Chapter 3

Regulation of endocytic recycling of transferrin receptor by optineurin, TBC1D17 and Rab8
3.1 Introduction

Rab GTPases are members of the largest family of Ras superfamily of small GTPases and play an important role in almost all the steps of vesicular trafficking in endocytosis and exocytosis (Nuoffer and Balch 1994; Somsel Rodman and Wandinger-Ness 2000; Agola et al. 2011). Close to 70 Rabs have been identified in humans till date, each believed to be specifically associated with a particular organelle or pathway (Stenmark 2009). Rab GTPases act as molecular switches as they exist in two states in the cell, a GTP bound active state, which is membrane associated and a GDP bound inactive state, which is cytoplasmic. In GTP bound state they bind to their effectors to mediate various processes like vesicle fusion, signal transduction and interaction with motor proteins to control motility along microtubule tracks (Hutagalung and Novick 2011). This cycling of active and inactive state of Rab GTPases is kept in tight control chiefly by two classes of proteins, guanine nucleotide exchange factors (GEFs), which activate Rabs and GTPase activating proteins (GAPs), which render Rabs inactive (Segev 2001).

Rab GAPs accelerate the conversion of GTP to GDP in Rabs. These are characterized by the presence of a conserved catalytic domain called TBC (Tre2/Bub2/Cdc16) domain (Bernards 2003). More than 40 Rab GAPs have been identified so far but no substrate specificity has been assigned to them except for a few (Fukuda 2011). It is likely that GAPs are redundant in their specificity towards Rabs, with a GAP possibly inactivating multiple Rabs. However, the mechanisms involved in targeting of Rabs to their GAPs are generally not known. Though direct interaction of TBC domains of GAPs with Rabs is known, this does not correlate with their activity towards those Rabs (Itoh et al. 2006; Fukuda 2011). TBC1D17 is one of the members of the Rab GAP family. A recent study has identified GAP activity of TBC1D17 (FLJ12168) towards several Rabs in vitro, like Rab1, Rab5, Rab8, Rab13 and Rab21 (Fuchs et al. 2007) whereas it showed direct interaction in yeast with Rab5 (Itoh et al. 2006). It inhibits the trafficking of Shiga toxin from plasma membrane to the Golgi apparatus through its catalytic activity (Fuchs et al. 2007). Although many
Rabs have been identified as putative targets of TBC1D17 in vitro, the cellular targets of TBC1D17 are not known.

Optineurin is an adaptor protein that interacts with numerous proteins including those involved in vesicular trafficking like huntingtin, Rab8 and myosin VI (Hattula and Peranen 2000; Anborgh et al. 2005; Sahlender et al. 2005; del Toro et al. 2009). It is involved in regulating many cellular functions such as vesicular trafficking from the Golgi to plasma membrane (Sahlender et al. 2005), endocytic trafficking (Au et al. 2007; Nagabhushana et al. 2010) and signaling to NF-κB activation (Zhu et al. 2007; Nagabhushana et al. 2011). Optineurin preferentially binds to the activated form of Rab8 (Hattula and Peranen 2000). Therefore, optineurin is considered to be an effector of some of the functions of Rab8 (Hattula and Peranen 2000). Rab8 is involved in regulating diverse trafficking pathways from the trans-Golgi network to the plasma membrane and in membrane trafficking at recycling endosomes (RE) (Huber et al. 1993b; Henry and Sheff 2008). It regulates endocytic trafficking of transferrin receptor (TfR) to RE (Hattula et al. 2006), and recycling of TfR from RE to plasma membrane (Sharma et al. 2009). Rab8 also has a role in establishment of cell polarity, ciliogenesis and translocation of GLUT4 vesicles to the plasma membrane (Watson and Pessin 2006; Nachury et al. 2007; Sato et al. 2007; Peranen 2011).

Though Rab8 has a role in controlling many functions, the regulatory mechanism controlling Rab8 activation and inactivation is not completely understood.

Though optineurin was identified as an effector of Rab8, the mechanisms by which optineurin regulates Rab8-mediated trafficking is not completely understood (Hattula and Peranen 2000; Peranen 2011). TBC1D17 was identified as an interacting partner of optineurin in a yeast two hybrid screening in our laboratory (Chalasani et al. 2009). In this study, the functional significance of the interaction between optineurin and TBC1D17 and their role in Rab8 mediated endocytic trafficking have been examined. The results presented in this chapter suggest that optineurin mediates interaction of TBC1D17, a Rab GAP, with Rab8 to regulate Rab8-mediated endocytic trafficking of TfR and recruitment of Rab8 to the tubules emanating from the endocytic recycling compartment (ERC).
3.2 Results

3.2.1 Optineurin interacts with a non-catalytic region of TBC1D17

TBC1D17 was identified as optineurin interacting protein in a yeast two hybrid screen using full length optineurin as bait (Chalasani et al. 2009). The cDNA clone obtained, codes for the full length protein (1-648 aa). TBC1D17 showed interaction with optineurin but not with the control plasmid (Figure 3.1A, upper panel) in a yeast two hybrid assay. Deletion analysis showed that the central region (amino acids 209-412) of optineurin is essential for its interaction with TBC1D17 (Figure 3.1A). The region of interaction of TBC1D17 with optineurin was in close proximity to the region of interaction of Rab8 (141-209 aa of optineurin) (Hattula and Peranen 2000) with optineurin (Figure 3.1B).

