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Supplementary Information for

Rhes, a Striatal-Enriched Protein, Promotes Mitophagy Via Nix.

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Other supplementary materials for this manuscript include the following:

Movies S1

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Supplementary Figures

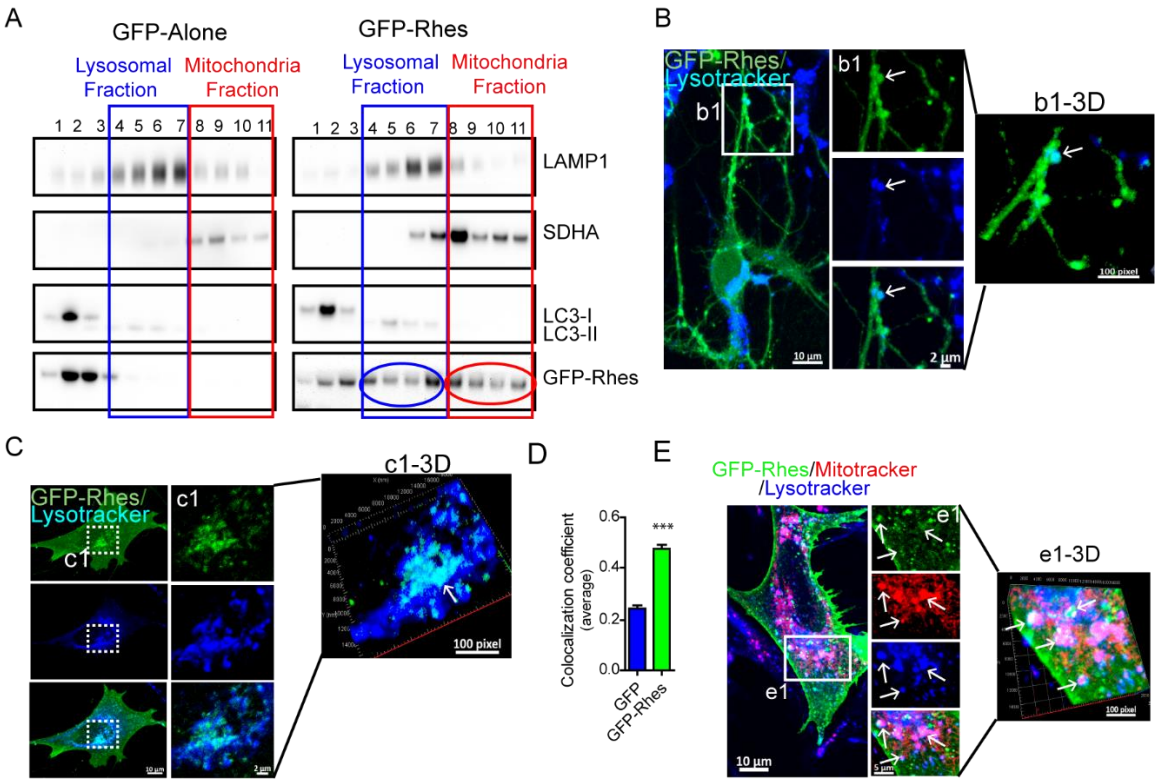

Fig. S1. Rhes associated globular mitochondria also positive for lysosomes. (A) indicating lysosomal (blue) and mitochondrial (red) proteins across different fractions. Representative confocal images (their respective insets or 3D rendered images) of primary striatal neuron (B), striatal neuronal cells (C) transfected with GFP-Rhes and co-stained with lysotracker (blue). Arrow represents the Rhes and lysosome colocalization. (D) Bar graph shows average Pearson's coefficient of colocalization (n=39-42 cells per group, Student's-t-test, ***p<0.001. Data mean ± SEM). (E) Confocal image, insets and 3D rendered image of striatal neuronal cells expressing GFP-Rhes co-stained with both mitotracker orange (red) and lysotracker (blue). Arrows indicates GFP-Rhes positive for mitochondria and lysosomes.

