Chapter 4

Role of TBC1D17 in defective endocytic trafficking by E50K-optineurin
4.1 Introduction

Mutations in optineurin are associated with certain glaucomas and amyotrophic lateral sclerosis (Rezaie et al. 2002; Maruyama et al. 2010). Rezaie et al. (2002) showed that certain mutations in the coding region of the gene OPTN are associated with 16.7% of the families with normal tension glaucoma (NTG), the only gene to be implicated in this sub-type of POAG. Most of these optineurin mutations are missense mutations. One of the mutations, in which glutamic acid at 50\textsuperscript{th} position is replaced by lysine (E50K), segregates with the disease in a large family affected with NTG (Rezaie et al. 2002). The E50K mutation was found in 13.5% of the affected families (Rezaie et al. 2002). Patients with this mutation present a severe phenotype with extensive loss of retinal ganglion cells (Aung et al. 2005). Subsequent studies have identified several other mutations in optineurin that are associated with adult onset NTG and in rare cases of juvenile onset glaucoma. However, the frequency of optineurin mutations in sporadic cases is low, generally less than 1%.

The exact molecular mechanisms of how mutations like E50K of optineurin would lead to pathology of glaucoma remain obscure. The E50K is a dominant mutation (Rezaie et al. 2002), which upon overexpression induces death of RGC-5 cells (a retinal ganglion cell line) in culture but not of other cell lines tested. None of the other glaucoma-associated mutants tested (H26D, H486R, R545Q) induced RGC death (Chalasani et al. 2007). This suggests that the E50K mutant causes glaucoma by directly inducing death of RGCs. Transgenic mice expressing E50K mutant showed apoptotic death of RGCs and hence showed degeneration of entire retina resulting in reduced thickness of retina (Chi et al. 2010). The E50K-induced death of RGCs is mediated by oxidative stress although the mechanism of induction of oxidative stress by E50K is not known. The oxidative stress is due to formation of reactive oxygen species probably produced by mitochondria because E50K-induced RGC death and production of reactive oxygen species were abolished by co-expression of mitochondrial superoxide dismutase (Chalasani et al. 2007).
One hypothesis given to explain the E50K-induced death of RGC-5 is that this cell death occurs because of defective endocytic trafficking of transferrin and its receptor (Nagabhushana et al. 2010). Endocytic membrane traffic is essential for the delivery of various membrane components, receptors and their ligands to their respective intracellular compartments (Maxfield and McGraw 2004). Iron is essential for survival of all the cells. Transferrin and transferrin receptor regulate iron uptake in almost all cell types, and are frequently used to study endocytosis and recycling. The iron bound transferrin is endocytosed after its binding to transferrin receptor (TfR) which is mostly clathrin dependent and delivered to peripheral early/sorting endosome (Maxfield and McGraw 2004). From the early endosomes most of the internalized transferrin receptor is recycled back either through a fast, direct step from early/sorting endosome, or a slower step via recycling endosome. Recycling endosome is a network of tubular structures that is juxtanuclear in most cells (Hopkins 1983; Hopkins and Trowbridge 1983; Yamashiro et al. 1984). It is a relatively long lived compartment and acts as a centre for recycling of cargo (Maxfield and McGraw 2004). Rab8 and Rab11 are two major Rab GTPases which regulate the endocytic traffic of cargo like transferrin receptor to recycling endosomes and from recycling endosome to plasma membrane in the slow recycling route (Ullrich et al. 1996; Chen et al. 1998; Ren et al. 1998; Hattula et al. 2006). The constant recycling of receptors between membrane and endosomes is vital for maintaining homeostasis of membrane components with recycling endosome serving as a transient store house of these membrane components (Maxfield and McGraw 2004). The E50K mutant inhibits endocytic trafficking and recycling of transferrin receptor leading to accumulation of transferrin receptor in large foci/vesicular structures (recycling endosomes, autophagosomes). This accumulation of TfR is because of defective recycling of TfR by E50K (Nagabhushana et al. 2010; Park et al. 2010). This defective TfR trafficking by E50K mutant is probably due to altered interaction of this mutant with Rab8 and transferrin receptor (Nagabhushana et al. 2010).