To test this interaction in mammalian cells, co-immunoprecipitations were carried out. GFP tagged TBC1D17 was coexpressed with HA-optineurin and immunoprecipitation was carried out with anti-HA antibody. GFP-TBC1D17 was seen in immunoprecipitate with HA antibody but not with control antibody (Figure 3.1C). Interaction of TBC1D17 with endogenous optineurin was tested by carrying out immunoprecipitation with optineurin antibody using cell lysates expressing GFP-TBC1D17. GFP-TBC1D17 was seen in the immunoprecipitate with optineurin antibody but not with control antibody (Figure 3.2B). The interaction of TBC1D17 with optineurin was also analysed by GST pulldown assay using GST-optineurin and cell lysates expressing GFP-TBC1D17. Western blot analysis suggested that TBC1D17 was seen in GST-optineurin pulldown lane but not in GST alone lane (Figure 3.2C).

TBC1D17 is a 648 aa protein with a TBC domain spanning 310-520 amino acid. It contains a proline rich region at its C-terminus (596-631aa) and NHL (NCL-1, HT-2A and LIN-41) repeat domain at the N-terminus (199-207 aa). The proline rich region and NHL repeat domains are known protein-protein interaction domains (Slack and Ruvkun 1998; Kay et al. 2000). To map the region of TBC1D17 interacting with optineurin, several deletion constructs of TBC1D17 were generated (Figure 3.2A). While Δ217 and 502N showed interaction with
Figure 3.1. Optineurin interacts with TBC1D17 in a region close to Rab8 binding site.

(A) Optineurin and its various deletion constructs cloned in pGBK7T prey vector were cotransformed with either TBC1D17 cloned in pACTII bait vector or pACTII control. Growth on selection plate Ade- indicated interaction. This deletion analysis showed that 209 to 412 amino acid region of optineurin is required for its interaction with optineurin. (B) Schematic of optineurin and its deletion constructs. Region of interaction of TBC1D17 with optineurin is also shown. (C) Hela cells were transfected with TBC1D17 fused to GFP and HA-optineurin. Immunoprecipitation was performed with anti-HA antibody or control antibody. Western blot was probed for GFP and HA which revealed that full length TBC1D17 immunoprecipitated with optineurin and not with control antibody.
Figure 3.2. Identification of region of interaction of TBC1D17 with optineurin.

(A) Various deletion constructs of TBC1D17 were generated by subcloning different domains of full length TBC1D17 to identify the region of interaction of TBC1D17 with optineurin. Full length TBC1D17 also shows its region of interaction with optineurin based on the interaction studies. (B) HEK293 cells were transfected with full length or Δ309 constructs of TBC1D17 fused to GFP and immunoprecipitation was performed with anti-optineurin antibody or control antibody. Western blot was probed for GFP and optineurin which revealed that full length TBC1D17 immunoprecipitated with endogenous optineurin while Δ309 did not. (C&D) To further narrow down the region of interaction, GST pull down approach was taken. GFP tagged TBC1D17, Δ217, Δ309 or 502N were transfected in Hela cells and lysates were pulled down by either GST or GST-optineurin. Probing the western blot for GFP revealed that apart from Δ309 all the other constructs of TBC1D17 interacted with GST-optineurin, indicating therefore that 218-309 amino acids region of TBC1D17 is sufficient for its interaction with optineurin.
GST-optineurin, Δ309 did not show interaction (Figure 3.2C,D). The deletion construct Δ309 showed no interaction with endogenous optineurin in co-immunoprecipitation experiment also (Figure 3.2B). These results suggest that a region spanning amino acids 218-309 of TBC1D17, close to the TBC domain, is required for its interaction with optineurin (Figure 3.2E).

Next, the colocalization of TBC1D17 with optineurin was examined. Hela cells were transfected with plasmid expressing GFP-TBC1D17 with or without HA-optineurin. The cells were fixed and stained for optineurin. When expressed alone, TBC1D17 showed diffuse localization in the cytoplasm with somewhat prominent staining in the juxtanuclear region (Figure 3.3A). However, when co-expressed with optineurin, TBC1D17 relocalized to vesicular structures formed by optineurin and showed strong colocalization with optineurin (Figure 3.3B).

Previously it has been shown that optineurin forms vesicles positive for TfR (Nagabhushana et al. 2010; Park et al. 2010). Hence we examined the distribution of TfR in the cells co-expressing optineurin and TBC1D17. Both the TfR and TBC1D17 were found together in the same vesicular structures that were positive for optineurin (Figure 3.4A). Quantitative analysis of colocalization was carried out by calculating correlation coefficients. This analysis showed that though TBC1D17 alone showed some colocalization with TfR, co-expression of optineurin significantly enhanced colocalization of TBC1D17 with TfR (Figure 3.4B). These results indicate that optineurin may have a role in recruiting TBC1D17 to TfR positive endosomes. In order to test this assumption Δ309 mutant of TBC1D17, which does not interact with optineurin, was coexpressed with optineurin and its colocalization with optineurin was examined. Δ309 showed a predominantly diffuse cytoplasmic distribution with some nuclear staining (Figure 3.3A). Consistent with its lack of interaction with optineurin, Δ309 showed significantly less colocalization with optineurin compared to full length TBC1D17 (Figure 3.3B,C). In contrast Δ217 mutant showed good colocalization with optineurin (Figure 3.3B,C). TBC1D17 also showed some colocalization with endogenous optineurin in RGC-5 cells (Figure 3.3D).
Figure 3.3. TBC1D17 colocalises with optineurin.

(A) Expression profile of TBC1D17 and its deletion constructs fused to GFP. Apart from Δ309, all the constructs show their presence predominantly in cytoplasm. Wild type TBC1D17 shows a prominent localisation in juxtanuclear region. 502N construct show a very different pattern in expression by its presence in vesicular structures. (B) When coexpressed with HA-optineurin, TBC1D17 and Δ217 colocalise with optineurin in vesicular structures formed by it. The region where they colocalised has been enlarged. Scale bar, 10μm. (C) A graph showing the comparision of Pearson’s correlation co-efficient of colocalisation of optineurin with TBC1D17 and its deletion constructs. **P<0.01, ***P<0.001. (D) GFP-TBC1D17 expressing cells were stained for endogenous optineurin in RGC-5 cells. Some colocalization was seen between TBC1D17 and endogenous optineurin.
**Figure 3.4.** Optineurin recruits TBC1D17 to TfR positive endosomes.

