

The neuropathy-linked protein TECPR2 is a Rab5 effector that regulates cargo recycling from early endosomes

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Mutations in the TECPR2 gene are a leading cause of autosomal recessive complex spastic paraplegia (HSP) or HSAN with autonomic dysfunction, respiratory failure, and intellectual disability. TECPR2 has been implicated in multiple endocytic transport pathways, including early secretion and autophagic degradation, though its precise role within subcellular compartments remains unclear. The study by Paul et al. provides a comprehensive analysis of the mechanisms by which TECPR2 regulates endosomal sorting, shedding light on the dynamic organization of the cell adhesion protein integrin under both physiological and pathological conditions.

This well-executed study presents evidence that TECPR2 functions in a Rab5-dependent manner, suggesting that TECPR2 may act as a Rab5 effector to facilitate the delivery of various intracellular cargo proteins. The authors propose a valuable model in which TECPR2 recruits SNX17 to selectively sort membrane proteins, a mechanism that may have broad implications, particularly for the dynamic regulation of neuronal receptors involved in neurodevelopmental and neurodegenerative diseases, as supported by previous studies and the authors' data. Intriguingly, the author identified the TECPR2 subcellular functional route as different from the previous work by Nalbach et al. 2023. It provided new avenues to assay the fundamental effects of TECPR2 mutation in pathogenesis. The study should, therefore, be of broad interest. A few points need revision, reformulation, or further experiments about the specificity of the identified mechanism.

Specific Comments:

1. The authors demonstrate that GFP-TECPR2 when weakly expressed, tends to localize to endosomal structures, though a substantial amount of TECPR2 appears diffusely distributed. This raises the question of whether GFP-tagged TECPR2 remains functional when overexpressed in cells. Additionally, the RFP-Rab5-transfected cells show almost exclusive colocalization with GFP-TECPR2 in Figure 2E, contrasting with the more moderate colocalization observed with endogenous Rab5 expression in Figure 1. How can these differences be explained? It would be helpful to test alternative tagged versions of TECPR2 or to perform rescue experiments in cells or zebrafish to assess functionality. Alternatively, split fluorescent protein tagging of TECPR2 and other endosomal molecules could be employed. This approach might help avoid potential issues introduced by large fluorophore tags and could provide improved resolution in colocalization studies.
2. Is Rab5 localization affected by the overexpression of mutant TECPR2 in Figure S2c-i? Testing additional early endosome markers could help. Concerning the two endosome populations, does the perinuclear population correspond to degrading lysosomes or MVB, given the rim-like structure resembling late degrading vesicles? Could EEA1 and Rab5 be redirected to the degradation pathway?
3. The author notes LAMP1 and HRS accumulation in SNX1-positive vesicles. Does TECPR2 also play a role in the degradation pathway? Specifically, does TECPR2 knockdown affect lysosome/late-endosome function, potentially contributing to observed phenotypes?
4. The author examined $\alpha 5 \beta 1$ recycling under TECPR2 depletion. How is cargo protein selectivity determined, and what key features guide cargo recognition with TECPR2 involvement?
5. The author attributes TfR and Rab11 relocalization to perinuclear regions upon TECPR2 deletion to cargo sorting/recycling issues. Could early secretory dysfunction in TECPR2 knockdown cells be an alternative explanation?
6. It seems that TECPR2 recruits multiple sorting endosome resident molecules such as SNX17, WASH, and Arp2/3 to early or recycling endosomes. It is still unclear whether the process depends on direct or indirect interactions. The author mentioned that no additive effect was observed upon co-depletion of TECPR2 and SNX17 and concluded that they may function in the same pathway. However, more attention should be paid to whether it is possible to generate accumulated phenotypes together with either of them and/or the other sorting molecules by performing a combination mutant assay.

7. Given β 1-integrin and SNX17's roles in neuronal differentiation and development, analyzing TECPR2's endocytic machinery in neurons or a zebrafish nervous system model could validate the proposed mechanisms.
8. Collapse of peripheral endosomal structures in TECPR2 depletion appeared to be the dominant causation for the major relocalization of Rab5-related proteins. What mechanism might lead to the severe deformation of the endosome structure?
9. In Figure 5o-q, the author concluded that colocalization between Itg β 1 and LAMP1 increased in TECPR2 knockdown cells. However, this conclusion seems unsupported by the representative image. Instead, in my observation, the correlation in control groups is even higher (uncropped cell), which suggests degradation of Itg β 1 was prevented. The quantification should be further validated.
10. SNX17, with its FERM and PX domains, sorts various membrane proteins and interacts with TECPR2. Cargo recycling involves both cargo recognition and tubule budding, suggesting that different cargo may engage distinct SNXs. For instance, SNX1, and Rme8, mediate retrograde transport. Rab7 activity precisely controls the delivery direction of a wide range of cargoes. The author should consider additional genetic/physical interactions beyond the SNX17/WASH axis.
11. In Figure 7, the author indicated that not all the WASH subunits decreased upon depletion of TECPR2 by FF-loaded assay. They also observed decreased WASH1 subunit on Rab5-positive endosomes. Thus, it may imply a novel unknown mechanism that links to Arp2/3 recruitment in addition to the well-known WASH function. It is worth checking the localization of the other WASH subunits in the same context.
12. Cell spreading decreases upon TECPR2 depletion, reasoned by integrin abnormalities. While TECPR2 recruits Arp2/3 on Rab5-positive endosomes, it's unclear if this specifically affects recycling endosome tubulation or overall actin polymerization. WASH co-depletion with TECPR2 might clarify these points.
13. The author speculated that depletion of TECPR2 can lead to defective locomotion in zebrafish. They visualized the pre- and post-synaptic regions. The nicotinic receptor level was reduced. This raises questions about physiological impacts, such as locomotion defects in zebrafish, as α -BTX, known to inhibit excitatory transmission, may exacerbate phenotypes in TECPR2 depletion contexts.

Minor points:

1. Some figure legends lack sufficient detail. For example, abbreviations such as "LE" and "SE" in western blot panels need clarification.
2. Terms like "enlarged" and "perinuclear" endosomes are used variably to describe both EEA1-positive and Itg β 1-positive endosomes. Aligning descriptions would clarify whether they refer to the same vesicle population influenced by TECPR2 depletion or Rab5(Q79L) transfection. Additionally, why did Rab5(Q79L) transfection enlarge early endosomes in Figure S6A?
3. In manuscript line 430, the supplementary S7A doesn't match the context.
4. SNX27 shares structural features with SNX17, but its role in the proposed model is unclear. Does it serve as a functional analog to SNX17 in TECPR2-regulated recycling?
5. Many figure legends are difficult to follow and should be reformulated. Legends should avoid conclusive statements.

Reviewer #2

(Remarks to the Author)

Paul et al.

TECPR2 is a Rab5 effector that regulates endosomal cargo recycling.

This paper has three primary sections:

- I. protein-protein interaction studies between TECPR2 and Rab5.
- II. cell biology (the largest section) involving the interaction of TECPR2 and Rab5.
- III. The role of TECPR2 in zebrafish.

The impact of the work is strong from a basic science perspective and from the perspective that mutations in the gene encoding TECPR2 has clinical implications in humans in they can lead to a rare neurological disease, HSN. Further, mutations in the gene encoding TECPR2 can cause neuroaxonal dystrophy in Spanish water dogs.

Part I. This paper provides strong evidence for the interaction of TECPR2 with Rab5 using both yeast two-hybrid approaches and biochemical approaches. Further the interaction is regulated by the state of Rab5 (GTP- or GDP-bound) and is weakened or absent when the TECPR2 protein is generated from disease-causing mutations.

Part II. Overall, the cell biology experiments are strong and characterize the molecular mechanisms of the TECPR2-Rab5 interaction in the context of endocytic recycling. The volume of work is impressive, and the overall quality is high.

Part III. The transition to the zebrafish work is a bit challenging as it doesn't fully encompass the molecular mechanisms from part II. However, in general, the quality of the zebrafish work is strong. In my mind the first two parts could stand alone as a nice cell biology publication and the zebrafish work (part III) could form the basis of a separate publication. That said, I wouldn't require removal of the zebrafish work for the publication of this article.

Some points needing to be addressed:

Part I. Overall, the binding assays between TECPR2 and Rab5 are quite solid, particularly given both the yeast two-hybrid data and the co-immunoprecipitations.

1. There is some apparent variability in the co-IPs between Fig. 1D and 1G and H in that TECPR2 in 1D binds equally well to Rab5 WT and Q79L, but the same is not true in 1G and 1H. The authors should comment on this. Is this due to the overexpression in 1D? Would they have a similar result immunoprecipitating endogenous TECPR2 with the WT, Q79L, and S34N versions of Rab5? Perhaps the N-terminus affords some binding in the context of the whole protein (compared to the truncated forms used in 1G and 1H)?

2. Ponceau stains of IPs, particularly for 1J and 1M would be helpful to verify equal amounts of antibody in the IPs.

Part II. Major point:

1. The authors state in a couple of sections that previous work using highly over-expressed TECPR2 made subcellular localization challenging and subject to artifacts. They then state that they use only weak to moderate TECPR2-expressing cells, levels close to endogenous. However, I could not find evidence for this claim in the paper. It should be quite simple for them to use their anti-TECPR2 antibody to assess the levels of epitope-tagged wild type and mutant versions of TECPR2 relative to the endogenous found in untransfected cells. They need to provide this to strengthen their results.

Part III. The authors first focus on the characterization of *tecpr2* morphant zebrafish. The use of morpholinos is sound given specificity controls including *tecpr2* mRNA rescues. Granted the results can represent mosaic effects, and while CRISPR-based approaches can be superior (when compensatory mechanisms don't emerge), this reviewer appreciates the morpholino work done in these experiments.

Major Points:

1. The expression data in 8C would be much stronger with sectioning and zooming in on the regions of interest, and if complemented with a sense probe.
2. The effect observed in 8L is a bit subtle. Do the authors suggest that this effect is the root cause of the motor defects observed in the morphants? A more developed discussion of this should be included.
3. The authors plan to continue to use zebrafish to model HSAN and similar diseases based on mutations in TECPR2. However, given the multiple comparisons between WT and mutant TECPR2 in the first two parts of the paper the authors should finish the paper with effects of HSAN mutants in mRNA rescues. Does mutant mRNA rescue the defects and developmental problems? Related, if zebrafish can be used as model, the authors should provide a multiple sequence alignment of TECPR2 (and Rab 5) proteins showing the zebrafish, human, dog and other vertebrate proteins in the alignment. They should indicate the position of the disease mutations and note which are conserved and which are not.

Minor points:

1. Typo: Page 5, line 62: "However, However"
2. This sentence is unclear and needs to be re-written for clarity: Page 7, lines 105-107: "Given that Rab5 is an extensively studied Rab protein, however, association of Rab5 with TECPR2 was not known, we wanted to confirm TECPR2 interaction with Rab5 and the significance of this interaction."
3. In the following sentence you cannot conclude TECPR2 is a Rab 5 effector. However, the rest of the sentence is fine: Page 14, line 265-267: "Taken together, we conclude that TECPR2 is a Rab5 effector and the C-terminal TECPR repeats are essential and sufficient for Rab5 binding and, consequently, for TECPR2 recruitment to early endosomes."
4. In the following sentence you cannot state this fully explains the loss-of-function phenotype. You should say "may explain the loss-of-function phenotype"...Page 14, lines 267-269. "Importantly, impaired Rab5 binding and lack of membrane localization explain the loss-of-function phenotype of the HSAN9-associated TECPR2 R1336W variant."
5. Sup. S4A. Not particularly worrisome, but is the anti-actin blot upside down?
6. TfR recycling should be referenced on Page 17, line 328-329: "As TECPR2-positive endosomes were accessible to endocytosed receptor such as transferrin receptor (TfR) which undergoes constitutive recycling..." [ref]... Further, it would be best to write, "...such as the transferrin ..."

Reviewer #3

(Remarks to the Author)

In this manuscript, Paul and colleagues investigated the role and mechanism of TECPR2 in endosomal trafficking. They demonstrated that TECPR2 is recruited to early endosomes by Rab5 and identified the C-terminal TECPR repeats and specific residues within these regions as essential for the direct interaction between TECPR2 and Rab5. Knockdown of TECPR2 (TECPR2 KD) led to the accumulation of early endosomes in the perinuclear area, increased localization of internalized integrins with late endosomes, and reduced levels of Integrin $\beta 1$. The authors also discovered that SNX17, members of the WASH complex, and SNX27 were present in TECPR2-containing protein complexes. This suggests that TECPR2 may facilitate the fission of recycling endosomes from early endosomes by recruiting SNX17 and the WASH complex, thereby promoting the movement of recycling endosomes toward the plasma membrane. Furthermore, in a zebrafish model, TECPR2 KD resulted in delayed motility and structural changes in the neuromuscular junction. Overall, the data on the interaction between TECPR2 and Rab5, as well as its recruitment to early endosomes, provide compelling evidence of TECPR2's novel role in endosomal trafficking. However, the mechanistic details remain preliminary and require further investigation.

Major Points:

1. The study used siRNA knockdown and transient addback approaches. However, to strengthen the findings presented in Figures 4–7, I recommend using CRISPR-based stable knockdown and stable rescue approaches for validation.