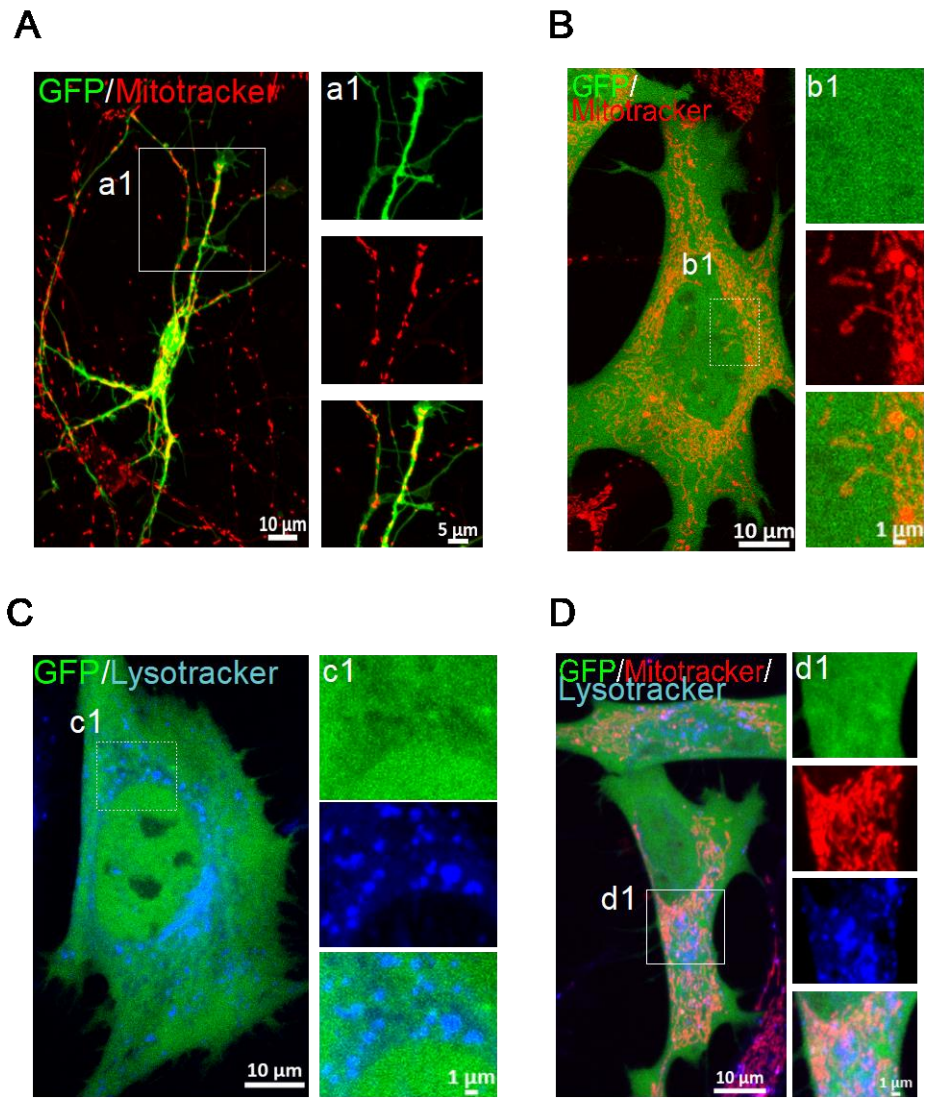

Fig. S2. GFP control does not interact with mitochondria or lysosome. (A) Representative confocal image of primary striatal neuron transfected with GFP vector. Cells were stained with mitotracker orange (red). a1 is inset from selected region. (B) Confocal image of striatal neuronal cells transfected with GFP. Cells were stained with mitotracker orange (red), b1 is inset from selected region. (C) Confocal image of GFP transfected striatal neuronal cell. Cells were stained with lysotracker (blue). c1 is magnified area from selected region. (D) Confocal image of striatal neuronal cells transfected with GFP vector. Cells were stained for mitotracker orange (red) and lysotracker (blue). d1 is magnified area from selected region.