(A) TBC1D17 was expressed in HeLa cells either alone or along with optineurin and cells were stained for TfR and optineurin after fixing. TBC1D17 was seen to be localising to TfR positive recycling endosomes in presence of optineurin. Scale bar, 10μm. (B) The graph shows correlation coefficient of colocalisation between TBC1D17 and TfR in absence and presence of optineurin. **P<0.01.**
3.2.2 Optineurin mediates interaction and colocalisation of TBC1D17 with Rab8

Optineurin preferentially interacts with activated form of Rab8 (Hattula and Peranen 2000) and like Rab8, is involved in regulating endocytic trafficking of transferrin and TfR (Hattula et al. 2006; Nagabhushana et al. 2010; Park et al. 2010). It has been reported that TBC1D17 (FLJ12168) does not show direct interaction with Rab8 in yeast two-hybrid assay (Itoh et al. 2006). Since optineurin directly interacts with Rab8 (Hattula and Peranen 2000) as well as TBC1D17 (this study, Figure 3.1), we hypothesized that optineurin may provide a link between these two proteins to regulate the function of Rab8. This possibility was tested by co-immunoprecipitation. Hela cells were infected with adenoviruses expressing shRNA against optineurin to knockdown endogenous optineurin or with control adenoviruses. After 48 hours of knockdown, the cells were transfected with plasmid expressing HA-TBC1D17. Cell lysates were made 24 hours after transfection and immunoprecipitation was carried out with HA-antibody or with control antibody. Endogenous Rab8 was seen in the HA-antibody immunoprecipitate from control cells but not in immunoprecipitate from optineurin knockdown cells (Figure 3.5A). These results indicate that optineurin, TBC1D17 and Rab8 exist as a multimolecular complex in the cell. More importantly, the results indicate that optineurin is required for interaction of TBC1D17 with Rab8.

Next, the role of optineurin in the localization of TBC1D17 and Rab8 was examined by knocking down endogenous optineurin. No significant difference was found in Rab8 distribution in control and optineurin knockdown cells with Rab8 seen in juxtanuclear region and also in the plasma membrane. Interestingly, the localization of TBC1D17 was altered in optineurin knockdown cells. While in control cells TBC1D17 showed a diffuse cytoplasmic distribution and showed some colocalization with Rab8 (Figure 3.5B), optineurin knockdown resulted in complete loss of colocalisation between TBC1D17 and endogenous Rab8 (Figure 3.5B,C). In fact in the absence of optineurin, most of the TBC1D17 was excluded from juxtanuclear region where Rab8 is present (Figure 3.5B). These observations strongly suggest that optineurin is essential for the
Figure 3.5. Optineurin is required for interaction and colocalisation of TBC1D17 with Rab8.

(A) Hela cells were infected with adenoviruses expressing shRNA against optineurin to knockdown optineurin, or control adenovirus. After 48 hours of infection, cells were transfected with HA-TBC1D17. After 24 hours of transfection, lysates were made and immunoprecipitation was carried out by anti-HA or control antibody. Immunoprecipitates were analysed by western blotting with anti-Rab8, anti-optineurin and anti-HA antibodies. WCL, whole cell lysate, 2%. (B) Hela cells seeded on coverslips were infected with control adenoviruses or adenoviruses expressing shRNA against optineurin. After 48 hours, cells were transfected with HA-TBC1D17 and stained with anti-Rab8 (shown in green) and anti-HA antibodies and observed by confocal microscopy for colocalisation. Scale bar, 10µm. (C) The graph shows correlation coefficient of colocalisation of TBC1D17 and Rab8 in optineurin knockdown and control cells. ***P<0.001.
localization and recruitment of TBC1D17 to Rab8. In accordance with this, overexpression of optineurin resulted in enhanced colocalisation of TBC1D17 with endogenous Rab8 (Figure 3.6A,B). Taken together, these results show that optineurin is essential for proper localization of TBC1D17, mediation of recruitment of Rab8 to TBC1D17 and interaction between Rab8 and TBC1D17.