Since optineurin functions as an adaptor protein to mediate negative regulation of Rab8 by the GTPase activating protein, TBC1D17, molecular mechanisms pertaining to defects in trafficking of TfR caused by its mutant, E50K and the role of TBC1D17 in it were examined. The results presented in this chapter suggest
that E50K impairs endocytic recycling of transferrin and its receptor from recycling endosomes to plasma membrane. Activated mutant of Rab8, Q67L as well as a catalytically inactive mutant of TBC1D17, R381A rescue the effect of E50K on transferrin trafficking. Knockdown of TBC1D17 by shRNA also rescues the effect of E50K on transferrin uptake. E50K inhibits recruitment of Rab8 on recycling tubular endosomes and co-expression of R381A as well as shRNA against TBC1D17 restores this effect of E50K on RTE formation by Rab8. The results of several experiments suggest that the E50K mutant causes impaired trafficking and recycling at the recycling endosomes due to enhanced inactivation of Rab8 by TBC1D17. These results also have implications for the pathogenesis of glaucoma caused by E50K mutation because endocytic recycling is vital for maintaining homeostasis of membrane components in the cell.

4.2 Results

4.2.1 E50K-optineurin inhibits recycling of transferrin receptor

E50K is a dominant glaucoma causing mutation of optineurin (Rezaie et al. 2002). Earlier studies have shown that E50K mutant of optineurin impairs endocytic trafficking of TfR resulting in accumulation of TfR in vesicular structures in the cytoplasm (Nagabhushana et al. 2010; Park et al. 2010) and hence reduced levels of surface TfR (Nagabhushana et al. 2010). Reduction in the levels of surface TfR by E50K indicated towards a defect in recycling. Therefore, the role of E50K-optineurin in recycling of TfR was examined. For this, transferrin recycling assay was carried out in cells expressing E50K or wild type optineurin. E50K-optineurin expressing cells were incubated with Alexa-546 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 E50K-expressing cells as compared to non-expressing cells (Figure 4.1A). Quantitative analysis showed that the expression of E50K inhibits recycling of transferrin whereas wild type optineurin had no significant effect.
Figure 4.1. E50K-optineurin inhibits endocytic recycling of transferrin.
(A) Hela cells grown on coverslips were transfected with GFP-tagged E50K or optineurin. After 24 hours, the cells were serum starved for 2 hours, incubated with Alexa 546-labelled transferrin for 30 min (pulse) and then fixed, or washed twice with PBS and incubated in complete medium for 45 min (chase) and then fixed. The fixed cells were examined by confocal microscopy. Scale bar, 10μm. (B) Graph shows the quantitative analysis carried out to calculate the percentage of transferrin remaining after the chase in expressing and nonexpressing (NE) cells.
E50K causes enhanced inactivation of Rab8

This result indicates that E50K-optineurin indeed inhibits recycling of TfR.

4.2.2 Defective trafficking of TfR by E50K-optineurin is mediated by Rab8

This defective trafficking and recycling is possibly due to altered interaction of the E50K mutant with Rab8 and also with TfR (Nagabhushana et al. 2010). Since optineurin mediates interaction of TBC1D17 with Rab8 and possibly Rab8 inactivation, we next examined whether the impaired trafficking caused by the E50K mutant is due to altered physical and/or functional interaction with TBC1D17. Co-immunoprecipitation experiments and yeast two hybrid assay revealed no noticeable differences in the interaction of TBC1D17 with E50K as compared to wild type optineurin (Figure 4.2A,B). Nevertheless colocalization of TBC1D17 with E50K mutant was significantly increased as compared to wild type optineurin (Figure 4.3A,B).

