Chapter 5

Regulation of clathrin independent endocytosis by TBC1D17 and optineurin
5.1 Introduction

To say that clathrin-dependent endocytosis is the only or the major route of entry of nutrients and ligands inside the cell is an overstatement, but undoubtedly, it is the most comprehensively studied mode of endocytosis in mammalian systems (Mellman 1996; Conner and Schmid 2003; Maxfield and McGraw 2004; Doherty and McMahon 2009; Grant and Donaldson 2009). However, mammalian cells endocytose a variety of proteins and lipids without utilizing clathrin-mediated route (Iversen et al. 2001; Lamaze et al. 2001; Nichols and Lippincott-Schwartz 2001; Puri et al. 2001). There are various pathways which are employed to bring these ligands inside the cell, such as, caveolae dependent, flotilin dependent, Arf6 dependent etc. These pathways are collectively known as clathrin-independent endocytosis (Mayor and Pagano 2007; Gong et al. 2008; Doherty and McMahon 2009; Hansen and Nichols 2009). As a general rule, these pathways are more cholesterol-sensitive, possibly because their receptors are generally lipids attached to some sugar moiety. One other major reason of this high cholesterol sensitivity can be that the structures formed on the plasma membrane through which these ligands are endocytosed, are in lipid raft rich regions (Nichols and Lippincott-Schwartz 2001). Detailed molecular mechanisms for clathrin-independent endocytosis are unclear. The Golgi complex appears to be one principal intracellular destination for these ligands (Nichols et al. 2001). Several bacterial toxins enter the cell by binding to cell-surface receptors, being endocytosed, and ultimately translocating into the cytosol from an intracellular compartment (Wernick et al. 2010). A number of these toxins are taken up independently of clathrin and hence provide valuable markers for clathrin-independent endocytosis (Falnes and Sandvig 2000). A case in perspective is that of cholera toxin. In this chapter, B-subunit of cholera toxin (CTxB) has been used to study clathrin independent endocytosis. Uptake of CTxB involves caveolae, small invaginations in the plasma membrane, marked by the presence of caveolin protein (Henley et al. 1998; Oh et al. 1998; Orlandi and Fishman 1998; Torgersen et al. 2001). However, the identity of intermediate organelles involved in plasma membrane to Golgi trafficking, as well as the function of
caveolins, defining protein components of caveolae, are unclear (Kurzchalia and Parton 1999; Razani and Lisanti 2001).

Cholera toxin, an intracellularly acting toxin produced from *Vibrio cholera*, is composed of six subunits of two classes and represents a structure AB$_5$. A-subunit is responsible for its virulence by virtue of its toxic, ADP-ribosylating activity while B-subunits are responsible for its entry inside the cell. In fact, B-subunit is required for the delivery of the toxin to the Golgi (Chinnapen et al. 2007). From the Golgi, the holo-toxin (AB$_5$) is trafficked to the endoplasmic reticulum (ER), where A-subunit is released into the cytosol by retrotranslocation. For studies in endocytosis, a non-virulent form of cholera toxin, CTxB is used which lacks the A-subunit (Lencer et al. 1999). The B-subunit mediates uptake of the holo-toxin into the cell by its binding to GM1, a glycosphingolipid found both in caveolae and elsewhere on the plasma membrane, within lipid rafts. The assumption that uptake of the cholera toxin B subunit directly and exclusively corresponds to budding of caveolae from the plasma membrane, though seems appealing but is not completely true. Cholera toxin B subunit and GM1 are also detected in clathrin-coated pits (Tran et al. 1987). Pharmacological studies on the uptake of cholera toxin suggest that agents that perturb clathrin function reduce the total amount of toxin internalized into cells (Orlandi and Fishman 1998; Schapiro et al. 1998; Shogomori and Futerman 2001). This approach also reveals a crucial difference between toxin internalization, which is in part clathrin dependent, and toxin activity, which is not clathrin dependent (Orlandi and Fishman 1998). Also, mutants of epsin and eps15 cause a partial decrease in the total amount of cholera toxin B subunit taken up into the cell, with no measurable effect on delivery to the Golgi apparatus (Nichols et al. 2001). For cholera toxin to be toxic, it must first reach the Golgi apparatus (Lencer et al. 1999), which implies that although cholera toxin can be taken up through multiple pathways, only non-clathrin pathways lead to delivery to the Golgi. However, the diversity of endocytic pathways and potential for simultaneous uptake through multiple routes frequently makes interpretation of the results complex. A brief account of itinerary of CTxB from the plasma membrane to the Golgi is shown in Figure 5.1 (Chinnapen et al. 2007). Thus, although there is now good evidence from several sources for a
Figure. 5.1. Itinerary of cholera toxin.
A schematic showing the various modes of entry of cholera toxin inside the cell and its subsequent journey. Once the holotoxin containing A and B subunit of cholera toxin reaches the endoplasmic reticulum (ER), the A subunit dissociates from the holotoxin and gets retro-translocated into the cytoplasm to show its toxic effects. CLIC, clathrin independent (noncaveolar/nonclathrin-mediated) carrier. Adapted and modified from Chinnapen et al., FEMS Microbiol Lett 266 (2007) 129–137.
clathrin-independent pathway from the plasma membrane to the Golgi apparatus, the extent of cross-talk between this pathway and conventional early and recycling endosomes, has not been estimated and needs to be investigated in more detail. Moreover, a fluorescent analogue of sphingomyelin, which, like cholera toxin B subunit, can be taken up by both clathrin-dependent and clathrin-independent mechanisms, is delivered to the Golgi through either of these two alternative uptake pathways (Puri et al. 2001).

