Supplemental material

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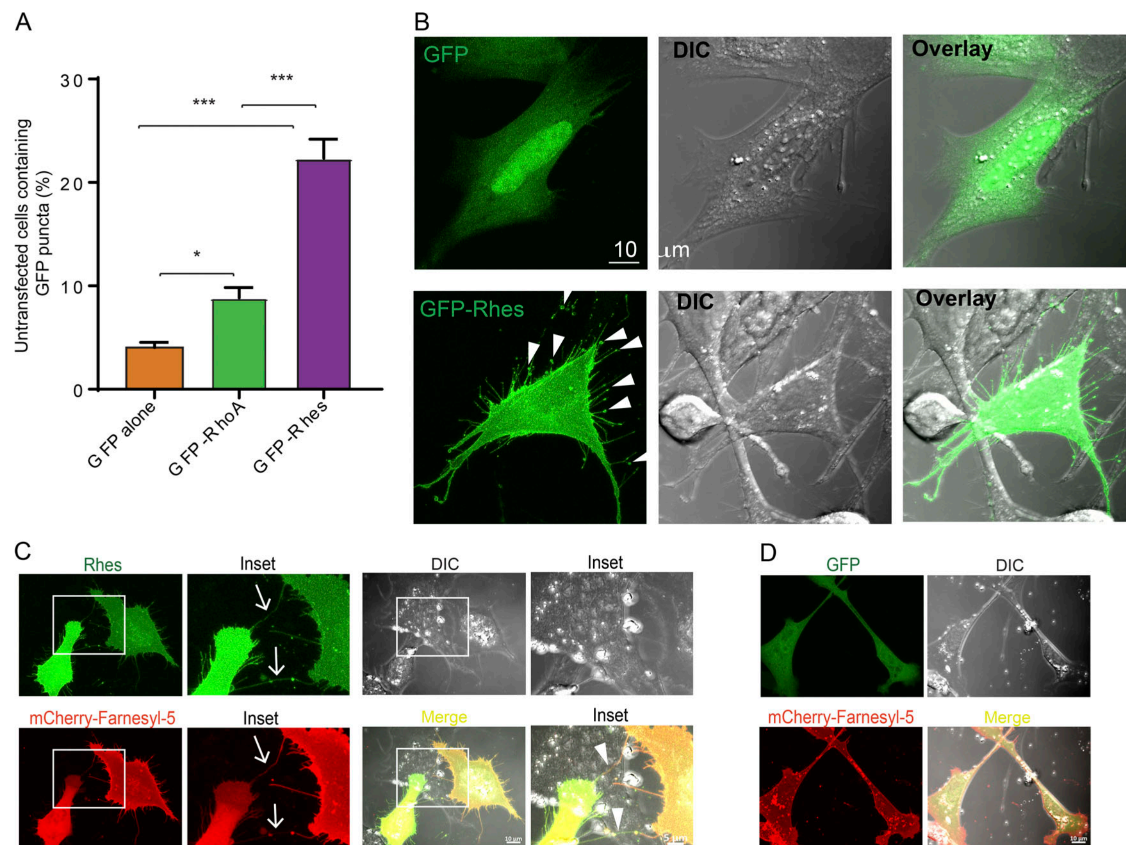

Figure S1. **Rhes induces TNT-like cellular protrusions, Rhes tunnels. (A)** Quantification of untransfected cells (%) containing GFP puncta from GFP (4.01 ± 1.80) or GFP-RhoA (8.84 ± 3.49) or GFP-Rhes (22.3 ± 6.43) transfected cells. Data are normalized to number of transfected cells and total number of cells (transfected and untransfected) per field. Bar graph shows mean ± SEM, one-way ANOVA (*, $P < 0.05$; ***, $P < 0.001$). GFP (n = 205), GFP-RhoA (n = 211), and GFP-Rhes (n = 207), total 12 fields/group. **(B)** Confocal and bright field (DIC) image of striatal neuronal cells expressing GFP alone (upper panel) or GFP-Rhes (lower panel). Arrowheads indicate Rhes-induced filopodia, with vesicles at their tip, similar to cytoneme in *Drosophila*, or TNTs in PC12, that are absent in GFP alone. **(C and D)** Confocal and bright field (DIC) image of striatal neuronal cells coexpressing GFP-Rhes (C) or GFP alone (D) with membrane marker mCherry-Farnesyl 5. Insets are shown at higher magnification with each panel. Arrows indicate Rhes-induced cellular protrusions that were absent in GFP alone–expressing cells.