In order to ascertain whether impaired Rab8 mediated trafficking of TfR by the E50K mutant is dependent on TBC1D17 function, the effect of expression of R381A mutant of TBC1D17 (which does not inactivate Rab8, keeping endogenous Rab8 in Rab8-GTP state), on E50K-dependent inhibition of transferrin uptake was analyzed. While cells expressing E50K mutant showed reduced uptake of transferrin, co-expression of R381A mutant significantly rescued the uptake of transferrin in these cells (Figure 4.4A,B). This reversal was not due to reduced expression of E50K in presence of R381A mutant as seen in the western blot (Figure 4.4C). E50K expression results in formation of comparatively bigger vesicles than wild type optineurin in Hela cells. When Hela cells were transfected with R381A alongwith E50K, size of these vesicles decreased significantly (Figure 4.4D). This indicates rescue of the phenotype of E50K by R381A. Co-expression of R381A was also able to restore the reduced surface TfR levels in E50K expressing cells (Figure 4.5). Next, the effect of co-expression of activated Rab8 (Q67L mutant) on E50K-optineurin mediated inhibition of transferrin uptake was examined. Similar to R381A mutant, co-expression of Q67L-Rab8 could significantly rescue the transferrin uptake by the
Figure 4.2. E50K-optineurin interacts with TBC1D17.
(A) HeLa cells were cotransfected with plasmids expressing GFP-TBC1D17 and HA-optineurin or HA-E50K. After 24 hours, cell lysates were prepared and immunoprecipitation was carried out using anti-HA antibody or control antibody. The immunoprecipitates were analyzed by western blotting. (B) Interaction of TBC1D17 cloned in pACTII with optineurin and its E50K mutant cloned in pGBK7T7 was analysed by yeast two hybrid assay. Growth on Ade- plate or colour development on x-Gal plate indicate interaction.
Figure 4.3. Colocalization of optineurin and its E50K mutant with TBC1D17.
(A) Hela cells grown on coverslips were transfected with GFP-TBC1D17 along with HA-optineurin or HA-E50K. After 24 hours, the cells were stained with anti-HA antibody and observed by confocal microscopy. Scale bar, 10µm. (B) The graph shows correlation coefficient of colocalisation between TBC1D17 and optineurin or E50K. **P<0.01.
**Figure 4.4. R381A-TBC1D17 can rescue the inhibitory effect of E50K-optineurin on transferrin uptake.**

(A) Hela cells were transfected with HA-E50K-optineurin alone, GFP-R381A-TBC1D17 alone or the two together. After 24 hours of transfection, transferrin uptake assay was performed and analysed by confocal microscopy. Scale bar, 10µm. (B) The graph shows relative fluorescence intensity of endocytosed transferrin by the cells expressing E50K alone, R381A alone or E50K and R381A together compared to non expressing cells. ***P<0.001. (C) Hela cells were transfected with GFP-R381A-TBC1D17 alone, HA-E50K-optineurin alone or the two together. Lysates were made, separated on SDS-PAGE and probed for GFP, HA and GAPDH. Western blot shows that R381A expression does not reduce E50K expression. (D) The graph shows size of the vesicles formed by the cells expressing E50K alone, E50K and R381A together or E50K and TBC1D17 together. ***P<0.001, *P<0.05.
Figure 4.5. R381A restores the reduced level of TfR at the cell surface in E50K-expressing cells.
Hela cells were transfected with GFP-E50K or GFP-R381A and HA-E50K. After 24 hours, labeling of surface transferrin receptor was done and examined by confocal microscopy. Scale bar, 10μm. In the lower panel, GFP-R381A expressing cells showing vesicles are representative of cells co-expressing GFP-R381A and HA-E50K. GFP-R381A, when expressed alone does not form vesicles.
E50K expressing cells (Figure 4.6A,B). This effect was not due to decrease in E50K expression upon co-expression of Q67L-Rab8 as seen in the western blot (Figure 4.6C). These results suggest that impaired trafficking of TfR by the E50K mutant is dependent on TBC1D17 and is possibly due to inactivation of Rab8.

4.2.3 E50K-optineurin causes enhanced TBC1D17 dependent inactivation of Rab8

It is likely that despite the lack of marked differences in interaction with TBC1D17, E50K mutant enhances catalytic function of TBC1D17 and hence inactivates Rab8. To test this hypothesis, Rab8-positive tubule formation assay was done. In accordance with the assumption, formation of Rab8-positive tubules by overexpressed Rab8 was strongly inhibited by the E50K mutant but not by wild type optineurin (Figure 4.7A,B). In fact, E50K-optineurin colocalised with Rab8 in vesicles whereas wild type optineurin colocalised with Rab8 on tubules as well as vesicles (Figure 4.7A). However, E50K showed significantly more colocalization with overexpressed Rab8 in vesicles (Figure 4.7A). Similarly, recruitment of endogenous Rab8 to the tubules was strongly inhibited by the E50K mutant whereas wild type optineurin showed significantly less inhibition (Figure 4.7D,E). Endogenous Rab8 also colocalised with E50K mutant in vesicles to a greater extent than wild type optineurin (Figure 4.7F). Colocalization of both endogenous as well as overexpressed Rab8 in E50K forming vesicles indicate towards trapping of Rab8 by E50K, rendering it less available for activation. Co-expression of R381A mutant of TBC1D17 resulted in reversal of E50K-optineurin dependent inhibition of endogenous Rab8 tubule formation (Figure 4.8A,B).

