Optineurin mediates a negative regulation of Rab8 by the GTPase-activating protein TBC1D17

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Summary

Rab GTPases regulate various membrane trafficking pathways but the mechanisms by which GTPase-activating proteins recognise specific Rabs are not clear. Rab8 is involved in controlling several trafficking processes, including the trafficking of transferrin receptor from the early endosome to the recycling endosome. Here, we provide evidence to show that TBC1D17, a Rab GTPase-activating protein, through its catalytic activity, regulates Rab8-mediated endocytic trafficking of transferrin receptor. Optineurin, a Rab8-binding effector protein, mediates the interaction and colocalisation of TBC1D17 with Rab8. A non-catalytic region of TBC1D17 is required for direct interaction with optineurin. Co-expression of Rab8, but not other Rabs tested, rescues the inhibition of transferrin receptor trafficking by TBC1D17. The activated GTP-bound form of Rab8 is localised to the tubules emanating from the endocytic recycling compartment. Through its catalytic activity, TBC1D17 inhibits recruitment of Rab8 to the tubules and reduces colocalisation of transferrin receptor and Rab8. Knockdown of optineurin or TBC1D17 results in enhanced recruitment of Rab8 to the tubules. A glaucoma-associated mutant of optineurin, E50K, causes enhanced inhibition of Rab8 by TBC1D17, resulting in defective endocytic recycling of transferrin receptor. Our results show that TBC1D17, through its interaction with optineurin, regulates Rab8-mediated endocytic recycling of transferrin receptor and recruitment of Rab8 to the endocytic recycling tubules. We describe a mechanism of regulating a Rab GTPase by an effector protein (optineurin) that acts as an adaptor to bring together a Rab (Rab8) and its GTPase-activating protein (TBC1D17).

Key words: Optineurin, Rab8, TBC1D17, Vesicular trafficking

Introduction

Rab GTPases are members of the largest group of the Ras superfamily of small GTPases and play an important role in almost all the steps of vesicular trafficking in endocytosis and exocytosis (Agola et al., 2011; Nuoffer and Balch, 1994; Somsel Rodman and Wandinger-Ness, 2000). Close to 70 Rabs have been identified in humans to date, and each is 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, in which they are membrane associated, and a GDP-bound inactive state, in which they are cytoplasmic. In the GTP-bound state they bind to their effectors to mediate various processes, such as vesicle fusion, signal transduction and interaction with motor proteins to control motility along microtubule tracks (Hutagalung and Novick, 2011). This cycling between the active and inactive state 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. They are characterised by the presence of a conserved catalytic domain called the TBC (Tre2/Bub2/Cdc16) domain (Bernards, 2003). More than 40 Rab GAPs have been identified so far, but only a few have a known substrate specificity (Fukuda, 2011). It is likely that GAPs are redundant in their specificity towards Rabs, with one GAP possibly inactivating multiple Rabs. However, the mechanisms involved in targeting of Rabs to their GAPs are generally not known. Although it has been shown that there can be a direct interaction between the GAP TBC domains and Rabs, this does not correlate with the activity of the GAP towards those Rabs (Fukuda, 2011; Itoh et al., 2006). TBC1D17 (also known as FLJ12168) is one of the members of the Rab GAP family. A recent study has identified GAP activity of TBC1D17 towards several Rabs *in vitro*, including Rab1, Rab5, Rab8, Rab13 and Rab21 (Fuchs et al., 2007), and, in yeast, it showed a direct interaction with Rab5 (Itoh et al., 2006). TBC1D17 also inhibits the trafficking of Shiga toxin from plasma membrane to the Golgi complex 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 conclusively.

Optineurin is an adaptor protein that interacts with numerous proteins including those involved in vesicular trafficking, such as huntingtin, Rab8 and myosin VI (Anborgh et al., 2005; del Toro et al., 2009; Hattula and Peranen, 2000; Sahlender et al., 2005). 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 leading to NF-κB activation (Nagabhushana et al., 2011; Zhu et al., 2007). Mutations in optineurin are associated with certain glaucomas and amyotrophic lateral sclerosis (Maruyama et al., 2010; Rezaie et al., 2002). Optineurin preferentially binds to the activated form of Rab8 (Hattula and Peranen, 2000). Therefore, optineurin is

considered 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 (REs) (Henry and Sheff, 2008; Huber et al., 1993). It regulates endocytic trafficking of transferrin receptor (TfR) to REs (Hattula et al., 2006) and recycling of TfR from REs to the 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 (Nachury et al., 2007; Peranen, 2011; Sato et al., 2007; Watson and Pessin, 2006). Although Rab8 has a role in controlling many functions, the regulatory mechanism controlling Rab8 activation and inactivation is not completely understood.

Although optineurin was identified as an effector of Rab8, the mechanisms by which optineurin regulates Rab8-mediated trafficking are not completely understood (Hattula and Peranen, 2000; Peranen, 2011). TBC1D17 was previously identified as an interacting partner of optineurin in a yeast two-hybrid screening in our laboratory (Chalasani et al., 2009). Here, we have examined the functional significance of the interaction between optineurin and TBC1D17 and their role in Rab8-mediated endocytic trafficking. Our results suggest that optineurin mediates the 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). A glaucoma-associated optineurin mutation, E50K, caused enhanced inhibition of Rab8 functions by TBC1D17, thus leading to defective endocytic recycling of TfR.

Results

Optineurin interacts with a non-catalytic region of TBC1D17

We previously identified TBC1D17 as an optineurin-interacting protein in a yeast two-hybrid screen using full-length optineurin as bait (Chalasani et al., 2009). The cDNA clone obtained encodes the full-length protein [amino acids (aa) 1–648]. TBC1D17 showed interaction with optineurin but not with the control plasmid (Fig. 1A) in yeast two-hybrid assay. Deletion analysis showed that the central region (aa 209–412) of optineurin is essential for its interaction with TBC1D17 (Fig. 1A,B). To test this interaction in mammalian cells, co-immunoprecipitations were carried out. The interaction between TBC1D17 and 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 (Fig. 1C). The interaction of TBC1D17 with optineurin was also analysed by using a GST pulldown assay with GST–optineurin and cell lysates expressing GFP–TBC1D17. Western blot analysis showed that TBC1D17 was seen in the GST–optineurin pulldown lane but not in the GST alone lane (Fig. 1E).

TBC1D17 is a 648-amino-acid protein with a TBC domain spanning amino acids 310–520. It contains a proline-rich region at its C-terminus (aa 596–631) and an NHL (NCL-1, HT-2A and LIN-41) repeat domain at the N-terminus (aa 199–207). The proline-rich region and NHL repeat domains are known to be protein interaction domains (Kay et al., 2000; Slack and Ruvkun, 1998). To map the region of TBC1D17 interacting with optineurin, several deletion constructs of TBC1D17 were generated (Fig. 1D). Whereas Δ217 and 502N showed interaction with GST–optineurin, Δ309 showed no interaction (Fig. 1E,F). The deletion construct Δ309 also showed no interaction with endogenous optineurin in co-immunoprecipitation experiment (Fig. 1C). 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.

We next examined the colocalisation of TBC1D17 with optineurin. 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 a diffuse localisation in the cytoplasm, with somewhat prominent staining in the juxtanuclear region (Fig. 1G). However, when co-expressed with optineurin, TBC1D17 relocalised to vesicular structures where optineurin was present and showed strong colocalisation with optineurin (Fig. 1H,I). Previously, it has been shown that optineurin is present in 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 (supplementary material Fig. S1A). Quantitative analysis of colocalisation was carried out by calculating correlation coefficients. This analysis showed that, although TBC1D17 alone showed some colocalisation with TfR, co-expression of optineurin significantly enhanced colocalisation of TBC1D17 with TfR (supplementary material Fig. S1B). These results indicate that optineurin might have a role in recruiting TBC1D17 to TfR-positive endosomes. In order to test this assumption, the Δ309 mutant of TBC1D17, which does not interact with optineurin, was coexpressed with optineurin and its colocalisation with optineurin was examined. Δ309 showed a predominantly diffuse cytoplasmic distribution with some nuclear staining. Consistent with its lack of interaction with optineurin, Δ309 showed significantly less colocalisation with optineurin compared to full length TBC1D17 (Fig. 1H,I). In contrast, the Δ217 mutant showed good colocalisation with optineurin (Fig. 1H,I).