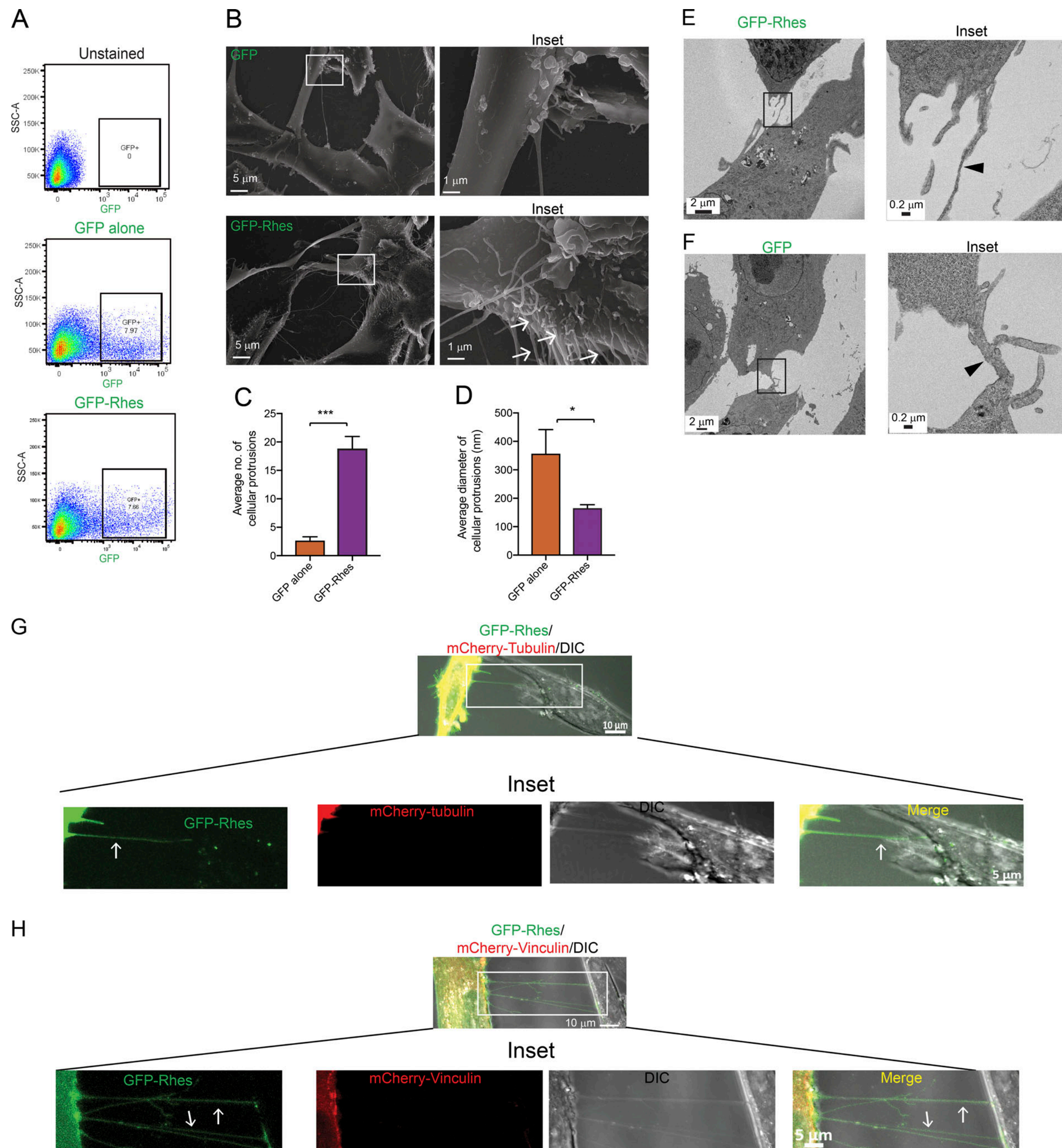

Figure S2. **Scanning EM and TEM images for Rhes-induced TNT-like cell protrusions and Rhes transportation to a neighboring cell. (A)** Representation of FACS plot of striatal neuronal cells sorted for unstained, GFP alone, or GFP-Rhes, and that were subsequently cultured for EM analysis. **(B)** Scanning EM images from FACS-sorted GFP alone or GFP-Rhes. Inset shows the boxed area at higher magnification. Arrows indicate numerous cellular protrusions in GFP-Rhes. **(C)** Bar graph represents quantification of average number of cellular protrusions per cell for GFP (2.72 ± 1.47) and GFP-Rhes (18.86 ± 5.16). **(D)** Bar graph shows the quantification of average diameter of cellular protrusions per cell or GFP (358.33 ± 203.51) and GFP-Rhes (166.67 ± 25.81) using scanning EM images. Data mean ± SEM; unpaired *t* test (*, $P < 0.05$; ***, $P < 0.001$; GFP: $n = 15$ cells; GFP-Rhes: $n = 13$ cells). **(E and F)** Rhes-induced TNT-like cell protrusions are negative for tubulin and vinculin. Representative TEM images from FACS-sorted GFP-Rhes (E) or GFP alone (F)–expressing cells. Magnified images are shown in insets from selected areas. Arrowheads indicate cellular protrusions that in GFP-Rhes are <150 nm and in GFP alone are >200 nm in diameter. **(G)** Confocal and bright field (DIC) image of striatal neuronal cells coexpressing GFP-Rhes and mCherry-Tubulin. Arrows indicate a Rhes-induced TNT-like process. **(H)** Confocal and bright field (DIC) image of striatal neuronal cells coexpressing GFP-Rhes and mCherry-Vinculin. Arrows show Rhes-induced TNT-like protrusions. Inset shows the boxed area at higher magnification.

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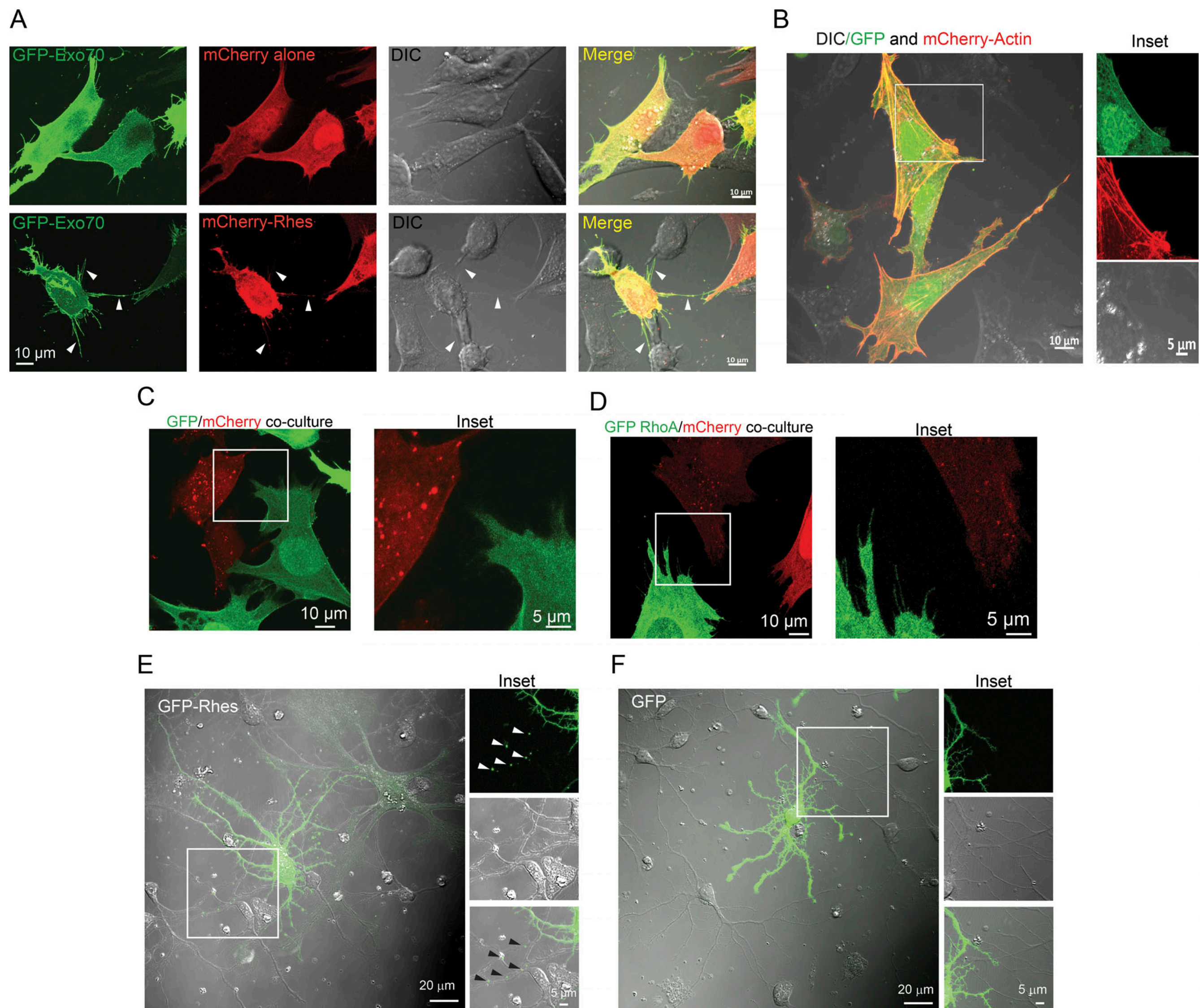

