction with other proteins. Several proteins have been shown to interact with optineurin (Figure 1.1). These interacting partners of optineurin are generally kept in four classes,

a) Involved in vesicular trafficking. Rab8, MyosinVI, huntingtin, transferrin receptor (TfR) fall in this class (Faber et al. 1998; Hattula and Peranen 2000; Sahlender et al. 2005; Nagabhushana et al. 2010).

b) Involved in TNF-α induced NF-κB pathway. E3-14.7K, ubiquitinated RIP (receptor interacting protein), CYLD, A20 etc. belong to this class (Li et al. 1998; Zhu et al. 2007; Chalasani et al. 2009).

c) Involved in autophagy. TBK1 (TANK binding kinase 1), LC3 (microtubule-associated protein 1 light chain 3) fall in this class (Morton et al. 2008; Wild et al. 2011).

d) Involved in cell cycle regulation. Plk1 (Polo-like kinase 1) and MYPT1 (myosin phosphatase target subunit 1) belong to this class (Kachaner et al. 2012a).

1.1.4 Functions of optineurin

Based on the diverse array of proteins interacting with optineurin, it is proposed to perform several functions inside the cells. The molecular mechanisms of the functions performed by optineurin are only beginning to be understood. Broadly, it is involved in regulating various cellular functions such as vesicular trafficking, Golgi organization, autophagy, NF-κB signalling, cell division, antiviral signalling, defence to bacterial infection and cell survival (Kachaner et al. 2012b; Ying and Yue 2012) (Figure 1.2).
Optineurin is involved in several cellular pathways. Schematic shows various functions performed by optineurin inside the cell. Proteins shown in the boxes are the ones involved in these pathways. Most of these proteins interact with optineurin.

1.1.4.1 Role of optineurin in vesicular trafficking

Since optineurin interacts with multiple proteins like Rab8, huntingtin, myosinVI, Tfr etc. that are involved in various intra-cellular trafficking pathways, its role in vesicular trafficking is evident. But the exact mechanisms by which optineurin performs its functions in trafficking are being uncovered only recently. MyosinVI is an actin based unusual (in the sense that it moves in the opposite direction when compared to other myosins) motor protein involved in various trafficking pathways (Wells et al. 1999). Optineurin in conjunction with myosinVI is required for maintenance of Golgi ribbon structure (Sahlender et al. 2005), polarized delivery of EGF receptor to the plasma membrane (Chibalina et al. 2010), sorting of AP-1B dependent cargo to the basolateral domain in polarized cells (Au et al.)
2007) and secretory vesicle fusion at the plasma membrane (Bond et al. 2010). Most of these processes are mediated by Rab8, also an optineurin interacting protein (Hattula and Peranen 2000). Optineurin was earlier identified as Huntingtin interacting protein (Faber et al. 1998). Later study showed that optineurin interacts with Rab8 through its N-terminus and recruits huntingtin to Rab8-positive vesicles (Hattula and Peranen 2000). One hypothesis being forwarded is that Rab8 recruits optineurin to link huntingtin and myosinVI to coordinate the movement of vesicles on microtubule and actin track (Sahlender et al. 2005). Mutations in huntingtin, as seen in Huntington’s disease, alter post Golgi trafficking to lysosomes by delocalising optineurin-Rab8 complex, which results in aberrant lysosomal function (del Toro et al. 2009).

1.1.4.2 Regulation of NF-κB by optineurin

Nuclear factor kappa B (NF-κB) is a family of inducible transcription factors, which is involved in regulating expression of genes involved in cell survival, immunity, inflammation, cell cycle, apoptosis etc. (Schreck et al. 1992; Gilmore 2006). Role of optineurin in TNFα and NF-κB signalling was long suspected, when it was observed that it shares 53% similarity to NEMO, which led to its earlier nomenclature, NRP (NEMO related protein) (Li et al. 1998). It is induced by TNFα and interacts with adenoviral protein E3-14.7K (Li et al. 1998). The role of optineurin in NF-κB signalling was shown by Zhu et al. (Zhu et al. 2007). Their work showed that optineurin acts as a negative regulator of TNFα–induced NF-κB signalling by binding to polyubiquitinated RIP (Zhu et al. 2007). Later, optineurin was shown to interact with CYLD (Chalasani et al. 2009), product of a tumor suppressor gene CYLD, involved in cylindromatosis or turban tumor syndrome. CYLD is a deubiquitinase which negatively regulates TNFα-induced NF-κB signalling by deubiquitinating polyubiquitinated RIP (Kovalenko et al. 2003; Trompouki et al. 2003; Sun 2010). By interacting with CYLD and also with polyubiquitinated RIP, optineurin facilitates deubiquitination of polyubiquitinated RIP by CYLD. In the absence of optineurin, CYLD is unable to deubiquitinate RIP leading to accumulation of polyubiquitinated RIP, even in the absence of any
stimulation, resulting in enhanced basal NF-κB activity. Thus, in NF-κB signalling, optineurin acts as an adaptor protein that brings together an enzyme (CYLD) and its substrate (polyubiquitinated RIP) together (Nagabhushana et al. 2011). A glaucoma-associated mutant of optineurin, H486R, is defective in inhibiting TNFα-induced NF-κB activation (Nagabhushana et al. 2011). The H486R mutant is associated with JOAG and POAG patients, and this mutant has not been found in any normal individual (Leung et al. 2003; Willoughby et al. 2004). This mutation lies in the ubiquitin-binding domain (Figure 1.3A). The H486R mutant shows drastically reduced interaction with CYLD and also shows somewhat reduced interaction with polyubiquitinated RIP (Nagabhushana et al. 2011). The inability of H486R mutant to inhibit TNFα-induced NF-κB activation is primarily due to defective interaction with CYLD although reduced interaction with RIP may also contribute to a small extent. This conclusion is supported by the finding that overexpressed CYLD was unable to deubiquitinate RIP and inhibit TNFα-induced NF-κB activity in presence of the H486R mutant. Thus it is clear that the interaction of optineurin with CYLD plays a crucial role in the regulation of TNFα-induced NF-κB activation (Nagabhushana et al. 2011).

1.1.4.3 Role of optineurin in autophagy

Autophagy is one of the intracellular quality control mechanisms for removing and degrading defective proteins and organelles in the lysosomes (Son et al. 2012). During induction of autophagy, specialized double membranous structures known as autophagosomes are formed which engulf the cargo (cytoplasmic components and organelles) and deliver it to lysosomes after fusion with the later (Yang and Klionsky 2009). Specificity in recruitment of cargo to autophagosomes is achieved by autophagy receptors. By definition, autophagy receptors have the ability to bind LC3 as well as ubiquitinated cargo simultaneously (Kirkin et al. 2009; Kraft et al. 2010). Proteins or organelles to be targeted for autophagy are tagged by ubiquitination, which then bind to autophagy receptors. LC3 is present in autophagosomal membranes, which serves as a marker for autophagosomes. Autophagy receptors, bound to cargo
to be degraded, interact with LC3 present on autophagosomes and are engulfed and fuse with lysosomes to be degraded (Tanida et al. 2004).

Optineurin was identified as an autophagy receptor and was shown to be involved in clearance of cytosolic Salmonella in macrophages (Wild et al. 2011). Optineurin directly interacts with ubiquitin and LC3 through well defined binding sites, and these interactions are necessary for autophagic clearance of bacteria (Wild et al. 2011). However, so far no specific protein of Salmonella has been identified as ligand for optineurin.