Small GTPases, which function through their ability to activate their effectors, have a profound role to play in both clathrin-dependent and clathrin-independent endocytic trafficking. GTPase activating proteins are important regulators of Rab GTPases, which inactivate the later and in turn down regulate the effector functions to maintain cellular homeostasis. Rab8 is known to have a role in the mediation of both clathrin-dependent and clathrin-independent endocytosis. Rab8 interference in clathrin-independent endocytosis is generally Arf6-dependent (Radhakrishna and Donaldson 1997; Brown et al. 2001; Rodriguez-Gabin et al. 2001; Hattula et al. 2006). Rab8 is also known to colocalize with caveolin, a protein involved in caveolin-mediated clathrin-independent endocytosis, on recycling tubular endosomes induced by Arf6 where EHD proteins also colocalize (Verma et al. 2010).

Considering that both optineurin and TBC1D17 regulate endocytic trafficking and recycling of transferrin and its receptor, Tfn/TfR (which is clathrin-dependent), their role in cholera toxin trafficking to the Golgi (which is primarily clathrin-independent) was explored. Knockdown of optineurin slowed down the trafficking of CTxB. Knockdown of optineurin also disrupted the trans-Golgi network (TGN). Ubiquitin-binding domain of optineurin is required for its role in CTxB trafficking. A glaucoma-associated mutant of optineurin, E50K, strongly inhibits uptake of CTxB inside the cell whereas wild type optineurin does not. TBC1D17 inhibited CTxB uptake but its catalytically inactive R381A mutant did not, indicating that this function of TBC1D17 is dependent on its catalytic activity. The results presented here suggest that optineurin as well as TBC1D17 regulate trafficking of cholera toxin (CTxB) from plasma membrane to the Golgi.
5.2 Results

5.2.1 TBC1D17 inhibits cholera toxin uptake

The results presented in Chapter 3 showed that TBC1D17 regulates trafficking and recycling of Tf/TfR that is mediated by clathrin-dependent pathway. To explore the role of TBC1D17 in other endocytic pathways, the role of TBC1D17 in regulating the trafficking of cholera toxin was examined by CTxB uptake assay. An engineered version of cholera toxin, which contains only the B-subunit (CTxB) labelled with Alexa fluor-555, was used for the experiments. Hela cells transfected with TBC1D17 or its mutants R381A and ∆309, were incubated with Alexa-555 labelled CTxB and the cells were allowed to undergo uptake for 25 minutes at 37°C. TBC1D17 expressing cells were seen to take up very less amount (16.2±11%) of CTxB (Figure 5.2A). R381A mutant also showed some reduction in uptake (73.5±26%) as compared to non-expressing cells (Figure 5.2B). The optineurin-binding deficient mutant, ∆309, also inhibited uptake of labelled cholera toxin (Figure 5.2A,B). Considering that majority of cholera toxin is taken inside the Hela cells via clathrin-independent route, these results indicate towards inhibition of clathrin-independent trafficking by TBC1D17. These results also suggest that this action of TBC1D17 is dependent on its catalytic activity and probably independent of its optineurin-binding potential.