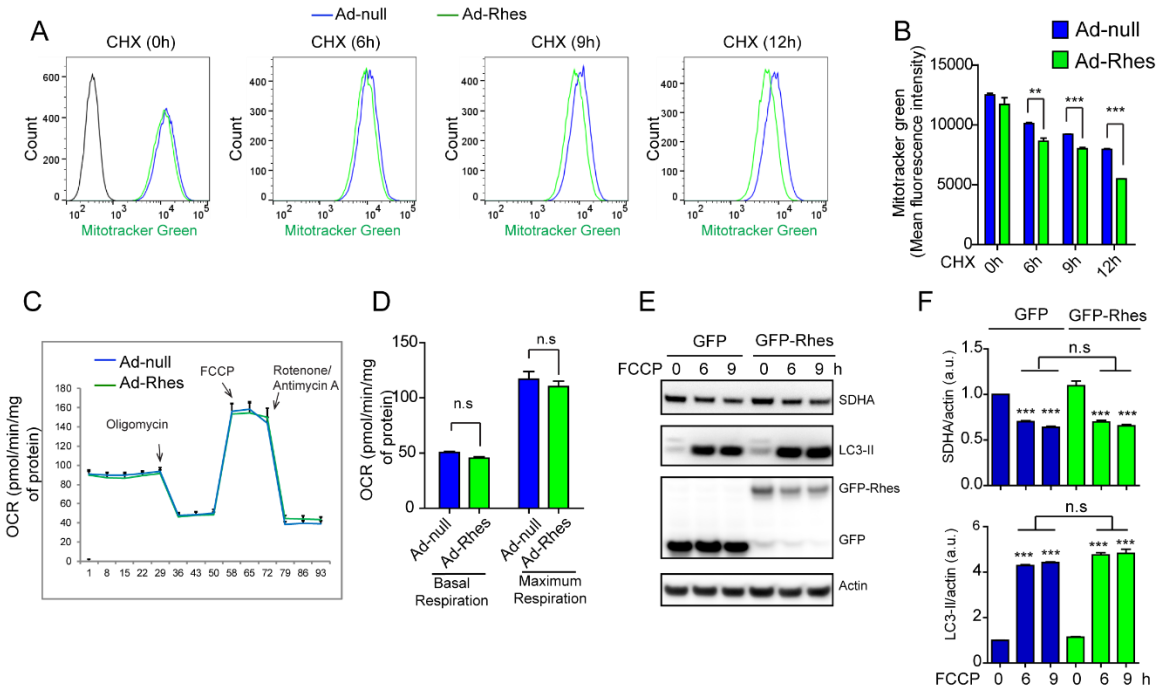

Fig. S3. Rhes promotes mitochondrial mass depletion in cycloheximide (CHX) chase experiment but does not affect mitochondrial respiration. (A) FACs plots indicating mitotracker green intensity in CHX chase experiment. Black histogram represents the unstained while blue and green indicates Ad-null and Ad-Rhes respectively. (B) Bar graph showing mean fluorescence intensity of mitotracker green for indicated samples in CHX (100 µM) treatment at indicated time points. (n=3, Two-way ANOVA-test followed by Bonferroni post hoc test, **p<0.01***p<0.001. Data mean ± SEM). (C) Seahorse assay in striatal neuronal cells infected with Ad-null or Ad-Rhes for 48hr to assess basal oxygen consumption rate (OCR) prior to any injections and after injection of the complex IV inhibitor oligomycin, the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and a combined injection of rotenone + antimycin A. (D) Bar graph shows the OCR at basal and maximum respiration for the indicated groups. (n = 6 per group). (E) Representative Western blot of indicated proteins and band intensity (F) from striatal neuronal cells expressing GFP or GFP-Rhes and treated with FCCP (1 µM) for the indicated time points. (n=3, One-way ANOVA test followed by Tukey post hoc test, ***p<0.001. n.s is not significant between indicated groups. Data mean ± SEM).