### 3.2.3 TBC1D17 inhibits endocytic trafficking and recycling of transferrin receptor

Since TBC1D17 forms a complex with Rab8 through optineurin, the possibility of regulation of Rab8 mediated functions by TBC1D17 was examined. As Rab8 is involved in regulating trafficking of TfR to RE and from RE to plasma membrane (Hattula et al. 2006; Sharma et al. 2009), the effect of overexpression of TBC1D17 on trafficking of transferrin was analyzed. Hela cells transfected with TBC1D17 were incubated with Alexa-546-conjugated transferrin. Expression of TBC1D17 strongly inhibited uptake of transferrin in most of the cells (Figure 3.7A). Quantitative analysis showed that there was 78% inhibition of uptake of labeled transferrin by TBC1D17 (Figure 3.7B). The specificity of the effect of a Rab GAP can be determined by using its catalytically inactive mutant in which the catalytic arginine is replaced by alanine (Pan et al. 2006; Fuchs et al. 2007). Expression of catalytically inactive R381A mutant of TBC1D17 showed only 11% inhibition in uptake of transferrin (Figure 3.7B). In the cells expressing TBC1D17, but not its R381A mutant, TfR showed more prominent staining in the perinuclear region as against juxtanuclear region (Figure 3.7A). The expression of optineurin binding deficient mutant Δ309 did not affect either the uptake of labeled transferrin or the distribution of TfR (Figure 3.7A). The expression of these constructs was checked by western blot to confirm that the observed effects are not due to differences in expression level or due to reduction of overall TfR level in TBC1D17 expressing cells (Figure 3.7C). Nevertheless, the level of surface TfR was reduced in TBC1D17 transfected cells but not in R381A or Δ309 transfected cells indicating that the reduced uptake of transferrin by the TBC1D17 expressing cells was possibly due to a reduction in TfR levels on the cell surface (Figure 3.8A). Taken together, these results suggest that TBC1D17
Figure 3.6. Optineurin enhances colocalization of TBC1D17 with Rab8.
(A) Hela cells grown on coverslips were transfected with GFP-TBC1D17 alone or alongwith HA-optineurin. After 24 hours, these cells were stained with anti-Rab8 and anti-HA antibodies and observed by confocal microscopy. Scale bar, 10μm. (B) The graph shows correlation coefficient of colocalisation between TBC1D17 and endogenous Rab8 in absence and presence of optineurin. **P<0.01.
Figure 3.7. TBC1D17 inhibits uptake of transferrin.
(A) Hela cells were transfected with GFP tagged TBC1D17, R381A or Δ309 constructs. After 22 hours, transferrin uptake assay was performed using Alexa-546 conjugated transferrin and cells were stained with anti-TfR antibody (shown in green) and analysed by confocal microscopy. GFP stained cells are artificially shown in blue. Scale bar, 10μm. (B) Graph shows the relative fluorescence intensity of endocytosed transferrin by TBC1D17, R381A and Δ309 expressing cells. ***P<0.001. (C) Hela cells were transfected with GFP tagged TBC1D17, R381A or Δ309. After 24 hours, lysates were made, separated on SDS-PAGE and analysed by western blotting using anti-GFP, anti-TfR and anti-tubulin antibodies.
Optineurin mediates Rab8 regulation by TBC1D17

inhibits endocytic trafficking of transferrin receptor. Both the catalytic activity of TBC1D17 and its interaction with optineurin is required for this inhibition of TfR trafficking by TBC1D17.

Reduced surface TfR levels in TBC1D17 expressing cells indicated towards its role in affecting TfR recycling. Therefore, the role of TBC1D17 in recycling of TfR was examined. Transferrin recycling assay was carried out in cells expressing TBC1D17 or its inactive mutant, R381A. Since TBC1D17 expressing cells show reduced uptake of transferrin, the cells were incubated with labeled transferrin for 30 min to allow sufficient uptake and then washed with complete medium for 45 min (chase). After the chase, much more transferrin was seen in TBC1D17-expressing cells as compared to non-expressing cells (Figure 3.8B). Quantitative analysis showed that the expression of TBC1D17 inhibits recycling of transferrin whereas R381A mutant does not (Figure 3.8C).

Several Rabs including Rab8, regulate endocytic trafficking and recycling (Hattula et al. 2006; Henry and Sheff 2008; Grant and Donaldson 2009; Sharma et al. 2009). Therefore, the inhibitory effect of TBC1D17 on transferrin receptor trafficking may be due to inhibition of Rab8 function or due to inhibition of function of some other Rab GTPase. This possibility was examined by analyzing the effect of coexpression of GFP-Rab8 (and other Rabs) on HA-TBC1D17-mediated inhibition of transferrin uptake. HA-TBC1D17 inhibited the uptake of Alexa-546 labeled transferrin as was the case with GFP tagged TBC1D17 (Figure 3.9A). GFP tagged Rab5, Rab8 or Rab21 did not show significant inhibition of transferrin uptake (Figure 3.9B). Coexpression of Rab8 resulted in significant reduction in inhibition of transferrin uptake by TBC1D17 (Figure 3.9C,D). This was not due to reduction in expression level of TBC1D17 in Rab8 overexpressing cells (Figure 3.9E). Coexpression of Rab5 or Rab21, which are known in vitro substrates of TBC1D17 (Fuchs et al. 2007), did not affect TBC1D17 mediated inhibition of transferrin uptake (Figure 3.9C,D). These results suggest that the inhibitory effect of TBC1D17 on transferrin uptake is largely due to inhibition of Rab8 function.
Figure 3.8. TBC1D17 overexpression reduces levels of surface TfR.

(A) HeLa cells transfected with GFP tagged TBC1D17, R381A or Δ309 were fixed after 24 hours and stained with anti-TfR antibody to label TfR on cell surface. Scale bar, 10µm. In cells expressing TBC1D17, reduction in surface TfR is seen as compared to untransfected cells. (B) HeLa cells grown on coverslips were transfected with GFP-tagged TBC1D17 or its R381A mutant as control. After 24 hours the cells were serum starved for 2 hours, incubated with Alexa 546-labelled transferrin for 30 min and then fixed, or washed twice with PBS and incubated in complete medium for 45 min (chase). The fixed cells were examined by confocal microscopy. (C) Quantitative analysis was carried out to calculate the percentage of transferrin remaining after the chase in expressing and nonexpressing (NE) cells.
**Figure 3.9. Effect of TBC1D17 on transferrin uptake is mediated by Rab8.**

Hela cells were transfected with HA-TBC1D17 alone (A), GFP tagged Rab5, Rab8 or Rab21 alone (B) or HA-TBC1D17 alongwith GFP tagged Rab5, Rab8 or Rab21 (C) and transferrin uptake assay was performed. Scale bar, 10μm. (D) Graph shows rescue of TBC1D17 dependent inhibition of transferrin uptake by Rab8, but not by Rab5 or Rab21. **P<0.01. (E) Hela cells were transfected with HA-TBC1D17 alone, GFP tagged Rab5, Rab8 or Rab21 and HA-TBC1D17 alongwith GFP tagged Rab5, Rab8 or Rab21. The cell lysates were analysed by western blotting and probing with anti-GFP, anti-HA and anti-GAPDH antibodies.
3.2.4 TBC1D17 regulates recruitment of Rab8 to the tubules emanating from the endocytic recycling compartment (ERC)

