Extended Data Figure Legends

Extended Data Fig.1 Axonal degeneration in NHE6-null rat brains.

a, Representative images of the brain sections from littermate WT and NHE6-null rat brains at 2 and 6 months. Brain sections were stained with SMI32 (axonal damage marker, grey). The increased SMI32 staining was detected in the 6-month NHE6-null rat brain section.

b, Representative electron microscopy images of the corpus callosum from littermate WT and NHE6-null rat brains at 6-7 weeks. The total number of axons, and the number of myelinated, demyelinated, and degenerating axons for each image were counted. Means from each independent experiment (big dots, WT = 3, Null = 3 animals) overlay the entire dataset (small dots, 5 images per each animal) and used for statistical analysis. Animals from same litters are color-coded. Scale bar, 2 μm. Two-way ANOVA with Tukey's HSD was performed. Data are represented as mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

Extended Data Fig.2 Rab5-positive early endosome defects were not detected in NHE6-null rats.

a, Representative images from the corpus callosum and cortex sections of littermate WT and NHE6-null rat brains at 2 months. Sections were stained with Rab5 (early endosomal marker, magenta). NeuN was used as a neuronal marker (green). No differences in size and number of enlarged Rab5 were detected between WT and NHE6-null rat brains. The number of swollen/enlarged Rab5 (diameter bigger than 1.5 μm) was divided by the total number of Rab5 puncta (%). Also, the average size of Rab5 puncta was measured. Means from each independent experiment (big dots, WT = 3, Null = 3 animals) overlay the entire dataset (small dots, 5 sections per each animal) and used for statistical analysis. Animals from same litters are color-coded. Two-tailed unpaired t-test with Welch's correction was performed. Scale bar, 10 μm.

b, Representative snapshots and kymographs from primary littermate WT and NHE6-null neurons show the movement of mEmerald-Rab5. Retrograde direction is from left (terminus) to right (cell body). Scale bar, 2 μm. There were no significant differences in the number of anterograde, retrograde, and stationary Rab5 endosomes in primary WT and NHE6-null neurons. Means from each animal (big dots, WT = 3, Null = 3 pups) overlay the entire dataset (small dots, n = 18-20 neurons from four independent experiments) and used for statistical analysis. Animals from same litters are color-coded. Ordinary one-way ANOVA with Tukey's HSD was performed.

c, Speed of anterograde and retrograde mEmerald-Rab5 endosomes in primary WT and NHE6-null neurons. Stationary Rab5 endosomes were not included for the calculation. There were no differences in speed.

d, RILP pull-down assay with Rab5. There were no differences in the amount of RILP-bound Rab5 (GTP-Rab5 pull-down) between WT and NHE6-null rat brains at 2 months. Lysates from WT and NHE6-null rat brains were incubated with His-RILP recombinant proteins for the pull-down assay. Once samples were eluted, Rab5 was detected by western blot. The densitometry of GTP-Rab5 was divided by the total Rab5 for the quantification (4 rat brains for each genotype). Two-tailed unpaired t-test with Welch's correction was performed.

e, GTP agarose assay with Rab5. There were no differences in the amount of GTP-bound Rab5 between littermate WT and NHE6-null rat brains at 2 months. Lysates from WT and NHE6-null rat brains were incubated with GTP agarose to enrich GTP-bound protein fractions. Rab5 was detected in the GTP-enriched fraction by western blot. The densitometry of GTP-bound Rab5 was divided by the total Rab7 for the quantification (3 rat brains for each genotype). Two-tailed unpaired t-test with Welch's correction was performed.

f, *In vitro* Rab5 GTPase assay shows that TBC1D5 does not efficiently hydrolyze Rab5. Recombinant Rab5 protein was pre-loaded with GTP. GTP-Rab5 was incubated with various concentration of recombinant TBC1D5. After the reaction, the phosphate release was measured. Three independent experiments were performed. Ordinary one-way ANOVA with Tukey's HSD was performed. Data are represented as mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

Extended Data Fig.3 Late endosomal pH measurement and speed and size of Rab7 endosomes.

a, The representative images from WT and NHE6-null neurons for late endosomal pH measurement. The late endosomal pH is decreased in primary NHE6-null neurons compared to WT neurons. Primary hippocampal neurons from WT and NHE6-null rat brains were transduced with Rab7-RFP (red) to label late endosomes at DIV 2. At DIV 4, neurons were loaded with pH-sensitive Oregon green-dextran (green) and pH-insensitive Alexa647-dextran (blue). Yellow arrows indicate Rab7-positive-late endosomes loaded with Oregon green-dextran and Alexa647-dextran. Each dot indicates each pup (WT = 14, Null = 13 pups, n = 10,00-4,000 neurons imaged

from each pup). Animals from the same litters are color-coded. Two-tailed unpaired t-test with Welch's correction. Scale bar: 10 μm.