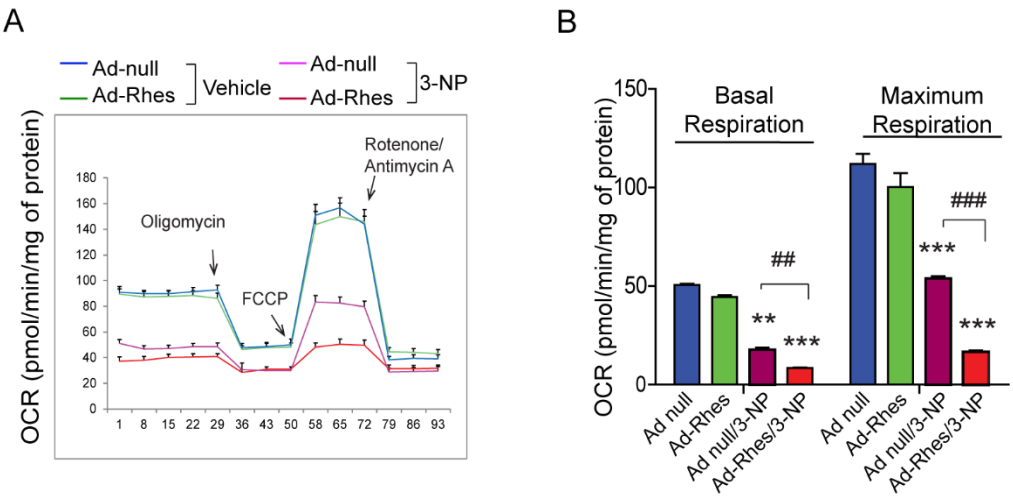

Fig. S4. Rhes affect mitochondrial respiration in presence of 3-NP. (A) Shows seahorse assay in striatal neuronal cells infected with Ad-null or Ad-Rhes viral particles with vehicle or 3-NP (10 mM for 2 hr). (B) Bar graph shows the OCR measurements at basal and maximum respiration among indicated groups. [**$p < 0.01$; ***$p < 0.001$, compared to Ad-null and vehicle treated; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$, between indicated groups, One-way ANOVA test followed by Tukey post hoc test ($n = 6$/group, data mean ± SEM)].