Microtubule dependent tubular structures emanating from the ERC play an important role in recycling of receptors from the ERC to the plasma membrane (Naslavsky and Caplan 2011). Earlier studies have shown that activated Rab8 along with MICAL-L1 and EHD-1 associates with these tubular structures to regulate recycling (Hattula et al. 2006; Sharma et al. 2009). Activated (GTP bound) Rab8 is preferentially present on these tubules as overexpressed Q67L mutant of Rab8 forms more prominent tubules (Hattula et al. 2006). First we confirmed that only the activated form of Rab8 (Q67L mutant) is recruited to these tubules. The inactive GDP bound form of Rab8 (T22N) was rarely seen on these tubules (Figure 3.10A,B). The results so far suggest that TBC1D17, in association with optineurin, inhibits Rab8 mediated trafficking of TfR. Since TBC1D17 is a Rab GAP, it is likely that TBC1D17 inactivates Rab8 function. In order to ascertain this, the role of TBC1D17 in recruitment of Rab8 to the tubular structures was examined. In untransfected HeLa cells, Rab8-positive tubular structures can be seen in about 15% cells. When HeLa cells expressing TBC1D17 were stained for endogenous Rab8, only 4.2±0.6% of TBC1D17 expressing cells showed Rab8-positive tubules. In contrast, 39.4±2.2% of the R381A expressing cells showed these tubules (Figure 3.11A,C). These tubular structures can be stabilized in most of the cells by using CytochalasinD (Hattula et al. 2006). When formation of these tubules was induced by treating the cells with 0.15 μM of cytochalasinD for 30 minutes, endogenous Rab8-positive tubules were found in 81.1±5% of untransfected cells whereas TBC1D17 expressing cells showed these tubules only in 13±7.1% of the cells (Figure 3.11B,C). In contrast, cells expressing R381A mutant showed tubules in 85.3±9.5% of the cells (Figure 3.11 B,C). These results show that TBC1D17, through its catalytic activity, inhibits recruitment of Rab8 to the tubular structures. Since only the GTP bound form of Rab8 is present on the tubules, these results suggest that TBC1D17 inactivates Rab8.
Figure 3.10. The activated form of Rab8 (Q67L) is selectively recruited to the tubules.

(A) Hela cells grown on coverslips were transfected with GFP-Q67L-Rab8 or GFP-T22N-Rab8. After 24 hours of transfection, these cells were fixed and observed by confocal microscopy. Scale bar, 10 μm. (B) The graph shows percentage of cells with Rab8-positive tubules in Q67L and T22N transfected cells from three independent experiments. ***P<0.001.
Figure 3.11. TBC1D17 overexpression inhibits Rab8-positive tubule formation. Hela cells were transfected with GFP tagged TBC1D17 or R381A and after 24 hours the cells were left untreated (A) or treated with 0.15 μM CytochalasinD for 30 minutes (B), fixed and stained with anti-Rab8 antibody. The cells were then observed by confocal microscopy for Rab8-positive tubules. Scale bar, 10μm. C. The graph shows the percentage of cells exhibiting Rab8-positive tubules in each scoring category. Data from three separate experiments are shown as the mean ± s.d. ***P<0.001, **P<0.01. (D) Graph shows the percentage of Rab8-positive tubules in Δ309 expressing cells in comparison to non-expressing or TBC1D17 expressing cells both under normal conditions or induced (with CytochalasinD) conditions.
3.2.5 Optineurin knockdown enhances recruitment of Rab8 to the tubules

We hypothesized that if optineurin recruits TBC1D17 to Rab8 to facilitate hydrolysis of Rab8-GTP, then knockdown of optineurin should result in increased formation of activated Rab8. To test this hypothesis Hela cells were infected with control adenoviruses or with optineurin shRNA-expressing adenoviruses. Staining for endogenous Rab8 showed that in 12.2±2.3% of control cells Rab8-positive tubules were present, whereas upon optineurin knockdown 38.4±2.8% cells showed these Rab8-positive tubules (Figure 3.12A,B). This effect was not due to change in the level of total endogenous Rab8 as seen in western blot (Figure 3.12C). Similarly, overexpression of HA tagged Rab8 in optineurin knockdown cells resulted in formation of more prominent and numerous tubules (comparable to those formed by the expression of Q67L mutant) (Figure 3.10A) as compared to control cells (Figure 3.12D,E). These results suggest that optineurin knockdown enhances recruitment of Rab8 to the tubules probably by shifting the equilibrium towards GTP bound form of Rab8.

3.2.6 TBC1D17 inhibits interaction and colocalization of TfR with Rab8

The results so far have suggested that TBC1D17 regulates Rab8 mediated TfR trafficking. Activated Rab8 is known to form a complex with TfR as shown by coimmunoprecipitation (Nagabhushana et al. 2010; Park et al. 2010). This is supported by preferential colocalization of activated Rab8 with TfR (Figure 3.13A,B). The possibility of using interaction of Rab8 with the cytoplasmic domain of TfR to develop an assay for Rab8 activity was explored. GST fusion protein of cytoplasmic domain of TfR showed stronger binding to Q67L mutant of Rab8 compared to T22N-Rab8 (Figure 3.14A,B). Quantitative analysis of the blot showed that the amount of Q67L-Rab8 was 8 fold more than T22N-Rab8 in the pulldown. This interaction of activated Rab8 with TfR is likely to be indirect because in yeast two-hybrid assay the cytoplasmic domain of TfR did not show any interaction with activated Rab8 (Figure 3.13C). However, these observations suggest that the active and inactive forms of Rab8 show differential binding to TfR, and can be used to assay Rab8 activity. The results so far suggest that
Figure 3.12. Knockdown of optineurin enhances Rab8-positive tubule formation.