2. In Figures 4A–4K, the EEA1-positive puncta in TECPR2 KD cells appeared larger and fewer, potentially due to increased Rab5 activity. I suggest quantifying the number and size of Rab5-positive puncta in control, TECPR2 KD, and TECPR2-rescued cells. Additionally, Rab5 activity should be assessed. Measurements of recycling and late endosome size and number, as well as Rab7 and Rab11 or Rab10 activity, should also be included to provide a more comprehensive analysis.
3. The method for quantifying the distance of early or recycling endosomes to the nucleus is unclear. Since TECPR2 KD cells exhibit altered size compared to control cells, distances should be normalized to cell size to avoid misleading conclusions.
4. In Figure 5, the cell density in TECPR2 KD cells appears higher than in control cells in Figures 5A–5C. The authors should ensure consistent plate density and assess whether TECPR2 KD impacts proliferation or viability. For Figures 5D–5I, the dynamics of focal adhesions (e.g., using a nocodazole-washout assay to monitor vinculin or paxillin puncta with phalloidin staining) should be analyzed instead of simply quantifying static paxillin puncta. For Figures 5J–5N, dynamic trafficking of integrins should be assessed by labeling live cells with anti-integrin antibodies on ice, followed by incubation at 37°C for various times. Co-staining with Rab5, EEA1, Rab7, Lamp1, or Rab11 antibodies would provide more insight. Cell surface integrin levels should be measured using FACS or confocal imaging. Similarly, in Figures 5O–5V, dynamic colocalization of internalized surface integrin with Lamp1 should be quantified, especially as Lamp1-positive vesicles appear larger in TECPR2 KD cells, suggesting increased Rab7 activity.
5. In Figure 6A, an additional TECPR2 KD lane should be included for better comparison of integrin changes. Clarification is needed on the criteria used to distinguish between the mature and immature forms of integrin. Notably, SNX17 KD resulted in an increased lower band, which needs explanation. Of note, in Figure 5R, single band of integrin was detected. In Figures 6D–6C, colocalization of SNX17 with Rab5 should be quantified. Similarly, quantification is needed for Figures 6E–6F. To monitor integrin trafficking dynamics, live cells should be labeled with anti-integrin antibodies on ice and then incubated at 37°C for various time points. This would provide a clearer picture of integrin trafficking from the cell surface to endosomal compartments.
- In Figures 7E–7F, in TECPR2 KD cells, SNX17 remains strongly associated with Rab5, but the size of SNX17- or Rab5-positive vesicles is larger. This difference should be further analyzed and compared to similar observations in Figures 6B and 6C. For Figures 6H–6I, the effects of TECPR2 KD on the association between WASHC1 and Rab5 should be evaluated in wild-type Rab5-expressing cells.
6. In Figures 7K–7L, TECPR2 KD appears to impair actin polymerization; however, the quality of the phalloidin staining is suboptimal and needs to be improved. The authors should evaluate the colocalization of p16-Arc with Rab5 in control and TECPR2 KD cells to provide further insights. Additionally, the effects of TECPR2 KD or WASH/p16-Arc KD on early endosome tubulation or fission should be assessed to better support the proposed role of TECPR2 in facilitating the transition of early endosomes to recycling endosomes through fission.
7. In Fig. S5F, it is notable that cell surface EGFR levels are lower in TECPR2 KD cells compared to control cells. Since most cell surface EGFR is typically sent to the lysosome for degradation upon ligand binding, this observation suggests that TECPR2 may play a role in regulating endolysosomal degradation of EGFR. To further explore this, the authors should assess whether TECPR2 inhibits Rab7 activity or impedes the transition of early endosomes to late endosomes by upregulating Rab5.

Minor Points:

1. All Western blot and immunoprecipitation data need to be quantified.
2. In Figures 4A and 4B, the cell edges should be shown in all images to provide better context.
3. Why TECPR2 KD failed to affect transferrin recycling as shown in Fig. S6B?
4. Show data of large and aberrant clustering of Rab5-positive vesicles in high TECPR2 expression cells (line 275).
5. Several typos and grammatical errors have been identified.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Having reviewed the revised manuscript, I find that the authors have effectively addressed my previous comments and concerns. The revisions reflect a comprehensive response to the feedback provided, enhancing both the clarity of the presentation and the strength of the conclusions. Through their detailed exploration of TECPR2's role as a Rab5 effector in endocytic recycling, along with the analysis of HSAN-associated variants and validation studies in zebrafish to establish disease relevance, the authors have produced a well-rounded study. I recommend accepting this manuscript for publication.

Reviewer #2

(Remarks to the Author)

The revised manuscript represents significant and substantial improvements. All of the points of concern raised by this reviewer during the initial review have been adequately addressed by the authors.

Reviewer #3

(Remarks to the Author)

The revised manuscript is much improved; however, I have one remaining concern regarding Figure 8J. The data show that

TECPR2 knockdown significantly reduces CapZ β puncta. Since CapZ normally functions to prevent actin filament polymerization, a decrease in CapZ puncta would be expected to enhance actin polymerization, rather than reduce it. This interpretation appears to contrast with the results in Figures 8E and 8J, where actin branching is inhibited in TECPR2 knockdown cells. To clarify this apparent discrepancy, the authors should examine whether TECPR2 knockdown alters the expression levels of relevant proteins, particularly CapZ and p16-Arc, and quantify overall actin filament density in the knockdown cells.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

No more comments

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Response to Reviewers Comments:

We thank the reviewers for their valuable comments and suggestions pertaining to our manuscript. Below we provide a point-by-point response (shown in blue) to how we have addressed the concerns raised by the reviewers (shown in black).

Reviewer #1 (Remarks to the Author)

Mutations in the TECPR2 gene are a leading cause of autosomal recessive complex spastic paraplegia (HSP) or HSAN with autonomic dysfunction, respiratory failure, and intellectual disability. TECPR2 has been implicated in multiple endocytic transport pathways, including early secretion and autophagic degradation, though its precise role within subcellular compartments remains unclear. The study by Paul et al. provides a comprehensive analysis of the mechanisms by which TECPR2 regulates endosomal sorting, shedding light on the dynamic organization of the cell adhesion protein integrin under both physiological and pathological conditions.

This well-executed study presents evidence that TECPR2 functions in a Rab5-dependent manner, suggesting that TECPR2 may act as a Rab5 effector to facilitate the delivery of various intracellular cargo proteins. The authors propose a valuable model in which TECPR2 recruits SNX17 to selectively sort membrane proteins, a mechanism that may have broad implications, particularly for the dynamic regulation of neuronal receptors involved in neurodevelopmental and neurodegenerative diseases, as supported by previous studies and the authors' data. Intriguingly, the author identified the TECPR2 subcellular functional route as different from the previous work by Nalbach et al. 2023. It provided new avenues to assay the fundamental effects of TECPR2 mutation in pathogenesis. The study should, therefore, be of broad interest. A few points need revision, reformulation, or further experiments about the specificity of the identified mechanism.

Response: We thank the reviewer for appreciating that this study adds new knowledge about TECPR2 subcellular role and that it is a step towards explaining the underlying mechanism disrupted in the HSAN9 neurological disorder.

Specific Comments:

1. The authors demonstrate that GFP-TECPR2 when weakly expressed, tends to localize to endosomal structures, though a substantial amount of TECPR2 appears diffusely distributed. This raises the question of whether GFP-tagged TECPR2 remains functional when overexpressed in cells. Additionally, the RFP-Rab5-transfected cells show almost exclusive colocalization with GFP-TECPR2 in Figure 2E, contrasting with the more moderate colocalization observed with endogenous Rab5 expression in Figure 1. How can these differences be explained? It would be helpful to test alternative tagged versions of TECPR2 or to perform rescue experiments in cells or zebrafish to assess functionality. Alternatively, split fluorescent protein tagging of TECPR2 and other endosomal molecules could be employed. This approach might help avoid potential issues introduced by large fluorophore tags and could provide improved resolution in colocalization studies.

Response: We thank the reviewer for this important comment. Our data shows that TECPR2 is a Rab5 effector and Rab5 is necessary and sufficient to recruit TECPR2 on membranes. Indeed, both the GUV assay and the Mito-tagging approach shown in Fig. 2E-

H demonstrate the sufficiency of Rab5 to target TECPR2 on membranes. Purification of ferrofluid-containing vesicles from cell homogenates showed that endogenous TECPR2 was also enriched in early endosome fractions as compared to late endosomes and lysosomes (**Fig. 2C**). GFP-TECPR2 punctae are positive for Rab5 and EEA1, which supports that GFP-tagging of TECPR2 does not affect its localization (**Fig. 2A**). However, as Rab5 expression is limiting under these conditions, GFP-TECPR2 is also present as a cytosolic pool. The data in **Fig. 2D** demonstrates that overexpression of Rab5 (WT) or (Q79L) promotes recruitment of GFP-TECPR2 to early endosomes. Nevertheless, to address this valid concern, we have rescued the effect of TECPR2 depletion on early endosomes using an siRNA-resistant GFP-TECPR2 construct. As shown in **Fig. 4A-D**, GFP-TECPR2 was able to partially rescue the size of early endosomes marked by EEA1 and Rab5 in TECPR2-depleted cells. Additionally, as mentioned by the reviewer, we expressed FLAG-tagged TECPR2 with and without co-expression of Rab5. As shown below in **Fig. A1**, FLAG-tagged TECPR2 is partly cytosolic when expressed by itself, but there is a dramatic recruitment to membranes upon co-expression of Rab5.

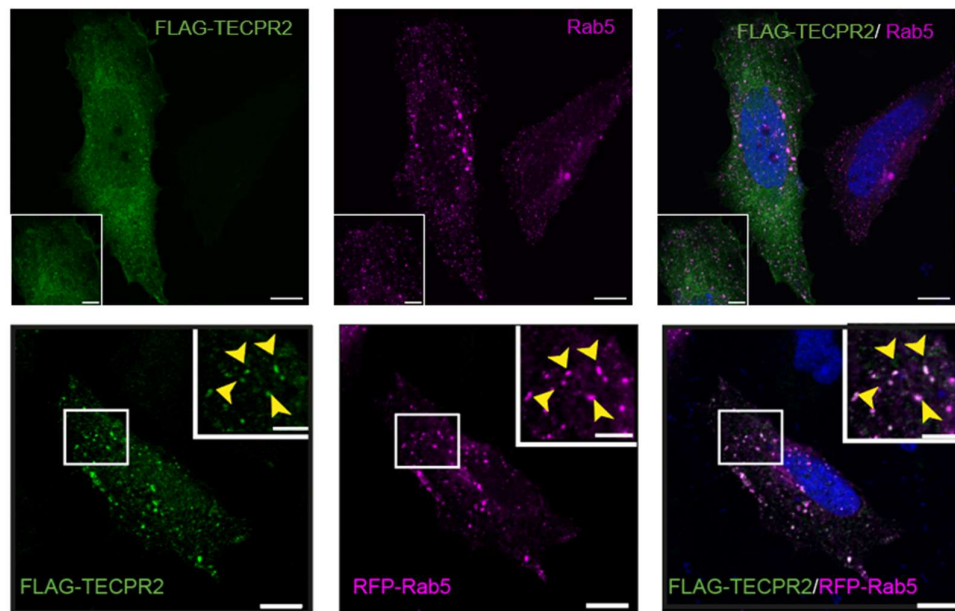


Figure A1: Rab5 recruits TECPR2 on early endosomes. Representative confocal images of HeLa cells expressing FLAG-TECPR2 or FLAG-TECPR2 with RFP-Rab5. The cells were fixed and immunostained using anti-Rab5 and anti-FLAG antibodies. Yellow arrowheads show the colocalized pixels for both the channels. Scale Bar: 10 μ m (main); 5 μ m (inset).

2. Is Rab5 localization affected by the overexpression of mutant TECPR2 in Figure S2c-i? Testing additional early endosome markers could help.

Response: To address reviewer's comment, we analyzed Rab5 distribution in cells expressing the HSN variant of TECPR2 namely, TECPR2 (D1000Y) and (R1336W), because these two variants have a better expression than TECPR2 (W1140G). Furthermore, we also assessed Rab5 distribution in TECPR2 (E1299A; Rab5 binding-defective mutant)-expressing cells. As shown in **Fig. A2**, TECPR2 (WT) punctae were colocalized with endogenous Rab5, whereas HSN9 variants and the TECPR2 (E1299A) mutant remained

cytosolic and did not show colocalization with Rab5. In a few TECPR2 (D1000Y) or TECPR2 (R1336W) transfected cells, we observed that Rab5 showed a perinuclear accumulation, but there was significant heterogeneity of Rab5 distribution between similarly expressing cells. We did not find a noticeable or consistent change in the endogenous Rab5 endosome distribution in cells expressing either FLAG-TECPR2 (D1000Y), FLAG-TECPR2 (R1336W) or FLAG-TECPR2 (E1299A) mutants. Thus, it appears that the three TECPR2 variants/mutants analyzed here are loss-of-function mutants and do not behave as dominant-negative mutants. Previous literature has also reported that these mutations occur in a *homozygous* manner in HSAN9 patients, and thus it is unlikely that the mutations analyzed here show a dominant-negative phenotype and disrupt the function of endogenous protein (Oz-Levi D et al, Am J Hum Genet. 2012, PMID: 23176824; Neuser S et al, Hum Mutat. 2021, PMID: 33847017).

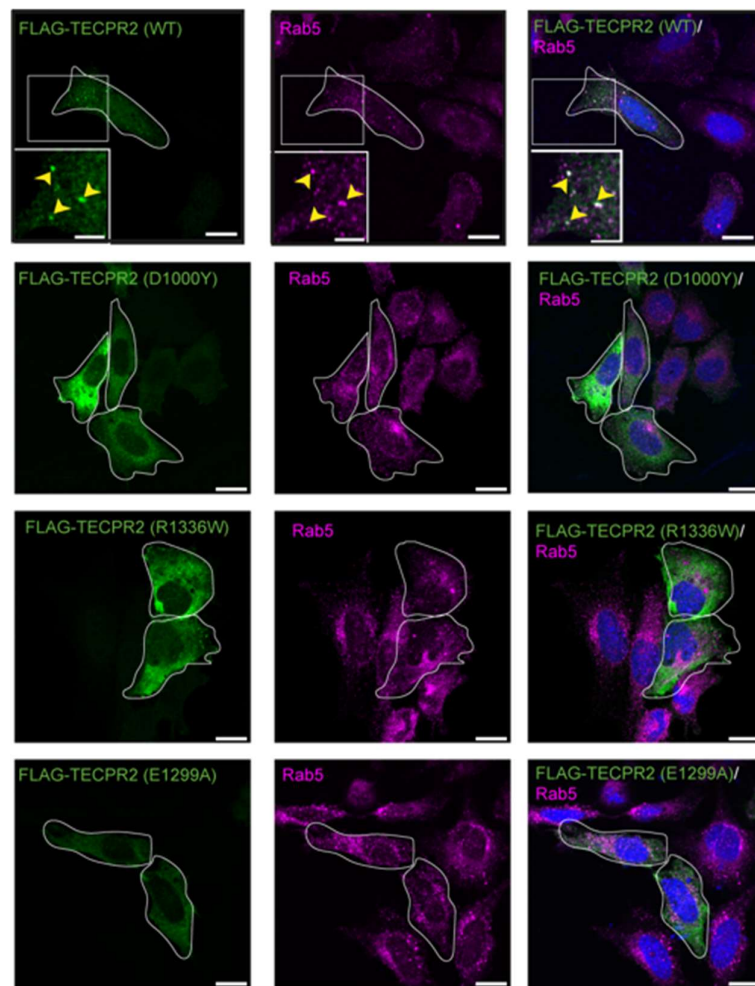


Figure A2: Rab5 distribution is not altered upon expression of TECPR2 (D1000Y), (R1336W) or (E1299A) mutants. Representative confocal images of HeLa cells expressing FLAG-TECPR2 (WT) or the indicated mutant and immunostained with anti-Rab5 antibodies. Yellow arrowheads show the colocalized pixels for both the channels. Scale Bar: 10 μ m (main); 5 μ m (inset).