Optineurin mediates the 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 (also known as FLJ12168) does not show direct interaction with Rab8 in a yeast two-hybrid assay (Itoh et al., 2006). Given that optineurin directly interacts with Rab8 (Hattula and Peranen, 2000), as well as TBC1D17 (this study), we hypothesised that optineurin might provide a link between these two proteins and could be involved in regulating 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 performed with anti-HA antibody or with control antibody. Endogenous Rab8 was seen in the anti-HA antibody immunoprecipitate from control cells but not in immunoprecipitate from optineurin-knockdown cells (Fig. 2A). These results indicate that optineurin is required for the interaction of TBC1D17 with Rab8. We then examined the role of optineurin in the localisation of TBC1D17 and Rab8 by knocking down endogenous optineurin. We did not find any significant difference

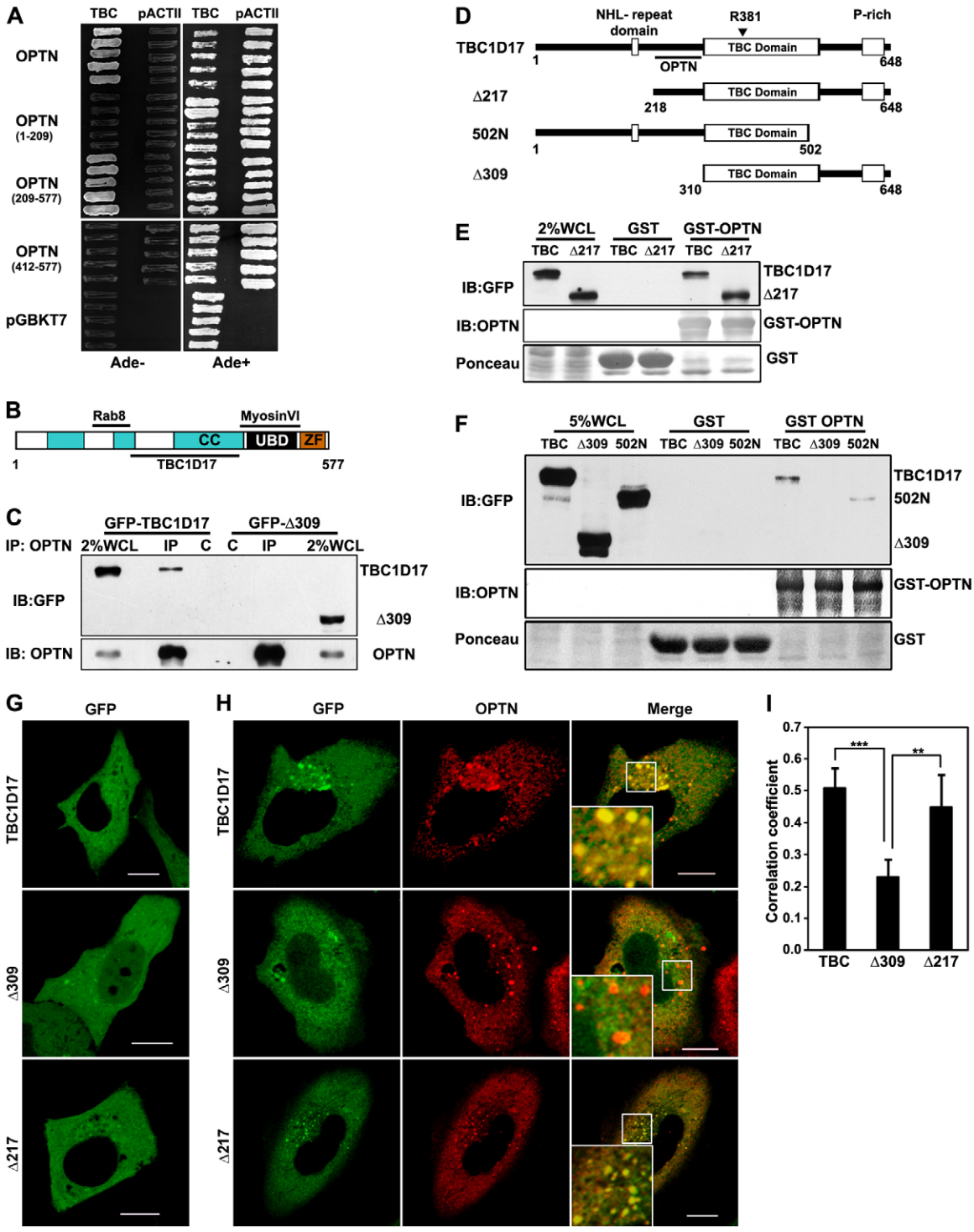

Fig. 1. Interaction and colocalisation of TBC1D17 with optineurin. (**A**) Interaction of optineurin (OPTN) and its deletion constructs, cloned in pGBKT7 vector, with TBC1D17, cloned in pACT2 vector. Transformants were plated on medium without (Ade–) or with (Ade+) adenine. Growth in the absence of adenine indicates an interaction between hybrid proteins. (**B**) Schematic representation of optineurin; the regions interacting with TBC1D17, Rab8 and myosin VI are shown. CC, coiled coil; UBD, ubiquitin-binding domain; ZF, zinc finger. (**C**) HEK293 cells were transfected with GFP–TBC1D17 or with a GFP-tagged deletion construct, Δ309 (aa 310–648), and immunoprecipitation (IP) was carried out using anti-optineurin antibody or control antibody (lane marked with C). The immunoprecipitates were analysed by western blotting (IB) with anti-GFP and anti-optineurin antibodies. WCL, whole-cell lysate. (**D**) Schematic representation of TBC1D17 and its deletion constructs. (**E**,**F**) HeLa cells were transfected with GFP-tagged TBC1D17 or its deletion constructs. After 24 hours of transfection, cell lysates were prepared and incubated with GST–optineurin or GST alone, as a control. The GST pulldowns were analysed by western blotting with anti-GFP and anti-optineurin antibodies. (**G**) Expression pattern of GFP-tagged TBC1D17 and its deletion constructs. (**H**) GFP-tagged TBC1D17 and its deletion constructs, Δ217 (aa 218–648) and Δ309 (aa 310–648) were co-transfected with HA–optineurin in HeLa cells. Cells were fixed after 24 hours and immunostained with anti-HA antibody (red) and observed by confocal microscopy. Scale bars: 10 μm. (**I**) Comparison of the correlation coefficient of colocalisation for optineurin with TBC1D17 and its deletion constructs. $^{**}P<0.01$; $^{***}P<0.001$.

in Rab8 distribution in control and optineurin-knockdown cells, with Rab8 seen in the juxtanuclear region and also in the plasma membrane. Interestingly, the localisation of TBC1D17 was altered in optineurin-knockdown cells. Whereas in control cells TBC1D17 showed a diffuse cytoplasmic distribution and showed some colocalisation with Rab8 (Fig. 2B), optineurin knockdown resulted in complete loss of colocalisation between TBC1D17 and endogenous Rab8 (Fig. 2B,C). In fact, in the absence of optineurin, most of the TBC1D17 was excluded from juxtanuclear region where Rab8 is present (Fig. 2B). These observations strongly suggest that optineurin is essential for the localisation and recruitment of TBC1D17 to Rab8. In accordance with this, overexpression of optineurin resulted in enhanced colocalisation of TBC1D17 with endogenous Rab8 (supplementary material Fig. S1C,D). Taken together, these results show that optineurin is essential for proper localisation of TBC1D17 and that it mediates the recruitment of, and the interaction between, Rab8 and TBC1D17.

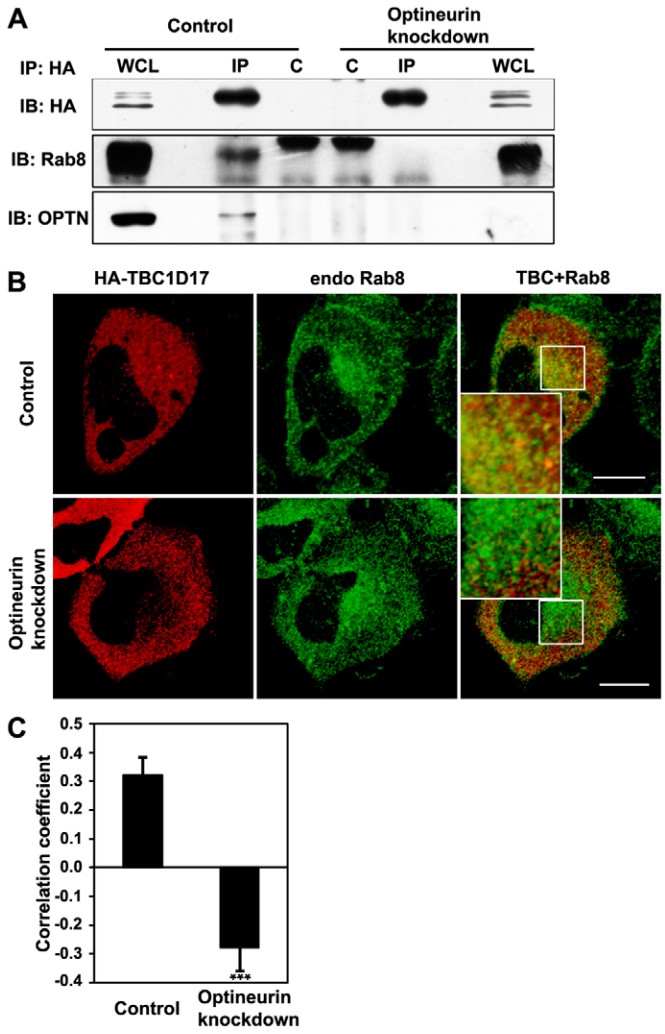

Fig. 2. Optineurin is required for interaction and colocalisation of TBC1D17 with Rab8. (**A**) HeLa cells were infected with adenoviruses expressing shRNA against optineurin to knockdown optineurin, or control adenovirus. After 48 hours of infection, cells were transfected with HA–TBC1D17. After 24 hours of transfection, lysates were made and immunoprecipitation (IP) was carried out by anti-HA or control antibody. Immunoprecipitates were analysed by western blotting (IB) 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 for colocalisation by using confocal microscopy. Scale bars: 10 µm. (**C**) The graph shows the correlation coefficient of colocalisation for TBC1D17 and Rab8 in optineurin-knockdown and control cells. ***$P<0.001$. endo Rab8, endogenous Rab8.