(A) Hela cells were infected with control adenoviruses or adenoviruses expressing shRNA against optineurin. After 72 hours, the cells were fixed and stained with anti-Rab8 antibody and analyzed by confocal microscopy. GFP is an indicator of infection by adenovirus. Scale bar, 10 μm. (B) The graph shows the percentage of cells with Rab8-positive tubules. Values are given as mean ± s.d. of percentage of cells from three separate experiments. (C) Lysates were made from Hela cells after 72 hours of infection with adenoviruses expressing shRNA against optineurin or control adenoviruses, separated on SDS-PAGE and analysed by western blotting using anti-Rab8, anti-optineurin and anti-GAPDH antibodies. (D) Hela cells were infected with control adenoviruses or adenoviruses expressing shRNA against optineurin. After 48 hours, the cells were transfected with HA-Rab8. After 24 hours of transfection, these cells were stained with anti-HA antibody and observed by confocal microscopy. Scale bar, 10 μm. (E) The graph shows the percentage of cells showing HA-Rab8 positive tubules. Data from three separate experiments are shown as the mean ± s.d. ***P<0.001.
**Figure 3.13. Colocalization of Rab8 mutants with TfR.**

(A) HeLa cells grown on coverslips were transfected with GFP-Q67L or GFP-T22N. After 24 hours, cells were stained with anti-TfR antibody and observed by confocal microscopy. Scale bar, 10 μm. (B) The graph shows correlation coefficient of colocalisation between Q67L or T22N and TfR. **P<0.01. (C) Interaction of Rab8 mutants (cloned in pGBK7T7 vector) with the cytoplasmic domain of TfR (cloned in pACT2 vector) in yeast two hybrid assay. Transformants were plated on medium without (Ade-) or with (Ade+) adenine. Growth on Ade- plate indicates interaction. As a positive control, interaction of TBC1D17 with optineurin is shown in lower panel.
Figure 3.14. TBC1D17 reduces interaction of Rab8 with transferrin receptor. (A) Schematic showing Tfr protein and cytoplasmic (Cyt) domain (1-67 aa) of Tfr fused to GST, used in the experiments. TM-transmembrane domain. (B) HEK293 cells were transfected with GFP tagged Q67L or T22N constructs. After 24 hours of transfection, cell lysates were prepared and incubated with GST fusion protein of Tfr or GST alone as control. The GST pulldowns were analysed by western blotting with anti-GFP antibody. (C) HEK293 cells were transfected with GFP-Rab8 along with GFP-TBC1D17 or R381A constructs. After 24 hours of transfection, cell lysates were prepared and incubated with GST-Tfr or GST alone as control. The GST pulldowns were analysed by western blotting with anti-GFP antibody and anti-Rab8 antibody.
TBC1D17 regulates Rab8 mediated trafficking possibly by inactivating it. Hence it was hypothesized that if TBC1D17 inactivates Rab8-GTP, then overexpression of TBC1D17 should reduce the level of activated form of Rab8. To test this, Rab8 and TBC1D17 or its R381A mutant were co-transfected in HEK293 cells, and cell lysates were incubated with GST-TfR (1-67aa) or GST. The amount of Rab8 bound to GST-TfR in TBC1D17-expressing cells was reduced as compared to those expressing R381A mutant (Figure 3.14C).

These observations were further validated by analyzing the effect of overexpression of TBC1D17 on colocalisation of Rab8 with TfR. In cells coexpressing TBC1D17 and Rab8, the amount of Rab8 colocalizing with TfR was significantly reduced (Figure 3.15A,B). This is dependent on catalytic activity of TBC1D17, since coexpression of R381A mutant did not decrease the colocalization of TfR with Rab8 (Figure 3.15A,B). These observations are similar to those seen for T22N-Rab8 that shows less colocalisation with TfR as compared to Q67L-Rab8 (Figure 3.13A,B). Taken together, these results strongly suggest that TBC1D17 renders Rab8 in an inactivated state resulting in its less colocalisation with TfR and less interaction with GST-TfR. The colocalization of TfR with Rab8 was seen more on the tubules in R381A coexpressing cells (Figure 3.15A). Percentage of cells showing Rab8-positive tubules and prominence of these tubules was also significantly enhanced in cells coexpressing R381A as compared to TBC1D17 coexpressing cells (Figure 3.15C).