Concerning the two endosome populations, does the perinuclear population correspond to degrading lysosomes or MVB, given the rim-like structure resembling late degrading vesicles? Could EEA1 and Rab5 be redirected to the degradation pathway?

Response: We thank the reviewer for this insightful comment. As shown below in **Fig. A3**, we did not observe colocalization of the enlarged and perinuclear EEA1- and Rab5-positive vesicles with acidic compartments (labeled by LysoTracker) in TECPR2-depleted cells, suggesting that early endosomal proteins, EEA1 and Rab5, are not lysosomally degraded. In line with this, EEA1 levels (as shown in **Fig. 7H-I**), were not altered upon TECPR2 depletion. However, Rab5 levels were significantly reduced upon TECPR2 knockdown (**Fig. 7H-I** and **Supplementary Fig. S7D**). Preliminary observations (as shown below in **Fig. A3**) indicate that inhibition of the proteasomal pathway by MG132 treatment partially restored Rab5 levels in TECPR2-depleted cells, suggesting that the cytosolic pool of Rab5 is subjected to proteasomal degradation. Previous studies have found Rab5 is ubiquitinated, and in particular, ubiquitination of the K134 residue might be relevant for its proteasomal degradation (Chen T, PLoS One. 2014, PMID: 24465676; Shin D et al., elife. 2017, PMID: 28968219). The detailed mechanism of Rab5 degradation upon TECPR2 depletion needs exploration in future studies.

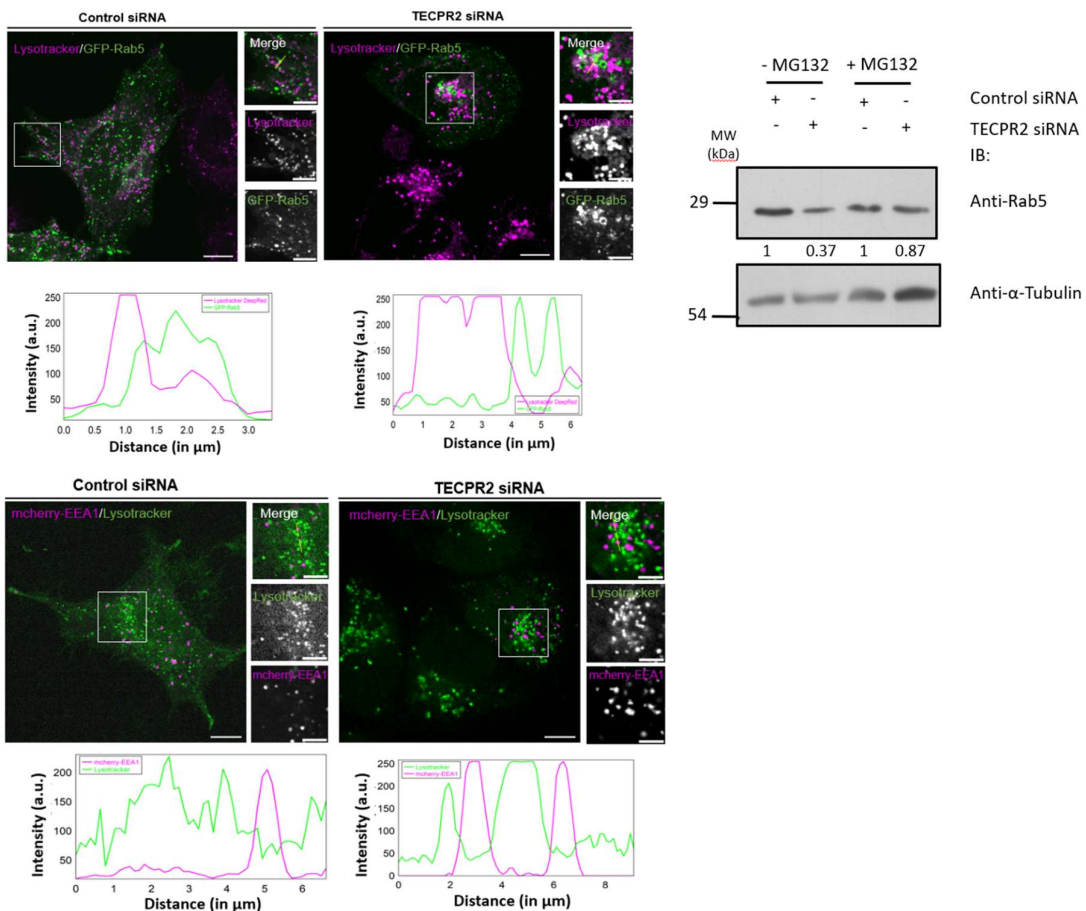


Figure A3: Enlarged Rab5 and EEA1-positive vesicles in TECPR2 depletion are not acidic compartments. *Left panel:* Representative single frame snapshot of HeLa cells treated with control or TECPR2 siRNA and expressing GFP-Rab5 or mcherry-EEA1 (as labeled) were incubated with LysoTracker Red DND-99. The line profiles indicate fluorescence intensity along the white lines for both channels. Bars: 10 μ m (main); 5 μ m (inset). *Right panel:* HeLa cell lysates transfected with control or TECPR2 siRNA, and treated with or without MG132 (2 μ M; 12 hr) were immunoblotted with anti-Rab5 and anti- α -tubulin antibodies. The values represent the densitometric analysis of Rab5 band signal intensity normalized to α -tubulin signal.

3. The author notes LAMP1 and HRS accumulation in SNX1-positive vesicles. Does TECPR2 also play a role in the degradation pathway? Specifically, does TECPR2 knockdown affect lysosome/late-endosome function, potentially contributing to observed phenotypes?

Response: We thank the reviewer for an insightful question. TECPR2 knockdown cells show a dramatically enlarged LAMP1-positive compartment. Both the previous and current versions of the manuscript show this data (**Supplementary Fig. S4M**). We have also assessed lysosome function in TECPR2-depleted cells by analyzing the extent of degradation of an endocytic probe, BODIPY-BSA, which becomes fluorescent upon lysosomal degradation. Our preliminary analysis depicts that TECPR2-depleted cells showed reduced mean fluorescence intensity of BODIPY-BSA, indicating that cargo delivery to lysosomes or lysosome composition is altered in these cells (as shown below in response **Fig. A4**). As lysosome biogenesis involves cargo trafficking of lysosomal proteins via early endosomes, we are currently investigating the potential role of TECPR2 on lysosome biogenesis and late endosome-lysosome fusion. (Luzio JP et al., Cold Spring Harb Perspect Biol. 2014, PMID: 25183830).

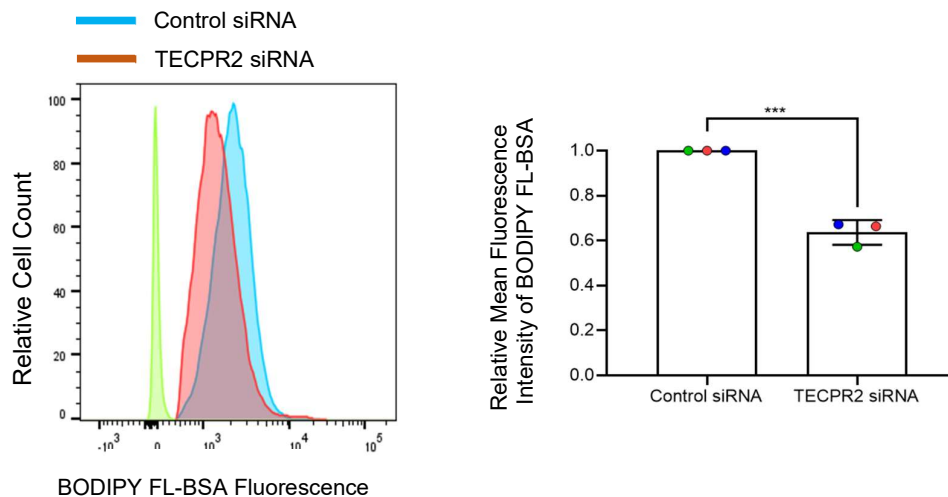


Figure A4: TECPR2 depletion impairs degradation of endocytic cargo in lysosomes. Representative histogram showing mean fluorescence intensity (MFI) of de-quenched BODIPY FL-BSA after 6 hr of incubation in control and TECPR2 siRNA-treated HeLa cells, as analyzed by flow cytometry. The bar graph on the right, represents the relative MFI signal for de-quenched BODIPY FL-BSA after 6hr of incubation in HeLa cells treated with control- or TECPR2 siRNA calculated from three independent experiments (*** $p = 0.0003$; two-tailed Student's t -test).

4. The author examined $\alpha 5\beta 1$ recycling under TECPR2 depletion. How is cargo protein selectivity determined, and what key features guide cargo recognition with TECPR2 involvement?

Response: Cargo receptors, including $\beta 1$ integrins, are sorted based on recognition of the specific signal motifs, such as the NPxY or YXX Φ motifs on the cytoplasmic tail of the receptors. As mentioned in the manuscript text, the Itg $\beta 1$ and Itg $\beta 5$ cytoplasmic tail regions contain the NPxY sorting motif, which is recognized by sorting adaptor SNX17 (Steinberg F, et al., J Cell Biol. 2012, PMID: 22492727; McNally KE et al., Nat Cell Biol. 2017, PMID: 28892079). Depletion of SNX17 leads to a defect in retrieval of $\beta 1$ integrin from the lysosomal degradation, a phenotype similar to TECPR2 depletion (Steinberg et al., J Cell Biol. 2012). In this study, we found that TECPR2 directly interacts with SNX17, and this interaction regulates SNX17 recruitment on endocytosed cargo (Itg $\beta 1$)-containing endosomes (**Figs. 6B-F, Fig. 7C and Supplementary Videos S8 and S10**). In the revised manuscript, we also show that TECPR2 interacts with SNX27, which, like SNX17, is an SNX-FERM family member. TECPR2-depleted cells showed reduced localization of SNX27 on early endosomes (**Supplementary Fig. S7E-I**). In summary, we propose that TECPR2 regulates recycling of cargoes recognized by SNX17 and SNX27 sorting adaptors. Importantly, both SNX17 and SNX27 also interact with the WASH complex, which generates branched actin-enriched subdomains on early endosomes, leading to cargo sequestration and formation of recycling transport carriers (Cullen PJ, Steinberg F. Nat Rev Mol Cell Biol. 2018, PMID: 30194414). TECPR2 promotes WASH recruitment to Rab5⁺ endosomes, and therefore, WASH-mediated actin generation and localization on the early endosomes is reduced upon TECPR2 depletion (**Fig. 8A-K and Supplementary Videos S11 and S12**). Thus, TECPR2 may regulate trafficking of cargoes that depend on a sequence-based slow recycling pathway, which involves the formation of a branched actin network on early endosomes. In contrast, recycling of cargo such as transferrin receptor (TfR) that traffics via an actin-independent bulk recycling pathway proceeds efficiently in TECPR2-depleted cells (**Supplementary Fig. S5B**).

5. The author attributes TfR and Rab11 relocalization to perinuclear regions upon TECPR2 deletion to cargo sorting/recycling issues. Could early secretory dysfunction in TECPR2 knockdown cells be an alternative explanation?

Response: We appreciate the reviewer's alternative explanation to explain the perinuclear TfR distribution in TECPR2-depleted cells. However, in the previous version of the manuscript, we had shown that the Alexa568-conjugated transferrin-TfR complex internalized from the cell surface is accumulated in a perinuclear compartment (**Supplementary Fig. S5C**). As the Alexa568-conjugated transferrin would label only the

surface TfR pool, this result suggests that the perinuclear pool in TECPR2-depleted cells is the endocytosed TfR and not the secretory pool. Furthermore, the perinuclear collapsed TfR endosomes were colocalized with the recycling endosomal marker Rab11 in TECPR2-depleted cells (**Supplementary Fig. S4L**). To further corroborate this data, we also performed FLAG pulldown from HeLa cell homogenates stably expressing 3x-FLAG-TfR and observed that Rab11 levels remain unchanged upon TECPR2 depletion (as shown below in **Fig. A5**). Thus, the data suggests that TfR is present in the Rab11⁺ recycling endosomes that are repositioned to the perinuclear region upon TECPR2 depletion, a phenotype also observed in WASH knockout cells (Gomez TS et al., Mol Biol Cell. 2012, PMID: 22718907).

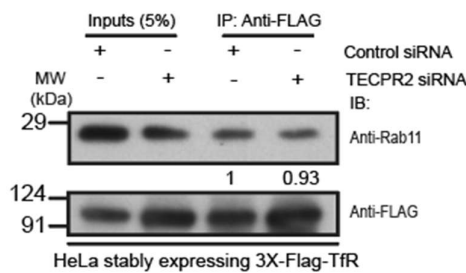


Figure A5: Rab11 remains associated with TfR membrane fractions upon TECPR2 depletion. Lysates of HeLa cells stably expressing 3X-FLAG-TfR were treated with control and TECPR2 siRNA and were immunoprecipitated with anti-FLAG antibody-conjugated beads followed by immunoblotting with the indicated antibodies. The values represent the densitometric analysis of Rab11 band intensity normalized to the input and direct IP of 3X-FLAG-TfR.

- It seems that TECPR2 recruits multiple sorting endosome resident molecules such as SNX17, WASH, and Arp2/3 to early or recycling endosomes. It is still unclear whether the progress depends on direct or indirect interactions. The author mentioned that no additive effect was observed upon co-depletion of TECPR2 and SNX17 and concluded that they may function in the same pathway. However, more attention should be paid to whether it is possible to generate accumulated phenotypes together with either of them and/or the other sorting molecules by performing a combination mutant assay.

Response: The multiple sorting endosome resident molecules analyzed in our study, including SNX17, WASH, and Arp2/3, participate in a sequential manner to mediate cargo retrieval from endosomes and are not functioning independently in separate cargo sorting pathways. SNX17 first binds to integrin sorting motifs, and this binding stabilizes the integrin in a specific membrane subdomain, thereby preventing ubiquitination and lysosomal degradation (Steinberg F, et al., J Cell Biol. 2012, PMID: 22492727). Cargo-bound SNX17 then interacts with the retriever complex (McNally KE, et al., Nat Cell Biol. 2017, PMID: 28892079). The retriever complex recruits the WASH complex to the endosomal membrane that promotes Arp2/3-dependent actin polymerization, which facilitates membrane remodelling and formation of recycling tubules for efficient recycling of integrin to the plasma membrane (McNally KE, et al., Nat Cell Biol. 2017, PMID: 28892079). Thus, the sequential actions of SNX17, the retriever, and the WASH complex result in cargo recognition, the formation of recycling tubules, cargo accumulation in tubular membranes, and membrane constriction for fission. We have shown that TECPR2 directly interacts with, and regulates, SNX17 association with Rab5⁺ early endosomes (**Fig.7A-I and Supplementary Video S10**). We also found that TECPR2 interacts with subunits of the WASH complex, and there was a strong decrease in WASH complex association and actin density on early endosomes upon TECPR2 knockdown (**Fig. 8A-K and Supplementary Videos S11 and S12**). Our study establishes a crucial link between

the master regulator Rab on early endosomes, Rab5, and the cargo recognition and retrieval machinery for receptors, such as integrins. Rab5 effector TECPR2 mediates the recruitment of the retrieval machinery on early endosomes. Nevertheless, it will be relevant to test whether TECPR2 directly interacts with the WASH complex or whether this interaction is dependent on SNX17. This is a new line of investigation that will require significant effort and time and is beyond the scope of current work.