Figure S3. **Rhes-induced TNT-like cell protrusions are positive for Exo70. (A)** Confocal and bright field (DIC) image of striatal neuronal cells coexpressing mCherry alone or mCherry-Rhes with GFP-Exo70. Arrowheads indicate Rhes-induced TNT-like protrusions. **(B)** Representative confocal and DIC image of striatal neuronal cells coexpressing GFP alone and mCherry-Actin. Inset shows the boxed area at higher magnification. **(C and D)** FACS-sorted GFP (C) or GFP-RhoA (D) expressing striatal neuronal cells (green) were co-cultured with FACS-sorted mCherry cells (red). Magnified images are shown in inset from selected area. GFP-Rhes expressing primary striatal neurons show numerous puncta in the neighboring cells. **(E and F)** Representative confocal and bright field DIC image of primary striatal neuronal cells, transfected with GFP-Rhes (E) or GFP (F). Insets are shown at higher magnification within each panel. Arrowheads indicate GFP-Rhes puncta in the neighboring untransfected primary striatal neuron, but no such puncta were observed in GFP control cells.

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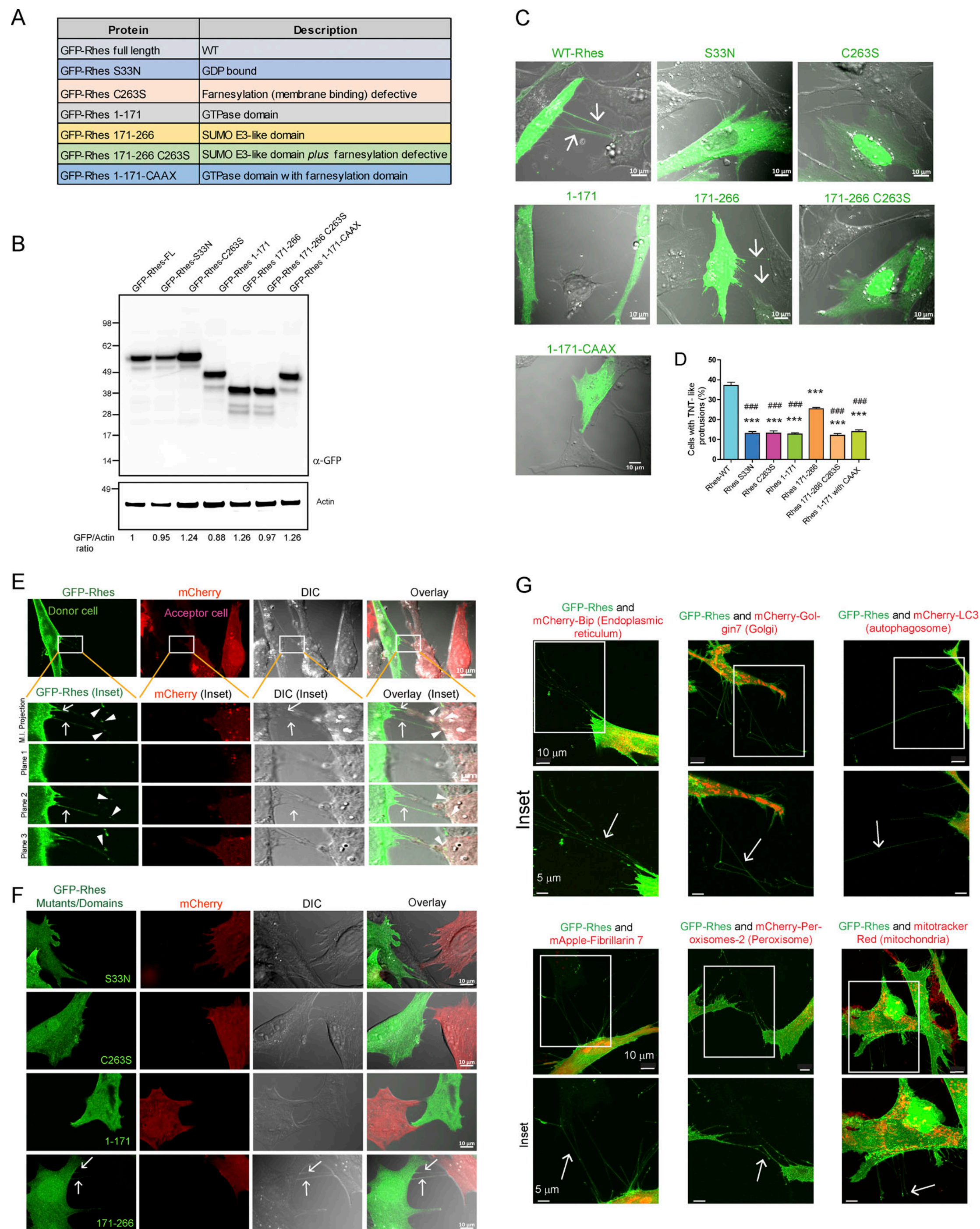