TBC1D17 inhibits endocytic trafficking of transferrin receptor

Because TBC1D17 forms a complex with Rab8 through optineurin, we examined the possibility of regulation of Rab8-mediated functions by TBC1D17. As Rab8 is involved in regulating trafficking of TfR to REs and from REs to plasma membrane (Hattula et al., 2006; Sharma et al., 2009), we analysed the effect of overexpression of TBC1D17 on trafficking of transferrin. HeLa cells transfected with TBC1D17 were incubated with Alexa-Fluor-546-conjugated transferrin. Expression of TBC1D17 strongly inhibited uptake of transferrin in most of the cells (Fig. 3A). Quantitative analysis showed that there was 78% inhibition of uptake of labeled transferrin upon expression of TBC1D17 (Fig. 3B). The specificity of the effect of a Rab GAP can be determined by using its catalytically inactive mutant in which the catalytic arginine residue is replaced by an alanine residue (Fuchs et al., 2007; Pan et al., 2006). Expression of the catalytically inactive R381A mutant of TBC1D17 showed only an 11% inhibition in uptake of transferrin (Fig. 3B). In the cells expressing TBC1D17, but not its R381A mutant, there was more prominent TfR staining in the perinuclear region (Fig. 3A). The expression of optineurin-binding deficient mutant Δ309 did not affect either the uptake of labeled transferrin or the distribution of TfR (Fig. 3A). The expression of these constructs was checked by western blot to confirm that the observed effects were not due to differences in expression level or a reduction in the overall TfR level in TBC1D17-expressing cells (Fig. 3C). 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 (Fig. 3D). Taken together, these results suggest that TBC1D17 inhibits the 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.

Several Rabs, including Rab8, regulate endocytic trafficking and recycling (Grant and Donaldson, 2009; Hattula et al., 2006; Henry and Sheff, 2008; Sharma et al., 2009). Therefore, the inhibitory effect of TBC1D17 on transferrin receptor trafficking might be due to inhibition of Rab8 function or inhibition of some other Rab GTPase. This possibility was examined by analysing the effect of coexpression of Rab8 on TBC1D17-mediated inhibition of transferrin uptake. Coexpression of Rab8 resulted in a significant reduction in the inhibition of transferrin uptake by TBC1D17-expressing cells (Fig. 3E). This was not due to reduction in expression level of TBC1D17 in Rab8-overexpressing cells (Fig. 3F). Coexpression of Rab5 or Rab21, which are known *in vitro* substrates of TBC1D17 (Fuchs et al., 2007) did not affect the TBC1D17-mediated inhibition of transferrin uptake (Fig. 3E). These results suggest that the

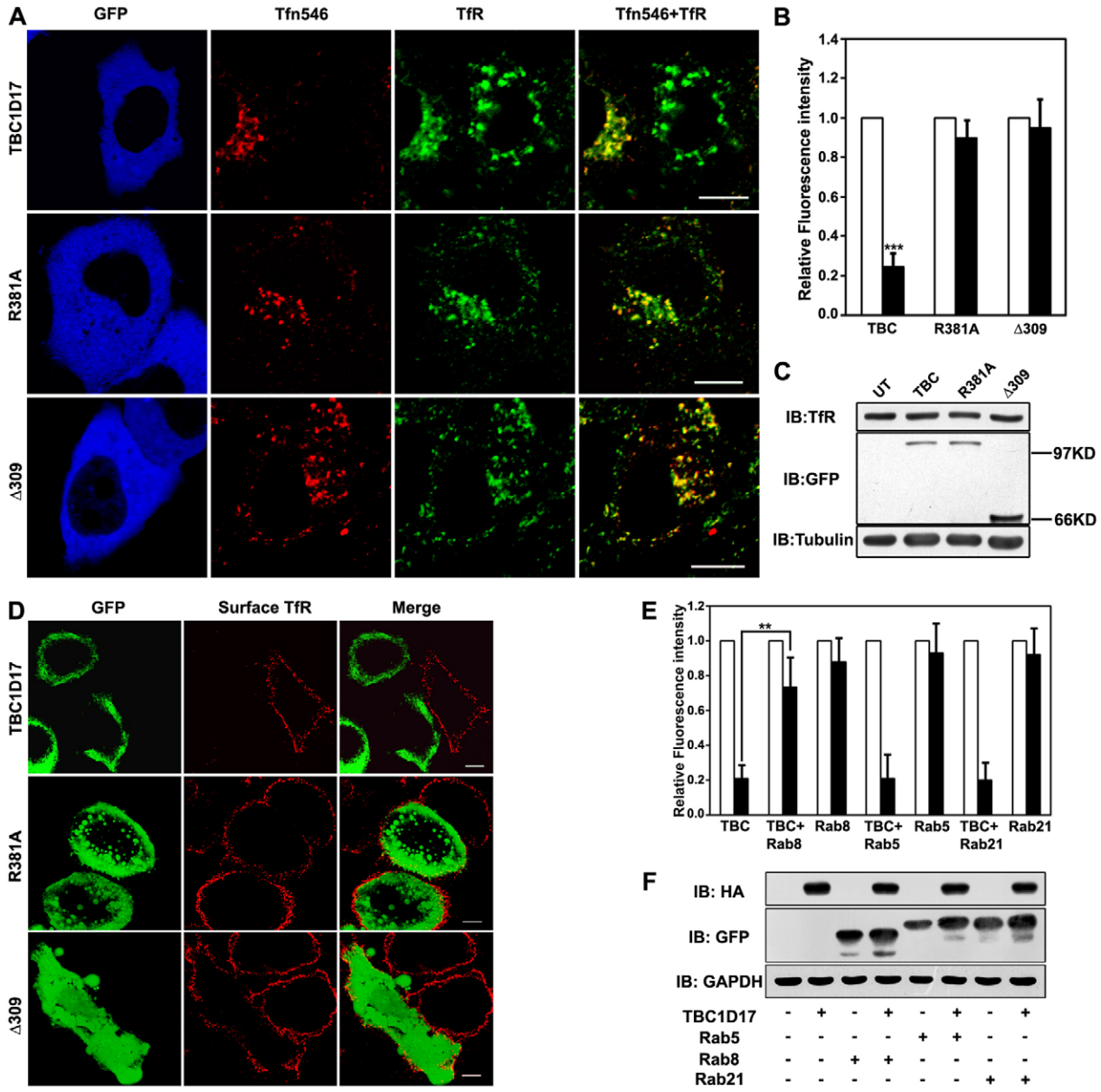

Fig. 3. TBC1D17 inhibits transferrin uptake. (**A**) HeLa cells were transfected with GFP-tagged TBC1D17, or its R381A or Δ309 constructs. After 22 hours, a transferrin uptake assay was performed using Alexa-Fluor-546-conjugated transferrin (Tfn546). Cells were stained with anti-TfR antibody (shown in green) and analysed by confocal microscopy. GFP-stained cells are artificially shown as blue. Scale bar: 10 μm. (**B**) Graph showing the relative fluorescence intensity of endocytosed transferrin in cells expressing TBC1D17 (labelled as TBC), or its mutants R381A and Δ309. ***$P<0.001$. (**C**) HeLa cells were transfected with GFP-tagged TBC1D17, R381A or Δ309. After 24 hours, lysates were made, separated by SDS-PAGE and analysed by western blotting (IB) using anti-GFP, anti-TfR and anti-tubulin antibodies. UT, untransfected cells. (**D**) 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 bars: 10 μm. (**E**) Graph showing rescue of TBC1D17-dependent inhibition of transferrin uptake by coexpression of Rab8 and not by Rab5 or Rab21. **$P<0.01$. (**F**) HeLa cells were transfected with HA–TBC1D17 and GFP-tagged Rab5, Rab8 or Rab21. The cell lysates were analysed by western blotting.

inhibitory effect of TBC1D17 on transferrin uptake is largely due to inhibition of Rab8 function.

TBC1D17 regulates recruitment of Rab8 to the tubules emanating from the ERC

Microtubule-dependent tubular structures emanating from the ERC play an important role in the 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 overexpression of the constitutively active Q67L mutant of Rab8 leads to the formation of 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 (supplementary material Fig. S2). Our results so far

suggest that TBC1D17, in association with optineurin, inhibits Rab8-mediated trafficking of TfR. Because TBC1D17 is a Rab GAP, it is likely that TBC1D17 inactivates Rab8 function. In order to ascertain this, we examined the role of TBC1D17 in recruitment of Rab8 to the tubular structures. In untransfected HeLa cells, Rab8-positive tubular structures could be seen in ~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 cells expressing TBC1D17 R381A had Rab8-positive tubules (Fig. 4A,C). These tubular structures can be stabilised in most of the cells by using cytochalasin D (Hattula et al., 2006). When formation of these tubules was induced by treating the cells with 0.15 μM of cytochalasin D for 30 minutes, 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 (Fig. 4B,C). In contrast, cells expressing the R381A mutant showed tubules in 85.3±9.5% of the cells (Fig. 4B,C). These results show that TBC1D17, through its catalytic activity, inhibits recruitment of Rab8 to the tubular structures. Given that only the GTP-bound form of Rab8 is present on the tubules, these results suggest that TBC1D17 inactivates Rab8.