3.2.7 TBC1D17 inhibits MICAL-L1 positive Recycling Tubular Endosomes

Recycling tubular endosomes (RTEs) which emerge from ERC, are important components of receptor recycling pathways. They facilitate the delivery of cargo (receptor) from the ERC to plasma membrane (Sharma et al. 2010; Rahajeng et al. 2012). MICAL-L1 and EHD1 have emerged as markers for RTEs (Naslavsky and Caplan 2011). MICAL-L1, which is an effector of Rab8, has been reported to recruit Rab8 to these EHD1 positive RTEs to regulate recycling (Sharma et al. 2009). Generally, Rabs recruit their effector to its target, but MICAL-L1 appears
Figure 3.15. TBC1D17 reduces colocalisation of Rab8 with transferrin receptor.
(A) HeLa cells were transfected with HA tagged Rab8 alone or along with GFP-TBC1D17 or GFP-R381A constructs. After 24 hours, cells were stained with anti-TfR (red) and anti-HA antibodies and observed by confocal microscopy. GFP staining is artificially shown in blue. Scale bar, 10μm. (B) The graph shows correlation coefficient of colocalisation between Rab8 and transferrin receptor in presence of TBC1D17 or R381A. **P<0.01. (C) The graph shows the percentage of cells exhibiting Rab8-positive tubules in each scoring category. The data are shown as the mean ± s.d. **P<0.01.
to be an unusual effector of Rab8. MICAL-L1 has been shown to have a role in recycling of TfR, as its knockdown inhibited the recycling of labeled transferrin (Sharma et al. 2009). Considering that cells expressing TBC1D17 inhibit TfR recycling, and also inhibits recruitment of Rab8 on RTEs, effect of TBC1D17 expression on these RTEs was analyzed. For this, Hela cells transfected with GFP alone or GFP tagged TBC1D17 or R381A were stained for endogenous MICAL-L1, a marker for RTEs (Figure 3.16A). Non-expressing and GFP transfected Hela cells showed MICAL-L1 positive tubules in 57±4.7% and 54.6±2.3% cells respectively, whereas TBC1D17 expressing cells showed MICAL-L1 positive tubules only in 18.6±4.5% of cells. The catalytically inactive R381A mutant of TBC1D17 did not show much effect, indicating that catalytic activity of TBC1D17 is required for its effect on MICAL-L1 positive tubules (Figure 3.16B). Overexpressed Rab8 forms tubules in about 25% cells. The tubules formed by overexpressed Rab8 show complete colocalization with endogenous MICAL-L1 (Figure 3.16C).

### 3.3 Discussion

The mechanism by which a Rab GAP specifically recognizes its substrate and targeted to is not completely understood. The catalytic domain of a TBC protein is likely to play a role in recognizing and interacting with a substrate Rab protein. This approach has been used successfully to identify a GAP for Rab5 (Haas et al. 2005). However, majority of the TBC domains did not show direct interaction with any of the Rab proteins (Itoh et al. 2006) indicating that non-catalytic sequences of TBC proteins are also likely to be involved in recognizing the target Rabs. The mechanisms by which non-catalytic sequences of TBC proteins recognize their target Rabs directly or indirectly are not known. Here, the mechanisms by which the Rab GAP TBC1D17 is recruited to Rab8 and regulates its function and activity have been investigated. The results presented here show that TBC1D17 interacts and colocalises with Rab8 through optineurin. The N-terminal non-catalytic domain of TBC1D17 is involved in direct interaction with optineurin. Recent studies suggest that optineurin might act as an adaptor protein facilitating assembly of multimolecular signaling complexes (Sahlender et
Figure 3.16. TBC1D17 inhibits formation of MICAL-L1 tubules.
(A) Hela cells transfected with TBC1D17 or its catalytically inactive R381A mutant were stained for endogenous MICAL-L1. Confocal microscopy revealed that expression of TBC1D17 led to reduction in cells showing MICAL-L1 tubules. Scale bar, 10 μm. (B) The graph shows the percentage of cells exhibiting MICAL-L1-positive tubules in each scoring category. Data from three separate experiments are shown as the mean ± s.d. ***P<0.001. (C) Hela cells transfected with GFP-Rab8 were stained for endogenous MICAL-L1. Rab8 showed colocalization with MICAL-L1 on tubules as well as in vesicles.
Optineurin mediates Rab8 regulation by TBC1D17

Optineurin mediates Rab8 regulation by TBC1D17 (al. 2005; del Toro et al. 2009; Nagabhushana et al. 2011; Wild et al. 2011). In accordance with its emerging role as an adaptor protein, our results show that the binding site of TBC1D17 on optineurin (amino acids 209-411) is close to the Rab8-binding site (amino acids 141-209) reported earlier. Optineurin is known to interact preferentially with the activated form of Rab8 (Hattula and Peranen 2000) and the proximity of binding sites of TBC1D17 and Rab8 is likely to facilitate the association of TBC1D17 with its probable substrate Rab8. We also provide evidence for the role of TBC1D17 in the regulation of Rab8 function in endocytic trafficking of TfR.

Rab8-positive tubules are involved in regulating recycling of TfR from the ERC to the plasma membrane (Sharma et al. 2009). Only the activated GTP bound form of Rab8 is present on these tubules (Hattula et al. 2006). This recruitment of active Rab8 on the tubules has been used as an assay to assess the activity of Rab8 in TBC1D17 and R381A overexpressing cells. The ability of TBC1D17, but not R381A mutant, to inhibit recruitment of Rab8 to the tubules strongly suggests that TBC1D17, through its catalytic activity negatively regulates Rab8 activation. A deletion mutant of TBC1D17 which is impaired in binding to optineurin, was unable to inhibit recruitment of Rab8 to the tubules (Figure 3.11D). However, when these tubules were induced by cytochalasinD, ∆309-TBC1D17 did show some inhibition of Rab8 positive tubules. These results suggest that TBC1D17 regulates Rab8 activity which is facilitated by optineurin. This hypothesis is further supported by the observation that endogenous as well as overexpressed Rab8 localised to the tubules more prominently in optineurin knockdown cells.