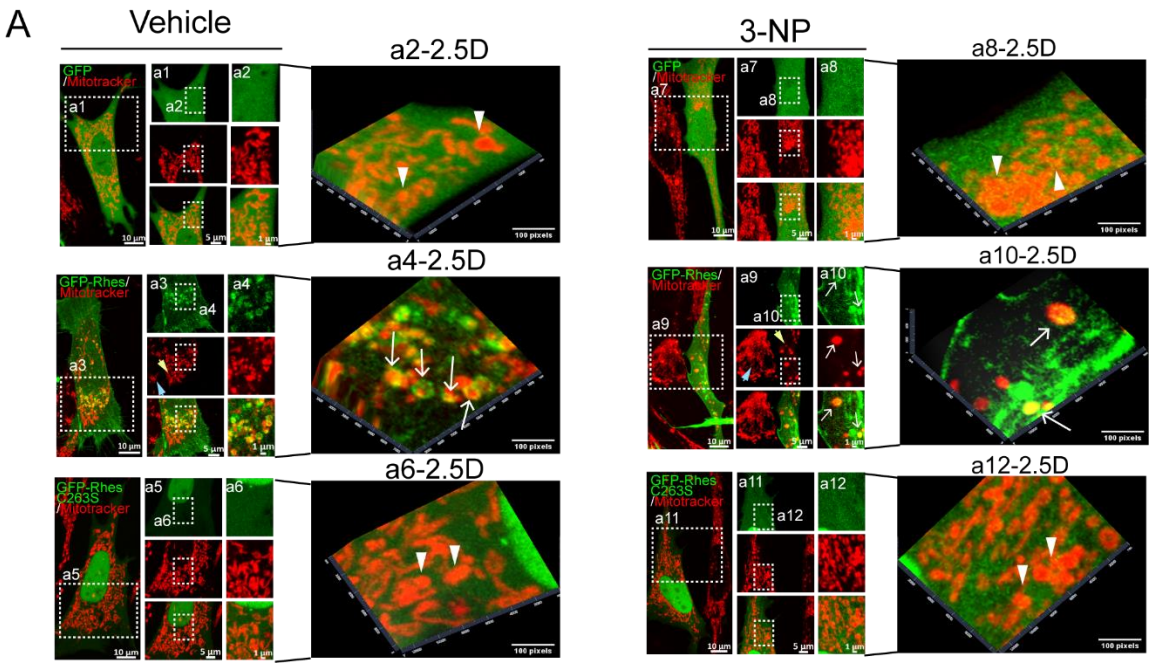

Fig. S5. Rhes affect mitotracker intensity in presence of 3-NP. (A) Representative confocal images of striatal neuronal cells transfected with GFP or GFP-Rhes or GFP-Rhes C263S treated with vehicle or 3-NP (2h and 10 mM) and corresponding insets or 2.5D rendered images. Arrows indicate globular mitochondria positive for GFP-Rhes. Yellow and blue arrowhead indicate GFP-Rhes transfected cell and untransfected cell respectively in the same field. White arrowheads show globular mitochondria negative for GFP or GFP-Rhes C263S.

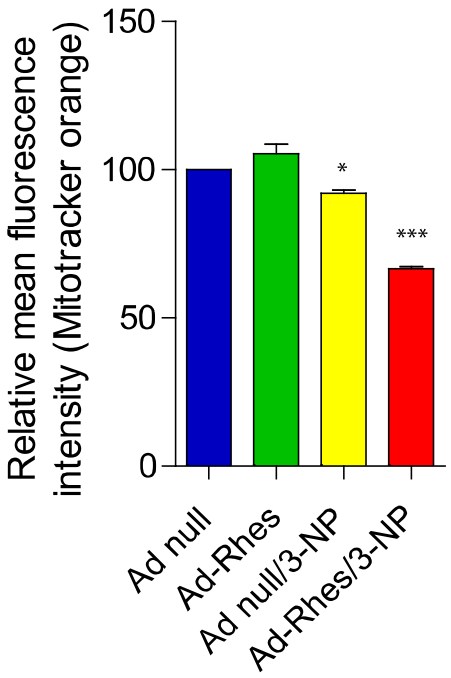

Fig. S6. Rhes expressing cells show reduced mitotracker intensity in 3-NP treatment. Bar graph shows relative mean fluorescence intensity of mitotracker orange in striatal neuronal cells infected with Ad-null or Ad-Rhes, treated with vehicle or 3-NP (10 mM for 2 hr). Statistical comparison was done between groups using One-way ANOVA followed by Tukey post hoc test (compared to Ad-null/vehicle) and significance values were set at *$p<0.05$, ***$p<0.001$. Data mean ± SEM.