Optineurin knockdown enhances recruitment of Rab8 to the tubules

We hypothesised 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 Rab8-positive tubules were present in 12.2±2.3% of control cells, whereas upon optineurin knockdown 38.4±2.8% cells showed these Rab8-positive tubules (Fig. 5A,B). This effect was not due to a change in the level of total endogenous Rab8, as seen by western blotting (Fig. 5C). Similarly, overexpression of HA-tagged Rab8 in optineurin-knockdown cells resulted in the formation of more prominent and numerous tubules (comparable to those formed by the expression of Q67L mutant) (supplementary material Fig. S2) as compared with that in control cells (Fig. 5D,E). These results suggest that optineurin knockdown enhances recruitment of Rab8 to the tubules, probably by shifting the equilibrium towards the GTP-bound form of Rab8.

TBC1D17 inhibits interaction and colocalisation of TfR with Rab8

Our results suggest 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 colocalisation of activated Rab8 with TfR (supplementary material Fig. S3A,B). We explored the possibility of using the interaction of Rab8 with the cytoplasmic domain of TfR to develop an assay for Rab8 activity. A GST fusion protein of the cytoplasmic domain of TfR showed stronger binding to the Q67L mutant of Rab8 than it did to T22N Rab8 (Fig. 6A,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 a yeast two-hybrid assay the cytoplasmic domain of TfR did not show any interaction with activated Rab8 (supplementary material Fig. S3C). However, these

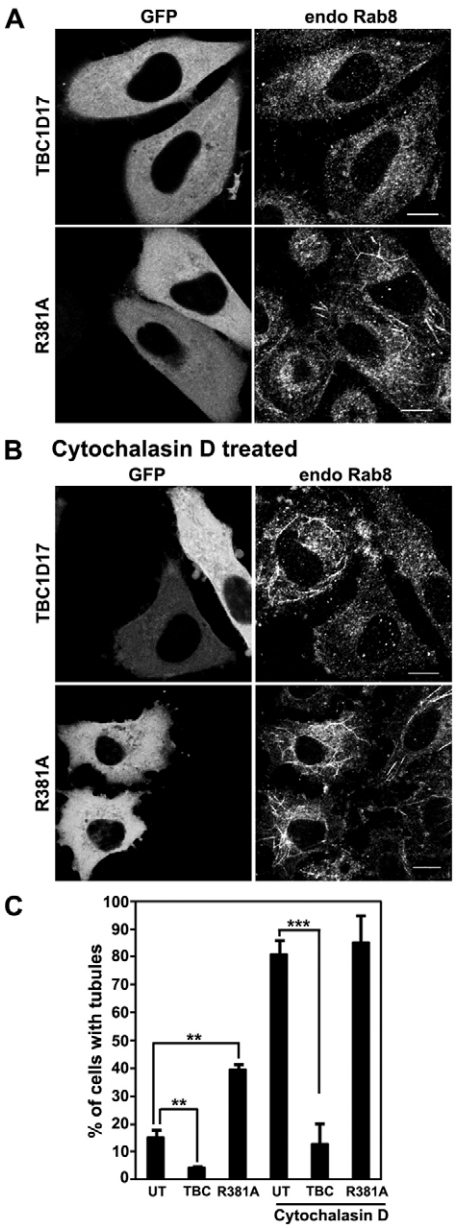

Fig. 4. Effect of TBC1D17 overexpression on Rab8-positive tubule formation. (**A**,**B**) HeLa cells were transfected with GFP-tagged TBC1D17 (TBC) or R381A and after 24 hours the cells were left untreated (A) or treated with 0.15 μM cytochalasin D (B) for 30 minutes, before being fixed and stained with anti-Rab8 antibody. The cells were then observed by confocal microscopy for Rab8-positive tubules. Scale bar: 10 μm. (**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 means±s.d. ***$P<0.001$, **$P<0.01$. UT, untransfected cells; endo Rab8, endogenous Rab8.

observations suggest that the active and inactive forms of Rab8 have differing binding affinities for TfR, and can be used to assay Rab8 activity. Our results so far suggest that TBC1D17 regulates Rab8-mediated trafficking, possibly by inactivating it. Hence, we hypothesised 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

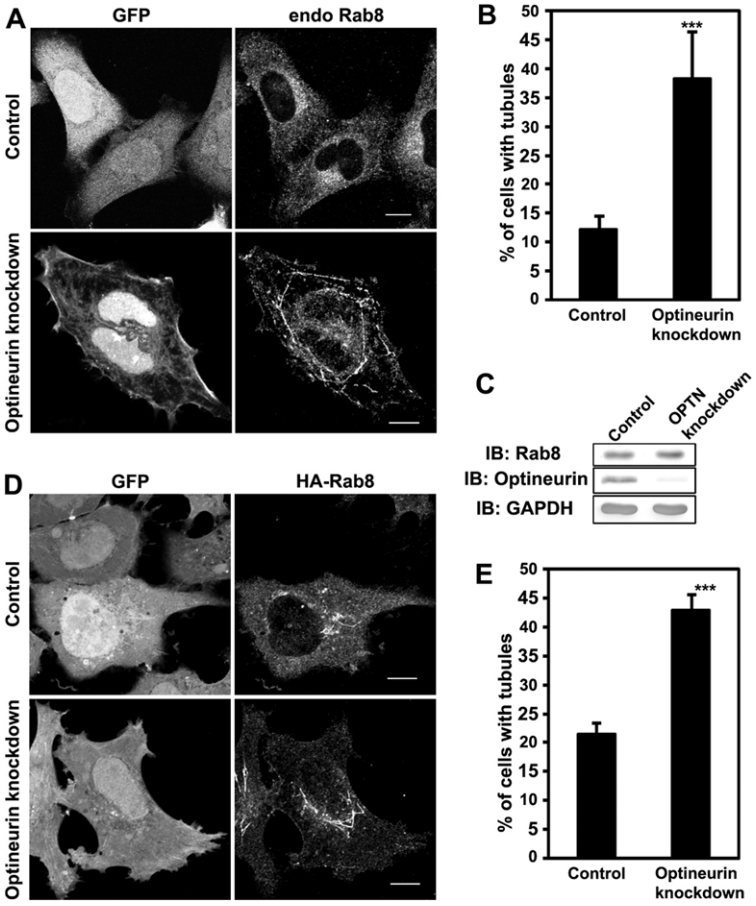

Fig. 5. Knockdown of optineurin enhances Rab8-positive tubule formation. (**A**) HeLa cells were infected with control adenoviruses or adenoviruses expressing shRNA against optineurin. After 72 hours, the cells were fixed and stained with anti-Rab8 antibody and analysed by confocal microscopy. The presence of GFP is an indicator of infection by adenovirus. Scale bars: 10 μm. (**B**) The graph shows the percentage of cells with Rab8-positive tubules. Values are percentages of cells given as means±s.d. 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 (IB) 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 means±s.d. ***$P<0.001$. endo Rab8, endogenous Rab8.

incubated with GST–TfR (aa 1–67) or GST. The amount of Rab8 bound to GST–TfR in TBC1D17-expressing cells was reduced as compared with that in those expressing the R381A mutant (Fig. 6C).

We further validated these observations by analysing the effect of overexpression of TBC1D17 on colocalisation of Rab8 with TfR. In cells coexpressing TBC1D17 and Rab8, the amount of Rab8 colocalising with TfR was significantly reduced (Fig. 6D,E). This is dependent on catalytic activity of TBC1D17, because coexpression of the R381A mutant did not decrease the colocalisation of TfR with Rab8 (Fig. 6D,E). These observations are similar to those seen for T22N-Rab8, which shows less colocalisation with TfR as compared to Q67L-Rab8 (supplementary material Fig. S3A,B). Taken together, these results strongly suggest that TBC1D17 renders Rab8 in an inactivated state resulting in less colocalisation with TfR and less interaction with GST–TfR. The colocalisation of TfR with Rab8 was also seen on the tubules in cells coexpressing the R381A mutant (Fig. 6D). The percentage of cells showing Rab8-positive tubules and prominence of these tubules was also significantly enhanced in cells coexpressing R381A as compared with cells coexpressing TBC1D17 (Fig. 6F).

E50K-optineurin inactivates Rab8 through TBC1D17

E50K is a dominant glaucoma-causing mutation of optineurin (Rezaie et al., 2002). Previous studies have shown that the 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). This defective trafficking is possibly due to an altered interaction of the E50K mutant with Rab8 and also with TfR. Because optineurin mediates the interaction between TBC1D17 and Rab8, and possibly inactivation of Rab8, we next examined whether the impaired trafficking caused by the E50K mutant is due to an altered physical and/or functional interaction with TBC1D17. Co-immunoprecipitation experiments and a yeast two-hybrid assay revealed no noticeable differences in the interaction of TBC1D17 with E50K as compared with that of wild-type optineurin (supplementary material Fig. S4). Nevertheless, colocalisation of TBC1D17 with the E50K mutant was significantly increased as compared with that for wild-type optineurin (supplementary material Fig. S5).