5.2.2 Knockdown of optineurin inhibits cholera toxin trafficking

To examine the role of optineurin in CTxB trafficking, optineurin was knocked down in Hela cells using adenoviruses expressing shRNA against optineurin and control adenoviruses. These cells were incubated with Alexa-555 labelled CTxB and uptake was performed for 18 minutes at 37°C. Much more CTxB was seen in the Golgi region in control cells compared to optineurin knockdown cells (Figure 5.3A). In knockdown cells, CTxB distribution was more peripheral compared to control cells. Efficacy of knockdown was ascertained by western blotting of the lysates prepared from control or optineurin knockdown cells (Figure 5.3B). To further check the specificity of role of optineurin in CTxB trafficking, a shRNA resistant mutant of optineurin was used which does not get
Figure 5.2. TBC1D17 inhibits uptake of cholera toxin.
(A) HeLa cells were transfected with TBC1D17 or its mutants R381A and Δ309, and cholera toxin uptake assay was performed. Cells were analysed with confocal microscopy. Scale bar, 10μm. (B) Graph shows relative fluorescence intensity of endocytosed CTxB by the cells expressing TBC1D17, R381A or Δ309 compared to non-expressing cells. ***P<0.001. (C) HeLa cells were incubated with Alexa fluor-555 labelled CTxB and cholera toxin uptake assay was performed. Cells were stained for TGN-38 after fixing. Scale bar, 10μm.
Figure 5.3. Optineurin is required for cholera toxin trafficking.
(A) Hela cells were infected with adenoviruses expressing two different shRNA against optineurin and control adenoviruses. After 72 hours, cholera toxin uptake assay was performed and cells were analysed by confocal microscopy. GFP is the indicator of infection by adenoviruses. (B) Cell lysates from optineurin knockdown cells and control cells were analysed by western blotting.
degraded by one of the shRNAs, 2H. Upon overexpression of shRNA resistant mutant of optineurin in the background of optineurin knockdown, the cells overexpressing this mutant were able to rescue the effect of optineurin knockdown as CTxB was again seen in the Golgi region (Figure 5.4, upper panel). Furthermore, overexpression of shRNA resistant D474N mutant of optineurin, which does not get degraded by shRNA against optineurin and is also defective in ubiquitin binding, again led to peripheral distribution of CTxB, indicating that ubiquitin-binding domain (UBD) of optineurin is required for its role in CTxB trafficking to the Golgi region (Figure 5.4, lower panel). These shRNA resistant mutants of optineurin have already been characterised in an earlier study in the laboratory (Nagabhushana et al. 2010).

5.2.3 E50K-optineurin inhibits uptake of cholera toxin

It appears that optineurin has a role to play in CTxB trafficking. In the previous chapter, a glaucoma-associated mutant of optineurin, E50K, was shown to impair Tfr recycling. Hence the effect of E50K on CTxB trafficking was examined. For this, Hela cells expressing E50K mutant or wild type optineurin were subjected to CTxB uptake assay. E50K expressing cells showed drastic reduction in uptake (16.8±9% uptake) as compared to non expressing or optineurin expressing cells (Figure 5.5A,B). A ubiquitin-binding deficient mutant of E50K, E50K-D474N did not show inhibition of CTxB uptake (Figure 5.5A,B). E50K-D474N has been earlier shown to be exhibiting less severe phenotype as compared to E50K such as smaller size of vesicles and reduced number of vesicles per cell (Nagabhushana et al. 2010). Taken together, these results suggest that E50K-optineurin inhibits uptake of CTxB and this requires its functional ubiquitin binding domain.