Figure S4. **The role of Rhes domain/mutants in production of Rhes-induced TNT-like cell protrusions. (A)** Rhes domains and Rhes mutants. **(B)** Western blot showing the expression of full-length GFP-Rhes WT or mutants or GFP-Rhes domains in striatal neuronal cells after 48 h of transfection. Membrane was blotted for anti-GFP antibody. Actin was used for loading control. GFP/Actin ratio was calculated by ImageJ software. **(C)** Representative confocal image of striatal neuronal cells expressing either GFP-tagged Rhes WT or its mutants. Arrows indicate TNT-like processes. **(D)** Quantification of the cells (%) with TNT-like protrusions for C. Bar graph shows mean ± SEM; one-way ANOVA (***, P < 0.001 compared with Rhes-FL; ###, P < 0.001 compared with Rhes 171–266). Data were quantified from four independent experiments, *n* = >200 cells/group. Cells exhibiting TNT-like protrusions more than 10 µm in length and <200 nm in diameter are included in quantification. Rhes WT (37.09 ± 3.43), Rhes S33N (13.03 ± 1.79), Rhes C263S (13.12 ± 2.36), Rhes 1–171 (12.68 ± 1.28), Rhes 171–266 (25.34 ± 1.40), Rhes 171–266 C263S (12.00 ± 2.01), and Rhes 1–171-CAAX (13.85 ± 1.99). **(E and F)** Assessment of various organelle markers in Rhes tunnels. Representative confocal and DIC image of FACS-sorted GFP-Rhes (E) or its mutants or domains (F) expressing striatal neuronal cells (donor cells) co-cultured with FACS-sorted mCherry cells (acceptor cells). Arrowheads indicate GFP-Rhes–positive vesicles in mCherry-expressing cells at different planes. Arrows indicate TNT-like protrusions induced by either GFP-Rhes or GFP-Rhes 171–266. **(G)** Representative confocal images of striatal neuronal cells expressing GFP-Rhes (green) and mCherry tagged (red) with the indicated cell organelle marker. Arrows indicate Rhes-induced TNT-like processes. For mitochondria staining, mitotracker was added after 48 h of GFP-Rhes transfection at 200 nM concentration, incubated at 37°C for 30 min, and imaged using confocal microscopy. Inset shows the boxed area at higher magnification.