In order to ascertain whether impaired Rab8-mediated trafficking of TfR by the E50K mutant is dependent on TBC1D17 function, we analysed the effect of expression of the R381A mutant of TBC1D17 on E50K-dependent inhibition of transferrin uptake. Whereas cells expressing the E50K mutant showed a reduced uptake of transferrin, coexpression of the R381A mutant significantly restored the uptake of transferrin in these cells (Fig. 7A). This reversal was not due to reduced expression of E50K in presence of the R381A mutant, as shown by western blot (Fig. 7B). Knockdown of TBC1D17 by a shRNA also partly restored the uptake of transferrin in E50K-expressing cells (Fig. 7C,D). However in GFP-expressing cells there was no significant effect of TBC1D17 knockdown on uptake of transferrin (Fig. 7D). These results suggest that impaired trafficking of TfR caused by the E50K mutant is dependent on

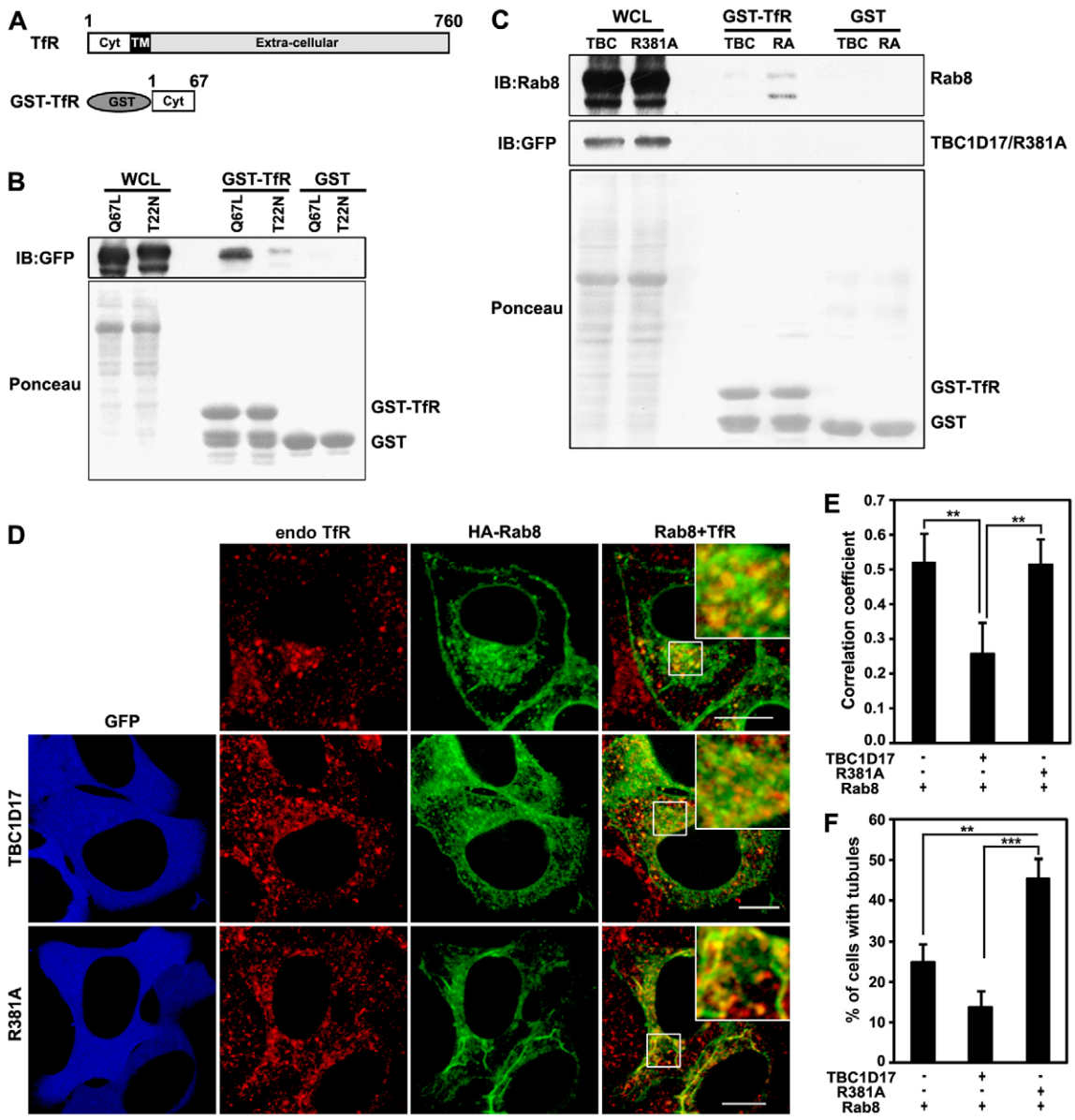

Fig. 6. TBC1D17 reduces interaction and colocalisation of Rab8 with transferrin receptor. (**A**) Schematic showing TfR protein and cytoplasmic domain (Cyt, aa 1–67) of TfR fused to GST, used in the experiments. (**B**) HEK293 cells were transfected with GFP-tagged Rab8 Q67L or T22N constructs. After 24 hours of transfection, cell lysates were prepared and incubated with GST–TfR or GST alone, as a control. The GST pulldowns were analysed by western blotting (IB) with anti-GFP antibody. (**C**) HEK293 cells were transfected with GFP–Rab8 along with GFP-tagged TBC1D17 or its R381A construct. After 24 hours of transfection, cell lysates were prepared and incubated with GST–TfR or GST alone, as a control. The GST pulldowns were analysed by western blotting with anti-GFP antibody and anti-Rab8 antibody. (**D**) HeLa cells were transfected with HA-tagged Rab8 either alone or along with GFP-tagged TBC1D17 or its R381A construct. After 24 hours, cells were stained with anti-TfR (red) and anti-HA antibodies and observed by using confocal microscopy. GFP staining is artificially shown in blue. Scale bars: 10 μm. (**E**) The graph shows the correlation coefficient of colocalisation between Rab8 and TfR in the presence of TBC1D17 or R381A. $^{**}P<0.01$. (**F**) The graph shows the percentage of cells exhibiting Rab8-positive tubules in each scoring category. The data are shown as the means±s.d. $^{**}P<0.01$.

TBC1D17. Next, we examined the effect of coexpression of activated Rab8 (Q67L mutant) on E50K-optineurin-mediated inhibition of transferrin uptake. Similar to with the R381A mutant, coexpression of Q67L-Rab8 significantly restored the transferrin uptake of the E50K-expressing cells (Fig. 7E). This effect was not due to a decrease in E50K expression upon coexpression of Q67L-Rab8, as shown in the western blot (Fig. 7F). These results suggest that impaired trafficking of TfR by the E50K mutant is dependent on TBC1D17 and is possibly due to inactivation of Rab8.

It is likely that despite the lack of marked differences in its interaction with TBC1D17, the E50K-optineurin mutant enhances the catalytic function of TBC1D17 and, hence, inactivates Rab8. In accordance with this assumption, formation of Rab8-positive tubules upon overexpression of Rab8 was strongly inhibited by the E50K mutant but not by wild-type optineurin (Fig. 8A,B).