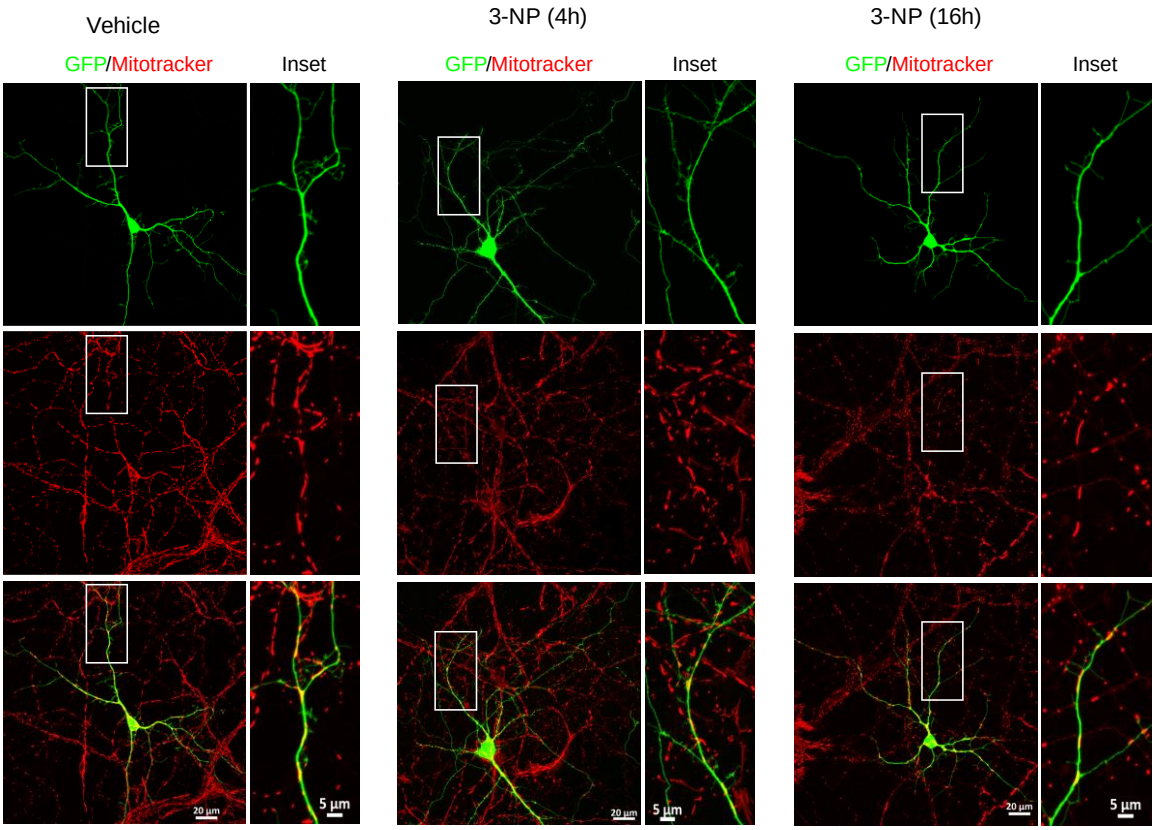

Fig. S7. GFP alone doesn't affect mitotracker staining in primary striatal neuron, treated with 3-NP. Confocal image of primary striatal neuron transfected with GFP vector, treated with vehicle or 3-NP (10 mM and for 4h or 16h). Corresponding insets show the magnified area from selected region.