5.2.4 Effect of TBC1D17 on trans-Golgi network

Biosynthesis of sphingolipids and their post-synthesis modification (addition of sugar moiety to the sphingolipid) occurs in the Golgi (Kent 1995; Henneberry et al. 2002). Consequently, these glycosphingolipids are packaged into transport
Figure 5.4. Functional ubiquitin-binding domain (UBD) of optineurin is essential for its role in cholera toxin trafficking.

shRNA resistant optineurin (upper panels) and shRNA resistant D474N mutant (lower panels) of optineurin were transfected in optineurin knockdown background in Hela cells and cholera toxin uptake assay was performed. Cells were analysed by confocal microscopy. GFP is shown in blue. Scale bar, 10μm. These mutants have earlier been characterised in the laboratory.
Figure 5.5. E50K-optineurin inhibits cholera toxin uptake.
(A) Hela cells were transfected with plasmids expressing optineurin and its mutants, E50K and E50K-D474N and cholera toxin uptake assay was performed. The cells were analysed by confocal microscopy. Scale bar, 10μm. (B) Graph shows relative fluorescence intensity of endocytosed CTxB by the cells expressing optineurin, E50K or E50K-D474N, compared to non-expressing cells. *P<0.05, ***P<0.001.
vesicles. These transport vesicles are then budded from the TGN to be transported to the PM. The fusion of transport vesicles with the PM leads to embedding of these sphingolipids in the PM. Receptor for cholera toxin GM1 is a glycosphingolipid and is synthesized in Golgi (Hajri and Abumrad 2002). Therefore, the reduced uptake by TBC1D17 or E50K expressing cells could be due to reduced amount of GM1 receptors in the plasma membrane. To test this hypothesis, effect of TBC1D17 on the Golgi integrity was examined. Hela cells expressing TBC1D17 or its catalytically inactive R381A mutant were stained for TGN-38, a marker for trans-Golgi network. TBC1D17-expressing cells showed a complete depletion of TGN in most of the cells while R381A expressing cells did not (Figure 5.6A,B). Expression of TBC1D17 or its catalytically inactive mutant, R381A, did not show any effect on staining of giantin, a cis-Golgi marker (Figure 5.6C,D). These results suggest that TBC1D17, through its catalytic activity, disrupts TGN but does not affect cis-Golgi.

5.2.5 Optineurin knockdown leads to disintegration of TGN
Optineurin is known to have a profound role in maintenance of Golgi structure by remodeling the actin and microtubule cytoskeleton (Sahlender et al. 2005). Golgi is the principle site for export of newly synthesized sphingolipids to the plasma membrane. So, the role of optineurin on depletion or disintegration of TGN was examined. For this, Hela cells were infected with adenoviruses expressing shRNA against optineurin or control adenoviruses and stained for TGN-38. Confocal microscopy revealed that optineurin knockdown cells showed disintegration of TGN but control cells did not (Figure 5.7A). When Hela cells expressing E50K and wild type optineurin were stained for TGN-38, E50K-optineurin expressing cells showed disintegration of TGN whereas wild type showed significantly less disintegration of TGN (Figure 5.7B,C).

5.3 Discussion
Mutations in OPTN gene which encodes optineurin protein have been linked to some glaucomas and ALS, both being neurodegenerative diseases (Rezaie et
Figure 5.6. TBC1D17 expression disrupts trans-Golgi network but not cis-Golgi.

(A&C) Hela cells were transfected with TBC1D17 or its R381A mutant, stained for TGN-38 (A) or giantin (C), and analysed by confocal microscopy. Scale bar, 10μm. (B&D) Graphs show percentage of cells with disruption or depletion of TGN (B) and cis-Golgi (D) in TBC1D17 or R381A expressing cells as compared to non-expressing (NE) cells. ***P<0.001.
Figure 5.7. Optineurin knockdown or E50K expression results in disruption of trans-Golgi network (TGN).
(A) Hela cells were infected with adenoviruses expressing shRNA against optineurin or control adenoviruses. After 72 hours of infection, cells were stained for TGN-38, a trans-golgi marker and cells were analysed by confocal microscopy. Scale bar, 10μm. (B) Hela cells transfected with wild type optineurin or its E50K mutant were stained for TGN-38 and analysed by confocal microscopy. Scale bar, 10μm. (C) Graph showing percentage of cells showing disruption of TGN.
TBC1D17 and optineurin regulate CIE