1.1.4.4 Regulation of mitosis by optineurin

Optineurin interacts with Polo-like kinase (Plk1) (Kachaner et al. 2012a). Plk1 is an important regulator of various events in cell division such as G2/M transition, centrosome maturation, chromosome segregation and cytokinesis. The precise control of these events depends on the kinase activity of Plk1 (van de Weerdt and Medema 2006; Petronczki et al. 2008; Song et al. 2012). During mitosis, optineurin is phosphorylated by Plk1 at Ser177 that leads to its relocalization to the nucleus from the Golgi. In the nucleus, optineurin enhances phosphorylation of MYPT1 (myosin phosphatase target subunit 1) by Cdk1 that leads to binding of MYPT1 with Plk1 and inactivation of Plk1. Knockdown of optineurin leads to defects in chromosome separation and formation of multinucleate cells (Kachaner et al. 2012a). Thus optineurin is involved in a feedback mechanism by which Plk1 modulates localization of optineurin that in turn regulates Plk1 activity and mitosis progression (Kachaner et al. 2012a).

Apart from these elaborative studies on optineurin, some studies have also implicated its role in antiviral signalling, gene expression, cell survival and cell death (Moreland et al. 2000; De Marco et al. 2006; Chalasani et al. 2007; Sudhakar et al. 2009; Mankouri et al. 2010; Sippl et al. 2011).
1.2 Optineurin and disease

The first indication that mutations in optineurin cause disease came from the work of Rezaie et al. in 2002. They showed that certain mutations in the coding region of the gene OPTN are associated with 16.7% of the families with normal tension glaucoma, the only gene to be implicated in this sub-type of glaucoma (Rezaie et al. 2002). Recently, certain mutations in optineurin have been shown to cause ALS (Maruyama et al. 2010; Millecamps et al. 2011; van Blitterswijk et al. 2011; Iida et al. 2012). These mutations are mostly different from those that cause glaucoma (Figure 1.3). Like glaucoma, ALS is also a progressive disease which involves degeneration of motor neurons in the primary cortex, brainstem and spinal cord (Maruyama et al. 2010).

![Figure 1.3. Disease associated mutations in optineurin.](image)

(A) Schematic showing various mutations in optineurin associated with POAG.

(B) Schematic shows mutations identified in ALS patients. Some mutations result because of deletion of certain exons, which generally leads to non-production of protein leading to reduction in protein levels.
1.2.1 Glaucoma

Glaucoma is a heterogeneous group of optic neuropathies characterized by the death of retinal ganglion cells (RGCs), leading to a deformation of the optic nerve, known as glaucomatous cupping, and progressive loss of the visual field. It is an irreversible disease of eye cupping and is the most common cause of irreversible blindness. This blindness is generally bilateral but not necessarily simultaneous (Quigley 1999). Worldwide, nearly 70 million people are affected by this optic neuropathy and 8.4 million are bilaterally blind (Resnikoff et al. 2004). It is associated generally but not necessarily with elevated intraocular pressure (IOP) because of accumulation of aqueous humour between iris and the lens (Vrabec and Levin 2007).

1.2.1.1 Types of glaucoma

There is debate over how the various forms of glaucoma be classified and several systems of classification exist but based on anatomical features two major types have been identified (Foster et al. 2002; Quigley 2011).

1.2.1.1.1 Open angle glaucoma

Primary open angle glaucoma (POAG) is the most common and clinically well defined subset of glaucoma (Weinreb and Khaw 2004). As the name suggests, in POAG, there is no anatomical hindrance to the flow of aqueous humor as the angle remains ‘open’. However, the drainage of humor is still inefficient resulting in an increase in IOP. Based on the age of onset, POAG can be juvenile (5-35 years) or adult onset (onset after 45 years) (Fan et al. 2006). POAGs are usually chronic and largely asymptomatic, with gradual elevation of IOP and consequent visual field loss.

Exfoliation glaucoma (XFG) is the most common identifiable form of open-angle glaucoma (Ritch and Schlotzer-Schrehardt 2001). It accounts for approximately 25% of all open angle glaucoma cases globally (Ritch 1994; Schlotzer-
In a significant proportion of patients with POAG, glaucoma occurs even in the absence of elevated IOP. This subtype of POAG is called normal tension glaucoma (NTG) (Rezaie et al. 2002).

### 1.2.1.1.2 Angle closure glaucoma

Angle closure glaucoma (ACG) is acute in incidence and is relatively rare in Caucasians. It is the most common form of glaucoma in Asian population (He et al. 2006; Friedman et al. 2012). In ACG, the iridocorneal angle is closed blocking the drainage of aqueous humor and resulting in elevation of IOP. Unlike POAGs, ACG is associated with symptoms like eye pain, blurred vision, headache, nausea, and hence is usually detected earlier (Libby et al. 2005). However, in Asian populations ACGs are generally asymptomatic (Foster et al. 2000; Quigley 2011).

Apart from these, one more subtype of glaucoma, developmental or congenital glaucoma is reported. In this form, developmental anomalies in tissues like trabecular meshwork and Schlemm’s canal cause blindness (Vasiliou and Gonzalez 2008).

### 1.2.1.2 Genetic basis of glaucoma

Considering that glaucoma is complex disease, both genetic heterogeneity and environmental factors complicate the disease. More so because genetic factors do not exhibit typical mendelian pattern of inheritance (Hewitt et al. 2006; Challa 2008). Over 20 chromosomal loci have been linked to glaucoma till date: GLC1A-1N, GLC3A-3C (Vasiliou and Gonzalez 2008). However, only five genes have so far been linked to glaucoma. While four genes – Myocilin/TIGR
(trabecular meshwork inducible glucocorticoid response), Optineurin, NTF4 (neurotrophin 4) and WDR36 (WD repeat 36), have been shown to be associated with POAGs, CYP1B1 (cytochrome p450-1B1) has been linked to congenital glaucoma (Fan et al. 2006; Hewitt et al. 2006; Vassiliou and Gonzalez 2008; Pasutto et al. 2009; Hilal et al. 2010). However, mutations in CYP1B1 have also been shown to be associated with POAG (Melki et al. 2004; Chakrabarti et al. 2007).

1.2.1.3 Optineurin in normal tension glaucoma

In the initial study of 54 families with autosomal dominantly inherited adult-onset POAG, OPTN (for optineurin) was identified as the causative gene for NTG. Sequence alterations in OPTN were found in 16.7% of families with hereditary POAG, including individuals with normal intraocular pressure (Rezaie et al. 2002). In the same study, optineurin mutations also led to an additional attributable risk factor of 13.6% in both familial and sporadic cases. The most common mutation found in optineurin was a missense mutation E50K (Glu-50-Lys) which was found in 13.5% of affected families (Rezaie et al. 2002). Later studies also showed that E50K is the most commonly encountered mutation in optineurin supporting the initial observations, though the frequency of optineurin mutations was much lesser in sporadic cases of POAG/NTG (Alward et al. 2003; Wiggs et al. 2003; Baird et al. 2004). Patients with this mutation have the most severe phenotype with extensive death of RGCs (Aung et al. 2005; Chalasani et al. 2007). Transgenic mouse with overexpression of E50K-optineurin shows thinning of retina because of massive apoptosis and degeneration of entire retina. This phenotype in E50K mutant mice was because of loss of RGCs and connecting synapses in the peripheral retina leading to a thinning of the nerve fiber layer at the optic nerve head at normal IOP (Chi et al. 2010).

Several other mutations in optineurin that are associated with adult onset NTG and in rare cases of juvenile onset glaucomas, have also been reported. Most of these optineurin mutations are missense mutations. A polymorphism in optineurin, M98K, is associated with glaucoma in some South Asian populations but not in Caucasians (Alward et al. 2003; Ayala-Lugo et al. 2007). One of the
rare mutations is an insertion in exon 5 which would lead to production of a truncated protein due to frameshift (Rezaie et al. 2002) (Figure 1.3A). H486R mutation has been found to be associated with JOAG (Willoughby et al. 2004). However, H486R mutation was also seen in POAG patients (Leung et al. 2003). Certain point mutations that do not cause a change in amino acid sequence, for example, V148V, T49T, P199P and T202T have also been reported (Leung et al. 2003). Considering that all the glaucoma-associated mutations of optineurin are single copy alterations, these are likely to be dominant. An alternate possibility is that these point mutations cause a loss of function and the resulting haploinsufficiency may cause the disease.