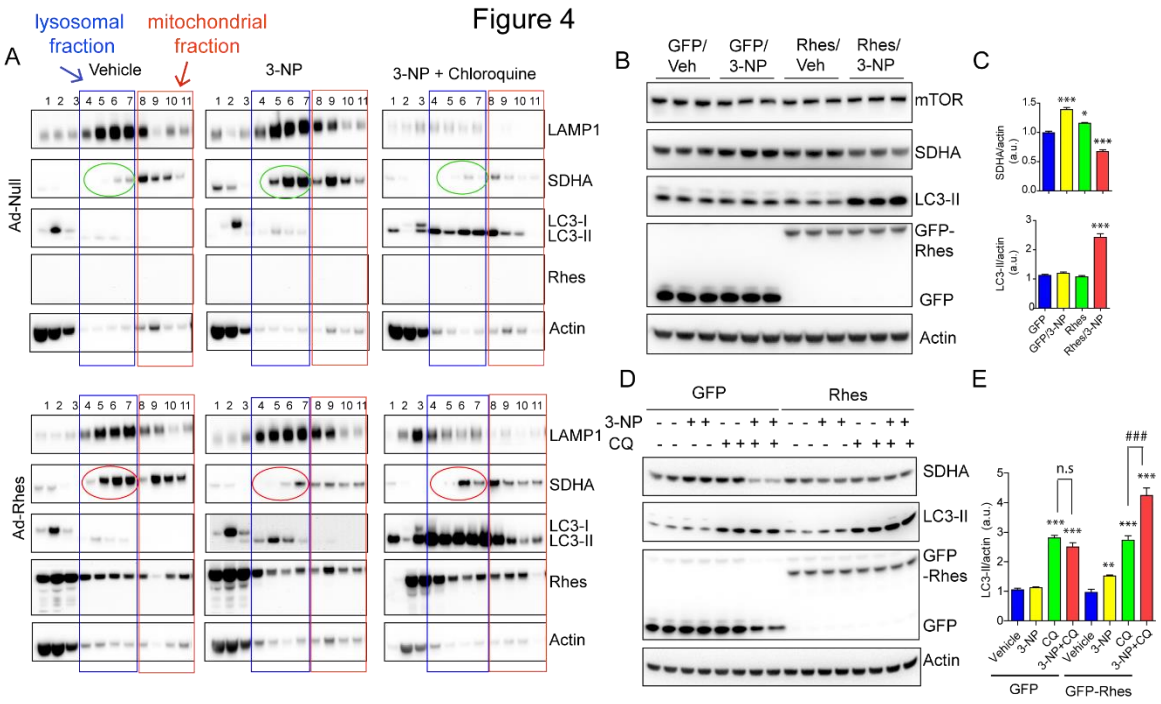

Fig. S8. Rhes increases the mitophagy flux. (A-E) Representative immunoblots of (A) sucrose gradient fractionations of striatal neuronal cells infected with Ad-null or Ad-Rhes or whole cell lysate (B and D) from GFP or GFP-Rhes transfected striatal neuronal cells treated with vehicle or 3-Nitropropionic acid (3-NP, 10 mM) for 2 h, with or without cholorquine (CQ, 50 µM), and probed for indicated proteins. (C and E) Bar graph shows the quantification of SDHA and LC3-II protein level normalized to actin from B and D. [(*$p < 0.05$; ***$p < 0.001$ compared to GFP/Veh (Vehicle) (n=6, Data mean ± SEM; One-way ANOVA test)].