7. Given β 1-integrin and SNX17's roles in neuronal differentiation and development, analyzing TECPR2's endocytic machinery in neurons or a zebrafish nervous system model could validate the proposed mechanisms.

Response: We thank the reviewer for a thoughtful and constructive suggestion. However, we would like to clarify that these experiments are beyond our expertise and the reagents and tools required for these studies are currently not present in our laboratory. In the future, we may collaborate with research groups having expertise in neuronal cell biology for future studies on TECPR2.

8. Collapse of peripheral endosomal structures in TECPR2 depletion appeared to be the dominant causation for the major relocalization of Rab5-related proteins. What mechanism might lead to the severe deformation of the endosome structure?

Response: We thank the reviewer for an insightful question. The perinuclear positioning of early and recycling endosomes was a striking phenotype observed in TECPR2-depleted cells (**Fig. 4A-E, Supplementary Fig. S5C and Fig. 8C**). Intriguingly, a similar phenotype of early endosome perinuclear positioning is also observed in WASH knockout cells as well as in cells knockdown of the early endosomal tether CORVET. Both of these complexes also regulate integrin recycling (Gomez TS et al., Mol Biol Cell. 2012, PMID: 22718907; Perini ED et al., Traffic. 2014, PMID: 2526629; Jonker CTH et al., Nat Commun. 2018, PMID: 29476049). In the *discussion* section, we have elaborated on the role of TECPR2 in mediating ER-early endosome contacts. In case TECPR2 depletion results in reduced contacts between ER and early endosomes, this may lead to a collapse of the endosomes in the perinuclear region, as contacts with ER promote organelle motility on microtubule tracks (Wang X, et al., Comput Struct Biotechnol J. 2023, PMID: 36860342). In addition, a recent preprint has shown that several Rab partner proteins, including Rab5 effector Rabaptin-5, can undergo liquid-liquid phase separation, allowing them to form condensates on membranes. The formation of these condensates serves to limit the Rab protein to specific membrane subdomains and ensure their function in membrane trafficking steps, such as in vesicle tethering and fusion (Jimenez et al., 2024 Bioarxiv). Intriguingly, TECPR2 contains a disordered region of ~400 a.a. in length, the function of which remains unknown. It is possible that TECPR2 also undergoes liquid-liquid phase separation, and TECPR2 condensates limit EEA1-based tethering and promote assembly of cargo retrieval machinery, including the SNX17-WASH-Arp2/3 complex. In the absence of TECPR2 condensates, cargo retrieval machinery is not recruited, and EEA1-based tethering could expand over the entire endosome, causing a faster maturation of early endosomes to MVBs or ILV-containing endosomes, leading to their perinuclear collapse. As part of future studies, we will investigate both of these hypotheses.

9. In Figure 5o-q, the author concluded that colocalization between Itg β 1 and LAMP1 increased in TECPR2 knockdown cells. However, this conclusion seems unsupported by the representative image. Instead, in my observation, the correlation in control groups is

even higher (uncropped cell), which suggests degradation of Itgβ1 was prevented. The quantification should be further validated.

Response: We appreciate the reviewer bringing this to our attention. To validate whether Itgβ1 is delivered to active and degradative lysosomes upon TECPR2 depletion, we performed live-cell imaging and analyzed colocalization of endocytosed Itgβ1 with SiR-Lysosome (that marks compartments with cathepsin D hydrolase activity). As shown in the new **Supplementary Fig. S6D-E** and **Supplementary Video S7**, there was an increased colocalization of endocytosed Itgβ1 and SiR-Lysosome in TECPR2-depleted cells. Taking this data along with other observations such as increased colocalization of Itgα5 and LAMP1 (**Fig. 5K-L**) and observed degradation of Itgβ1 in TECPR2-depleted cells (**Fig. 5I-J**), we conclude that TECPR2 knockdown impairs the retrieval of endocytosed Itgα5β1 from lysosomal degradation.

10. SNX17, with its FERM and PX domains, sorts various membrane proteins and interacts with TECPR2. Cargo recycling involves both cargo recognition and tubule budding, suggesting that different cargo may engage distinct SNXs. For instance, SNX1, and Rme8, mediate retrograde transport. Rab7 activity precisely controls the delivery direction of a wide range of cargoes. The author should consider additional genetic/physical interactions beyond the SNX17/WASH axis.

Response: We agree with the reviewer that there are other sorting adaptors that we have not tested that regulate cargo trafficking from early endosomes. This study aimed to elucidate the subcellular function of TECPR2, which is not understood till date. TECPR2 localization was not characterized, nor was it understood how it is recruited to any intracellular membranes. We identified that TECPR2 is a Rab5 effector that localizes to early endosomes. The data led us to the conclusion that TECPR2 interacts with SNX17 (integrin sorting adaptor) and its associated protein complexes and regulates their recruitment to Rab5⁺ early endosomes. In the revised manuscript, we also show that TECPR2 interacts with SNX27, which, like SNX17, is an SNX-FERM family member. TECPR2-depleted cells show reduced localization of SNX27 on early endosomes and also lead to decreased cell surface localization of copper transporter ATP7A, an SNX27 cargo (**Supplementary Fig. S7E-K**). Our study establishes a crucial link between the master regulator Rab protein, Rab5, and the cargo recognition and retrieval machinery on early endosomes. We predict that TECPR2 regulates trafficking of cargoes that rely on a sequence-based, slow recycling pathway that involves formation of a branched actin network on early endosomes. As mentioned in the discussion section, it will be relevant to test whether TECPR2 directly interacts with the WASH complex or whether this interaction is dependent on SNX17. As part of future studies, we plan to analyze the role of TECPR2 in cargo trafficking from early endosomes to the TGN, which is also regulated by the WASH complex. This is a new line of investigation that will require significant effort and time and is beyond the scope of current work.

11. In Figure 7, the author indicated that not all the WASH subunits decreased upon depletion of TECPR2 by FF-loaded assay. They also observed decreased WASH1 subunit on Rab5-positive endosomes. Thus, it may imply a novel unknown mechanism that links to Arp2/3 recruitment in addition to the well-known WASH function. It is worth checking the localization of the other WASH subunits in the same context.

Response: We thank the reviewer for this important suggestion. WASH is a pentameric complex consisting of WASHC1, WASHC2 (FAM21), WASHC3 (coiled-coil domain-containing protein 53 or CCDC53), WASHC4 (SWIP) and WASHC5 (Strumpellin). We have now included the analysis of four of the five WASH subunits, for which we were able to standardize antibodies for Western blotting and immunofluorescence applications (refer to **Fig. 8A-D**). We observed significantly reduced levels of Strumpellin and WASHC1 in early endosomal membrane fractions whereas FAM21C and SWIP appeared to be minimally affected in TECPR2-depleted homogenates as compared to control (**Fig. 8A-B**). In accordance with this, we found reduced punctate localization of Strumpellin and WASHC1, as well as reduced colocalization of WASHC1 with Rab5 in TECPR2-depleted cells (**Fig. 8C-F** and **Supplementary Video S11**). As observed with the ferrofluid-loaded early endosomal fractions, FAM21C continued to be membrane-bound in TECPR2-depleted cells (**Fig. 8C**). The finding that FAM21C is independent of TECPR2 for its membrane localization is consistent with previous data showing that Vps35, a core subunit of retromer, recruits FAM21C on membranes (Harbour ME et al., *Biochem J.* 2012, PMID: 22070227). FAM21C also interacts with SWIP, which in turn directly binds to phosphoinositides PI(3,5)P₂ and PI3P present on early endosomes and this interaction is also required for FAM21C membrane localization (Dostál V et al., *Traffic.* 2023, PMID: 36995008). Indeed, SWIP levels in early endosome fractions were only modestly reduced upon TECPR2 depletion (**Fig. 8A-B**). As part of future studies, we will investigate whether TECPR2 directly interacts with the WASH complex or whether this interaction is dependent on SNX17. This is a new line of investigation that will require significant effort and time and is beyond the scope of current work.

12. Cell spreading decreases upon TECPR2 depletion, reasoned by integrin abnormalities. While TECPR2 recruits Arp2/3 on Rab5-positive endosomes, it's unclear if this specifically affects recycling endosome tubulation or overall actin polymerization. WASH co-depletion with TECPR2 might clarify these points.

Response: The cell spreading defects in TECPR2-depleted cells could be explained in part by the impaired assembly of focal adhesions, which in turn was due to a reduced retrieval of endocytosed integrin from lysosomal degradation (**Fig. 5**). We found that TECPR2 is a Rab5 effector that localizes to early endosomes and regulates integrin sorting adaptor SNX17 association with Rab5⁺ early endosomes (**Fig. 6** and **Fig. 7**). TECPR2 also interacts with and recruits the WASH complex on early endosomes, which in turn regulates Arp2/3-dependent actin polymerization on early endosomes (**Fig. 8**). Based on the data that TECPR2 is an early endosomal protein, it appears less likely that actin polymerization in the cytosol or on other membranes would be affected by TECPR2 expression, although we cannot rule it out. A defect in actin polymerization would impair receptor endocytosis of TfR or integrins, which was not observed upon TECPR2 depletion. Reduced endocytosis would result in an increase in the surface level of these receptors upon TECPR2 depletion. Instead, we have observed that surface levels of TfR and integrins is reduced upon TECPR2 depletion (**Supplementary Fig. S5A** and **S6C**). The images in **Fig. 5C-D** and **Fig. 8G** show that cortical actin immunostaining is similar in both control and TECPR2-depleted cells. However, endosomal actin (as observed by Arp2/3 subunit p16-Arc punctae quantification, p16-Arc and Rab5 colocalization and actin capping factor CapZ β punctae quantification) was strikingly reduced upon TECPR2 depletion (**Fig. 8G-I**). Considering the evidence of TECPR2 localization on early endosomes and its interaction with endosomal nucleation-promoting factor WASH, it is unlikely that TECPR2 depletion leads to changes in overall

actin polymerization. Co-depletion of WASH and TECPR2 is also unlikely to resolve this question, as WASH might be involved in other TECPR2-independent processes as well.

13. The author speculated that depletion of TECPR2 can lead to defective locomotion in zebrafish. They visualized the pre- and post-synaptic regions. The nicotinic receptor level was reduced. This raises questions about physiological impacts, such as locomotion defects in zebrafish, as α -BTX, known to inhibit excitatory transmission, may exacerbate phenotypes in TECPR2 depletion contexts.

Response: We want to clarify that the control and *Tecpr2*-morphants were first fixed with paraformaldehyde, permeabilized and then stained with Alexa Fluor-488 conjugated α -bungarotoxin (BTX) to detect the postsynaptic acetylcholine receptor (AChR). The α -BTX probe was not employed on live embryos and thus would not have any effect on neurotransmission in control or *Tecpr2*-morphants. We want to emphasize that in the revised manuscript we have now performed experiments using anti-SV2 antibodies and α -BTX on sectioned embryos, and the new data clearly shows that in *tecpr2* (201+202) morphants, the neuromuscular synaptic morphology was altered, and they appeared mostly punctate and smaller compared to the WT control neuromuscular junction (NMJ). The change was also reflected in the significantly reduced "percentage area" of SV2 and acetylcholine receptor signal in *tecpr2* (201+202) morphants as compared to WT control (**Fig. 9G-I**).

Minor points:

1. Some figure legends lack sufficient detail. For example, abbreviations such as "LE" and "SE" in western blot panels need clarification.

Response: We thank the reviewer for bringing this to our attention. We have now clarified these abbreviations in the figure legends.

2. Terms like "enlarged" and "perinuclear" endosomes are used variably to describe both EEA1-positive and Itg β 1-positive endosomes. Aligning descriptions would clarify whether they refer to the same vesicle population influenced by TECPR2 depletion or Rab5(Q79L) transfection. Additionally, why did Rab5(Q79L) transfection enlarge early endosomes in Figure S6A?

Response: We thank the reviewer for bringing this to our attention. We have now removed the confusing word (enlarged in this case) and provided a better description of why the active mutant of Rab5 was employed in these experiments (line # 452-457). Rab5 (Q79L) is a constitutively active mutant of Rab5, which implies that it cannot undergo hydrolysis from the GTP to GDP state. GTP-bound Rab5 recruits EEA1, a tethering factor that promotes homotypic fusion of early endosomes, therefore, Rab5 (Q79L) expressing cells show ring-like enlarged early endosomes (Bucci C, et al., Cell. 1992, PMID: 1516130). The increase in endosome size enables better visualization of membrane subdomains even at the resolution of confocal microscopy. Indeed, using the Rab5 (Q79L) mutant, we observed that TECPR2 was colocalized with SNX17, WASH subunits (Strumpellin and FAM21), and the Arp2/3 complex on specific membrane subdomains, supporting that TECPR2 recruits the cargo retrieval machinery (**Supplementary Fig. S7A and Supplementary Fig. S8**).

3. In manuscript line 430, the supplementary S7A doesn't match the context.

Response: We thank the reviewer for bringing this to our attention. We have now corrected the figure numbering in the text (now **Supplementary Fig. S9C**).

4. SNX27 shares structural features with SNX17, but its role in the proposed model is unclear. Does it serve as a functional analog to SNX17 in TECPR2-regulated recycling?