1.2.2 Amyotrophic lateral sclerosis (ALS)

Like glaucoma, ALS is also a progressive, neurodegenerative disease which involves degeneration of motor neurons in the primary cortex, brainstem and spinal cord (Kiernan et al. 2011). The average age at onset is 60 years with disease duration of only 3 years between symptom onset and death, usually due to respiratory failure (Shaw et al. 2001). Majority (~90%) of the ALS cases are sporadic (SALS), but remaining cases, which are familial (FALS), show mendelian pattern of inheritance (Beleza-Meireles and Al-Chalabi 2009).

1.2.2.1 Genetic basis of ALS

Till date 13 loci have been linked to ALS (Beleza-Meireles and Al-Chalabi 2009; Maruyama et al. 2010). Of the known loci, mutations in five genes, SOD1 (encodes copper/zinc ion-binding superoxide dismutase), TARDBP/TDP-43 (encodes TAR DNA binding protein), FUS/TLS (encodes fusion in sarcoma/translated in liposarcoma), ANG (encodes angiogenin, a ribonuclease of RNase A family), and OPTN (encodes optineurin) have been shown to cause a typical clinical phenotype. SOD1 mutations account for 20% of familial ALS and 5% of sporadic disease. TARDBP mutations account for 5–10%, mutations in FUS for 5%, and mutations in ANG for about 1% of familial ALS (Rosen et al. 1993;
Several mutations in optineurin have been linked to FALS (Figure 1.3B). In the first study by Maruyama et al., implicating role of optineurin mutation in ALS, three mutations were identified- a homozygous deletion of exon 5, a homozygous Q398X nonsense mutation and a heterozygous E478G missense mutation within its UBD (Maruyama et al. 2010). Later studies have identified several other mutations, a heterozygous nonsense 382_383insAG (2-bp “AG” insertion, also called 691_692insAG) mutation and a novel missense mutation R96L were reported in French familial ALS patients (Millecamps et al. 2011). In the screening of a large Dutch cohort of sporadic ALS patients, a nonsense Gln-165-stop (Q165X) and a missense Gln-454-Glu (Q454E) mutations were identified to be linked to SALS (van Blitterswijk et al. 2011). In a recent study in Japanese ALS patients, optineurin mutations were shown to be responsible for 3.8% of FALS cases and 0.29% of SALS cases (Iida et al. 2012). This study also identified three deletions in optineurin one being homozygous and two of them heterozygous. They also suggested that OPTN deletion mutation in ALS is not infrequent and the prevalence of the OPTN mutation in Japanese sporadic ALS is considerably high (Iida et al. 2012).

1.2.2.2 Types of ALS

Though the ALS phenotype might seem similar across populations, there are subtle differences in their clinical presentation. The clinical hallmark of ALS is the presence of UMN (upper motor neuron) and LMN (lower motor neuron) features involving brainstem and multiple spinal cord regions of innervations (Kiernan et al. 2011). Broadly, ALS can be classified into four types:

a) limb-onset ALS with a combination of upper and lower motor neuron (UMN and LMN) signs in the limbs. It is the most common form of ALS with an incidence of 70%.
b) **bulbar-onset ALS** with speech and swallowing difficulties and with limb features developing later in the course of the disease. It occurs in 25% of the cases.

c) **primary lateral sclerosis** with pure UMN involvement. It is less common.

d) **progressive muscular atrophy** with pure LMN involvement.

In more complex cases patients can present with bulbar-onset disease or limb-onset disease or with initial trunk or respiratory involvement, subsequently spreading to involve other regions (Kiernan et al. 2011).

### 1.2.3 Other diseases

Optineurin has also been seen to be localized in pathological structures in neurofibrillary tangles and dystrophic neurites in Alzheimer’s disease, Lewy bodies and Lewy neurites in Parkinson’s disease, ballooned neurons in Creutzfeldt–Jakob disease, glial cytoplasmic inclusions in multiple system atrophy, and Pick bodies in Pick disease (Osawa et al. 2011). However, it is not known whether optineurin present in these pathological aggregates is mutated or not. In addition, optineurin has been identified as one of the genetic risk factor for Paget’s disease of bone (PDB) (Albagha et al. 2010; Chung et al. 2010). Despite its association with glaucoma almost a decade ago and more recently in other neurodegenerative diseases, the cellular functions of optineurin and how its mutations alter these functions are beginning to be understood only now.

### 1.3 Vesicular trafficking

Vesicular trafficking is one of the most fundamental processes of eukaryotic cells, which more likely originated as a result of cellular compartmentalization and the resultant need to move things between them. It ensures supply of nutrients and signals to various compartments of the cell, crosstalk between various organelles inside the cell, secretion and exocytosis (Mellman 1996; Jahn and Sudhof 1999). Typically, vesicular traffic in a cell involves four basic steps: selection of cargo and budding of a vesiculo-tubular transport intermediate,
movement of this vesicle on a cytoskeletal track, tethering or docking with an appropriate target compartment and finally fusion of the vesicle with the target membrane (Zerial and McBride 2001) (Figure 1.4).

### 1.3.1 Mechanism of vesicular trafficking

Cargo selection is accomplished by recognition of specific signal sequences by coat proteins either directly or through their adaptor proteins. Apart from cargo selection, coat protein complexes also assist in vesicle budding (Kirchhausen 2000b; Bonifacino and Lippincott-Schwartz 2003; Bonifacino and Glick 2004; McMahon and Mills 2004; Cai et al. 2007). Coat proteins concentrate cargo molecules and cause deformation of donor membrane resulting in the formation of a bud which is ultimately pinched off to form a vesicle. The pinching off of the bud is mediated mostly by dynamin (Hinshaw 2000; Wiejak and Wyroba 2002). After their budding, vesicles are transported to their target sites along actin and/or microtubule cytoskeleton or less commonly by diffusion. The movement of a vesicle along actin or microtubule track is facilitated by ‘molecular motors’ like myosin, kinesin and dynein (Hirokawa et al. 1998; Vale 2003). The next critical step is tethering, which brings a vesicle and its target membrane in close proximity. Once the acceptor and the donor membrane are brought together, actual fusion of the vesicles takes place (Figure 1.4). Tethering and fusion are accomplished by concerted action of SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins, tethers and Rab GTPases (Cai et al. 2007).

### 1.3.2 Types of vesicular trafficking

Vesicular transport pathways can be broadly classified into two categories depending on its direction in relation to cell membrane, biosynthetic or secretory (exocytic) pathway and endocytic pathway.
Figure 1.4. Overview of various steps of vesicular trafficking.
Coat proteins along with adaptors concentrate cargo molecules and cause deformation of donor membrane to form a bud, which is eventually pinched off to form a vesicle (Sorting). These processes are regulated by Rab GTPases apart from other factors. Then coat proteins of these transport vesicles are lost (Uncoating). Vesicular transport is effected by motor proteins. The motor proteins are recruited to these vesicles along cytoskeletal tracks by Rab GTPases either by recruiting motor adaptors or by binding directly to motors (Motility). The incoming vesicle is docked in close proximity to its target membrane by tethering factors and SNAREs that are again recruited by Rabs (Tethering). Concerted action of SNAREs, tethers and Rabs facilitate the fusion of the donor and acceptor membranes (Fusion). Modified from Stenmark H., Nat. Rev. Mol. Cell Biol 10: 513-525, (2009)
1.3.2.1 Biosynthetic or secretory pathway

The biosynthetic or secretory pathway is undertaken by the proteins destined for secretion to outside the cell, plasma membrane or various compartments of endomembrane system from the time of its synthesis in the ER. Typically a membrane or a secretory protein targets its nascent polypeptide chain to rough endoplasmic reticulum (rER) membrane. Once synthesized in the rER these proteins are recognized by ‘cargo-sorting’ machinery and are incorporated into transport vesicles for their delivery into the Golgi. These proteins then move through the cisternae of Golgi, are modified post-translationally and eventually reach the trans-Golgi network (TGN). The TGN acts as a sorting station targeting a protein to its right destination, plasma membrane for membrane proteins or extracellular space for secretory cargo or to endosomal compartments like lysosomes (Derby and Gleeson 2007).