al. 2002; Maruyama et al. 2010). The glaucoma causing E50K mutant of optineurin is a dominant mutation which causes death of RGCs possibly by production of reactive oxygen species and defective trafficking of TfR (Chalasani et al. 2007; Chi et al. 2010; Nagabhushana et al. 2010; Park et al. 2010). Optineurin is an adapter protein which has a diverse role to play in the cell. These include, endocytic trafficking, autophagy, organization of Golgi, regulation of TNF-α induced NF-κB regulation etc. (Sahlender et al. 2005; Zhu et al. 2007; Nagabhushana et al. 2010; Park et al. 2010; Wild et al. 2011; Kachaner et al. 2012a). Defect in anyone or more than one of these processes can potentially lead to the disease phenotype observed in E50K patients. It appears that defect in trafficking is a more pronounced phenotype in cell culture conditions by E50K-optineurin which might contribute to RGC death. After establishing the role of optineurin and TBC1D17 in clathrin mediated trafficking, their role in clathrin independent endocytic trafficking was investigated. Knockdown of optineurin slowed down the trafficking of CTxB from plasma membrane to Golgi pointing towards importance of optineurin in this pathway too. Requirement of UBD of optineurin was also established in this process. But the precise step where optineurin acts in this pathway is yet to be investigated.

TBC1D17 is a GTPase activating protein which inactivates Rab8 that is mediated by optineurin (this study). Rab8 regulates both clathrin dependent as well as clathrin independent endocytic trafficking albeit by differential dependency on associated proteins. So it is pertinent to think of a role of TBC1D17 in CTxB trafficking. In fact, overexpression of TBC1D17 inhibited uptake of CTxB. However this action of TBC1D17 appears to be independent of optineurin since optineurin binding deficient deletion mutant of TBC1D17, ∆309 also inhibited the uptake of CTxB. Rab GAPs are known to be redundant in their actions (Bernards 2003; Fukuda 2011; Frasa et al. 2012), which implies that they can act on more than one Rab GTPase. Hence, it is likely that TBC1D17 inactivates some other Rabs to regulate clathrin-independent endocytic pathway.

E50K-optineurin, a glaucoma-associated dominant mutant of optineurin causes cell death by production of reactive oxygen species and defects in trafficking and recycling of transferrin and its receptor (Chalasani et al. 2007; Nagabhushana et
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al. 2010; Park et al. 2010). Results presented in this chapter indicate that clathrin-independent endocytic trafficking is also inhibited by E50K. Although clathrin-independent endocytosis appears to be a preferred mode of entry of bacterial toxins, it is also important for replenishment of some of the membrane proteins and lipids in the plasma membrane (Sandvig et al. 2008; Donaldson et al. 2009). So, its alteration, as seen in E50K-optineurin expressing cells, can have potential implications in membrane homeostasis and disease.

Ubiquitin is a conserved 76 amino acid polypeptide, which possesses the ability to be covalently attached to different proteins (Kerscher et al. 2006) and thereby acts as a tag. Optineurin is one such molecule which binds ubiquitinated proteins through its ubiquitin-binding domain (UBD). Ubiquitination of a protein and its consequent binding to proteins like optineurin has been shown to regulate various cellular processes, like NF-κB regulation, autophagy and endocytic trafficking of transferrin receptor (Zhu et al. 2007; Nagabhushana et al. 2010; Wild et al. 2011). Earlier study in the laboratory has demonstrated that UBD of optineurin is required for its role in trafficking of TfR (Nagabhushana et al. 2010). In this chapter, we show that UBD of optineurin is also required for its role in cholera toxin trafficking, as a mutation in the UBD of optineurin, D474N, was unable to simulate the rescue shown by shRNA resistant mutant of optineurin in optineurin knockdown background. Also, an additional mutation of D474N in E50K mutant rescued the inhibition of uptake of cholera toxin shown by E50K. These results suggest a role for UBD in optineurin mediated cholera toxin trafficking and defective trafficking of cholera toxin by E50K mutant. However, further characterization of involvement of UBD in these processes is yet to be done.