Response: The reviewer is correct that SNX27 is a functional analog of SNX17 for a different set of cargo receptors. We have now included data demonstrating the interaction between TECPR2 and SNX27, as well as the significance of their binding for the localization of SNX27 on early endosomes (**Supplementary Fig. S7E-I**). As now stated in the manuscript text line #504 “SNX27, a member of the SNX-FERM family (as SNX17), was previously reported to be a potential binding partner for TECPR2 (Nalbach et al., Nat Commun 2023, PMID: 36797266; Stadel et al., Mol. Cell 2015, PMID: 26431026). We validated this interaction by GST pulldown assay, where endogenous TECPR2 showed binding to GST-tagged SNX27 but not GST (used as a control) (**Supplementary Fig. S7E**). Furthermore, endogenous SNX27 was co-immunoprecipitated with epitope-tagged TECPR2 (WT) but not the HSN9-associated Rab5 binding-defective form of TECPR2 (R1336W), suggesting that membrane localization of TECPR2 is required for interaction with SNX27 (**Fig. 7D**). Consistent with their binding, endogenous SNX27 was colocalized with GFP-TECPR2 on early endosomes (**Supplementary Fig. S7F-G**). Importantly, SNX27 was reduced in the FF-loaded early endosome fractions upon TECPR2 depletion, suggesting that TECPR2 acts as a linker for both SNX17 and SNX27 to mediate their association with Rab5⁺ early endosomes (**Supplementary Fig. S7H-I**). SNX27 is a sorting adaptor for cargoes, including copper transporter ATP7A and glucose transporter GLUT1, that contain a PDZ-binding motif, which is recognized by the N-terminal PDZ domain of SNX27. Depletion or knockout of SNX27 result in reduced surface levels of these cargo proteins and eventually their lysosomal degradation (Steinberg et al., Nat Cell Biol 2013, PMID: 23563491). In accordance with the decreased association of SNX27 with early endosomes, we found that surface levels of ATP7A, an SNX27 cargo, were also reduced upon TECPR2 depletion (**Supplementary Fig. S7J-K**).

5. Many figure legends are difficult to follow and should be reformulated. Legends should avoid conclusive statements.

Response: We thank the reviewer for bringing this to our attention. We have now corrected the figure legends and have removed the concluding statements.

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Reviewer #2 (Remarks to the Author)

Paul et al.

TECPR2 is a Rab5 effector that regulates endosomal cargo recycling.

This paper has three primary sections:

- I. protein-protein interaction studies between TECPR2 and Rab5.
- II. cell biology (the largest section) involving the interaction of TECPR2 and Rab5.
- III. The role of TECPR2 in zebrafish.

The impact of the work is strong from a basic science perspective and from the perspective that mutations in the gene encoding TECPR2 has clinical implications in humans in they can lead to a rare neurological disease, HSAN. Further, mutations in the gene encoding TECPR2 can cause neuroaxonal dystrophy in Spanish water dogs.

Part I. This paper provides strong evidence for the interaction of TECPR2 with Rab5 using both yeast two-hybrid approaches and biochemical approaches. Further the interaction is regulated by the state of Rab5 (GTP- or GDP-bound) and is weakened or absent when the TECPR2 protein is generated from disease-causing mutations.

Part II. Overall, the cell biology experiments are strong and characterize the molecular mechanisms of the TECPR2-Rab5 interaction in the context of endocytic recycling. The volume of work is impressive, and the overall quality is high.

Part III. The transition to the zebrafish work is a bit challenging as it doesn't fully encompass the molecular mechanisms from part II. However, in general, the quality of the zebrafish work is strong. In my mind the first two parts could stand alone as a nice cell biology publication and the zebrafish work (part III) could form the basis of a separate publication. That said, I wouldn't require removal of the zebrafish work for the publication of this article.

Response: We thank the reviewer for appreciating our work and providing a succinct and apt summary of the study. We have made significant efforts to include additional relevant data as suggested by the reviewer in the zebrafish section of the manuscript. The details of these new experiments are provided in our responses to the reviewer comments in Part III. We believe that with the addition of this data, the zebrafish part is now more aligned with the TECPR2 functional data from mammalian cells.

Some points needing to be addressed:

Part I. Overall, the binding assays between TECPR2 and Rab5 are quite solid, particularly given both the yeast two-hybrid data and the co-immunoprecipitations.

1. There is some apparent variability in the co-IPs between Fig. 1D and 1G and H in that TECPR2 in 1D binds equally well to Rab5 WT and Q79L, but the same is not true in 1G and 1H. The authors should comment on this. Is this due to the overexpression in 1D? Would they have a similar result immunoprecipitating endogenous TECPR2 with the WT, Q79L, and S34N versions of Rab5? Perhaps the N-terminus affords some binding in the context of the whole protein (compared to the truncated forms used in 1G and 1H)?

Response: We thank the reviewer for bringing this to our attention. We have consistently found that, similar to other Rab5 effectors, TECPR2 binds to the Rab5 (Q79L) mutant more strongly (qualitatively) than Rab5 (WT). This result is observed in the yeast two-hybrid

assay, the affinity pulldown from cell lysates, and the purified protein-protein interaction assay (**Fig. 1C** and **1G-H**). We have now replaced the experiment presented in the earlier version of Fig. 1D with a new data where we probed the immunoprecipitates of GFP-Rab5 (WT) and mutants for endogenous TECPR2. As shown in the new Fig. 1D, we find that TECPR2 has a higher affinity for the Rab5 Q79L (constitutively GTP-bound) mutant than Rab5 (WT) and a highly reduced binding with the Rab5 S34N (constitutively GDP-bound) mutant (**Fig. 1D**).

2. Ponceau stains of IPs, particularly for 1J and 1M would be helpful to verify equal amounts of antibody in the IPs.

Response: We thank the reviewer for bringing this to our attention. We have now included the ponceau images for **Fig. 1I** and **Fig. 1K** (originally Fig. 1J)

Part II. Major point:

1. The authors state in a couple of sections that previous work using highly over-expressed TECPR2 made subcellular localization challenging and subject to artifacts. They then state that they use only weak to moderate TECPR2-expressing cells, levels close to endogenous. However, I could not find evidence for this claim in the paper. It should be quite simple for them to use their anti-TECPR2 antibody to assess the levels of epitope-tagged wild type and mutant versions of TECPR2 relative to the endogenous found in untransfected cells. They need to provide this to strengthen their results.

Response: We thank the reviewer for raising this concern. As suggested by the reviewer, we measured the corrected total cellular fluorescence intensity of GFP and GFP-TECPR2 (WT) and GFP-TECPR2 (R1336W) transiently transfected cells and immunostained these cells with anti-TECPR2 antibody. Taking only cells with weak to moderate expression, we observed on average a ~5–6-fold higher signal with anti-TECPR2 antibody per cell when transfected with GFP-TECPR2 (WT) and 3–4-fold higher in GFP-TECPR2 (R1336W) mutant. The average CTCF values were normalized to the average CTCF values in GFP-transfected cells (as shown below **Fig. A6**). Keeping this analysis in mind, we now use the phrase “moderately expressing cells” (see line # 199 and 281) in the manuscript text to better describe the GFP-TECPR2 expressing cells chosen for analysis. We want to highlight that co-expression of Rab5 with GFP-TECPR2, recruits TECPR2 from cytosol to vesicles, which in live cells are motile and undergo membrane remodelling such as fission and fusion as one would expect for early endosomes (**Fig. 3** and **Supplementary Video 3**). Furthermore, endogenous TECPR2 is also enriched in early endosome fractions as compared to late endosomes and lysosomes, as shown by analysis of ferrofluid-containing membranes from cell homogenates (**Fig. 2C**).

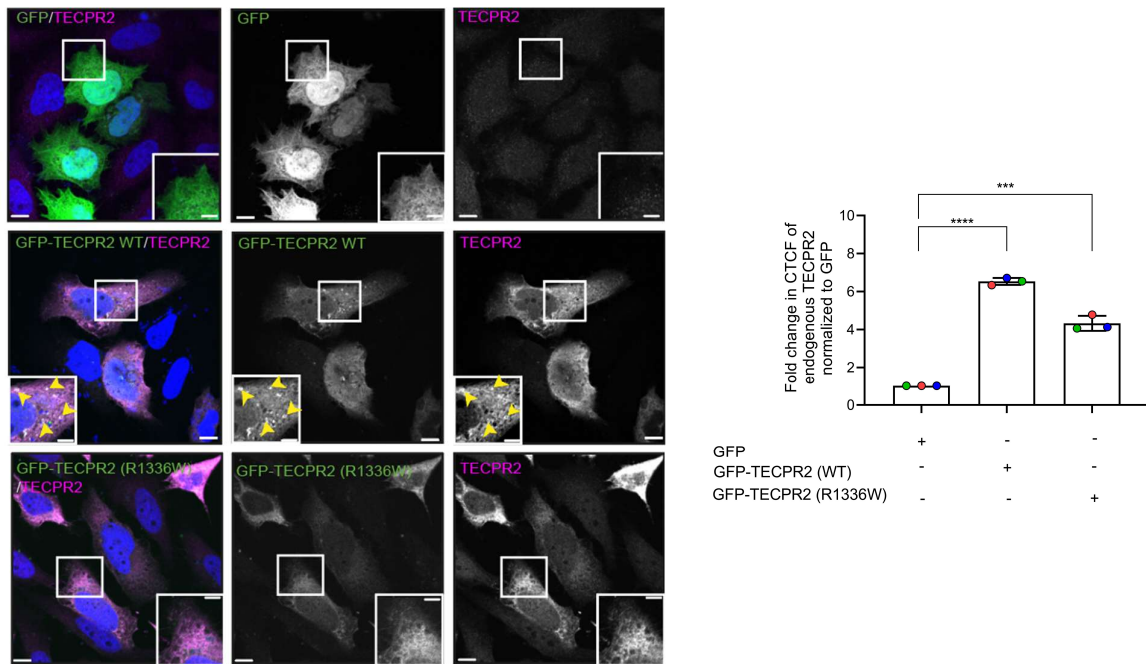


Figure A6: Estimation of levels of TECPR2 in TECPR2-overexpressing cells. Representative confocal images of HeLa cells transfected either with GFP, GFP-TECPR2 (WT) or GFP-TECPR2 (R1336W) followed by immunostaining with anti-TECPR2 antibodies. Yellow arrowheads indicate colocalized pixels in both channels. Scale Bar: 10µm (Main); 5µm (Inset). The graph on the right shows the fold-change in the average CTCF of red channel (showing immunostaining with anti-TECPR2 antibodies) in GFP-TECPR2 WT or R1336W expressing cells normalized to the average CTCF values in GFP-only expressing cells (used as a control). Data are presented as mean $\pm$ SD from n=45 cells for each sample across three independent experiments (**** $p < 0.0001$; *** $p = 0.0001$ unpaired two-tailed Student's t test).

Part III. The authors first focus on the characterization of *tecpr2* morphant zebrafish. The use of morpholinos is sound given specificity controls including *tecpr2* mRNA rescues. Granted the results can represent mosaic effects, and while CRISPR-based approaches can be superior (when compensatory mechanisms don't emerge), this reviewer appreciates the morpholino work done in these experiments.

Major Points:

1. The expression data in 8C would be much stronger with sectioning and zooming in on the regions of interest, and if complemented with a sense probe.

Response: We thank the reviewer for the suggestion and have accordingly shown the data of sectioned samples with zoomed in images (Supplementary Fig. S9B). We have included now *in-situ* hybridization of the embryos with sense probe (Fig. 9C).

2. The effect observed in 8L is a bit subtle. Do the authors suggest that this effect is the root cause of the motor defects observed in the morphants? A more developed discussion of this should be included.

Response: We thank the reviewer for this comment. To achieve a more detailed observation, we performed sectioning of the zebrafish embryos instead of staining whole-mount embryos, as limited antibody penetration into the deeper tissues during whole-mount staining can compromise the quality of the data. We found that in *tecpr2* (201+202) morphants, the neuromuscular synaptic morphology was altered, and they appeared mostly punctate and smaller compared to WT control NMJ (**Fig. 9G**). The change was also reflected in the significantly reduced “percentage area” of SV2 and acetylcholine receptor signal in *tecpr2* (201+202) morphants as compared to WT control (**Fig. 9G-I**). Importantly, the neuromuscular synaptic morphology was rescued in embryos injected with *tecpr2*-201 (WT) mRNA. Moreover, the branched and elongated appearance of NMJs in the “rescue” was more frequent as compared to the control, which was also reflected in the “percentage area” of SV2 and the postsynaptic AChRs signal (**Fig. 9G-I**). In contrast, there was no discernible rescue observed upon expression of *tecpr2*-201 (R1234W) mRNA, and neuromuscular synaptic morphology was similar to the *tecpr2* (201+202) morphants (**Fig. 9G-I**).

Thus, we suggest that zebrafish *Tecpr2* regulates the formation of NMJ, which in turn is essential for normal motility (also see **Supplementary Video S13**). In the revised version of our manuscript, we have also included additional lines in the discussion section describing these findings in the zebrafish model system and the implications of these results on understanding of the HSAN9 disorder.

3. The authors plan to continue to use zebrafish to model HSAN and similar diseases based on mutations in *TECPR2*. However, given the multiple comparisons between WT and mutant *TECPR2* in the first two parts of the paper the authors should finish the paper with effects of HSAN mutants in mRNA rescues. Does mutant mRNA rescue the defects and developmental problems?

Response: We thank the reviewer for this important suggestion. We have now included the data showing that the R1234W mutation, which is orthologous to the residue R1336W in human *TECPR2*, disrupts binding to the zebrafish Rab5 ortholog, indicating that the region and mode of Rab5 binding in *TECPR2* might be conserved across different vertebrate species (**Fig. 9A**). Furthermore, we have now included data of *tecpr2*-201+202 morphants co-injected with the *tecpr2*-201 (R1234W) mRNA. As shown in **Fig. 9E-F**, neither the embryo viability nor the morphological defects of *tecpr2* (201+202) morphants were rescued by co-injection of *tecpr2*-201 (R1234W) mRNA, indicating that, similar to other vertebrate orthologs, the R1234W mutation disrupts *Tecpr2* function in zebrafish as well. Whole-mount immunostaining of the HA-epitope confirmed the translation of the injected *tecpr2*-201 and *tecpr2*-201 (R1234W) mRNA (**Supplementary Fig. S9F**). The immunofluorescence of HA-tagged *Tecpr2* in the 5 dpf embryos revealed the myotome-restricted expression in the trunk region of the WT protein. Interestingly, the mutant *tecpr2* mRNA-injected embryos revealed a diffused expression of the HA-tagged *Tecpr2* (R1234W), which is consistent with its lack of binding to Rab5 and reminiscent of the cytoplasmic expression of the orthologous human mutant in HeLa cells (**Supplementary Fig. S9F**). Similarly, we have also employed this mutant to rescue the impaired and suboptimal motility in *tecpr2* (201+202) morphants and the altered neuromuscular synaptic morphology of the *tecpr2* (201+202) morphants. Indeed, as shown in **Fig. 9G-I** and **Supplementary Video S13**, *Tecpr2* (R1234W) expression was unable to rescue the neuromuscular synaptic morphology or the larval motility defects of *tecpr2* (201+202) morphants.

Related, if zebrafish can be used as model, the authors should provide a multiple sequence alignment of TECPR2 (and Rab 5) proteins showing the zebrafish, human, dog and other vertebrate proteins in the alignment. They should indicate the position of the disease mutations and note which are conserved and which are not.