1.3.2.2 Endocytic trafficking

Endocytosis can be best described as a means of taking up materials inside the cells from the extra cellular environment. Endocytosis probably evolved as a means of taking up nutrients in eukaryotic cells and was perhaps the first vesicular trafficking event to emerge during the pro- to eukaryotic transition (Conner and Schmid 2003). Endocytosis encompasses multiple mechanisms by which cells internalize extracellular solutes and nutrients, membrane components like lipids and proteins, receptors and their bound ligands and their delivery to various intracellular destinations. Homeostasis of membrane composition, nutrient uptake, response to various environmental cues like metabolic and proliferative signals, intercellular and intracellular communication, recognition and protection against pathogens and transmission of neuronal signals are vital for existence and maintenance of multicellular metazoan organization and endocytosis has a role in all these processes at some or the other place (Mellman 1996; Sorkin and von Zastrow 2009). Endocytosis can be broadly divided into phagocytosis, which is the uptake of large solid particles, and pinocytosis, which involves uptake of fluid and small solid particles. While
phagocytosis is restricted to lower metazoans and certain specialized mammalian cell types, pinocytosis, commonly considered as endocytosis, occurs in almost all the cell types (Soldati and Schliwa 2006).

Generally, endocytosis is receptor mediated and involves identification of the nutrient/signal by receptor at the plasma membrane, its internalisation, its release inside the cell where it performs its various function, and recycling of the receptor back to the plasma membrane for further cycles to be performed or alternatively receptor degradation (Pastan and Willingham 1981; Mellman 1996; Platta and Stenmark 2011). These processes are assisted by several proteins like coat proteins, adaptor proteins, small GTPases (Rab, Rho, Arf etc.), SNARE proteins, EHD proteins etc.

1.3.3 Modes of endocytic trafficking

Once a ligand is bound to its receptor on the plasma membrane it gets internalised by invagination of the plasma membrane. Based on the proteins which assist in this process of invagination, two major modes of receptor mediated endocytosis have been classified (Doherty and McMahon 2009).

a) Clathrin dependent endocytosis (CDE): CDE is the most comprehensively studied mode of endocytosis. As the name suggests, clathrin assists at the point of internalisation of receptor-ligand complex. After binding of ligands to their specific receptors, receptor-ligand complexes generally aggregate in the clathrin rich region on the plasma membrane which gets invaginated into clathrin coated pits. With the help of the AP-2 adapter complex, clathrin triskelia form a cage like structure around invaginated membrane, and the activity of the dynamin, a GTPase, results in vesicle scission (Doherty and McMahon 2009; McMahon and Boucrot 2011). Various tools to study clathrin dependent endocytosis are available like labelled transferrin, labelled anthrax toxin etc. In our study, we have used Alexa-546 labelled transferrin, which is predominantly endocytosed via CDE in Hela cells.
b) **Clathrin independent endocytosis (CIE):** Some of the ligand receptor complexes are internalised without any assistance of clathrin. In contrast to CDE, CIE is relatively less studied and only in recent years these pathways have gained interest. Considering that there is no common theme and a diverse array of proteins are involved in it, these have been named variously based on the proteins involved (Mayor and Pagano 2007; Doherty and McMahon 2009; Hansen and Nichols 2009).

(i) Caveolin dependent endocytosis (Nichols 2002; Hansen and Nichols 2010).

(ii) GEEC/CLIC pathway- GPI-AP enriched early compartment/clathrin independent carrier (Chadda et al. 2007).

(iii) Arf6 dependent endocytosis (Palacios et al. 2002).

(iv) Flotillin dependent endocytosis (Glebov et al. 2006).

(v) IL2Rβ pathway (Tong et al. 2009).

### 1.3.4 Mechanism of endocytic trafficking

No matter what is the mode of entry of the cargo (receptor-ligand complex), the invaginated membrane now in the form of vesicles, reach early endosome (EE) or sorting endosome (SE) where sorting of the cargo takes place. Ligand is released at this point because of the acidic pH of EE and the receptor is freed to take either of the two routes, degradative or recycling pathway. Some receptors, like epidermal growth factor receptor (EGFR), are degraded whereas others, like transferrin receptor (TfR) and LDL receptor, are recycled back to plasma membrane (Wells 1999; Ceresa 2006; Grant and Donaldson 2009). The vesicles containing receptors destined to be degraded mature into Rab7 positive late endosome (LE) which later fuse with lysosomes where it gets degraded (Figure 1.5). On the other hand, vesicles containing receptors to be recycled can take one of the routes (Maxfield and McGraw 2004; Mayle et al. 2012):

(i) **Fast recycling route,** which is mediated chiefly by Rab4. Vesicles containing the receptors emerging from EE directly
Figure 1.5. Endocytic trafficking and recycling pathways.
Receptor bound to ligand is endocytosed by either clathrin mediated or clathrin independent route. After internalisation, receptor-ligand complex in the vesicle fuses to early or sorting endosome (EE/SE) where due to acidic pH of EE the complex is broken and ligand is released. Receptors can have two fates, degradation or recycling. Receptors to be degraded form multivesicular bodies (MVB) and can be degraded either by its fusion to lysosome or by exosome. Receptors to be recycled can take either of the two routes i.e. fast recycling mediated by Rab4, which is more direct or slow recycling mediated by Rab11 and Rab8, which involves intermediates like recycling endosome (RE) and recycling tubular endosomes (RTEs).
fuse with plasma membrane and the receptors are returned back to PM. It is comparatively fast with a $t_{1/2}$ of 2-3 minutes (Maxfield and McGraw 2004).

(ii) **Slow recycling route**, which is mediated mainly by Rab11. Vesicles containing receptors to be recycled emerging from EE fuse with an intermediate, the recycling endosome (RE) or endocytic recycling compartment (ERC) from where receptors are delivered to plasma membrane. It is relatively slow with a $t_{1/2}$ of ~11 minutes (Ciechanover et al. 1983; Dautry-Varsat et al. 1983). This mode of recycling will be discussed further in this study.

### 1.3.5 Proteins involved in endocytic trafficking and recycling

Several proteins are involved at the various stages of endocytic trafficking. Some of them are common to all the pathways of endocytic trafficking whereas a few of them are quite specific to a particular pathway. We have studied trafficking of transferrin and its receptor in this study.

Transferrin is a very important molecule for the cellular homeostasis. It binds two iron ($\text{Fe}^{3+}$) ions and is now ready to be internalised by the cell. It is then recognised by its receptor, TfR at the cell surface. After binding to TfR, transferrin is internalised and iron is delivered to the cell. Later, TfR is recycled back to the cell surface for further cycles of internalisation (Mayle et al. 2012). Here, some of the proteins involved in TfR trafficking are discussed.

#### 1.3.5.1 Clathrin as coat protein

Clathrin is a multimeric protein consisting of three heavy chains and two light chains that coat the endocytic vesicles by forming typical cage like structures (Kirchhausen 2000a). Clathrin was the first coat protein identified. Clathrin
coated vesicles arise mainly from the plasma membrane and trans-Golgi network (Pearse 1975; Robinson and Pearse 1986).