Receptor for CTxB, GM1 is a glycosphingolipid, which is synthesised and processed in the Golgi. It is packaged in transport vesicles in the TGN before being delivered to the plasma membrane (Kent 1995; Henneberry et al. 2002). Optineurin is known to play a role in maintenance of Golgi ribbon structure (Sahlender et al. 2005). Optineurin knockdown led to the disintegration of the TGN, the principal organelle in maturation of CTxB receptor, GM1. E50K-optineurin expression also led to disintegration of TGN. This lends support to the
hypothesis that disrupted Golgi leads to reduced production and/or reduced export of GM1 which is manifested as reduced uptake of CTxB. However, the molecular mechanism of these defects needs to be probed further to ascertain the Rab GTPase(s) involved in it, precise point of action of optineurin etc.

In conclusion, this study provides evidence for a role for optineurin and TBC1D17 in clathrin-independent endocytosis. The precise roles of optineurin and TBC1D17 in this process are yet to be defined. The glaucoma-causing mutant of optineurin, E50K, inhibits not only the clathrin-mediated endocytosis but also inhibits clathrin-independent endocytosis.
SUMMARY OF RESULTS, CONCLUSIONS AND SIGNIFICANCE

CHAPTER 3

Summary

✓ A domain close to TBC domain in the non-catalytic region of TBC1D17 interacts with optineurin in a region close to Rab8 binding site.
✓ Optineurin recruits TBC1D17 to TfR positive vesicles.
✓ TBC1D17 causes defective recycling of transferrin receptor leading to inhibition of transferrin uptake.
✓ Optineurin is required for localisation of TBC1D17 to juxtanuclear region and for interaction and colocalisation of TBC1D17 with Rab8.
✓ TBC1D17 regulates recruitment of Rab8 to the tubules emanating from the endocytic recycling compartment (ERC).
✓ Optineurin knockdown enhances recruitment of Rab8 to the tubules.
✓ TBC1D17 inhibits interaction and colocalization of TfR with Rab8.
✓ TBC1D17 inhibits MICAL-L1 positive recycling tubular endosomes.

Conclusion

TBC1D17, through its interaction with optineurin, regulates Rab8-mediated endocytic recycling of transferrin receptor and recruitment of Rab8 to the recycling tubular endosomes.

Significance

➢ The study suggests a function for TBC1D17 and Rab8 in TfR trafficking.
➢ The study also provides a rather unexplored function for an effector and a novel and simple mechanism of Rab8 GTPase regulation by a GTPase activating protein.
CHAPTER 4

Summary

✓ E50K-optineurin causes defective recycling of transferrin receptor.
✓ Defective trafficking of TfR by E50K-optineurin is mediated by Rab8.
✓ E50K-optineurin causes enhanced TBC1D17 dependent inactivation of Rab8.
✓ Knockdown of TBC1D17 rescues the effect of E50K on TfR trafficking and inhibition of Rab8-positive tubule formation.
✓ E50K-optineurin inhibits MICAL-L1 positive tubules.

Conclusion

Impaired trafficking of TfR caused by E50K, a glaucoma-associated mutant of optineurin, is due to TBC1D17 dependent enhanced inactivation of Rab8.

Significance

The study provides a mechanism for defective trafficking of transferrin receptor by E50K-optineurin.

CHAPTER 5

Summary

✓ TBC1D17 inhibits uptake of cholera toxin which is dependent on its catalytic activity.
✓ Knockdown of optineurin inhibits cholera toxin trafficking.
✓ Ubiquitin-binding domain (UBD) of optineurin is required for its role in mediating cholera toxin trafficking.
✓ E50K-optineurin inhibits uptake of cholera toxin, which is again dependent on a functional UBD.
Optineurin knockdown as well as TBC1D17 expression leads to disintegration of trans-Golgi network.

Conclusions

Apart from clathrin-dependent endocytosis, optineurin and TBC1D17 are involved in clathrin-independent endocytosis too.

Significance

The study implicates a role for optineurin and TBC1D17 in clathrin-independent endocytosis and provides new horizons to be explored in E50K-optineurin mediated defects.