Response: We have now included the multiple sequence alignment of TECPR2 and Rab5 proteins from various vertebrate species, including zebrafish, human, dog and other vertebrate proteins in **Supplementary Table IV**.

Minor points:

1. Typo: Page 5, line 62: “However, However”

Response: We appreciate the reviewer bringing this to our attention. We have made the necessary changes to the text.

2. This sentence is unclear and needs to be re-written for clarity: Page 7, lines 105-107: “Given that Rab5 is an extensively studied Rab protein, however, association of Rab5 with TECPR2 was not known, we wanted to confirm TECPR2 interaction with Rab5 and the significance of this interaction.”

Response: We appreciate the reviewer bringing this to our attention. We have made the necessary changes to the text.

3. In the following sentence you cannot conclude TECPR2 is a Rab5 effector. However, the rest of the sentence is fine: Page 14, line 265-267: “Taken together, we conclude that TECPR2 is a Rab5 effector and the C-terminal TECPR repeats are essential and sufficient for Rab5 binding and, consequently, for TECPR2 recruitment to early endosomes.”

Response: We agree with the reviewer and have made the necessary changes to the text.

4. In the following sentence you cannot state this fully explains the loss-of-function phenotype. You should say “may explain the loss-of-function phenotype”...Page 14, lines 267-269. “Importantly, impaired Rab5 binding and lack of membrane localization explain the loss-of-function phenotype of the HSAN9-associated TECPR2 R1336W variant.”

Response: We agree with the reviewer and have made the necessary changes to the text.

5. Sup. S4A. Not particularly worrisome, but is the anti-actin blot upside down?

Response: We appreciate the reviewer bringing this to our attention. The blot is not inverted. The samples in the gel ran in that manner. We have now included the complete blot in the source file. As you may be able to appreciate, the other lanes in the same X-ray film are showing typical bands. We have included the image below for your reference (**Fig. A7**). The red box marks the area shown in **Supplementary Fig. S4A**.

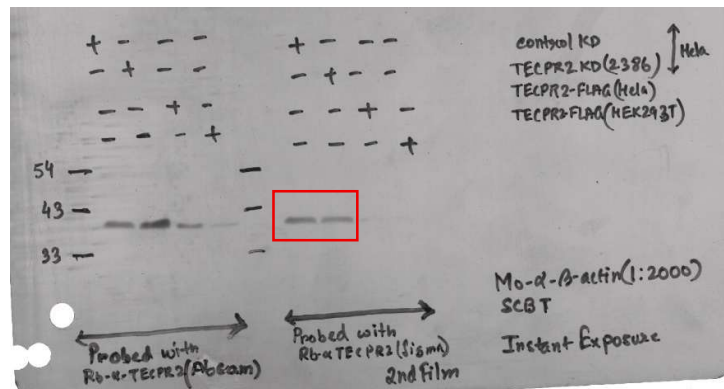


Figure A7: Source data of Supplementary Fig. S4A

6. TfR recycling should be referenced on Page 17, line 328-329: “As TECPR2-positive endosomes were accessible to endocytosed receptor such as transferrin receptor (TfR) which undergoes constitutive recycling...” [ref]...

Further, it would be best to write, “...such as the transferrin ...”

Response: We appreciate the reviewer bringing this to our attention. We have made the necessary changes to the text.

Reviewer #3 (Remarks to the Author):

In this manuscript, Paul and colleagues investigated the role and mechanism of TECPR2 in endosomal trafficking. They demonstrated that TECPR2 is recruited to early endosomes by Rab5 and identified the C-terminal TECPR repeats and specific residues within these regions as essential for the direct interaction between TECPR2 and Rab5. Knockdown of TECPR2 (TECPR2 KD) led to the accumulation of early endosomes in the perinuclear area, increased localization of internalized integrins with late endosomes, and reduced levels of Integrin $\beta 1$. The authors also discovered that SNX17, members of the WASH complex, and SNX27 were present in TECPR2-containing protein complexes. This suggests that TECPR2 may facilitate the fission of recycling endosomes from early endosomes by recruiting SNX17 and the WASH complex, thereby promoting the movement of recycling endosomes toward the plasma membrane. Furthermore, in a zebrafish model, TECPR2 KD resulted in delayed motility and structural changes in the neuromuscular junction. Overall, the data on the interaction between TECPR2 and Rab5, as well as its recruitment to early endosomes, provide compelling evidence of TECPR2's novel role in endosomal trafficking. However, the mechanistic details remain preliminary and require further investigation.

Response: We thank the reviewer for appreciating our work and finding that this study adds new knowledge about TECPR2 localization and subcellular function. We have added new experiments that shed mechanistic insights into the mechanism of TECPR2 function in regulating endocytic recycling.

Major Points:

1. The study used siRNA knockdown and transient addback approaches. However, to strengthen the findings presented in Figures 4–7, I recommend using CRISPR-based stable knockdown and stable rescue approaches for validation.

Response: We appreciate the suggestion to validate our findings through CRISPR-based gene silencing or knockout. Our multiple attempts to generate a complete knockout of *TECPR2* in HeLa cells failed and did not yield viable cells. The surviving cell population when assayed using anti-TECPR2 antibodies showed a band at the size of endogenous TECPR2 (as shown below in **Fig. A8**). The technique of gene knockdown using CRISPR (as mentioned by the reviewer) is not standardized in our laboratory, and to achieve this in the time frame of revision was not feasible for us.

To validate our major conclusions regarding TECPR2 function, we have employed two additional siRNA sequences against TECPR2 and found similar results, such as perinuclear clustering of early endosomes and lysosomal degradation of endocytosed integrin receptors (**Supplementary Fig. S4G and S6F**). We have performed rescue experiments to show the specificity of the phenotypes observed upon TECPR2 knockdown, such as an increase in the size of early endosomes and altered distribution of early and recycling endosomes (**Figs. 4A–D, 4E and 4G**). Additionally, several experiments provide a mechanistic explanation of the above-mentioned phenotypes, observed in TECPR2-depleted cells. These include direct interaction of TECPR2 with integrin sorting adaptor SNX17, which in turn regulates its early endosome localization (**Fig. 7**). Also, new evidence in the revised manuscript shows that SNX17 interaction with Rab5 is dependent upon TECPR2 expression (**Fig. 7G**). Thus, taken together, multiple lines of investigation support that TECPR2 regulates trafficking of cargoes that rely on a sequence-based slow recycling pathway that involves the formation of a branched actin network on early endosomes.

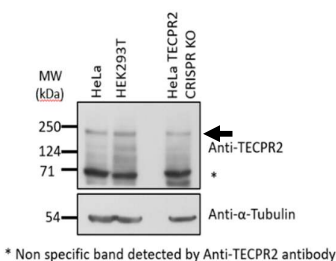


Figure A8: Validating TECPR2 knockout in HeLa cells: Immunoblotting of cell lysates from HeLa, HEK293T and TECPR2 CRISPR knockout HeLa cells probed with the indicated antibodies. The band of endogenous TECPR2 is marked with an arrowhead, and the (*) asterisk indicates a non-specific band detected by the anti-TECPR2 antibody.

2. In Figures 4A–4K, the EEA1-positive puncta in TECPR2 KD cells appeared larger and fewer, potentially due to increased Rab5 activity. I suggest quantifying the number and size of Rab5-positive puncta in control, TECPR2 KD, and TECPR2-rescued cells.

Response: To address this comment, we have quantified the size of Rab5 and EEA1-positive puncta in HeLa cells treated with control siRNA, TECPR2 siRNA, and TECPR2 siRNA transfected with siRNA-resistant GFP-TECPR2 construct. As shown in **Fig. 4A–C**, the average

area of early endosomes marked by Rab5 and EEA1 was increased upon TECPR2 depletion, and this phenotype was partially rescued upon re-expression of TECPR2 (WT). The number of Rab5⁺ early endosomes was quite variable across the cell population (as shown below in **Fig. A9**). Nevertheless, it was obvious that the Rab5⁺ endosomes were significantly reduced upon TECPR2 knockdown. As shown below, we observe a partial but significant rescue of this phenotype following the re-expression of wild-type TECPR2.

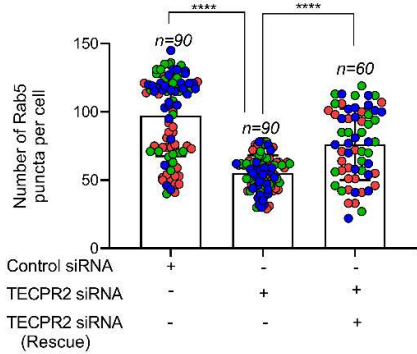


Figure A9: TECPR2 depletion results in a decrease in the number of Rab5⁺ endosomes. Quantification of number of Rab5 puncta per cell in HeLa cells treated with the indicated siRNAs and transfected with siRNA-resistant TECPR2 (Rescue) construct. Experiments are color-coded, and each dot represents the individual data points from each experiment. The total number of cells analyzed is indicated on the top of each data set graph (**** $p < 0.0001$; unpaired two-tailed Student's t test).

Additionally, Rab5 activity should be assessed.

Response: To address the reviewer's comment on whether there is any change in the status of active Rab5 upon TECPR2 depletion, we have performed GST-Rabaptin-RBD (Rab5 Binding Domain) pulldown to capture the active or GTP-bound Rab5 (refer to the **Fig. A10** below). Although total Rab5 levels were reduced upon TECPR2 depletion (as described in the line # 498-500) of manuscript text; also see response to comment #3 of reviewer #1), active Rab5 pulled down by GST-Rabaptin was not significantly altered when normalized to the input levels and when compared to control. These results suggest that TECPR2 depletion does not impact Rab5 activation.

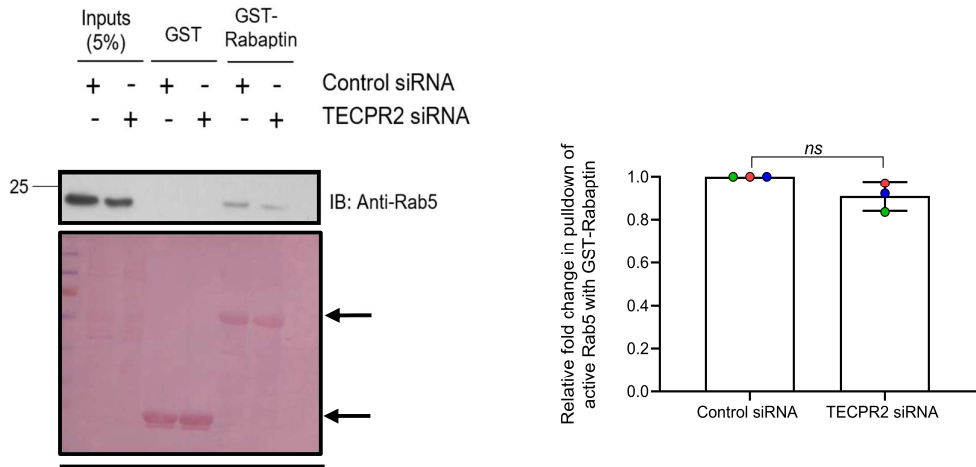


Figure A10: Levels of active Rab5 are not altered upon TECPR2 depletion. Recombinant GST and GST-Rabaptin proteins immobilized on glutathione-conjugated beads were incubated with lysates of indicated siRNA treated HEK293T cells. The precipitates were IB with anti-Rab5 antibody, and Ponceau S staining was done to visualize the purified proteins. The graph represents relative fold-change in levels of active Rab5 pulldown with GST-Rabaptin upon TECPR2 siRNA treatment normalised to input and to control siRNA from three independent experiments.

Measurements of recycling and late endosome size and number, as well as Rab7 and Rab11 or Rab10 activity, should also be included to provide a more comprehensive analysis.

Response: We would like to mention that Rab11⁺ endosomes are too numerous to obtain reliable data from confocal resolution images. Furthermore, Rab11 shows a dramatic perinuclear collapse upon TECPR2 knockdown, and unlike EEA1 or Rab5 where individual endosomes are apparent, it is not possible to obtain data of individual endosomes from these images. We have quantified the number of cells displaying Rab11⁺ tubules, which was reduced upon TECPR2 depletion (**Fig. 4E-F**). As mentioned in the discussion section, we speculate that TECPR2 interaction with BLOC-1 complex (that promotes biogenesis and tubulation of recycling endosomes) may explain the effect of TECPR2 depletion on tubular recycling endosomes (line # 686-702).

Regarding quantification of Rab7⁺ endosomes, indeed, we have observed a dramatic increase in the size of late endosomes and lysosomes marked by Rab7 and LAMP1 (**Supplementary Fig. S4M**). This phenotype has been described in the manuscript text as well (line # 346-349). To better understand how TECPR2 regulates the late endocytic pathway requires a significant effort and diverges from the major findings of this manuscript. As part of an ongoing study in our laboratory, we are exploring whether lysosome biogenesis is regulated by TECPR2. Indeed, degradation of endocytic cargo such as BODIPY-BSA, which becomes fluorescent upon lysosomal degradation, is reduced upon TECPR2 depletion (see comment #4 from reviewer #1). We are also exploring TECPR2's role in retrieval of cation-independent mannose-6-phosphate receptor (CI-M6PR) from early endosomes to the trans-Golgi network (TGN). Indeed, a defect in CI-M6PR retrieval from early endosomes to the TGN will result in mis-sorting of lysosomal hydrolases and reduced hydrolytic activity of late endocytic compartments. This, in turn, would result in accumulation of cargo and subsequent enlargement of late endosomes and lysosomes. Our previous work on mechanisms of CI-M6PR sorting will be helpful in carrying out this study (Rawat et al., JCB 2023, PMID: 36282215). We have not explored the effect of TECPR2 depletion on Rab10, as the focus of this work became a detailed analysis of the SNX17 cargo retrieval pathway due to similarity of the integrin degradation phenotype observed upon both TECPR2 and SNX17 depletion.

3. The method for quantifying the distance of early or recycling endosomes to the nucleus is unclear. Since TECPR2 KD cells exhibit altered size compared to control cells, distances should be normalized to cell size to avoid misleading conclusions.

Response: We agree with the reviewer that the methodology used to analyze endosome distribution upon TECPR2 knockdown should be normalized to the cell size. Indeed, in the previous version of the manuscript itself, we employed the fractional distance method to determine the distribution of early/recycling markers (refer to the **Fig. 4D** and **4G** in the revised version). Briefly, as described in the manuscript method section, a line is drawn from the center

of the cell to the cell periphery using the “line tool” of Fiji software in the confocal micrograph. The plot profile tool is then used, which selects all the endosomal marker fluorescent intensities along with their corresponding distance values on the drawn line. The background pixels and their corresponding distance values are not considered for analysis. The remaining distances corresponding to the endosomal pixels are then converted to fractional distances. This is done by dividing all these values by the total distance of the line drawn. Thus, the spatial distribution of the endosomes is independent of the cell size. Please see other studies for reference where the fractional distance method is employed to study organelle distribution, taking into account differing cell size (Kumar G, et al., Nat Commun. 2022, PMID: 35314681; Jongsma ML, et al., Cell. 2016, PMID: 27368102).