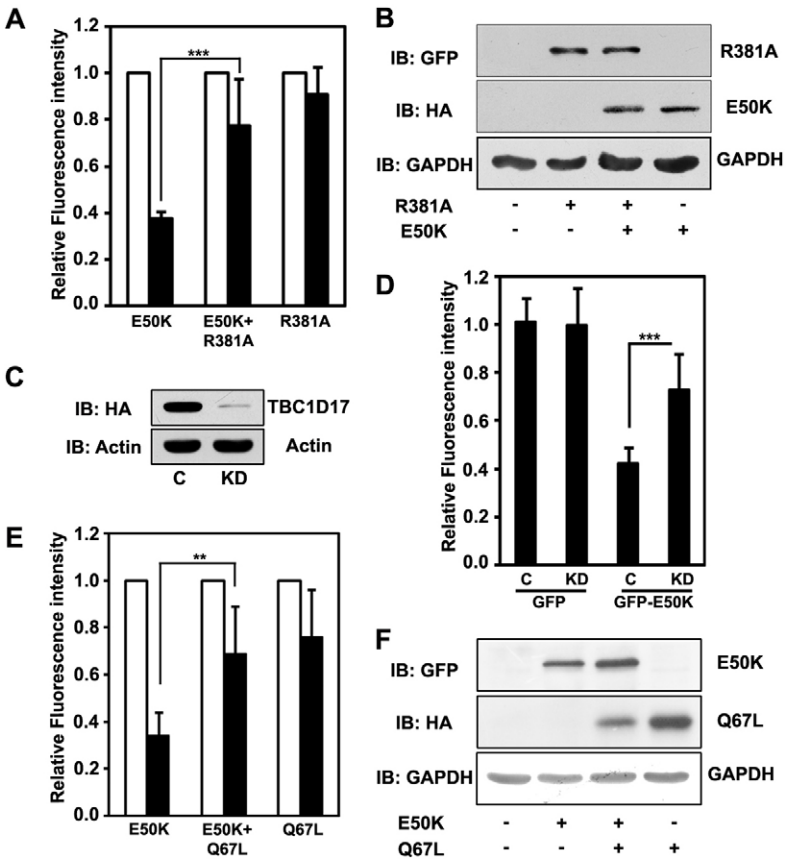

Fig. 7. R381A-TBC1D17 or Q67L-Rab8 can rescue the inhibitory effect of E50K-optineurin on transferrin uptake. (**A**) HeLa cells were transfected with HA–E50K-optineurin alone, GFP–R381A-TBC1D17 alone or the two together. After 24 hours of transfection, a transferrin uptake assay was performed. The graph 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. ***$P<0.001$. (**B**) Western blot (IB) to show that R381A expression does not reduce E50K expression. (**C**) HeLa cells were transfected with HA–TBC1D17 along with an shRNA expression plasmid against TBC1D17 (KD) or a control plasmid (labelled C). After 24 hours, cell lysates were made and analysed by western blot to test the efficacy of shRNA mediated knockdown. (**D**) HeLa cells were transfected with GFP–E50K-optineurin or GFP along with TBC1D17-directed shRNA expression plasmid or control plasmid. After 40 hours of transfection, a transferrin uptake assay was performed. The graph shows the relative florescence intensity of endocytosed transferrin by cells expressing GFP or GFP–E50K-optineurin. ***$P<0.001$. (**E**) HeLa cells were transfected with GFP–E50K-optineurin alone, HA–Q67L-Rab8 alone or the two together. After 24 hours, a transferrin uptake assay was performed. The graph shows the relative florescence intensity of endocytosed transferrin by the cells expressing E50K alone, Q67L alone or E50K and Q67L together compared with that in non-expressing cells (white bars). **$P<0.01$. (**F**) Western blot to show that HA–Q67L-Rab8 expression does not reduce GFP–E50K-optineurin expression.

Similarly, recruitment of endogenous Rab8 to the tubules was strongly inhibited by the E50K mutant, whereas wild-type optineurin showed significantly less inhibition (Fig. 8C,D). Coexpression of the R381A mutant of TBC1D17 resulted in reversal of E50K-optineurin-dependent inhibition of endogenous Rab8 tubule formation (Fig. 8E,F). Knockdown of TBC1D17 by shRNA also resulted in reversal of E50K-mediated inhibition of tubule formation by endogenous Rab8 (Fig. 8G). In GFP-expressing control cells, knockdown of TBC1D17 resulted in an increase in Rab8-positive tubules (Fig. 8G). Taken together, these results suggest that the impaired trafficking of TfR observed in E50K-expressing cells is possibly due to an enhanced TBC1D17-dependent inhibition of Rab8 function.

TBC1D17 inhibits recycling of transferrin receptor

Our results show that TBC1D17 inhibits TfR trafficking and also inhibits formation of the Rab8-positive tubules that are involved in endocytic recycling. Therefore, we examined the role of TBC1D17 in recycling of TfR. We carried out a transferrin recycling assay in cells expressing TBC1D17 or its inactive mutant R381A. Given that TBC1D17-expressing cells show reduced uptake of transferrin, we incubated the cells with labelled transferrin for 30 minutes to allow sufficient uptake and then washed with complete medium for 45 minutes (chase). After the chase, much more transferrin was seen in TBC1D17-expressing cells as compared with that in non-expressing cells (Fig. 9A). Quantitative analysis showed that the expression of TBC1D17 inhibits recycling of transferrin, whereas the R381A mutant does not (Fig. 9B). Expression of the E50K mutant of optineurin also inhibited recycling of transferrin, whereas wild-type optineurin had no significant effect (Fig. 9C,D).

Discussion

The mechanism by which a Rab GAP specifically recognises its substrate and is targeted to it, is not completely understood. The catalytic domain of a TBC protein is likely to play a role in recognising 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 do 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 recognising the target Rabs. The mechanisms by which non-catalytic sequences of TBC proteins recognise their target Rabs directly or indirectly are not known. Here, we have investigated the mechanism by which the Rab GAP TBC1D17 is recruited to Rab8 and regulates its function and activity. Our results 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 can act as an adaptor protein facilitating assembly of multimolecular signaling complexes (del Toro et al., 2009; Nagabhushana et al., 2011; Sahlender et al., 2005; Wild et al., 2011). In accordance with its emerging role as an adaptor protein, our results show that the binding site of TBC1D17 for optineurin (aa 209–411) is close to the Rab8-binding site (aa 141–209) reported earlier (Hattula and Peranen, 2000). Optineurin is known to interact preferentially with the activated form of Rab8 (Hattula and Peranen, 2000) and the proximity of the binding sites for 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.

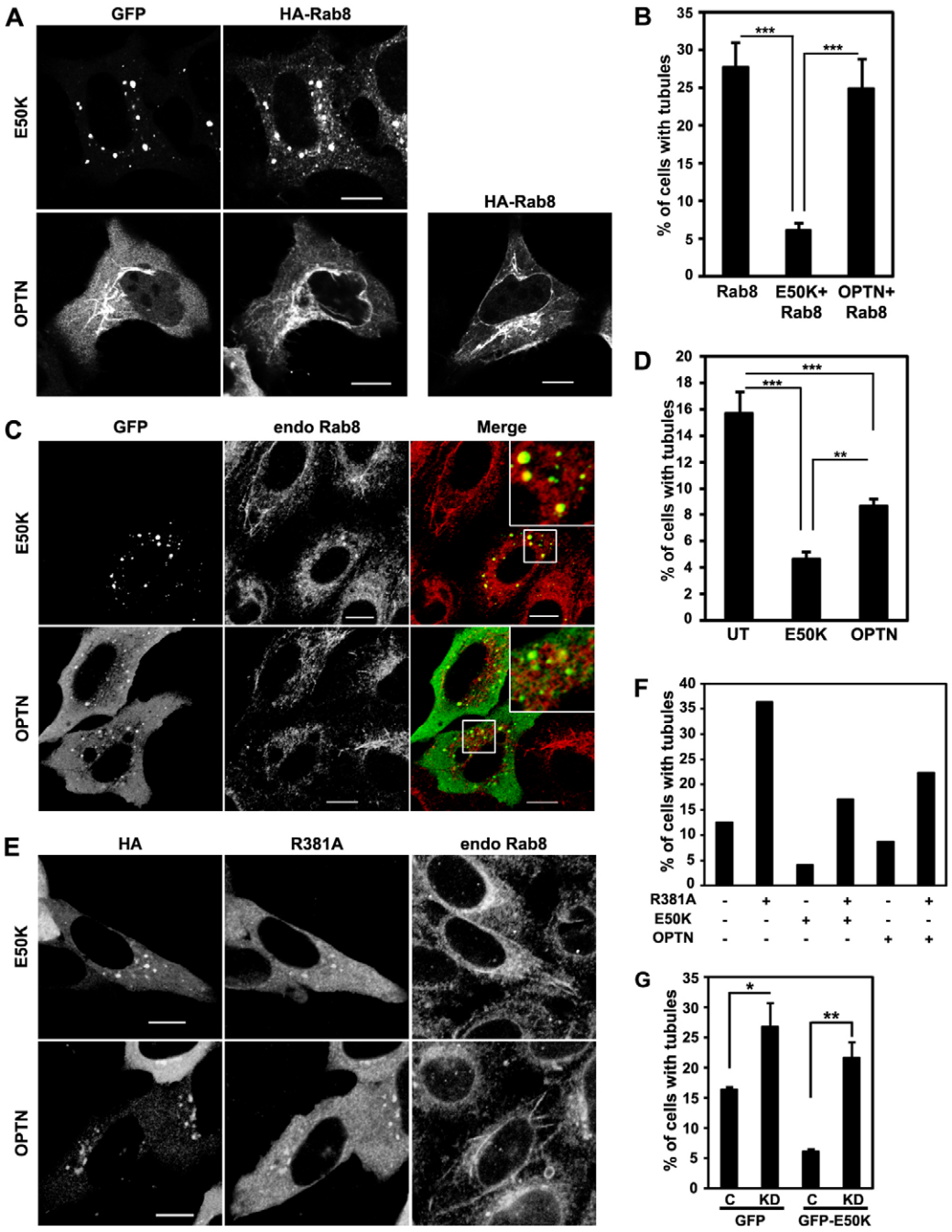

Fig. 8. The E50K mutant of optineurin inhibits Rab8-positive tubule formation. (**A**) HeLa cells grown on coverslips were transfected either with HA-tagged Rab8 alone or along with plasmids encoding GFP-tagged E50K mutant or wild-type optineurin. After 24 hours of transfection, these cells were stained with anti-HA antibody and observed by confocal microscopy. (**B**) Cells were scored for the presence of Rab8-positive tubules. The graph shows percentage of cells containing Rab8-positive tubules. (**C**) HeLa cells grown on coverslips were transfected with the GFP-tagged E50K mutant or wild-type optineurin. After 24 hours of transfection, cells were stained with anti-Rab8 antibody and observed by confocal microscopy. (**D**) The graph shows percentage of cells containing Rab8-positive tubules. (**E**) HeLa cells were transfected with the GFP-tagged R381A mutant of TBC1D17 along with either HA-tagged E50K or wild-type optineurin. After 24 hours of transfection, these cells were stained for HA and Rab8, and were observed with a confocal microscope to score for Rab8-positive tubules. (**F**) The graph shows the percentage of cells that had Rab8-positive tubules. (**G**) HeLa cells grown on coverslips were transfected with GFP–E50K-optineurin or GFP along with TBC1D17-directed shRNA expression plasmid (KD) or control plasmid (C). After 40 hours, the cells were stained for Rab8 and examined by confocal microscopy to score for Rab8-positive tubules. *$P<0.05$; **$P<0.01$; ***$P<0.001$. endo Rab8, endogenous Rab8. Scale bars: 10 µm.