4.2.4 Knockdown of TBC1D17 rescues the effect of E50K on TfR trafficking

The specificity of involvement of a protein in a given process can be studied by its depletion by knockdown approach. For knocking down TBC1D17, shRNA
Figure 4.6. Activated Rab8 rescues the inhibition of transferrin uptake by E50K.

(A) Hela cells were transfected with GFP-E50K-optineurin alone, HA-Q67L-Rab8 alone or the two together. After 24 hours, transferrin uptake assay was performed using Alexa-546 conjugated transferrin and cells were analysed by confocal microscopy. Scale bar, 10μm. (B) Graph shows relative florescence intensity of endocytosed transferrin by the cells expressing E50K alone, Q67L alone or E50K and Q67L together compared to non-expressing cells. **P<0.01. (C) Hela cells were transfected with GFP-E50K-optineurin alone, HA-Q67L-Rab8 alone or the two together. Lysates were made, seperated on SDS-PAGE and probed for GFP, HA and GAPDH. Western blot shows that HA-Q67L-Rab8 expression does not reduce GFP-E50K-optineurin expression.
Figure 4.7. E50K-optineurin inhibits Rab8-positive tubule formation.

(A) Hela cells grown on coverslips were transfected either with HA-tagged Rab8 alone or alongwith GFP tagged E50K mutant or wild type optineurin. After 24 hours, these cells were stained with anti-HA antibody and observed by confocal microscopy. Scale bar, 10µm. (B) The graph shows percentage of cells containing Rab8-positive tubules. (C) Graph shows comparision of colocalization between HA-Rab8 and GFP tagged optineurin or E50K. (D) Hela cells grown on coverslips were transfected with GFP tagged E50K mutant or wild type optineurin. After 24 hours, cells were stained with anti-Rab8 antibody and observed by confocal microscopy. Scale bar, 10µm. (E) The graph shows percentage of cells containing Rab8 positive tubules. (F) Graph shows comparision of colocalization between endogenous Rab8 and GFP tagged optineurin or E50K. **P<0.01; ***P<0.001.
Figure 4.8. R381A-TBC1D17 rescues the inhibitory effect of E50K-optineurin on Rab8-positive tubule formation.

(A) Hela cells were transfected with GFP-tagged R381A mutant of TBC1D17 along with either HA-tagged E50K or optineurin. After 24 hours of transfection, these cells were stained for HA and Rab8 and observed by a confocal microscope to score for Rab8-positive tubules. Scale bar, 10 µm. (B) The graph shows percentage of cells showing Rab8-positive tubules.
(short hairpin RNA) approach was taken. Plasmid vectors expressing shRNA against various regions of TBC1D17 were made and their efficiency was checked by western blot. One of the shRNAs tested, shB, caused drastic reduction in the amount of TBC1D17 protein (Figure 4.9A) and was subsequently used for knockdown experiments. To test the requirement of TBC1D17 in E50K mediated inhibition of transferrin uptake, GFP tagged E50K was co-expressed with shRNA against TBC1D17 or control shRNA. Knockdown of TBC1D17 by shRNA partly restored the uptake of transferrin in E50K expressing cells (Figure 4.9B,C). However, in GFP expressing cells there was no significant effect of TBC1D17 knockdown on uptake of transferrin (Figure 4.9C). These results suggest that impaired trafficking of TfR caused by the E50K mutant is dependent on TBC1D17. Knockdown of TBC1D17 by shRNA also resulted in reversal of E50K mediated inhibition of tubule formation by endogenous Rab8 (Figure 4.9D,E). In GFP expressing control cells, knockdown of TBC1D17 resulted in an increase in Rab8-positive tubules (Figure 4.9E). Taken together, these results show that TBC1D17 mediates enhanced inactivation of Rab8 by E50K that leads to defective Rab8 mediated TfR trafficking and recycling.