Endocytic trafficking and recycling of transferrin and its receptor has been studied extensively and several Rab proteins are involved in controlling distinct steps of this trafficking (Mayle et al. 2012). After endocytosis, Tfn/TfR complex moves from primary endocytic vesicles to early endosomes which is regulated by Rab5 (Sonnichsen et al. 2000). Trafficking of TfR from early endosome to recycling endosome requires Rab8 and recycling from recycling endosome to the plasma membrane is mediated by Rab11 (Ullrich et al. 1996; Hattula et al. 2006). Expression of TBC1D17 inhibited uptake of transferrin by the cells due to a block in trafficking and/or recycling of TfR to the plasma membrane.
Optineurin mediates Rab8 regulation by TBC1D17

Restoration of transferrin uptake upon co-expression of Rab8 but not other Rabs tested, suggests that TBC1D17 mediated inhibition of transferrin uptake is primarily due to impairment of Rab8 function by TBC1D17. Δ309-TBC1D17, which is defective in binding to optineurin, was not able to inhibit transferrin uptake, suggesting therefore that binding of TBC1D17 to optineurin is required for inhibition of transferrin uptake. The inability of the catalytically inactive TBC1D17 mutant to inhibit transferrin uptake indicates that the GTP hydrolyzing catalytic activity of TBC1D17 is responsible for impairment of Rab8 function. Various lines of evidence indicate that Rab8 is a substrate of TBC1D17. TBC1D17 expression results in decreased interaction and colocalization of Rab8 with TfR, a feature of T22N or inactive Rab8. Expression of TBC1D17 suppresses the association of Rab8 with the tubular structures, a feature of inactive Rab8. Disruption of the catalytic activity of TBC1D17 by a point mutation reverses most of the phenotypes of TBC1D17. The enhanced recruitment of Rab8 on the tubules, and colocalization of a fraction of TfR with these tubules in R381A mutant-expressing cells, but not in TBC1D17 expressing cells, indicate the presence of active Rab8 associating with its ‘cargo’ on the tubules. Overall, the results presented here suggest that TBC1D17 inhibits transferrin receptor trafficking primarily due to inhibition of Rab8 function.

Tubular membrane structures that emanate from the ERC, are involved in endocytic recycling of membrane proteins (Naslavsky and Caplan 2011). MICAL-L1 is present on these tubules and it links both EHD1 and Rab8 to these structures. Depletion of MICAL-L1 leads to loss of Rab8 from these tubules and also inhibits recycling of TfR (Sharma et al. 2009) indicating a role for Rab8 recruitment to the tubules in recycling of TfR. Our results show that Rab8 recruitment to the tubules is inhibited by TBC1D17, which also inhibit TfR recycling. This provides support to the suggestion that Rab8 recruitment to the tubules plays a role in TfR recycling. Further support for this suggestion is provided by the observation that TfR-positive vesicles seem to follow Rab8-positive tubules in cells expressing catalytic mutant of TBC1D17 (Figure 3.15A, lower panel). This is similar to the situation described by Roland et al. where Rab11-positive vesicles are associated with the Rab8-specific tubules (Roland et al. 2007).
All cells forming Rab8 positive tubules in Rab8 expressing cells also formed and colocalised with MICAL-L1 tubules. TBC1D17 inhibited and R381A did not inhibit tubules by Rab8 as well as MICAL-L1. This suggests that Rab8 has some role in the formation of MICAL-L1 positive tubules. However, formation of endogenous Rab8 tubules is seen only in ~15% of non-expressing cells whereas MICAL-L1 positive tubules are seen in 55-60% cells suggesting that tubules can be formed without Rab8 being there on the tubules. MICAL-L1 is highly unlikely to be affected directly by TBC1D17. Thus it seems that the relationship between Rab8 and MICAL-L1 positive tubules is somewhat complex. One possible explanation is that the presence of Rab8 on tubules is transient while that of MICAL-L1 is less transient. In other words, Rab8-GTP recruits MICAL-L1 on the tubules and then gets inactivated leading to its release from the tubules. At present, the data indicates that MICAL-L1 can form Rab8 dependent as well as Rab8-independent tubules.

Some other TBC domain proteins have been shown to inactivate Rab8 in restricted niches. AS160 (TBC1D4) has been shown to be a GAP for Rab8 in muscle cells and adipocytes during insulin stimulated GLUT4 vesicle translocation (Zeigerer et al. 2004; Miinea et al. 2005; Randhawa et al. 2008). TBC1D30 functions as a GAP for Rab8 in primary cilium formation in RPE cells (Yoshimura et al. 2007). Recently, EPI64, an apical microvillar protein with a TBC domain, has been shown to inactivate Rab8 and regulate membrane recycling through the effector protein JFC1 (Hokanson and Bretscher 2012). Our results suggest that TBC1D17 could be a new GAP of Rab8 involved in regulating trafficking of TfR. Since Rab8 has been implicated in diverse membrane trafficking pathways, it is likely that several TBC proteins are involved in regulating specific functions of Rab8. The mechanisms that determine this specificity remains to be investigated.

In conclusion, the results presented in this chapter show that optineurin mediates interaction between a Rab GTPase activating protein, TBC1D17 and its target, Rab8. TBC1D17 is possibly a GAP of Rab8 involved in regulating trafficking and recycling of TfR, and recruitment of Rab8 on the tubules. We describe a novel mechanism of regulating a Rab GTPase through interaction with an effector.
Optineurin mediates Rab8 regulation by TBC1D17 protein (optineurin), which brings together a Rab (Rab8) and its GAP (TBC1D17) (Figure 3.17). Since activation of Rab GTPases is generally a transient event, effectors of Rabs might be involved in feedback mechanisms by facilitating the recruitment of GAPs to their Rabs.
Figure 3.17. A model showing the optineurin mediated regulation of Rab8 by TBC1D17.
Activated form of Rab8 (Rab8-GTP) binds to optineurin on the membrane. TBC1D17 is then recruited to this complex of Rab8-GTP and optineurin. This brings TBC1D17 in close proximity to Rab8-GTP in the complex, resulting in the conversion of Rab8-GTP to Rab8-GDP. Inactive Rab8 (Rab8-GDP) then dissociates from the membrane and the molecular complex also dissociates.