b, The speed of Rab7 endosomes. Stationary Rab7 endosomes were not included for the calculation. Primary neurons from WT and NHE6-null rats were transfected with mEmerald-Rab7. 4 independent experiments from 4 different litters were conducted (WT = 5, Null = 5 pups). Two-way ANOVA with Tukey's HSD was performed.

c, Representative confocal images of primary neurons from WT and NHE6-null rat brains. Primary neurons were transfected with mEmerald-Rab7 a day prior to the imaging. The size of mEmerald-Rab7 in NHE6-null neurons was larger than that of WT neurons. Scale bar, 5 μm.

d, Quantification of % swollen mEmerald-Rab7 in primary WT and NHE6-null neurons. The number of swollen mEmerald-Rab7 puncta was divided by the total number of mEmerald-Rab7 puncta found within the 50 μm from the AIS. Rab7 endosomes were considered to be swollen if mEmerald-Rab7 puncta's diameter size is larger than 1.5 μm. Two-tailed unpaired t-test with Welch's correction was performed.

e, Quantification of the number of swollen mEmerald-Rab7 per μm in primary WT and NHE6-null neurons. The number of swollen mEmerald-Rab7 was divided by 50 μm, to present how many swollen mEmerald-Rab7 exists per μm. Two-tailed unpaired t-test with Welch's correction was performed.

f, Size of endogenous Rab7 is larger in primary NHE6-null neurons than WT neurons (WT = 5, Null = 6 animals). The data was obtained in high-content imaging. Two-tailed unpaired t-test with Welch's correction was performed. Data are represented as mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

Extended Data Fig.4 Decreased active GTP-bound Rab7 in NHE6-null rat brains.

a, Lysates from NHE6-null and WT littermate rat brains at 2 months were incubated with His-RILP recombinant proteins with non-hydrolysable GTPγS analog or GDP for the pull-down assay. NHE6-null rat brain lysates incubated with GTPγS showed the decreased RILP-bound Rab7, while NHE6-null rat brain lysates incubated with GDPβS showed the increased (3 rat brains for each genotype). Two-tailed unpaired t-test with Welch's correction.

b, The increase in cytosolic Rab7 and TBC1D5 was detected in NHE6-null brain lysates compared to WT. Through the series of centrifugations, post-nuclear supernatant (PNS),

cytosolic fraction, and membrane fraction were harvested from lysates of WT and NHE6-null rat brains at 2 months. The following markers were used: vinculin (cytosolic marker), NHE6 (membrane marker), GAPDH (cytosolic/membrane, loading control). Two-way ANOVA with Tukey's HSD was performed for both quantifications. Data are represented as mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

c, Yeast two-hybrid assay validated the interaction of NHE6 with TBC1D5 through its cytoplasmic tail. The positive control grew normally on plates made with SD medium without Trp and Leu (referred as to -Trp/-Leu) and plates with SD medium without Trp, Leu, His, and Ade (referred as to -Trp/-Leu/-His/-Ade). The negative control grew only on -Trp/-Leu plates. The bait plasmids and prey plasmids were co-transformed, and they grew on -Trp/-Leu plates, indicating that the co-transformation was successful. However, on plates without Trp, Leu, and His (referred as to -Trp/-Leu/-His) and on -Trp/-Leu/-His/-Ade plates, the bait #1 (NHE6, cytosolic loop between transmembrane domain 7 and 8) and prey protein (a full length human TBC1D5) were not activated, indicating no interaction. In contrast, the bait #2 (NHE6, cytoplasmic tail) and the prey were activated and grown on both plates, indicating the interaction. Positive control (pGBKT7-p53+pGADT7-T), negative control (pGBKT7-lam+pGADT7-T), bait #1 pGBKT7-bait1(NHE6, 345-371 a.a.), bait #2 (NHE6, 536-701 a.a.), prey (a full length human TBC1D5).

d, Computational analysis with the molecular mechanics/Poisson–Boltzmann surface area method[70] predicted that F351 and Y610 of NHE6 directly interact with TBC1D5. Both residues contribute to binding, with Y610 showing the most negative free energy, suggesting it plays a dominant role in stabilizing the NHE6–TBC1D5 interaction.