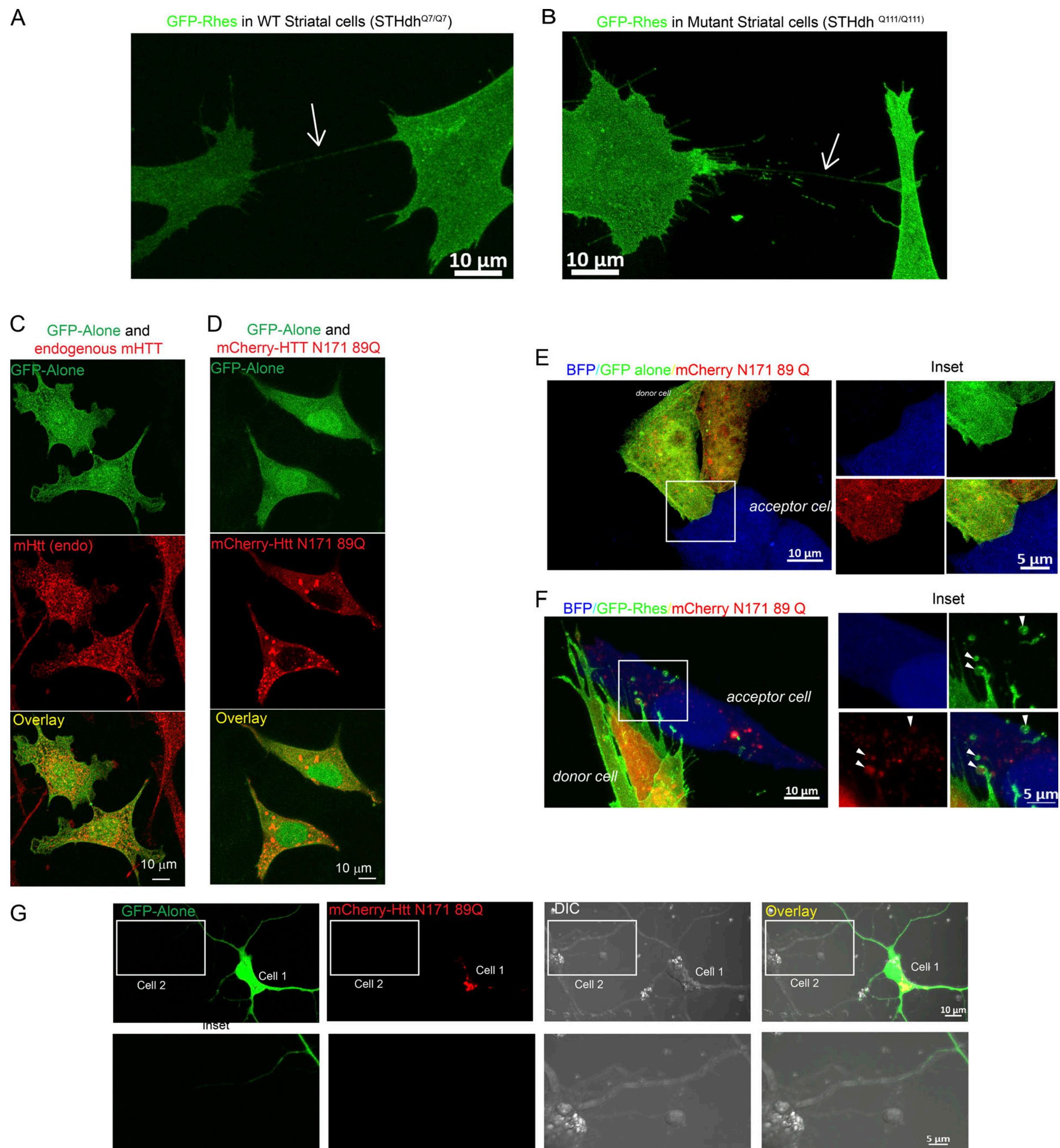


Figure S5. **mHTT N171-89Q transportation via Rhes-induced TNT-like Rhes tunnels. (A and B)** Representative confocal image of (A) WT (STHdh$^{Q7/Q7}$) or (B) mHTT (STHdh$^{Q111/Q111}$) expressing striatal neuronal cells, transfected with GFP-Rhes. GFP-Rhes induces TNT-like processes in both the cells. Arrows indicate Rhes-induced TNT-like protrusions. **(C and D)** Representative confocal image of STHdh$^{Q111/Q111}$ neuronal cells (expressing endogenous FL-mHTT [endo]), transfected with GFP alone (C) and immunocytochemistry performed with MAB2166 HTT antibody. **(D)** Representative confocal image of STHdh$^{Q7/Q7}$ striatal neuronal cells expressing GFP alone and mCherry-HTT N171-89Q (mHTT). **(E and F)** Representative confocal image of FACS-sorted BFP expressing striatal neuronal cells (acceptor cell) co-cultured with (E) GFP alone + mCherry N171-89Q or (F) GFP-Rhes + mCherry N171-89Q (double-positive cells, donor cell). Arrowheads indicate mHTT colocalized with Rhes-positive vesicle-like structures in acceptor cells. Magnified images are shown in the inset from the selected area for each panel. **(G)** Representative confocal or DIC image of primary striatal neurons, cotransfected with GFP alone (green) and mCherry N171-89Q (mHTT; red). No apparent mHTT transport was observed. Insets are shown at higher magnification in each panel.