Apart from clathrin, two non-clathrin coats- COPI (coat protein complex/coatomer protein I) and COPII (coat protein complex/coatomer protein II) mediate transport between the Golgi and ER and from ER to the Golgi, respectively (Waters et al. 1991; Barlowe et al. 1994; Letourneur et al. 1994).

1.3.5.2 Adaptor proteins

The recruitment and assembly of clathrin coat to plasma membrane or Golgi membrane depends on certain adaptor protein (AP) complexes (Owen et al. 2004). AP complexes also mediate selective inclusion of membrane-anchored proteins into budding coated pits. The adaptor protein AP1 functions at the Golgi while AP2 at the plasma membrane (Mellman 1996; Owen et al. 2004).

1.3.5.3 Rab GTPases

Central in ensuring that cargoes are delivered to their correct destinations are the Rab GTPases. They are the largest family of the Ras superfamily of small GTPases. Rab GTPases are small monomeric proteins of 20 to 29 KD having intrinsic enzymatic activity to hydrolyze GTP to GDP (Colicelli 2004). More than 70 of them have been reported till date, indicating the diversity and complexity of endocytic trafficking in mammalian cells (Colicelli 2004; Stenmark 2009). They are critical regulators of membrane traffic and mediate almost all the steps of endocytic trafficking, including control of organelle identity and vesicle budding, uncoating, motility and fusion. A vital aspect of the Rab function is the specific localization of each Rab to a particular subcellular compartment and regulation of a specific transport event (Zerial and McBride 2001).

Rab GTPases function as molecular switches in the cell as they exist in two different forms, a GTP bound active form that is membrane associated, and a GDP bound inactive form that is cytoplasmic (Pfeffer and Aivazian 2004; Seabra
Rab GTPases mediate their functions mainly through effector proteins, which by definition, bind to the activated form of Rabs and not with their inactive form (Goody et al. 2005; Grosshans et al. 2006). The specific localization of a Rab itself is controlled, at least in part, by its interaction with the effector proteins and lipids (Zerial and McBride 2001). Many of the tethering factors that aid in docking are effectors of Rabs. Similarly, Rabs regulate vesicle fusion by indirectly regulating SNARE function through their effectors. Rabs have been shown to directly interact with actin based motors, myosins or microtubule based motors like kinesins or dyneins and dynactins (Hammer and Wu 2002; Seabra and Coudrier 2004). Several lines of evidence indicate a role for Rabs and its effectors in cargo selection and vesicle formation (de Hoop et al. 1994; Diaz and Pfeffer 1998; McLauchlan et al. 1998; Carroll et al. 2001). The versatility of Rabs in regulating virtually all the events of vesicular transport is due largely to their specific localization and activation along with their ability to signal through a diverse array of effectors which they interact with (Zerial and McBride 2001; Pfeffer and Aivazian 2004).

1.3.5.4 SNAREs and tethers

One of the key players in the membrane fusion events are the SNAREs (Soluble N-ethylmaleimide sensitive-factor-Attachment Protein Receptors), which provide specificity in membrane trafficking. It is believed that the specific pairing of t-SNAREs (present on target membrane) and v-SNAREs (present on incoming vesicles) drives vesicle fusion (Sollner et al. 1993; Rothman 1994; Ungar and Hughson 2003). Fidelity of membrane fusion is ensured by additional players particularly tethering factors and Rabs (Pfeffer 1999; Guo et al. 2000; Waters and Hughson 2000; Pfeffer 2001; Whyte and Munro 2002).

Tethers are postulated to function by acting as structural bridges. A tether on a target membrane recognizes and binds a specific determinant on the incoming vesicle thereby forming a molecular link holding the membranes together (Ungar and Hughson 2003; Cai et al. 2007). Many tethers are recruited onto specific
Figure 1.6. Rab GTPases act as molecular switches.

Rab GTPases exist in two forms inside the cells. GTP-bound or active state is membrane bound and is generally regarded as ON state. GDP-bound or inactive state is generally cytosolic and is called OFF state. The cycle between active and inactive states is effected by two classes of proteins, GAPs and GEFs. GAPs (GTPase activating proteins) accelerate the hydrolysing capacity of Rab-GTP and its conversion to Rab-GDP. Conversely, GEFs (guanine nucleotide exchange factors) replace GDP to add GTP on Rabs (A). A part of (A) which deals with the inactivation of Rabs has been elaborated in (B). In its active GTP-bound state Rab binds to its effector and performs its various functions listed. To counteract the constitutive effect, GAP is recruited to this Rab-effector complex and GTP is hydrolysed to GDP to bring the Rab to its inactive state. GDI (GDP dissociation inhibitor) present in the cytosol bind to these Rab-GDP to keep them in the inactive state. When Rab-GDP has to undergo cycle of activation, another protein GDF (GDI dissociation factor) binds to GDI and make Rab-GDP available for activation by GEF. Newly synthesised Rabs undergo geranyl-gerenylation by GGT (geranyl-geranyl transferase) to enter the cycle.
compartments and are downstream effectors of Rabs (Grosshans et al. 2006; Cai et al. 2007).

1.3.5.5 MICAL-L1 and EHD proteins

MICALs are large, multi-domain proteins and have been implicated in cytoskeletal regulation, exocytosis, apoptosis and neural development (Zhou et al. 2011). MICAL stands either for molecules interacting with CasL or microtubule associated CH and LIM domain containing proteins (Kolk and Pasterkamp 2007). MICAL-like (MICAL-L) proteins are a class of MICAL proteins that have an overall domain organization similar to MICALs but they lack the conserved N-terminal region and are implicated in recycling of receptors (Sharma et al. 2010). These generally act as adaptors and associate with tubular recycling membranes where EHD proteins also localizes. Of the several members of MICAL-L family, MICAL-L1 is of particular interest as it has been implicated in recycling of TfR and β1-integrin (Sharma et al. 2009; Rahajeng et al. 2010) and endocytosis of EGF receptor (Abou-Zeid et al. 2011). It serves as a linker between Rab8 and EHD1 (Sharma et al. 2009). The interaction of MICAL-L1 with GTP-bound Rab8 facilitates bridging of an indirect interaction between Rab8 and EHD1. MICAL-L1 is considered an effector of Rab8 as it interacts with the activated form of Rab8 (Sharma et al. 2009). Moreover, depletion of MICAL-L1 causes dissociation of Rab8 from the tubular membranes, which suggests that MICAL-L1 functions as an unusual effector of Rab8, because it recruits Rab8 to EHD1-containing tubular REs (Sharma et al. 2009).

EHD stands for C-terminal Eps15 homology domain containing proteins, a class of proteins belonging to dynamin superfamily. These are ATPases i.e. cytoplasmic EHD proteins bind ATP and dimerize. EHD dimerization causes the formation of a membrane binding site and the EHD proteins associate with tubular membranes, where they undergo further oligomerization (Naslavsky and Caplan 2011). Upon ATP hydrolysis, the membranes are destabilized, leading to scission of vesicles containing concentrated cargo/receptors, thus facilitating vesicular transport. EHDs have been linked to a number of Rab proteins through
their association with mutual effectors, suggesting a coordinated role in endocytic regulation and highlighting the significance of EHD proteins in these processes (Roland et al. 2007; Naslavsky and Caplan 2011).

1.4 Rab8

Rab8 is one of the most versatile Rab GTPases considering the diversity of functions it is involved in. Chavrier et al. isolated and reported about Rab8 as a mammalian homologue of fission yeast Ypt2 protein (Chavrier et al. 1990). Later, it was identified as a protein localizing to golgi, vesicular structures and basolateral plasma membrane (Huber et al. 1993b). Amongst mammalian Rabs, Rab8 is most similar to Rab10 and Rab13. RAB8 gene can complement the YPT2 gene of fission yeast, Schizosaccharomyces pombe and is also closely related to the SEC4 gene of budding yeast, Saccharomyces cerevisiae (Craighead et al. 1993). Most Rab proteins undergo double geranylgeranylation within CC or CXC motifs. Rab8 belongs to a subset of Rabs that contain a CAAX motif undergoing carboxyl methylation at the ER followed by single geranylgeranylation, and finally getting targetted to their destination (Leung et al. 2007).