4. In Figure 5, the cell density in TECPR2 KD cells appears higher than in control cells in Figures 5A–5C. The authors should ensure consistent plate density and assess whether TECPR2 KD impacts proliferation or viability.

Response: We thank the reviewer for the comment. We have repeated the experiment and replaced the earlier images with new images showing an almost equal cell seeding density in control and TECPR2 knockdown (**Supplementary Fig. S6A**). TECPR2-depleted cells show a dramatic cell spreading defect, grow in clusters, and do not form membrane projections that are apparent in control cells (likely due to reduced integrin-based attachment to extracellular matrix). Thus, it may appear that cell density is different between the two samples. We want to also emphasize that the defect in the cell area has been quantified across three independent experiments by measuring the area of 80 cells over three experiments (please refer also to the source data file). Furthermore, as shown in **Fig. 5A**, the cell spreading defect was more obvious in TECPR2-depleted cells that were trypsinized and allowed to adhere to the fibronectin-coated culture dish for a limited period of time. The number of focal adhesions measured across three independent experiments provide a convincing proof of a defect in cell spreading upon TECPR2 depletion (**Fig. 5B**). To determine whether TECPR2 knockdown affects cell viability or proliferation, we performed Alamar Blue assay that measures the metabolic activity that acts as an indicator of proliferating and viable cells. HeLa cells were treated with control or TECPR2 siRNA and the fluorescence of Alamar blue was measured for the indicated days. As shown in **Fig. A11**, a significant reduction in the fluorescence was observed in TECPR2 siRNA treated cells at day 2 but especially at day 3 of knockdown as compared to control. Thus, there is a reduced cell proliferation or cell viability upon TECPR2 siRNA treatment. Indeed, a decrease in the recycling of mitogenic receptors, such as EGFR (as observed in **Supplementary Fig. S6G-I**) may explain the reduced cell viability observed upon TECPR2 knockdown.

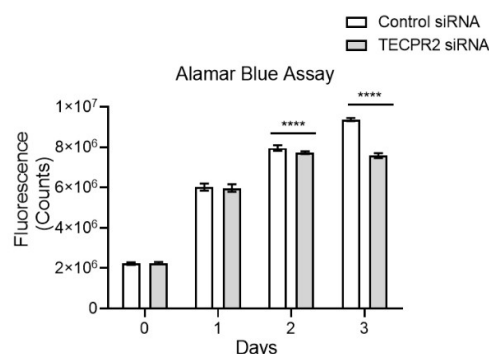


Figure A11: TECPR2 depletion leads to reduced cell viability. Graph showing the quantification of cell viability at different days post control and TECPR2 siRNA treatment. The cell viability was assessed based on the fluorescence intensity of Alamar Blue that progressively decreased over time in TECPR2 siRNA treated cells compared to control siRNA treatment. Data represents mean $\pm$ SD from $n=3$ independent experiments (**** $p<0.0001$; unpaired two-tailed Student's t test).

For Figures 5D–5I, the dynamics of focal adhesions (e.g., using a nocodazole-washout assay to monitor vinculin or paxillin puncta with phalloidin staining) should be analyzed instead of simply quantifying static paxillin puncta.

Response: We thank the reviewer for this important suggestion. To address this comment and gain insights into the dynamics of focal adhesions (FAs) upon TECPR2 knockdown, we performed the nocodazole washout assay wherein FAs, stabilized by nocodazole-mediated microtubule depolymerization, were observed for their microtubule regrowth-dependent disassembly and then reassembly during the washout phase. As shown in **Fig. 5C-D**, TECPR2-depleted cells showed smaller focal adhesions than control cells under basal conditions, which were stabilized upon nocodazole treatment. While disassembly of FAs was observed during the first 30 minutes of the washout phase, FA reassembly by 120 minutes post-washout was significantly delayed upon TECPR2 depletion as compared to control cells (**Fig. 5C-D**). Consistent with a lack of FA assembly, TECPR2-depleted cells also showed a dramatic absence of actin cables or stress fibers that were prominently observed in the control cells during the FA reassembly phase (**Fig. 5C**). These findings are in line with previous work showing that integrin recycling defect regulates kinetics of focal adhesion reassembly (Nader GP, et al., Nat Cell Biol. 2016, PMID: 27043085).

For Figures 5J–5N, dynamic trafficking of integrins should be assessed by labeling live cells with anti-integrin antibodies on ice, followed by incubation at 37°C for various times. Co-staining with Rab5, EEA1, Rab7, Lamp1, or Rab11 antibodies would provide more insight.

Response: We thank the reviewer for this important suggestion. To address this comment, we investigated the colocalization of endocytosed active Itgβ1 with various markers in control and TECPR2-depleted cells. The colocalization analysis revealed that the internal non-recycled pool of Itgβ1 was present in HRS⁺ endosomes in TECPR2-depleted cells, while Itgβ1 colocalization with Rab5 and EEA1 was reduced (**Fig. 5G-H**). As mentioned above, HRS, a subunit of ESCRT-0, marks the degradative subdomain of early endosomes from where cargo is sorted in intraluminal vesicles for their eventual degradation.

To address the concern of whether Itgβ1 is delivered to late endocytic compartments marked by Rab7 and LAMP1, we have instead employed live-cell imaging to analyze and measure the colocalization of endocytosed Itgβ1 with SiR-Lysosome, a dye that marks compartments with cathepsin D hydrolase activity (i.e., it marks degradative lysosomes). As shown in the new **Supplementary Fig. S6D-E** and **Supplementary Video S7**, there was a significantly increased colocalization of endocytosed Itgβ1 and SiR-Lysosome over time in TECPR2-depleted cells. Taking this data along with other observations such as increased colocalization of Itgα5 and LAMP1 and observed degradation of Itgβ1 in TECPR2-depleted cells that was rescued upon Bafilomycin A1 treatment (**Fig. 5I-J**), we conclude that TECPR2 knockdown impairs the retrieval of endocytosed Itgα5β1 from lysosomal degradation.

Cell surface integrin levels should be measured using FACS or confocal imaging.

Response: We had shown the surface levels of active Itgβ1 in the previous submission as well. This is now shown in **Supplementary Fig. S6C**.

Similarly, in Figures 5O–5V, dynamic colocalization of internalized surface integrin with Lamp1 should be quantified, especially as Lamp1-positive vesicles appear larger in TECPR2 KD cells, suggesting increased Rab7 activity.

Response: We thank the reviewer for this important suggestion. As described above, we have employed live-cell imaging to analyze and measure the colocalization of endocytosed Itg β 1 with SiR-Lysosome. As shown in the new **Supplementary Fig. S6D-E** and **Supplementary Video S7**, there was a significantly increased colocalization of endocytosed Itg β 1 and SiR-Lysosome over time in TECPR2-depleted cells.

5. In Figure 6A, an additional TECPR2 KD lane should be included for better comparison of integrin changes.

Response: We thank the reviewer for the suggestion. We have repeated the experiment and included the sample of only TECPR2 knockdown (refer to revised **Fig. 6A**).

Clarification is needed on the criteria used to distinguish between the mature and immature forms of integrin. Notably, SNX17 KD resulted in an increased lower band, which needs explanation. Of note, in Figure 5R, single band of integrin was detected.

Response: We thank the reviewer for this comment. The two forms of β 1 integrin arise due to the differential mobility of precursor and mature β 1 integrins that differ in their N-linked glycosylated pattern. The 105-110 kDa form is the precursor form, typically the ER resident form, and the ~130 kDa form is the mature β 1 integrin present at the cell surface (as one of the examples showing the two forms of β 1 integrin, please refer to Seales EC, et al., *Oncogene*, PMID: 14562042). We typically see both forms of β 1 integrin in Western blots with the anti- β 1 integrin antibodies (BD Biosciences; Cat #:610467); however, the intensity of the band depends upon the exposure time and concentration of cell lysates in the experiment. For better consistency, we are now showing another experiment where both the precursor and mature forms of β 1 integrins are visible (refer to the new **Fig. 5I**). The increase in the precursor β 1 integrin form upon SNX17 knockdown was not consistently observed in all experiments. In the revised **Fig. 6A** (with TECPR2-single siRNA also included), we are showing a representative experiment. We did not observe a noticeable increase in the precursor form of β 1 integrin either in SNX17 knockdown or combined knockdown of SNX17 and TECPR2.

In Figures 6D–6C, colocalization of SNX17 with Rab5 should be quantified. Similarly, quantification is needed for Figures 6E–6F. To monitor integrin trafficking dynamics, live cells should be labeled with anti-integrin antibodies on ice and then incubated at 37°C for various time points. This would provide a clearer picture of integrin trafficking from the cell surface to endosomal compartments.

Response: We thank the reviewer for this comment. We have measured the colocalization between SNX17 and Rab5 (refer to the graph shown in **Fig. 6D**) and colocalization between SNX17 and endocytosed integrin (refer to the graph shown in **Fig. 6F**). Additionally, as mentioned in the previous responses, we have employed SiR-Lysosome to analyze the colocalization of endocytosed Itg β 1 with acidic and degradative late endocytic compartments (**Supplementary Fig. S6D-E** and **Supplementary Video S7**). We have also measured the colocalization of endocytosed active Itg β 1 with various markers in control and TECPR2-depleted cells. The colocalization analysis revealed that the internal non-recycled pool of Itg β 1 was present in HRS⁺ endosomes in TECPR2-depleted cells, while Itg β 1 colocalization with Rab5 and EEA1 was reduced (**Fig. 5G-H**). As mentioned above, HRS, a subunit of ESCRT-0, marks the degradative subdomain of early endosomes from where cargo is sorted in intraluminal vesicles for their eventual degradation.

In Figures 7E–7F, in TECPR2 KD cells, SNX17 remains strongly associated with Rab5, but the size of SNX17- or Rab5-positive vesicles is larger. This difference should be further analyzed and compared to similar observations in Figures 6B and 6C.

Response: We would like to emphasize that we have analyzed SNX17 localization to early endosomes in multiple ways, and data from independent approaches support the conclusion that “TECPR2 regulates SNX17 localization to Rab5⁺ early endosomes.” First, there was a decrease in the number of SNX17 punctae and colocalization of SNX17 and Rab5 was reduced in TECPR2-depleted cells under endogenous conditions (**Fig. 6B–D**). Importantly, live-cell imaging of SNX17 and Rab5 showed a significant reduction in their colocalization in TECPR2-depleted cells (**Fig. 7E–F** and **Supplementary Video S10**). As is evident in the data and the intensity profile shown in **Fig. 7E**, less recruitment of SNX17 was observed on Rab5⁺ endosomes in TECPR2 knockdown. Second, we investigated whether TECPR2 acts as a linker between Rab5 and SNX17. To this end, we employed GST-tagged SNX17 as a bait and tested pull down of endogenous Rab5 in control and TECPR2-depleted cells (**Fig. 7G**). We found that endogenous Rab5 and TECPR2 both interact with SNX17, and Rab5 binding to SNX17 was reduced upon TECPR2 depletion, suggesting that TECPR2 acts as a linker between SNX17 and Rab5 (**Fig. 7G**). Third, there was a modest but significant decrease in SNX17 in the early endosome fractions, whereas no significant change in EEA1 was observed upon TECPR2 depletion (**Fig. 7H–I**). Thus, taking all of these multiple data into account, along with the observed direct binding of TECPR2 to SNX17 allows us to conclude that TECPR2 regulates SNX17 localization to early endosomes.

For Figures 6H–6I, the effects of TECPR2 KD on the association between WASHC1 and Rab5 should be evaluated in wild-type Rab5-expressing cells.

Response: We thank the reviewer for this comment. We have visualized and quantified WASHC1 colocalization with wild-type Rab5 using live-cell imaging. As shown in **Fig. 8E–F**, we found a reduced colocalization of WASHC1 with wild-type Rab5 in TECPR2-depleted cells (also refer to **Supplementary Video S11**).

6. In Figures 7K–7L, TECPR2 KD appears to impair actin polymerization; however, the quality of the phalloidin staining is suboptimal and needs to be improved. The authors should evaluate the colocalization of p16-Arc with Rab5 in control and TECPR2 KD cells to provide further insights.

Response: We thank the reviewer for this comment. We have now shown better representative images of phalloidin (refer to revised **Fig. 8G**). Additionally, the colocalization of p16-Arc and Rab5 is included now as **Fig. 8I**. To corroborate the result that TECPR2 depletion reduces actin on early endosomes, we also visualized endosomal actin indirectly by live-cell imaging of actin capping protein CapZ, which localizes to early endosomes and binds to the barbed ends of actin filaments to prevent their elongation. We found a dramatic reduction in CapZ β -GFP punctate localization upon TECPR2 depletion, confirming that TECPR2 depletion abrogates actin nucleation on early endosomes (**Fig. 8J–K** and **Supplementary Video S12**).

Additionally, the effects of TECPR2 KD or WASH/p16-Arc KD on early endosome tubulation or fission should be assessed to better support the proposed role of TECPR2 in facilitating the transition of early endosomes to recycling endosomes through fission.

Response: We thank the reviewer for this important suggestion. SNX1 is one of the few markers that one can employ to visualize early endosome tubulation. Fission of SNX1⁺ tubules is generally regarded as the process of conversion of early endosomes to recycling/retrieval tubules. Indeed, SNX1 tubules are found to be more persistent in knockdown of Strumpellin, one of the subunits of the WASH complex (Harbour ME, et al., J Cell Sci. 2010, PMID: 20923837). SNX1 is part of the ESCPE-1 complex that recognizes cargo destined for retrieval back to the TGN, such as the cation-independent mannose-6-phosphate receptor (Simonetti B, et al., Nat Cell Biol. 2019, PMID: 31576058). In this study, we have focused on characterizing TECPR2 localization and analyzed its function in recycling of integrin to the cell surface, thus we have done an in-depth analysis of integrin cargo retrieval machinery upon TECPR2 knockdown. Nevertheless, to address the reviewer's comment, we have done a preliminary analysis of the dynamics of SNX1⁺ tubulation in control and TECPR2-depleted cells. As shown below in response **Fig. A12**, we found that the average length of SNX1⁺ tubules was increased by ~2-fold, signifying decreased fission of the tubular endosomes in TECPR2 depletion. We are currently investigating the role of TECPR2 in CI-M6PR trafficking, and as part of this study, we are investigating if WASH and actin recruitment to SNX1 tubules is dependent upon TECPR2.