Extended Data Fig.5 Validation and characterization of the NHE6 exchanger-defective (NHE6-ED) mouse line.

a, Alignment of amino acid sequences of human mouse NHE6 from a part of the transmembrane domain. The glutamic acid [E] at position 235 in mouse NHE6 corresponds to the glutamic acid [E] at position 255 in human NHE6 (both in red font). The aspartic acid [D] at position 240 in mouse NHE6 corresponds to the aspartic acid [D] in human NHE6 260 (both in red font).

b, Schematic of the targeted exon of mouse *Slc9a6* (NHE6). The target sequence for CRISPR/Cas9 genome editing is displayed (wild-type (WT), top; NHE6 E235Q/D260N,

bottom). Red font indicates the E235Q point mutation (G > C) and the D260N mutations (G>A, C>T). The targe sequences of guide RNA and the protospacer adjacent motif (PAM) are underlined.

c, Sanger sequences of WT and NHE6-ED mutant mice. Genomic DNA sequence, isolated from mice tail biopsy, presents the sequence in the WT mice and the substitution on the corresponding positions. The presence of the (G > C) point mutation along with (G>A & C>T) mutations were confirmed in samples from NHE6-ED mouse lines.

d, Western blot of mouse brain lysates from WT and NHE6-ED mutant mice. The expression of NHE6 was detected in brain lysates from both WT and mutant mice for the NHE6-ED mouse line. Quantification of NHE6 densitometry was performed separately for bands at 70 kDa, indicating monomer form of NHE6 and 140 kDa, indicating dimer forms of NHE6. NHE6 expression was normalized to actin. No significant difference in NHE6 levels was detected between WT versus mutant mice. Each dot indicates each animal (WT = 4, Null = 4 pups, n = 10,000-14,000 neurons imaged from each pup). Two-tailed unpaired t-test with Welch's correction was performed.

e, Colocalization of Rab7-labeled late endosomes (magenta) and NHE6 (cyan) in primary neurons from littermate WT and NHE6-ED mouse hippocampi. The quantification showed the Mander's coefficient (NHE6 overlapping Rab7) from the high-content imaging. No significant difference was detected. Each dot indicates a mean of each pup from 3 different litters (WT = 6, Null = 6 pups, n = 10,000-14,000 neurons imaged from each pup). Two-tailed unpaired t-test with Welch's correction was performed. Scale bar, 5 μm.

f, Size of endogenous Rab7 is larger in primary NHE6-ED neurons than WT neurons (WT = 10, Null = 11 animals). The data was obtained in high-content imaging. Two-tailed unpaired t-test with Welch's correction was performed. Data are represented as mean ± SEM.

g, Immunoprecipitation from WT, NHE6-ED and NHE6-null mouse brain lysates shows the interaction of NHE6 with TBC1D5. No significant differences were detected between WT and NHE6-ED brains. Brains were immunoprecipitated with anti-NHE6 antibody and immunoblotted against NHE6 and TBC1D5 (n = 3 animals per each genotype). NHE6-null mouse brains were used as a negative control. The immunoprecipitated TBC1D5 is divided by the immunoprecipitated NHE6 for the quantification. Ordinary ANOVA with Dunnet's test was performed.

h, Speed of anterograde and retrograde Rab7 endosomes in primary WT and NHE6-ED neurons. No significant differences were detected between WT and NHE6-ED neurons. Two-tailed unpaired t-test with Welch's correction was performed. Data are presented as the mean ± SEM. Data are represented as mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

Extended Data Fig.6 Intrinsic hydrolytic activity and TBC1D5-accelerated hydrolytic activity of Rab7 at pH 4.5-8.5.

a, Initial rate of intrinsic GTP hydrolysis on Rab7 as a function of pH. In the absence of *Caenorhabditis elegans* TBC1D5 (RBG-3), the rate of intrinsic GTP hydrolysis of Rab7 was very slow ($6x10^{-6}$) and barely changed in the pH range 4.5-8.5.

b, Intrinsic tryptophan fluorescence time courses of Rab7-GTP hydrolysis in the absence and presence of the *Caenorhabditis elegans* TBC1D5 orthologue (RBG-3) at pH 4.5-8.5. Solid lines are fits of the global exponential model to the experiment data. This assay was repeated for H166N and H166S mutants in the pH range 4.5-8.5 to determine k_{obs}. H166 of worm TBC1D5 orthologues corresponds to H207 of human TBC1D5.

c, Normalized PDFs as a function of the fixed parameter values. Symbols and lines represent observed probability density values and fitted Gaussian models, respectively. Tabulated values correspond to the pH range analyzed and maximum likelihood values for the mean and standard deviation (SD) of the fitted Gaussian model.

d, Parameter values for fits to 100 Monte Carlo synthetic data sets generated with the maximum likelihood parameters from the fits to the experimental data calculated at the same pH values as the experimental data and combined with Gaussian noise having a standard deviation (SD) equal to that of the experimental data. Tabulated values correspond to the pH range, mean, and SD of the fitted parameter values.

e, Normalized PDFs as a function of the fixed parameter values for a simultaneous fit in which the pK_a was treated as a global parameter whereas the constant and amplitude were treated as local parameters. Symbols and lines represent observed probability density values and fitted Gaussian models, respectively. Tabulated values correspond to the pH range analyzed and maximum likelihood values for the mean and standard deviation (SD) of the Gaussian model.