Rab8 does not have a unique localization and its localization is context as well as activity dependent. It has been shown to localize to centrosome, juxtanuclear region, primary cilium, tracking tail of the cell, macropinosomes, leading edge of the cell and recycling tubular endosomes (RTEs) (Peranen 2011) (Figure 1.7).

1.4.1 Functions of Rab8

The first function to be assigned for Rab8 came from the work of Peranen et al. in 1996. They showed that in fibroblasts, Rab8 reorganises actin and microtubule machinery to promote polarized membrane transport (Peranen et al. 1996). Over the years, various functions for Rab8 have been assigned, which include cell migration, neuronal differentiation, cell polarization, ciliogenesis, cholesterol removal, insulin induced GLUT4 trafficking in muscle cells, exocytosis etc. (Chen and Wandinger-Ness 2001; Hattula et al. 2006; Watson and Pessin 2006; Linder et al. 2007; Nachury et al. 2007; Sato et al. 2007;
Figure 1.7. Localization of Rab8.
Apart from its juxtanuclear localisation, Rab8 localises to various other microdomains inside the cell which reflects its diverse functions. Localization to filopodium indicates its role in cell migration whereas its localization in primary cilium and centrosome point towards its function in sensing the environmental cues. Rab8 also localises to macropinosomes indicating its role in cell adhesion. Localization of Rab8 in vesicles and recycling tubular endosomes (RTEs) indicates its role in endocytic trafficking and recycling. Adapted and modified from Peranen J., Cytoskeleton, 68:10 (527–539), (2011).
Ishikura and Klip 2008; Peranen 2011). At the molecular level these functions of Rab8 are performed in part by its property to modulate various aspects of membrane trafficking. Rab8 is involved in regulating diverse trafficking pathways from the trans-Golgi network to the plasma membrane and in membrane trafficking at the RE (Huber et al. 1993b; Henry and Sheff 2008). It regulates endocytic trafficking of transferrin receptor to RE (Hattula et al. 2006), and recycling of TfR from RE to plasma membrane (Sharma et al. 2009; Rahajeng et al. 2010).

Studies with Rab8 conditional knockout mouse have revealed that Rab8 is essential for localization of apical proteins in intestinal epithelial cells. Apical peptidases and transporters mislocalize to lysosomes in small intestine of Rab8 null mouse leading to their degradation and this leads to a marked reduction in absorption of nutrients, ultimately leading to death of mouse in 3-4 weeks (Sato et al. 2007). In another study using knockout mouse (Rab8-/-), Rab8 was shown to be essential for apical localization of oligopeptide transporter PEPT1/SLC15A1 and sodium/glucose co-transporter SGLT1/SLC5A1 in small intestinal epithelial cells. Mislocalization and reduced expression of these transporters led to reduced gastrointestinal absorption of a beta-lactam antibiotic, cefixime, and alpha-methyl-d-glycopyranoside (α-MDG), respectively (Kato et al. 2009).

1.4.1.1 Cell Migration

Cell migration is a crucial event for various processes like embryonic development, metastasis, wound healing and inflammation. It is a complex process which is brought about by multiple cellular events including reorganization of the cytoskeleton, turnover of cell adhesions, and membrane trafficking. Rab8 expression induces the formation of actin containing filopodia and lamellipodia, structures known to mediate cell migration (Peranen et al. 1996; Hattula et al. 2006). Membrane turnover is important for cell migration. Rab8 helps in internalization of membrane from the cell surface ruffles that no longer participate in adhesion. It also helps in formation of new protrusions which
require recycling of membrane consisting of structural (adhesion receptors) and regulatory components (Arf6, Ras, and Rho GTPases) (Hattula et al. 2006). A cessation of this recycling pathway would promote cell surface stabilization and cell–cell adhesion, as seen in Rab8 deficient cells (Hattula et al. 2006). Thus, Rab8 appears to link membrane trafficking and recycling to cytoskeletal reorganisation to bring about cell migration.

1.4.1.2 Neuronal differentiation
Two basic phenomena in neuronal differentiation are formation of axonal and somatodendritic cell surface domains. Besides actin and microtubules, membrane trafficking plays an important role at different stages of neuronal differentiation i.e. neurite outgrowth, axon and dendrite formation, and synapse formation. In fully polarized hippocampal neurons, Rab8 has been shown to be localized to the somatodendritic domain, and it was suggested that Rab8 regulates the transport of newly synthesized Semliki Forest virus envelope proteins to dendrites, but not to the axon (Huber et al. 1993a). Rab8 is required for the delivery of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (AMPAR) to the dendritic spine surface (Brown et al. 2007). AMPA receptors are non-NMDA glutamate receptors that mediate fast synaptic transmission in the central nervous system (McGee and Bredt 2003). In the spine, Rab8 alongwith Rab11 and EHD1 mediates constitutive and regulated cycling of AMPARs, and controls spine size (Gerges et al. 2004; Park et al. 2004; Brown et al. 2007). Rab8 is linked to huntingtin by optineurin and the complex participates in signaling by mGluR5 that localizes to the dendritic spine (Anborgh et al. 2005). Overexpression of Rab8 in diverse cells induces the formation of long cell surface protrusions (Armstrong et al. 1996; Peranen et al. 1996; Hattula and Peranen 2000; Hattula et al. 2006). Taken together, these studies support a role for Rab8 in neurogenesis, especially in the formation of dynamic cell surface domains.

1.4.1.3 Ciliogenesis
Primary cilium is an antenna-like structure, present on almost every cell type (Singla and Reiter 2006). It acts as a sensory organelle which senses and
transmits signals from the extracellular environment. Defects in cilia have been implicated in numerous human developmental disorders (Lancaster and Gleeson 2009). In a screen with GFP tagged Rabs, Rab8 was the only Rab identified to be present in the cilia (Yoshimura et al. 2007). Endogenous Rab8 was also shown to be present in cilia (Nachury et al. 2007). Rab8 was conclusively shown to be required for ciliogenesis in activity dependent manner (Yoshimura et al. 2007; Westlake et al. 2011). Entry of Rab8-GTP to the primary cilium has been shown to promote extension of the ciliary membrane (Nachury et al. 2007). Rabin8 is a crucial component of this whole process, which acts as a Guanine nucleotide exchange factor (GEF) for Rab8. Rabin8 is a Rab11 effector, which activates Rab8 to bring about ciliogenesis (Westlake et al. 2011).

1.4.1.4 Epithelial polarization
Epithelial cells are polarized, which means that they have distinct domains, namely apical domain and basolateral domain. This classification is based not only on the geography of the cells but also on functional and structural differences (i.e. protein and lipid composition) between the two domains of plasma membrane. Apical domain, which faces the lumen, is generally secretory in nature whereas basolateral domain serves the function of anchorage apart from other functions. Rab8 overexpression was shown to disrupt epithelial polarity during the ongoing event of cell polarization (Henry and Sheff 2008). Knockout of Rab8 was also shown to cause accumulation of apical surface components in vacuoles of epithelial cells in intestine indicating a role of Rab8 in recycling apical cell surface components (Sato et al. 2007). Exocyst mediates membrane trafficking during the early stages of epithelial lumenogenesis (a process of formation of lumen in the small intestine). It is an octameric protein complex, which mediates vesicle tethering at plasma membrane for exocytosis. Rab8 is required for exocyst formation (Das and Guo 2011). A major event in lumen formation is polarity inversion through a massive transport of apical membrane, which is mediated by Rab8 (Bryant et al. 2010). Moreover, depletion of MyoVb also inhibits lumenogenesis by disrupting the Rab8/Rab11 vesicle transport route (Roland et al. 2011). However, inhibition of Rab8 does not seem
to affect the polarity of already polarized epithelial cells in confluent monolayers (Ang et al. 2003; Henry and Sheff 2008).