4.2.5 E50K-optineurin inhibits MICAL-L1 positive tubules

Recycling tubular endosomes are an important component of the recycling machinery inside the cell. These are long, tubular structures which act as carriers of cargo (in this case TfR) from ERC to plasma membrane. Considering that TBC1D17 inhibits the formation of RTEs (Figure 3.16B, Chapter 3) it is likely that E50K mutant might be doing so. Therefore, the effect of E50K-optineurin on recruitment of MICAL-L1, on to tubules was tested. For this, Hela cells overexpressing GFP tagged optineurin and its E50K mutant were stained for endogenous MICAL-L1 and scored for MICAL-L1 positive tubules. Confocal microscopy revealed that E50K-optineurin strongly inhibited recruitment of MICAL-L1 to RTEs as compared to non-expressing or optineurin expressing cells (Figure 4.10A,B). Optineurin overexpression also inhibited MICAL-L1 recruitment to these tubules albeit to a lesser extent (Figure 4.10A,B). This might
Figure 4.9. Knockdown of TBC1D17 reverses the phenotype of E50K overexpression.

(A) Hela cells were transfected with HA-TBC1D17 along with TBC1D17 directed shRNA expression plasmid (KD) or control plasmid (C). After 24 hours, cell lysates were made and analysed by western blot to test the efficacy of shRNA mediated knockdown. (B) Hela cells were transfected with GFP-E50K-optineurin along with TBC1D17-directed shRNA expression plasmid or control plasmid. After 40 hours of transfection, transferrin uptake assay was performed and analysed by confocal immunofluorescence microscopy. (C) The graph shows relative fluorescence intensity of endocytosed transferrin by the cells expressing GFP or GFP-E50K-optineurin. ***P<0.001. (D) Hela cells grown on coverslips were transfected with GFP-E50K-optineurin or GFP along with TBC1D17-directed shRNA expression plasmid or control plasmid. After 40 hours, the cells were stained for endogenous Rab8 and examined by confocal microscopy. Scale bar, 10 μm. (E) The graph shows percentage of cells containing Rab8-positive tubules.*P<0.05; **P<0.01.
Figure 4.10. E50K-optineurin inhibits MICAL-L1 tubule formation.
(A) Hela cells transfected with GFP tagged E50K or wild type optineurin were stained for endogenous MICAL-L1 and analysed by confocal microscopy. Scale bar, 10µm. (B) Cells were scored for the presence of MICAL-L1-positive tubules. The graph shows percentage of cells containing MICAL-L1-positive tubules. The data are shown as the mean ±s.d. ***P<0.001.
be because optineurin overexpression also serves to concentrate endogenous TBC1D17 and Rab8 in a niche so as to facilitate inactivation of Rab8. These results suggest that E50K mutant of optineurin inhibits the formation of RTEs.

4.3 Discussion

E50K is a dominant mutation which causes normal tension glaucoma by directly inducing the death of retinal ganglion cells (RGCs) (Chalasani et al. 2007; Chi et al. 2010). But the molecular mechanism(s) of RGC death by E50K is not known. Earlier studies have shown that oxidative stress due to production of reactive oxygen species (ROS) by mitochondria cause death of RGCs in E50K-expressing cells (Chalasani et al. 2007). Upon overexpression, E50K-optineurin inhibits endocytic trafficking of TfR, resulting in accumulation of TfR in large E50K positive structures/foci (Nagabhushana et al. 2010; Park et al. 2010). E50K mutant shows altered interactions with Rab8 and TfR to impair Rab8 mediated trafficking but the molecular mechanism of this defective endocytic trafficking of TfR by E50K is not known. Here we have shown that similar to TBC1D17, the expression of E50K mutant inactivates Rab8 as seen by nearly complete loss of Rab8 from the tubules. Interestingly, co-expression of R381A mutant of TBC1D17 or knockdown of TBC1D17 restored formation of these tubules in E50K expressing cells. In addition, E50K-dependent inhibition of transferrin uptake was also partially restored by co-expression of R381A-TBC1D17 or knockdown of TBC1D17. These results suggest that the E50K mutant causes strong inactivation of Rab8 by endogenous TBC1D17. Inactivation of Rab8 by E50K possibly contributes to inhibition of TfR trafficking and recycling. This is supported by the observation that the expression of activated Rab8 (Q67L) partly restored E50K-mediated inhibition of transferrin uptake.