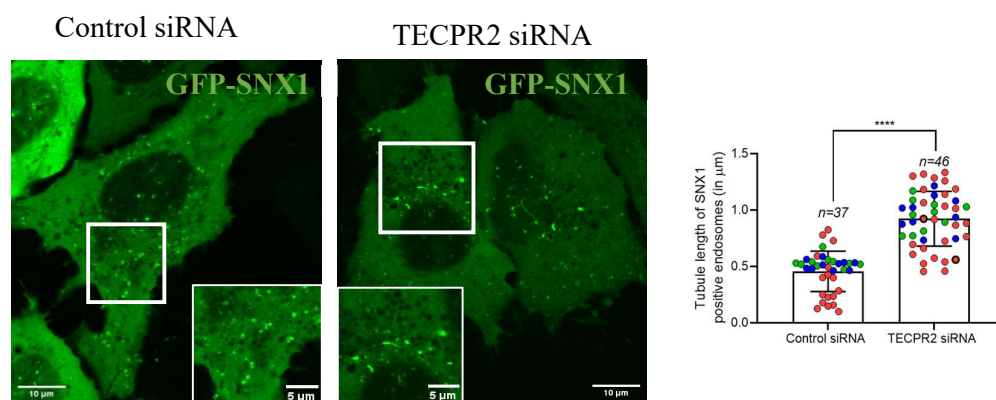


Figure A12: TECPR2 depletion leads to increased length of SNX1-positive endosomal tubules. Representative single-frame confocal images of HeLa cells transfected with either control or TECPR2 siRNA and overexpressing GFP-SNX1. Cells depleted of TECPR2 show longer SNX1⁺ endosomal tubules compared to control cells. Scale bar: 10 μm (main image); 5 μm (inset). The graph on the right quantifies the average length of SNX1-positive tubules (in μm) and showing a significant increase upon TECPR2 knockdown. Data are presented as mean ± SD. n indicates the total number of cells over three independent experiments. Experiments are color-coded, and each dot represents the individual data points from each experiment (*****p*<0.0001; unpaired two-tailed Student's *t* test).

7. In Fig. S5F, it is notable that cell surface EGFR levels are lower in TECPR2 KD cells compared to control cells. Since most cell surface EGFR is typically sent to the lysosome for degradation upon ligand binding, this observation suggests that TECPR2 may play a role in regulating endolysosomal degradation of EGFR. To further explore this, the authors should assess whether TECPR2 inhibits Rab7 activity or impedes the transition of early endosomes to late endosomes by upregulating Rab5.

Response: We want to clarify that analysis of EGFR surface levels was done at steady state and not in the presence of its ligand EGF. EGFR in the absence or low concentration of its

ligand (EGF) is sorted from early endosomes for recycling back to the plasma membrane (Bakker et al., J Cell Sci 2017, PMID: 29180516). Indeed, we found that, similar to the integrin receptor, total levels of EGFR were reduced upon TECPR2 depletion, which was partially rescued upon bafilomycin A1 (lysosomal V-ATPase inhibitor) treatment, suggesting that TECPR2 regulates retrieval of EGFR from a lysosomal degradation fate and recycled to the cell surface (**Supplementary Fig. S6H-I**). Furthermore, as stated in the previous response to comment # 2 of the reviewer, we are analysing the mechanistic basis of the dramatic increase in the size of late endosomes and lysosomes marked by Rab7 and LAMP1 upon TECPR2 depletion.

Regarding the reviewer's comment to analyze Rab7 activity, we are exploring how late endocytic membrane trafficking players are regulated by TECPR2. Nevertheless, to address the reviewer's comments, we have analyzed the GTP-bound Rab7 levels in control and TECPR2-depleted cells using the well-established assay of the GST-RILP Rab7 binding domain employed as bait to pull down Rab7 in its GTP-bound state. As shown in the figure below in **Fig. A13**, we found no significant difference in Rab7-GTP levels upon TECPR2 depletion.

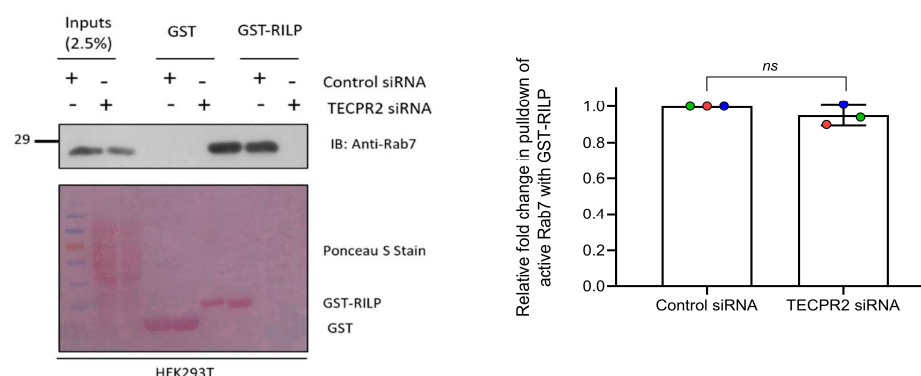


Figure A13: Levels of GTP-bound Rab7 are not altered upon TECPR2 depletion. Recombinant GST and GST-RILP proteins immobilized on glutathione-conjugated beads were incubated with lysates of indicated siRNA treated HEK293T cells. The precipitates were IB with anti-Rab7 antibodies, and Ponceau S staining was done to visualize the purified proteins. The graph represents relative fold change in levels of active Rab7 pulldown with GST-RILP upon TECPR2 siRNA treatment normalised to input and to control siRNA.

Minor Points:

1. All Western blot and immunoprecipitation data need to be quantified.

Response: We appreciate the reviewer bringing this to our attention. We have now included the densitometric analysis of all the Western blot data in the manuscript.

2. In Figures 4A and 4B, the cell edges should be shown in all images to provide better context.

Response: We thank the reviewer for this useful suggestion and have now marked the outline of cells in **Fig. 4A**.

3. Why TECPR2 KD failed to affect transferrin recycling as shown in Fig. S6B?

Response: We thank the reviewer for this important question. It is appreciated in previous studies that transferrin receptor (TfR) recycles via an actin-independent bulk membrane flow to the cell surface (Puthenveedu MA et al., Cell. 2010, PMID: 21111236). For instance, TfR recycling is unaffected in the WASH knockout cells, although, similar to TECPR2 depletion, TfR is accumulated in collapsed endosomes in the perinuclear region of the cell (Gomez TS et al., Mol Biol Cell. 2012, PMID: 22718907). In agreement with previous studies, we also propose that TECPR2 regulates trafficking of cargoes that rely on a sequence-based slow recycling pathway that involves the formation of a branched actin network on early endosomes. In contrast, recycling of cargo such as transferrin receptor (TfR) that traffics via an actin-independent bulk recycling pathway proceeds efficiently in TECPR2-depleted cells.

4. Show data of large and aberrant clustering of Rab5-positive vesicles in high TECPR2 expression cells (line 275).

Response: Please refer to the image **Fig. A14** shown below. Furthermore, the same phenotype can be appreciated in **Supplementary Fig. S2B**. Please compare the distribution of GFP-Rab5 alone and in cells co-expressing FLAG-TECPR2 (WT). We have now added the reference figure in the manuscript text as well (line # 232-238).

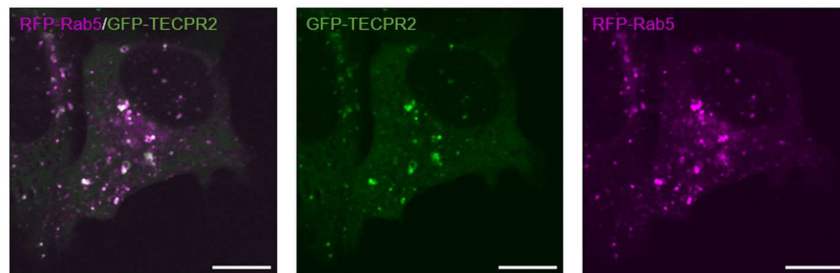


Figure A14: Rab5-positive vesicles are enlarged and perinuclearly clustered in cells with higher over expression of TECPR2. Representative confocal micrographs of HeLa cells co-transfected with GFP-TECPR2 and RFP-Rab5. Scale Bars: 10 μ m.

5. Several typos and grammatical errors have been identified.

Response: We appreciate the reviewer bringing this to our attention and apologize for these errors. We have tried our best to correct all typos and grammatical errors in the manuscript.

Response to Reviewers Comments:

Reviewer #1:

Having reviewed the revised manuscript, I find that the authors have effectively addressed my previous comments and concerns. The revisions reflect a comprehensive response to the feedback provided, enhancing both the clarity of the presentation and the strength of the conclusions. Through their detailed exploration of TECPR2's role as a Rab5 effector in endocytic recycling, along with the analysis of HSN-associated variants and validation studies in zebrafish to establish disease relevance, the authors have produced a well-rounded study. I recommend accepting this manuscript for publication.

Reviewer #2:

The revised manuscript represents significant and substantial improvements. All of the points of concern raised by this reviewer during the initial review have been adequately addressed by the authors.

Reviewer #3:

The revised manuscript is much improved; however, I have one remaining concern regarding Figure 8J. The data show that TECPR2 knockdown significantly reduces CapZ β puncta. Since CapZ normally functions to prevent actin filament polymerization, a decrease in CapZ puncta would be expected to enhance actin polymerization, rather than reduce it. This interpretation appears to contrast with the results in Figures 8E and 8J, where actin branching is inhibited in TECPR2 knockdown cells. To clarify this apparent discrepancy, the authors should examine whether TECPR2 knockdown alters the expression levels of relevant proteins, particularly CapZ and p16-Arc, and quantify overall actin filament density in the knockdown cells.

Response: We thank all the reviewers for their time and efforts in providing a constructive and thorough review that greatly helped in strengthening our manuscript. We are glad to note that the revised version of the manuscript addresses the previous concerns of all the reviewers.

We thank reviewer #3 for the insightful comment on the role of CapZ as an actin capping factor and the observed decrease in its early endosomal localization upon TECPR2 depletion. Further, we appreciate the suggestion to measure the overall F-actin density in TECPR2-depleted cells and to measure the levels of p16-Arc and CapZ. Please find our detailed response below, which addresses these points, and we have highlighted all changes in yellow in the uploaded manuscript text file.

The results shown in **Figs. 7 and 8** of the revised manuscript show that TECPR2 interacts with the subunits of the WASH complex (**Fig. 7D**), and TECPR2 depletion reduces the recruitment of the WASH complex on early endosomes, a conclusion supported by independent approaches of isolation of early endosomal membranes (**Fig. 8A-B**), localization analysis of WASH subunits (**Fig. 8C-D**), and live-cell imaging of WASHC1 with Rab5 (**Fig. 8E-F** and **Supplementary Video S11**). Furthermore, we found that TECPR2-depleted cells show a dramatic decrease in early endosomal recruitment of the Arp2/3 complex (as assessed by visualization of the p16-Arc subunit), which, upon activation by the WASH complex, mediates branched actin polymerization on early endosomes (**Fig. 8G-I**).

As suggested by the reviewer, we have now measured the total levels of p16-Arc in control and TECPR2-depleted cell lysates. As shown in the new **Supplementary Fig. S8B**, we did not observe any change in total levels of p16-Arc, indicating that TECPR2 regulates the

recruitment of p16-Arc on early endosomes, while its protein levels remain unaffected. In line with reduced localization of actin nucleation machinery on early endosomes, we also observed reduced labeling of phalloidin (that binds to actin filaments) on early endosomes in TECPR2-depleted cells (**Fig. 8G**). As suggested by the reviewer, we have now measured the overall actin filament density in control and TECPR2-depleted cells by measuring the CTCF (corrected total cell fluorescence) of phalloidin. As shown in the new **Supplementary Fig. S8C**, there was an approximately 1.7-fold decrease in phalloidin CTCF upon TECPR2 depletion, reinforcing that TECPR2 regulates actin nucleation on early endosomes.

Previous studies have shown that the heterodimeric actin capping protein CapZ (a constitutive heterodimer of two closely related subunits, α and β) interacts with Rab5 and Rab5 GEF, Rabaptin-5, and Rabex-5. CapZ was preferentially localized to early endosomes but not on recycling endosomes or late endosomes (Wang D et al., *eLife* 2021, PMID: 34796874). Taking this study into consideration, we employed GFP-tagged CapZ β as a probe for visualizing actin density on early endosomes and found GFP-CapZ β to be an excellent probe for visualizing endosomal actin in live cells. We consistently observed cytosolic localization of CapZ β in TECPR2-depleted cells, as also reflected by its significantly reduced punctae number (**Fig. 8J-K** and **Supplementary Video S12**). A potential hypothesis that explains our data is that CapZ dynamically associates with early endosomes by binding to Rab5 and to the barbed ends of branched actin filaments. As Rab5 levels and WASH and ARP2/3-mediated actin nucleation on early endosomes are impaired upon TECPR2 depletion, this leads to reduced CapZ localization on early endosomes. Importantly, the current literature does not define the mechanism of CapZ recruitment to early endosomes, and such a study would require a detailed investigation of various domains and interaction partners of CapZ, which is beyond the scope of this manuscript.

It is worth noting that most phenotypes observed in TECPR2-depleted cells, such as reduced actin on early endosomes, enlarged and perinuclear early endosomes, and impaired integrin recycling, are observed in cells with loss or depletion of the WASH complex (Derivery et al., 2009, *Dev Cell* PMID: 19922875; Gomez et al., *MBoC* 2012, PMID: 22718907; Zech T et al., 2011 *JCS*, PMID: 22114305). In contrast, cells lacking the actin capping factor (such as upon gene knockout of the CapZ β) have the opposite phenotype to depletion of TECPR2 or WASH, i.e., increased actin density on early endosomes, accumulation of small and peripheral early endosomes, and increased cell size (Wang D et al., *eLife* 2021, PMID: 34796874). Thus, it appears that loss of CapZ from endosomal membranes to cytosol (see **Supplementary Video S12**) as observed in TECPR2-depleted cells does not mimic a complete loss of CapZ function. Rather, an increase in CapZ cytosolic localization may lead to sequestration of WASH subunits in the cytosol (as CapZ interacts with the WASH complex- Jia D et al., *Proc Natl Acad Sci U S A.* 2010, PMID: 20498093) and to secondary defects on actin polymerization on early endosomes. These possibilities will need to be investigated in future studies and are not the focus of the current work.

Once again, we thank all the reviewers for their valuable comments and suggestions pertaining to our manuscript.