1.4.2 Rab8 in endocytic trafficking and recycling

Role of Rab8 in membrane trafficking and recycling is central to the various functions performed by it. Initially, Rab8 was implicated in post Golgi trafficking and secretory pathway (Craighead et al. 1993; Au et al. 2007; Henry and Sheff 2008). Later work showed that Rab8 links actin and microtubules and reorganises the two to coordinate the vesicle movements and these two events are not mutually exclusive. Rab8 synchronizes multiple events in transport from budding to fusion. Movement of vesicles inside the cell is achieved by cytoskeletal based motors. Rab8 links these different types of motors with the aid of associated proteins like optineurin, huntingtin etc. (Sahlender et al. 2005; Ying and Yue 2012).

In recent years, another role of Rab8 has emerged. Rab8 has been shown to bring cargo, which is to be recycled, to recycling endosomes. It is required for transport of TfR containing vesicles to RE (Hattula et al. 2006). Rab8 has been shown to localize to recycling tubular endosomes (RTEs) as evidenced by its colocalisation with MICAL-L1 and EHD1, which are markers for the same (Sharma et al. 2009). RTEs are prerequisite to the delivery of membrane and protein to the plasma membrane. This recent advancement in the knowledge about Rab8 has led us to an important conclusion that Rab8 is required for recycling of receptors.

1.4.3 Involvement of Rab8 in disease

So far no mutation in the RAB8 genes has been linked to any disease, but Rab8 knockout mouse does not survive beyond the age of 3-4 weeks due to reduced absorption of nutrients in small intestine (Sato et al. 2007; Kato et al. 2009). Given the fact that Rab8 is such an important player in vesicular trafficking, it can affect so many cellular pathways and its deregulation can cause disease
phenotypes. In fact, several diseases have been linked to deregulation of Rab8. This deregulation can be manifested in its enhanced or reduced interaction with the mutants of its interacting proteins. A case in citation is that of optineurin where the E50K mutant shows loss of direct interaction with Rab8 and leads to progressive retinal degeneration in mice (Chi et al. 2010). In another case Rab8 is linked to huntingtin by optineurin, and mutant huntingtin impairs post-Golgi transport by delocalizing optineurin and Rab8 (Hattula and Peranen 2000; del Toro et al. 2009). Altered Rab8 interaction has also been shown to be implicated in Bardet-Biedl syndrome (BBS), which is a pleiotropic ciliopathic human disorder (Nachury et al. 2007; Knodler et al. 2010). Mutations in the Rab8-interacting protein CEP290 are associated with the Meckel-Gruber syndrome (Kim et al. 2008; Tsang et al. 2008). Ahi1 (Abelson helper integration site 1) binds Rab8 and mutation of the former has been implicated in Joubert Syndrome (Hsiao et al. 2009). Rab8 also associates with OCRL1, a protein linked to the Lowe syndrome (Lowe 2005; Hou et al. 2011). Rab8 has also been linked to the human autosomal recessive polycystic kidney disease, through its association with fibrocystin (Follit et al. 2010). It has been shown that loss-of-function of MyoVb is the major cause of microvillus inclusion disease (Muller et al. 2008; Ruemmele et al. 2010). Rab8 interacts with MyoVb and knockout of Rab8 in the intestine produces a similar inclusion phenotype suggesting that function of Rab8 may be affected in the microvillus inclusion disease (Sato et al. 2007; Roland et al. 2011). Mutations in the alpha-synuclein gene cause familial Parkinson’s disease. Rab8 interacts with mutant alpha synuclein (Dalfo et al. 2004). Moreover, overexpression of Rab8, suppressed alpha-synuclein induced toxicity in neuronal models of Parkinson’s disease (Gitler et al. 2008).

1.4.4 Rab8 activation and inactivation

The assembly and disassembly of Rab GTPases and their effectors at specialized membrane domains are tightly controlled by their activation and inactivation. These regulators modulate specific cellular processes to prevent effector mislocalization, and to recruit Rabs to distinct intracellular compartments (Frasa et al. 2012). However, the regulation of the Rab GDP–GTP cycle is
comparatively less well understood than that of other small GTPase families, such as Ras and Rho GTPases. Thus, understanding the mechanisms of activation and inactivation of Rabs will provide insights into the spatiotemporal specificity of a given cellular process.

Regulation of Rab proteins is generally achieved by two classes of proteins—guanine nucleotide exchange factors (GEFs), which activate Rabs, and GTPase activating proteins (GAPs), which inactivate Rabs. GEFs exchange GDP of inactive Rabs for cellular GTP and thereby activate Rabs whereas the GAPs inactivate Rabs by hydrolysing GTP to GDP (Barr and Lambright 2010). There is consensus about the GEF for Rab8 and Rabin8 is the GEF for Rab8 (Hattula et al. 2002; Knodler et al. 2010; Westlake et al. 2011). But despite the identification of Rab8 almost two decade ago, establishment of a GAP for Rab8 is not conclusive. Several lines of evidence indicate involvement of multiple GAPs in inactivating Rab8 (Zeigerer et al. 2004; Yoshimura et al. 2007; Randhawa et al. 2008; Hokanson and Bretscher 2012). This is not uncommon among Rabs and there are examples where multiple GAPs inactivate one Rab or one GAP inactivates multiple Rabs (Fukuda 2011). This can be because of pathway specificity of GAPs.

1.5 GTPase activating proteins (GAPs)

Once effector of a Rab GTPase has performed its function, the effector needs to be down regulated. This is achieved by inactivation of Rab GTPase by GTPase activating proteins (Figure 1.6 B). GAPs bring Rab GTPases in an inactive Rab-GDP state, which leads to its dissociation from the membrane and the effector, and hence down regulation of effector functions. Cytosolic GDI (GDP dissociation inhibitor) binds to Rab-GDP to keep it in inactive state. In the event of next cycle to be performed, GDF (GDI dissociation factor) funnels away GDI and Rab-GDP is ready to undergo next cycle of activation and inactivation (Goody et al. 2005) (Figure 1.6B).
1.5.1 Rab GAPs are TBC domain proteins

Rab GAPs are fairly large proteins, which contain a prominent catalytic domain apart from other protein-protein interaction domains. Except for Rab3-GAP, GTPase activating proteins invariably contain a TBC (Tre2/Bub2p/Cdc16) domain (Fukui et al. 1997; Nagano et al. 1998; Fukuda 2011). This nomenclature is based on the fact that this domain is common to these three (Tre2, Bub2p and Cdc16) classes of proteins. TBC domain is a stretch of ~200 amino acids and serves as the catalytic domain. In humans, 44 TBC domain proteins have been identified till date (Bernards 2003; Frasa et al. 2012). Despite having a unifying theme in their primary structure and possibly secondary and tertiary structure, it has been an uphill task to assign GAPs to individual Rabs (Fukuda 2010). Now there seems to be consensus on specificity of the RabGAPs to various trafficking routes (Fukuda 2011). An extension to this statement is that one GAP might inactivate several Rabs and several GAPs might inactivate a single Rab.