Extended Data Fig. 7: Mathematical modeling of local pH within the NHE6-TBC1D5-Rab7 complex

a, Proton diffusion pathway through the NHE6-TBC1D5-Rab7 complex. Proton diffusion domain (pink cylinder) overlaid on model of the TBC1D5 (green, teal)/Rab7 (gray, light gray)/NHE6 (violet, cyan) dimeric complex. Unstructured loops that were predicted with low confidence by AlphaFold 3 were omitted from the model[1]. The approximate position of the membrane is indicated by light-gray planes. A possible proton path from the proton-releasing residue D260 in NHE6 to the proton sensor H207 in TBC1D5 is shown as red spheres (partially hidden inside the cylinder). H207 is located at 7.5 nm, while the total length of the cylinder is 9.5 nm. The cylinder starts inside NHE6 and ends at the surface of TBD1D5 (Image rendered with VMD[2]).

b, Mathematical modeling of local pH suggests the existence of an acidic microenvironment in the NHE6-TBC1D5-Rab7 complex. Local pH profile for the cylindrical diffusion model along the path axis (z in nm), assuming a kinetically limited transporter with a minimal pH of 5.5 near the cytosolic proton release site of NHE6 and bulk pH 7.2. The approximate position of H207 along the diffusion path is indicated where the estimated local pH is 6.15.

See the Supplementary Equation for more details of the modeling and calculations.

Extended Data Fig.8 Representative images of TBC1D5 staining after TBC1D5 shRNA transduction.

Transduction of TBC1D5 shRNA decreased the expression of TBC1D5 but did not affect the expression of mEmerald-Rab7 in primary neurons, as compared to scrambled shRNA control transductions where there was no effect on TBC1D5. Scrambled shRNA and TBC1D5 shRNA (MOI = 2) were transduced in primary neurons at DIV 2 and incubated for 72 hours. Primary neurons were transfected with mEmerald-Rab7 a day prior to the imaging, and stained with anti-mEmerald (stained with anti-GFP antibody, green), TBC1D5 (magenta), and MAP2 (blue; neuronal marker) antibodies. Scale bar, 5 μm.

Extended Data Fig.9 Knock-down of the Rab7 GAP TBC1D5 rescues endosomal phenotypes in NHE6-ED neurons.

a, Representative kymographs from primary littermate WT and NHE6-ED neurons with scrambled or TBC1D5 shRNA show the movement of mEmerald-Rab7 endosomes. Retrograde direction is indicated. Scale bar, 1 μm. The number of retrograde Rab7 endosomes in primary NHE6-ED neurons transduced with TBC1D5 shRNA was increased compared to NHE6-ED neurons transduced with scrambled shRNA. Also, the number of stationary Rab7 endosomes was decreased in primary NHE6-ED neurons transduced with TBC1D5 shRNA. Means from each animal (big dots, WT = 3, ED = 3 pups) are overlay the entire dataset (small dots, n = 20-33 neurons from three independent experiments) and used for statistical analysis. Animals from same litters are color-coded. Ordinary one-way ANOVA with Tukey's HSD.

b, The FRAP recovery rate of mEmerald-Rab7 in primary NHE6-ED neurons transduced with TBC1D5 shRNA exhibited significant increase relative to WT neurons. The recovery rate of mEmerald-Rab7 in NHE6-ED neurons transduced with TBC1D5 shRNA was comparable to WT neurons or NHE6-ED neurons with scrambled shRNA (4 pups in total for each genotype, n = 21-30 neurons from 3 experiments). Non-linear mixed model was conducted.

c, The increased endosomes-lysosomes fusion in primary NHE6-ED neurons transduced with TBC1D5 shRNA. Shown are still images at different time points from live imaging of the endosome-lysosome fusion assay on primary littermate WT and NHE6-ED neurons transduced with scrambled or TBC1D5 shRNA at different time points. The level of endosome-lysosome fusion is presented as the percentage fold change in overlap between the endosome label (magenta) and the lysosome label (green), from time point 0 for the same animal. Yellow arrows indicate the fusion events. (WT = 3, ED = 3 pups, n = 10,000-14,000 neurons imaged from each pup). Non-linear mixed model was conducted. Scale bar, 5 μm. Data are represented as mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

References

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2 Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J Mol Graph* **14**, 33-38, 27-38 (1996). https://doi.org/10.1016/0263-7855(96)00018-5