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). We have used this recruitment of active Rab8 on the tubules as an assay to assess the activity of Rab8 in cells overexpressing TBC1D17 and its R381A mutant. The ability of TBC1D17, but not its R381A mutant, to inhibit recruitment of Rab8 to the tubules strongly suggests that

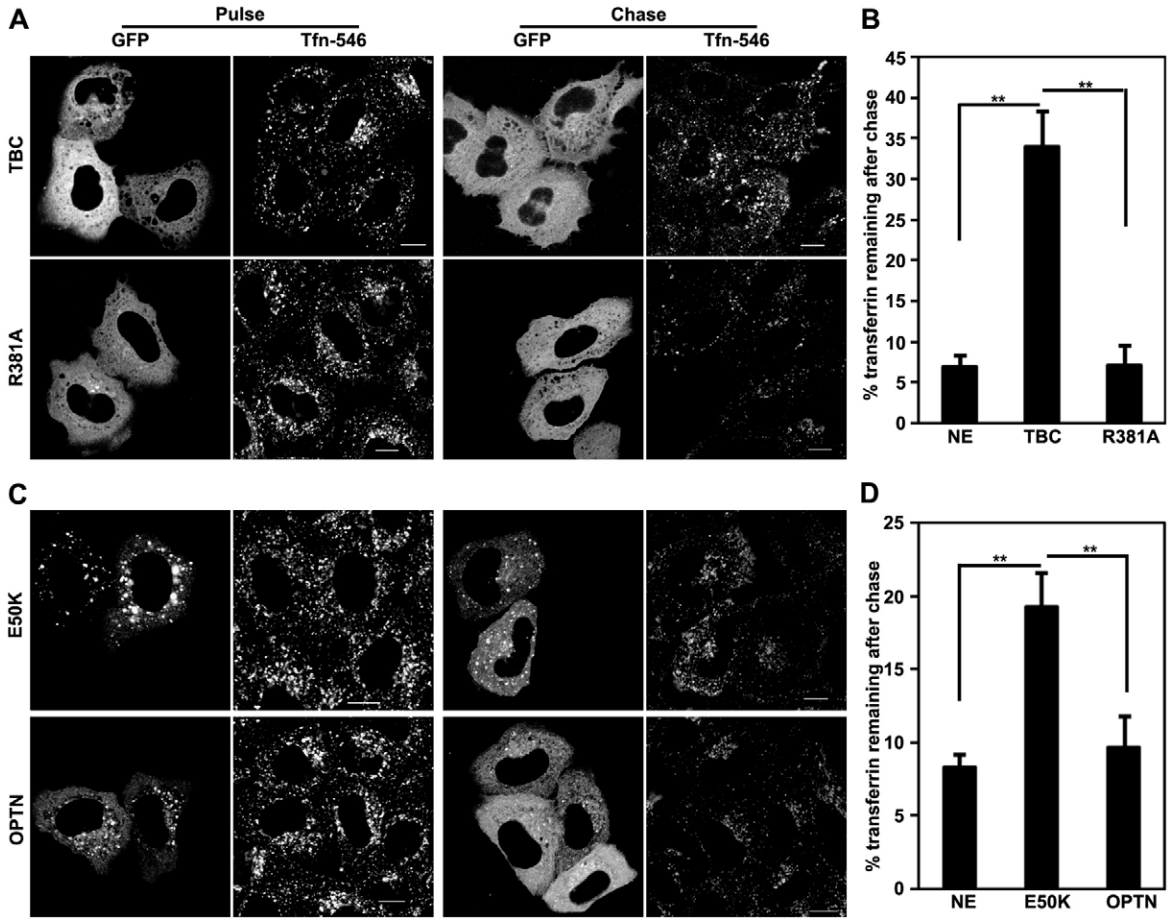

Fig. 9. TBC1D17 inhibits endocytic recycling of transferrin. (**A**) HeLa cells grown on coverslips were transfected with GFP-tagged TBC1D17 (TBC) or its R381A mutant. After 24 hours, the cells were serum starved for 2 hours, incubated with Alexa-546-labelled transferrin (Tfn-546) for 30 minutes and then fixed, or washed twice with PBS and incubated in complete medium for 45 minutes (chase). The fixed cells were examined by confocal microscopy. (**B**) Quantitative analysis was carried out to calculate the percentage of transferrin remaining after the chase in expressing and non-expressing (NE) cells. (**C**) HeLa cells were transfected with GFP-tagged E50K mutant or wild-type optineurin, and a transferrin recycling experiment was carried out as described in A. (**D**) Quantitative analysis was carried to calculate the percentage of transferrin remaining after the chase in expressing and non-expressing cells. Scale bars: 10 μm.

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. These results lead us to suggest that TBC1D17 regulates Rab8 activity in a manner that is facilitated by optineurin. This hypothesis is further supported by the observation that endogenous, as well as overexpressed, Rab8 localises to the tubules more prominently in optineurin-knockdown cells.

Endocytic trafficking and recycling of transferrin and its receptor have been studied extensively, and several Rab proteins are involved in controlling distinct steps of this trafficking (Mayle et al., 2011). After endocytosis, the transferrin–TfR complex moves from primary endocytic vesicles to early endosomes in a step that is regulated by Rab5 (Sonnichsen et al., 2000). Trafficking of TfR from early endosomes to recycling endosomes requires Rab8, and recycling from recycling endosomes to the plasma membrane is mediated by Rab11 (Hattula et al., 2006; Ullrich et al., 1996). 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. The finding that transferrin uptake was restored upon coexpression of Rab8, but not the 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, therefore suggesting 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 GAP 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 colocalisation 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 the colocalisation of a fraction of TfR with these tubules in cells expressing the mutant R381A TBC1D17, but not in TBC1D17-expressing cells, indicates that there is active Rab8 associating with its ‘cargo’ on the tubules. Overall our results 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 Kaplan, 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 and E50K-optineurin, which also both 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 associate with Rab8-positive tubules in cells expressing the catalytic mutant of TBC1D17 (Fig. 6D). 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).

Mutations in the coding region of optineurin cause certain glaucomas and amyotrophic lateral sclerosis (Maruyama et al., 2010; Rezaie et al., 2002). E50K is a dominant mutation that causes glaucoma by directly inducing the death of retinal ganglion cells (Chalasani et al., 2007; Chi et al., 2010). Upon overexpression, E50K-optineurin inhibits endocytic trafficking of TfR, resulting in accumulation of TfR in large E50K-positive structures or foci (Nagabhushana et al., 2010; Park et al., 2010). This optineurin mutant shows altered interactions with Rab8 and TfR, and impairs Rab8-mediated trafficking, but the molecular mechanism of the defect in endocytic trafficking of TfR is not known. Here, we have shown that, similar to with TBC1D17, the expression of E50K mutant inactivates Rab8, as seen by the nearly complete loss of Rab8 from the tubules. Interestingly, co-expression of the 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. As optineurin acts as an adaptor protein to mediate the assembly of larger complexes, it is likely that altered interactions of optineurin mutants, such as E50K, might result in functional defects. Previously, we have shown that Rab8, optineurin and TfR form a multimolecular complex (Nagabhushana et al., 2010). The E50K mutant of optineurin forms a stronger complex with Rab8, as well as TfR, as seen in immunoprecipitation experiments. E50K-optineurin also showed stronger colocalisation with Rab8 in TfR-positive structures and vesicles. However, direct interaction between Rab8 and E50K-optineurin is lost, as determined by a yeast two-hybrid assay (supplementary material Fig. S6). This loss of direct interaction between the E50K mutant and Rab8 has also been shown in mammalian cells (Chi et al., 2010). These observations suggest that in the multimolecular complex, direct interaction between Rab8 and E50K is lost, although indirect interaction is enhanced. This might alter the functional positioning of the molecules in the complex in such a way that it leads to constitutive or increased inactivation of Rab8 by TBC1D17. Co-expression of the R381A mutant of TBC1D17 might prevent this inactivation by displacing endogenous TBC1D17. This hypothesis is supported by the following observations: (1) as compared with wild-type optineurin, the E50K mutant shows stronger colocalisation with TBC1D17 and Rab8; (2) coexpression of the R381A mutant or knockdown of TBC1D17 reverses the inhibitory effect of E50K mutant on transferrin uptake and Rab8-positive tubule formation.

Some other TBC domain proteins have been shown to inactivate Rab8 in restricted niches. AS160 (also known as TBC1D4) has been shown to be a GAP for Rab8 in muscle cells and adipocytes during insulin stimulated GLUT4 vesicle translocation (Miinea et al., 2005; Randhawa et al., 2008; Zeigerer et al., 2004). 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. Given that 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 remain to be investigated.