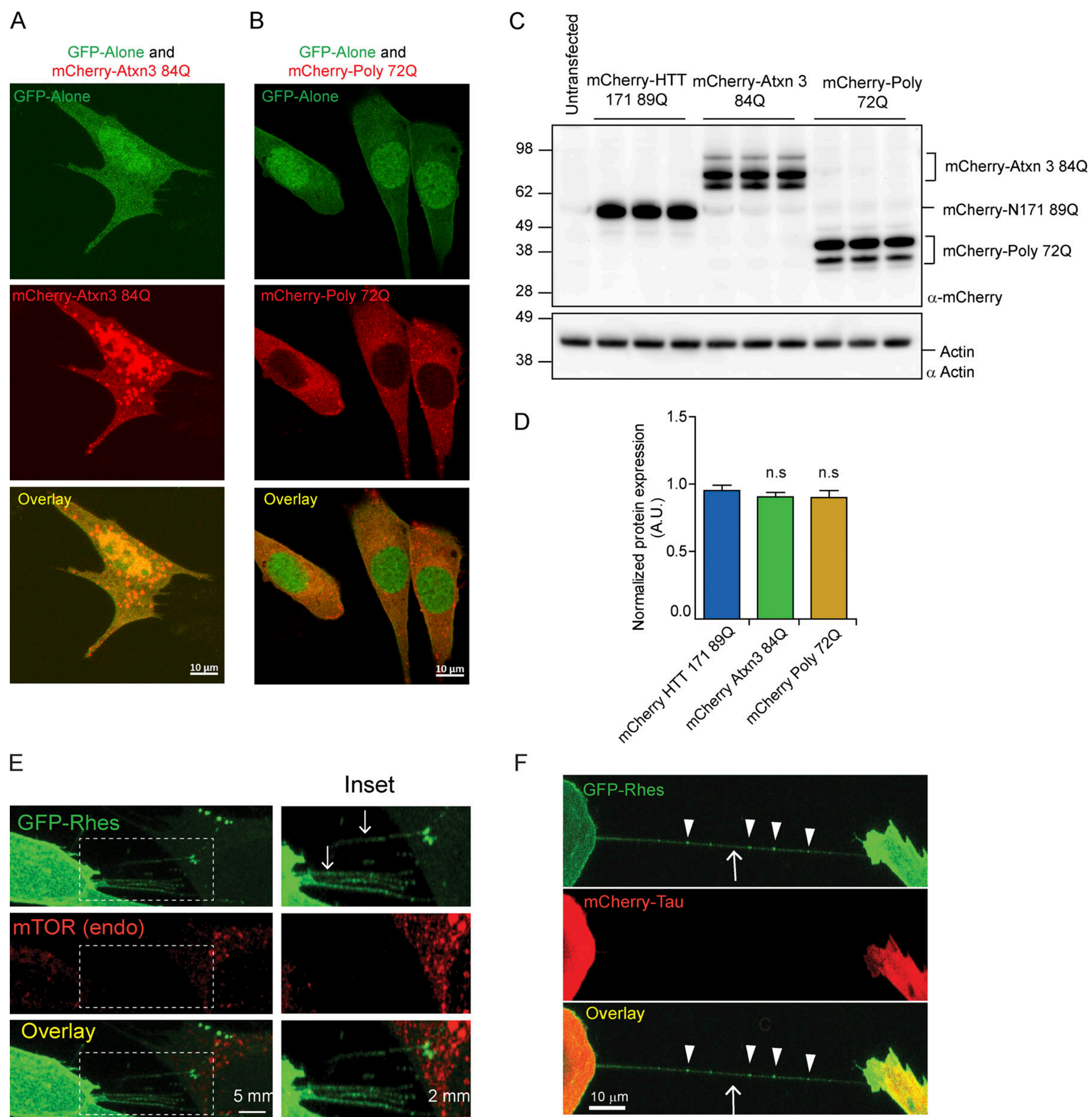
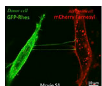
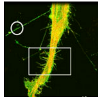


Figure S6. **Rhes tunnels are negative for mTOR and wtTau proteins. (A and B)** Representative confocal image of striatal neuronal cells, cotransfected with (A) GFP alone and mCherry-Atxn3 84Q, and (B) GFP alone and mCherry-Poly 72Q for 48 h. **(C and D)** Representative Western blot (C) showing the expression of mCherry-HTT 171 89Q or mCherry-Atxn3 84Q or mCherry-Poly 72Q in striatal neuronal cells after 48 h of transfection. Membrane was probed for mCherry antibody. Actin was used as loading control. **(D)** Quantification of the indicated proteins (normalized to actin). Bar graph shows mean ± SEM; one-way ANOVA (n.s, not significant), *n* = 3 independent experiments. **(E)** Representative image of striatal neuronal cells, expressing GFP-Rhes (green) and costained for mTOR by immunocytochemistry (red), as described in Materials and methods. Arrows indicate TNT-like protrusions. **(F)** Representative confocal image of striatal neuronal cells, cotransfected with GFP-Rhes and mCherry-Tau. Arrows indicate TNT-like protrusions, and arrowheads indicate GFP-Rhes–positive vesicles.