1.5.2 Mechanism of inactivation of Rab GTPases by GAPs

Small GTPases like Rho and Ras are inactivated by their GAPs by a common mechanism called ‘Dual finger mechanism’ and this event is well characterised and supported by structural studies (Zhang et al. 1999; Fidyk and Cerione 2002). Rab GTPases are also thought to be inactivated by the same considering the homology among the GAPs of all the small GTPases both structurally and functionally. In fact, Rab GTPase homologues in yeast, the Ypt proteins, are known to employ dual finger mechanism for their inactivation by Gyps (GAP for Ypt proteins) (Albert et al. 1999; Rak et al. 2000; Segev 2001). Majority of the GAPs contain the crucial arginine (R) and glutamine (Q) in the respective motifs in their catalytic TBC domain. This arginine is a part of IxxDxxR motif and glutamine is a part of YxQ motif. When a Rab GTPase approaches its GAP for inactivation, glutamine (Q) from its DxxGQ aligns with the Q of the GAP, which facilitates insertion of R from IxxDxxR motif of GAP resulting in stabilisation of GTP hydrolysis state (Pan et al. 2006; Frasa et al. 2012).
1.5.3 TBC1D17

TBC1D17 is a member of TBC domain family of proteins that serve as Rab GAPs. It has been shown to possess a functional TBC domain (Fuchs et al. 2007). It belongs to the RQ subfamily of TBC domain proteins, which means that it employs arginine and glutamine finger to bring about inactivation of Rab GTPase by dual finger mechanism (Fuchs et al. 2007; Frasa et al. 2012). In humans, it is located on chromosome 19 (19q13.33) in a glaucoma associated locus 19q12-14 (Wiggs et al. 2000). It is a 648 amino acid protein and was identified as an optineurin interacting protein in a yeast two hybrid screen in our laboratory (Chalasani et al. 2009). The first report on TBC1D17 by Fuchs et al. showed that it inhibits shiga toxin trafficking from plasma membrane to Golgi. In the same study, in an in vitro assay several Rabs like Rab1, Rab5, Rab8, Rab13 and Rab21 were identified as its targets (Fuchs et al. 2007). A more recent study showed that TBC1D17 interacts with LC3 and GABARAPL1, two of the markers of autophagy, indicating its involvement in autophagy (Popovic et al. 2012). But the functional implications of these interactions are yet to be investigated.

1.8. Schematic showing various domains of TBC1D17.

TBC1D17 is a 648 amino acid protein with a prominent TBC domain spanning 310 to 520 amino acids. On its both sides, protein-protein interaction domains are present, NHL repeat domain on its N-terminus and proline rich domain on its C-terminus. The two catalytically important amino acid residues arginine (R) at 381 position and glutamine (Q) at 418 position are also shown.
Bioinformatic analysis of polypeptide of TBC1D17 has revealed presence of a prominent TBC domain and two poorly defined protein-protein interaction domains, namely NHL repeat domain (199-207 aa) and a proline rich domain (596-631 aa) (Figure 1.8). TBC domain of TBC1D17 is a 210 aa sequence with arginine and glutamine in their respective catalytic motif. Mutating these residues to alanine has shown that indeed these residues are important for catalysis (Pan et al. 2006; Fuchs et al. 2007). NHL repeat domain is N-terminus to TBC domain. It has been characterised as protein-protein interaction domain (Slack and Ruvkun 1998). Proline rich domain is on the C-terminus to the TBC domain in TBC1D17 and is a well characterised protein-protein interaction domain (Kay et al. 2000).

1.6 Background and objectives

Mutations in optineurin are associated with certain forms of glaucoma, a neurodegenerative disease that causes blindness and amyotrophic lateral sclerosis, a motor neuron disease. Optineurin has been implicated to play a role in vesicular trafficking, autophagy, regulation of mitosis and in NF-κB regulation. Earlier work from our laboratory has shown that E50K, a glaucoma-associated dominant mutation of optineurin causes cell death in mouse retinal ganglion cells (RGC-5) (Chalasani et al. 2007). It was also shown that optineurin is required for trafficking of TfR and the E50K mutant of optineurin causes defective trafficking of TfR (Nagabhushana et al. 2010; Park et al. 2010). However, the mechanism by which E50K mutant causes defective trafficking of TfR is not known. A yeast-two hybrid screen carried out in the laboratory led to the identification of several novel interacting proteins of optineurin, one of which is TBC1D17, a GTPase activating protein (Chalasani et al. 2009). But the functional significance of interaction of optineurin with TBC1D17 is not known. Interestingly, TBC1D17 is located in a glaucoma associated locus (Wiggs et al. 2000). Considering that optineurin interacts with diverse array of proteins like huntingtin, myosinVI and Rab8 and is considered to be an effector of Rab8, these observations point to a new and complex role played by optineurin in vesicular trafficking. In the present study we have tried to decipher the role of optineurin and TBC1D17 in endocytic
vesicular trafficking and provide an insight into mechanism of alteration caused by E50K mutant of optineurin. The main objectives of this study are

(i) To investigate the role of TBC1D17 and optineurin in transferrin receptor (TfR) trafficking and recycling, and Rab8 regulation.

(ii) To study the role of Rab8 and TBC1D17 in defective trafficking caused by a glaucoma-associated mutant of optineurin, E50K.

(iii) To study the role of TBC1D17 and optineurin in other endocytic pathways.

Chapter one introduces the relevant topics and provides background information about concepts pertaining to this study.

Chapter two lists the reagents used and detailed description of methods used in this study.

Chapter three describes the role of TBC1D17 and optineurin in the endocytic trafficking and recycling of transferrin receptor. Rab GTPases regulate various membrane trafficking pathways but the mechanisms by which GTPase activating proteins recognize specific Rabs are not clear. Rab8 is involved in controlling several functions including the trafficking of transferrin receptor from early endosome to recycling endosome. Here we provide evidence to show that TBC1D17, a Rab GTPase activating protein, through its catalytic activity, regulates Rab8-mediated endocytic trafficking of transferrin receptor. shRNA mediated knockdown of optineurin revealed that optineurin, a Rab8-binding effector protein, is required for localisation of TBC1D17 to juxta-nuclear region and its interaction with Rab8. A non-catalytic region of TBC1D17 is required for its direct interaction with optineurin. Co-expression of Rab8, but not other Rabs tested, rescues the inhibition of transferrin receptor trafficking by TBC1D17. Activated GTP-bound form of Rab8 is localized to the tubules emanating from the endocytic recycling compartment. Through its catalytic activity, TBC1D17 inhibits recruitment of Rab8 to the tubules and reduces colocalisation between transferrin receptor and Rab8. Knockdown of optineurin or TBC1D17 resulted in enhanced recruitment of Rab8 to the tubules. The results of several independent
experiments show that TBC1D17, through its interaction with optineurin, regulates Rab8-mediated endocytic recycling of transferrin receptor and recruitment of Rab8 to the tubules. This study describes a novel mechanism of regulating a Rab GTPase by an effector protein (optineurin) that acts as an adaptor to bring together a Rab (Rab8) and its GTPase activating protein (TBC1D17).

**Chapter four** describes results of experiments carried out to understand the mechanism of defective recycling of transferrin receptor caused by a glaucoma associated mutant of optineurin, E50K. E50K-optineurin causes defective trafficking and recycling of transferrin and its receptor. Co-expression of an activated form mimicking mutant of Rab8, Q67L rescues the effect of E50K on TfR trafficking. A catalytically inactive mutant of TBC1D17 as well as knockdown of TBC1D17 rescues this effect of E50K on TfR trafficking. E50K-optineurin also inhibits Rab8-GTP tubule formation and knockdown of TBC1D17 reverses this effect. These results suggest towards enhanced inactivation of Rab8 by TBC1D17 in presence of E50K. This also suggests that defective trafficking by E50K is mediated by TBC1D17.

**Chapter five** attempts to understand the role of TBC1D17 and optineurin in clathrin-independent endocytic pathway. The results suggest that TBC1D17, through its catalytic activity, inhibits this pathway. Optineurin knockdown results in inhibition of trafficking in this pathway. These results indicate that optineurin as well as TBC1D17 are involved in regulating clathrin-independent endocytic trafficking.