In conclusion, our results show that optineurin mediates an interaction between the Rab GTPase activating protein TBC1D17 and its target, Rab8. TBC1D17 is possibly a GAP of Rab8, and is involved in regulating trafficking and recycling of TfR, and recruitment of Rab8 on the tubules. The defective trafficking of TfR by the E50K glaucoma-causing mutant of optineurin is due to the TBC1D17-mediated inactivation of Rab8, and this might be involved in the etiopathogenesis of glaucoma caused by this mutation. We describe a mechanism of regulating a Rab GTPase through interaction with an effector protein (optineurin) which brings together a Rab (Rab8) and its GAP (TBC1D17) (supplementary material Fig. S7). While this paper was being revised, Hokanson and Bretscher (Hokanson and Bretscher, 2012) described a similar mechanism of regulating Rab8 that was mediated by EPI64 through the effector protein JFC1. Because 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.

Materials and Methods

cDNA constructs and reagents

TBC1D17 was amplified by PCR from the clone (FLJ12168) obtained from the human placental cDNA library used in yeast two-hybrid screening. It was cloned in pEGFP-C1 (Clontech, CA, USA), pcDNA3.1-HA and pACT2 (which contains a GAL4 activation domain) vectors (Clontech, CA, USA). Deletion constructs of TBC1D17 were generated by PCR. Point mutations in TBC1D17 were created by a PCR-based site-directed mutagenesis strategy following the protocol described in QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Plasmid vectors for expressing human optineurin and its mutant (E50K) with a HA and GFP tag have been described previously (Chalasani et al., 2007; Nagabhushana et al., 2010). A GST fusion protein of optineurin was made by subcloning it in pGEX-5X2 vector (GE Healthcare, Uppsala, Sweden). Optineurin and its deletion constructs were cloned in pGBKT7 (which contains a GAL4 DNA-binding domain) vector (Clontech, CA, USA). Human Rab8a (referred to as Rab8) and its mutants (Q67L and T22N) cloned in pEGFPC3 and pcDNA3.1-HA vectors have been described previously (Nagabhushana et al., 2010). Human Rab5 was amplified by PCR from IMR32 cell RNA and cloned in pEGFP-C3 (Clontech, CA, USA) vector. GFP–Rab21 was a kind gift from Arwyn T. Jones (Welsh School of Pharmacy, Cardiff University, Cardiff, UK) and has been described previously (Simpson et al.,

2004). The cytoplasmic domain of human transferrin receptor (aa 1–67) cDNA was amplified by PCR and cloned in pGEX4T3 vector (GE Healthcare, Uppsala, Sweden).

Mouse monoclonal anti-Rab8 antibody was from BD Biosciences (San Jose, CA, USA), rabbit polyclonal anti-optineurin antibody and mouse monoclonal anti-transferrin receptor antibody, used for surface transferrin receptor labelling, were from Abcam (Cambridge, UK). Mouse monoclonal anti-transferrin receptor antibody was from Zymed (San Francisco, CA, USA). Mouse monoclonal anti-HA and protease cocktail inhibitor were from Roche Applied Biosystems (Indianapolis, USA). Rabbit polyclonal anti-HA, mouse monoclonal anti-tubulin, mouse monoclonal anti-GFP, and normal IgG control antibodies were from Santa Cruz Biotechnology (CA, USA). Glutathione–agarose beads, mouse monoclonal anti-GAPDH antibody and cytochalasin D were from Sigma (St. Louis, Missouri, USA). Alexa-Fluor-546-conjugated transferrin was from Molecular Probes (Invitrogen Corporation, Carlsbad, CA, USA).

Cell culture and transfections

HeLa cells were grown as monolayers in a humidified atmosphere of 5% CO_2 at 37℃ in DMEM (Dulbecco's minimal essential medium) containing 10% fetal calf serum. Transfections were performed using Lipofectamine Plus™ reagent or Lipofectamine™ 2000 (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions.

Indirect immunofluorescence and confocal microscopy

For immunofluorescence, cells were grown on coverslips, transfected with the required plasmids, fixed, permeabilised and stained with appropriate antibodies, as described previously (Gupta and Swarup, 2006). For staining of endogenous Rab8, cells were fixed with 3.7% formaldehyde for 10 minutes, and permeabilised for 6 minutes with 0.5% Triton X-100 and 0.05% Tween-20 (Sigma) in PBS. Permeabilised cells were incubated with blocking solution (2% BSA in PBS) for 1 hour at room temperature. The cells were then incubated with anti-Rab8 antibody (1:200 dilution) in blocking solution for 2 hours at room temperature followed by 10–12 hours at 4℃. For staining of surface TfR, the cells were fixed at 4℃ with 2% formaldehyde for 4 minutes and, after blocking with BSA, staining with primary anti-TfR antibody was performed at 4℃. For analysis of colocalisation, cells were observed using a LSM 510 NLO confocal microscope (Carl Zeiss Microimaging, Jena, Germany). For imaging GFP, Cy3 and Alexa Fluor 633, a 488 nm argon laser, 561 nm DPSS laser and 633 nm HeNe laser were used, respectively. Serial optical sections in the *z*-axis of the cells were collected at 0.35 µm intervals with a 63× oil immersion objective lens (NA 1.4). Generally, two serial optical sections were projected and colocalisation was observed using the LSM 510 software (version 3.2). Quantitative analysis of colocalisation was performed by calculating Pearson's correlation coefficients using the LSM 510 software. Images were further processed by using Adobe Photoshop software.

Analysis of Rab8-positive tubules

For quantitative analysis of Rab8-positive tubules, cells, after transfection with the required plasmid or infection with adenoviruses, were stained for Rab8 and images were acquired using the LSM 510 NLO confocal microscope. Cells containing a minimum of three distinct tubules per cell with a length greater than 10 µm were counted (Hattula et al., 2006). At least 100 cells were counted for each set of experiments.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed as described previously (Gupta and Swarup, 2006). Briefly, yeast strain PJ69-4A was co-transformed with the required plasmids by the lithium-acetate method. The transformants were selected by growth in minimal media (Trp−, Leu−). Yeast colonies obtained on Trp−, Leu− plates were patched on adenine deficient selection plates (Trp−, Leu−, Ade−) to assay activation of reporter gene and hence interaction. The interactions were also tested by the activation of β-galactosidase gene by patching the colonies on plates supplemented with β-galactosidase substrates (X-gal+ plates). Growth on Ade− plate or colour on X-Gal plate indicated interaction.

Knockdown of optineurin and TBC1D17

Endogenous optineurin was downregulated by using adenoviral vectors expressing shRNAs as described previously using pAdEASY system (He et al., 1998; Sudhakar et al., 2009). Cells grown on coverslips or dishes were infected with adenoviruses and processed after 72 hours for efficient knockdown. The shRNA expression vector targeting human TBC1D17 was made using a plasmid vector with a U6 promoter as described previously (Jain et al., 2005; Yu et al., 2002). The TBC1D17 sequence targeted by shRNA was from nucleotides 69 to 87 of the coding region (GenBank accession no. NM_024682). A plasmid expressing shRNA of an unrelated sequence of the same length was used as a control.

Immunoprecipitation, western blotting and GST pulldown assay

Immunoprecipitations were carried out essentially as described previously (Muppirala et al., 2011). Briefly, cells were washed with ice-cold PBS and lysed in lysis buffer [25 mM Tris-HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 0.1% BSA, 1 mM PMSF and protease inhibitor cocktail (Roche)]. The cell lysates were cleared by centrifugation and supernatants were used for immunoprecipitation with 2 µg of appropriate antibodies for 8 hours at 4℃. The immunoprecipitated proteins were washed three times with wash buffer [20 mM HEPES pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF and protease inhibitors], eluted by boiling in SDS sample buffer and resolved in by SDS-PAGE (8–12% gels). The proteins were transferred onto nitrocellulose membrane for western blot analysis as described previously (Jain et al., 2005).

For GST pulldown assays, GST and GST fusion proteins were expressed in *E. coli* and conjugated to Sepharose beads as described previously (Paliwal et al., 2007). These beads were incubated for 6–8 hours with lysates of HeLa or HEK293 cells transiently transfected with the indicated plasmids. To remove non-specific binding, beads were washed three times with wash buffer (20 mM HEPES pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF and protease inhibitors). Bound proteins were eluted by boiling in SDS sample buffer and subjected to immunoblotting.

Transferrin uptake

The transferrin uptake assay was performed essentially as described previously (Nagabhushana et al., 2010). HeLa cells were grown on coverslips and washed and pre-incubated with serum-free DMEM for 2 hours. These cells were then incubated with 10 µg/ml of Alexa-Fluor-546-conjugated transferrin in serum-free medium for 1 hour at 4℃. The cells were then shifted to 37℃ for 20 min to allow uptake of transferrin, washed with PBS twice and fixed in 3.7% formaldehyde. For quantitative analysis of transferrin uptake, the fluorescence intensity of internalised transferrin was measured using ImageJ software (National Institutes of Health, Bethesda, USA). The fluorescence intensities of the transfected cells were normalised to that of non-expressing cells. For the transferrin recycling assay, the serum-starved cells were incubated with Alexa-Fluor-546-labelled transferrin for 30 minutes and then fixed, or washed twice with PBS and incubated in complete medium for 45 minutes (chase).

Statistical analysis

Graphs show means±s.d. Statistical differences were calculated using Student's *t*-test. When significant differences were observed, *P*-values for pair-wise comparisons were calculated by using two-tailed *t*-tests. $P<0.05$ was considered significant.

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