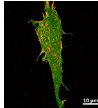
Figure S7. **The experimental design for various experiments and SUMO 1/2/3 protein depletion. (A)** Experimental design for FACS and co-culture experiments for Fig. 10, C–F. **(B)** Striatal neuronal cells, transfected with control CRISPR/Cas9 plasmid or SUMO1/2/3 CRISPR/Cas9 and sorted, then lysed to check SUMO1 depletion using anti-SUMO1 antibody. Western blot showing SUMO2/3 depletion in SUMO 1, 2, and 3–depleted cells. Actin was used as loading control. **(D and E)** Quantification of SUMO1 or SUMO2/3 protein depletion (normalized to actin) in SUMO1/2/3 KO cells versus control cells. Bar graph shows mean ± SEM; Student's *t* test (**, $P < 0.01$; ***, $P < 0.001$; $n = 3$ independent experiments). **(F)** Experimental design for FACS and co-culture experiments (Fig. 10, G–J).



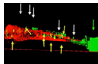
Video 1. **Striatal neuronal cells, positive for GFP-Rhes or mCherry-Farnesyl 5, were FACS-sorted and co-cultured (related to Fig. 2 B, inset, b1–b3).** The image demonstrates that the GFP-Rhes–induced TNT-like protrusion from the donor cell is connected to mCherry-Farnesyl 5 expressing the acceptor cell. Time-lapse imaging indicates the retraction of one of the TNT-like cellular protrusions, Rhes tunnels, after delivery of Rhes-positive vesicles to an acceptor cell. A total of four Z-stacks (planes) were acquired and presented as maximum intensity projection. Total time of the video file is 14 min. 29 cycles were collected at a rate of 29 s per cycle.



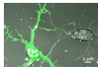
Video 2. **Striatal neuronal cells, coexpressing GFP-Rhes, and mCherry-actin were treated with DMSO for 8 h (related to Fig. 2 G).** Time-lapse imaging was performed, and a total of seven Z-stacks were collected and represented as maximum intensity projection for cells treated with DMSO. The length of the video is ∼27 min (29 cycles) and 15 min (14 cycles). The interval between each period is 57–60 s.



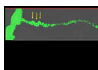
Video 3. **Striatal neuronal cells were exposed to or cytochalasin D (2 µg/ml) for 8 h (related to Fig. 2 G).** Time-lapse imaging was performed as in Video 2.



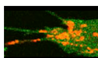
Video 4. **Deconvolved 3D animation of Fig. 3 D shows white arrows depicting GFP-Rhes vesicle-like puncta on the surface and yellow arrows depicting GFP-Rhes vesicle-like puncta inside the lumen of the cell.**



Video 5. **Time-lapse imaging was acquired in the primary striatal neuron, expressing GFP-Rhes (related to Fig 3 I).** GFP-Rhes–positive vesicle delivery to an adjacent cell (arrow in Video 6) and retraction of a TNT-like protrusion are shown in real time. A total of five Z-stacks were acquired and represented as maximum intensity projection. The length of the video is 13 min, and 22 cycles were collected with a rate of 36 s per cycle.



Video 6. **Animation of Video 5 (Fig. 3 I, inset) showing delivered vesicles (white arrow) and vesicles (yellow arrows) in the TNT-like Rhes tunnels.**



Video 7. **Time-lapse imaging was acquired in striatal neuronal cells, coexpressing GFP-Rhes and m-Cherry HTT 171 89Q (related to Fig. 6 E).** A total of four Z-stacks was collected and represented as maximum intensity projection. The length of the video is ∼7 min, and five cycles were acquired at a rate of 84 s per cycle.

Video 8. **Time-lapse imaging was acquired in striatal neuronal cells, co-cultured with FACS-sorted GFP-Rhes and m-Cherry HTT 171 89Q and FACS-sorted cell-brite cells (related to Fig. 6 F).** A total of four Z-stacks were collected and represented as maximum intensity projection. The video is 3 min; slow motion was applied after the 13th point (for the same video), using video software Movavi.