The molecular basis of the functional defects caused by E50K and other mutations in optineurin has not been completely elucidated. It appears that the molecular mechanism of defective TfR trafficking by the E50K mutant is somewhat complex. As optineurin acts as an adaptor protein to assemble larger
E50K causes enhanced inactivation of Rab8

complexes, it is likely that altered interactions of optineurin mutants like E50K might result in functional defects. Optineurin forms a multi-molecular complex containing Rab8 and TfR as seen by co-immunoprecipitation (Nagabhushana et al. 2010; Park et al. 2010). The E50K mutant forms a stronger complex with transferrin receptor and Rab8. Stronger colocalization of E50K mutant with Rab8 and transferrin receptor in the same structures/foci provides support for this suggestion (Nagabhushana et al. 2010). But, direct interaction between E50K mutant and Rab8 is lost as shown in mammalian cells (Chi et al. 2010) and also by yeast two-hybrid assay (study in the laboratory by Dr. Ch. Madhavi). Based on these observations it appears that in the multi-molecular complex, direct interaction between E50K mutant and Rab8 is lost but indirect interaction is increased. Therefore, it is likely that the functional positioning of these proteins in the multi-molecular complex is altered in such a way that the inactivation of Rab8 by TBC1D17 is increased in E50K-expressing cells. This is depicted schematically as a model in Figure 4.11. Co-expression of R381A may prevent this inactivation by displacing endogenous TBC1D17. This hypothesis is supported by the following observations: (a) As compared to wild-type optineurin, the E50K mutant shows stronger colocalization with TBC1D17 and Rab8; (b) Coexpression of R381A mutant or knockdown of TBC1D17 reverses the inhibitory effect of E50K mutant on transferrin uptake and Rab8-positive tubule formation.

Recycling tubular endosomes emerging from ERC are important structures, which serve as tracks for recycling of membrane proteins especially receptors. Receptors for various ligands are vital for survival of cells as they help in endocytosis of the required ligand inside the cell to effect different signaling pathways. Recycling of these receptors serves as an important mechanism to maintain homeostasis in the cells. Recruitment/presence of MICAL-L1 to RTEs is an indicator of formation of active RTEs in the cell. Overall the results suggest that E50K mutant of optineurin causes impaired recycling of Tfn/TfR by inhibiting Rab8 and MICAL-L1 positive recycling tubular endosomes.

In conclusion, the E50K mutant recruits TBC1D17 more efficiently to the multi-molecular complex leading to enhanced inactivation of Rab8 by TBC1D17. This
Figure 4.11. A model showing regulation of Rab8 by optineurin and its defective regulation by the E50K mutant.
(A) GTP-bound active Rab8 performs its various functions by its interaction with effector proteins. Optineurin, an effector of Rab8, binds to the activated form of Rab8. Upon binding to activated Rab8, optineurin recruits a GAP, TBC1D17, in close proximity to Rab8. This leads to inactivation of Rab8 and thus maintenance of homeostasis. (B) E50K-optineurin causes enhanced inactivation of Rab8 by recruiting TBC1D17 more efficiently.
E50K causes enhanced inactivation of Rab8

leads to inhibition of Rab8-mediated TfR trafficking and recycling. This hypothesis is supported by the observation that E50K-optineurin dependent inhibition of transferrin receptor trafficking can be prevented by knockdown of TBC1D17 or by expressing a catalytically inactive mutant of TBC1D17. A constitutively active mutant of Rab8, Q67L also reverses E50K-optineurin induced inhibition of transferrin receptor trafficking. Whether E50K-induced TBC1D17 mediated Rab8 inactivation, or defective TfR trafficking, play a role in RGC death, is yet to be investigated. However, the results presented in this chapter do provide a basis for exploring the role of TBC1D17 and Rab8 in E50K induced ROS production and death of retinal ganglion cells.