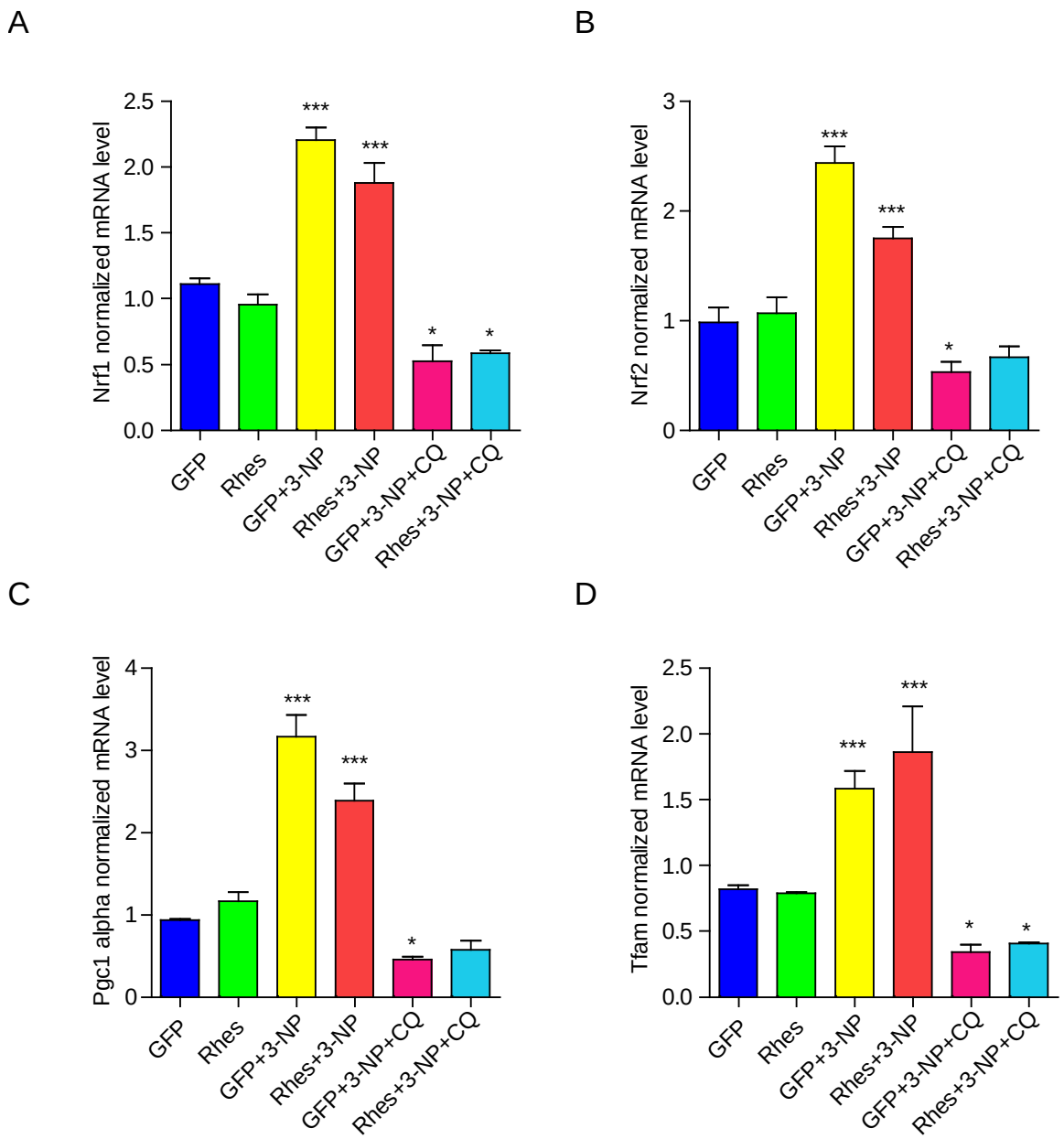

Fig. S9. 3-NP upregulates the expression of genes involved in mitochondrial biogenesis. (A-D) Bar graph showing the relative gene expression level of (A) Nrf1, (B) Nrf2, (C) Pgc1α and (D) Tfam in GFP or GFP-Rhes transfected striatal neuronal cells in vehicle or 3-NP (10 mM, 2h) or 3-NP + chloroquine (CQ) (50 µM) treatment. mRNA levels were normalized to Gapdh. Statistical comparison was done between groups using One-way ANOVA followed by Tukey post hoc test (compared to GFP/vehicle) and significance values were set at *p<0.05, ***p<0.001. Data mean ± SEM.

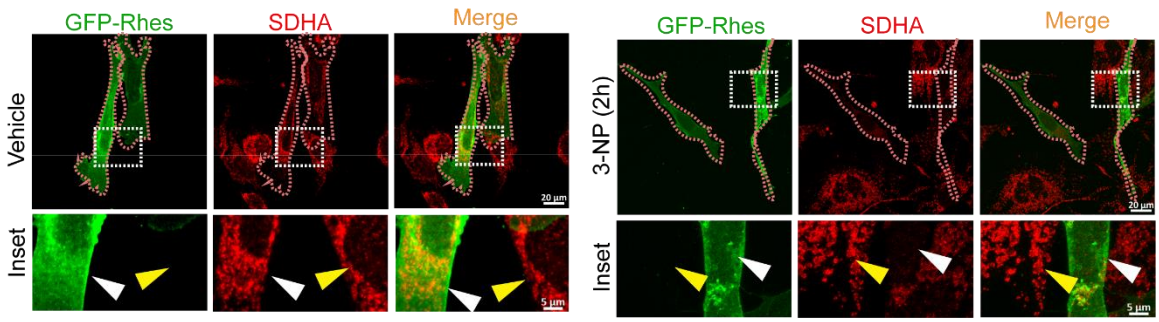

Fig. S10. Rhes diminishes SDHA signal in presence of 3-NP. Representative confocal image of striatal neuronal cells transfected with GFP-Rhes, treated with vehicle or 3-NP (10 mM, 2h) and stained for endogenous SDHA (a mitochondrial protein). GFP-Rhes transfected cell periphery was marked by dashed line (brown). Insets show the magnified region from the selected area. White arrowhead represents the GFP-Rhes transfected cell and yellow arrowhead shows the untransfected cell in the same area.

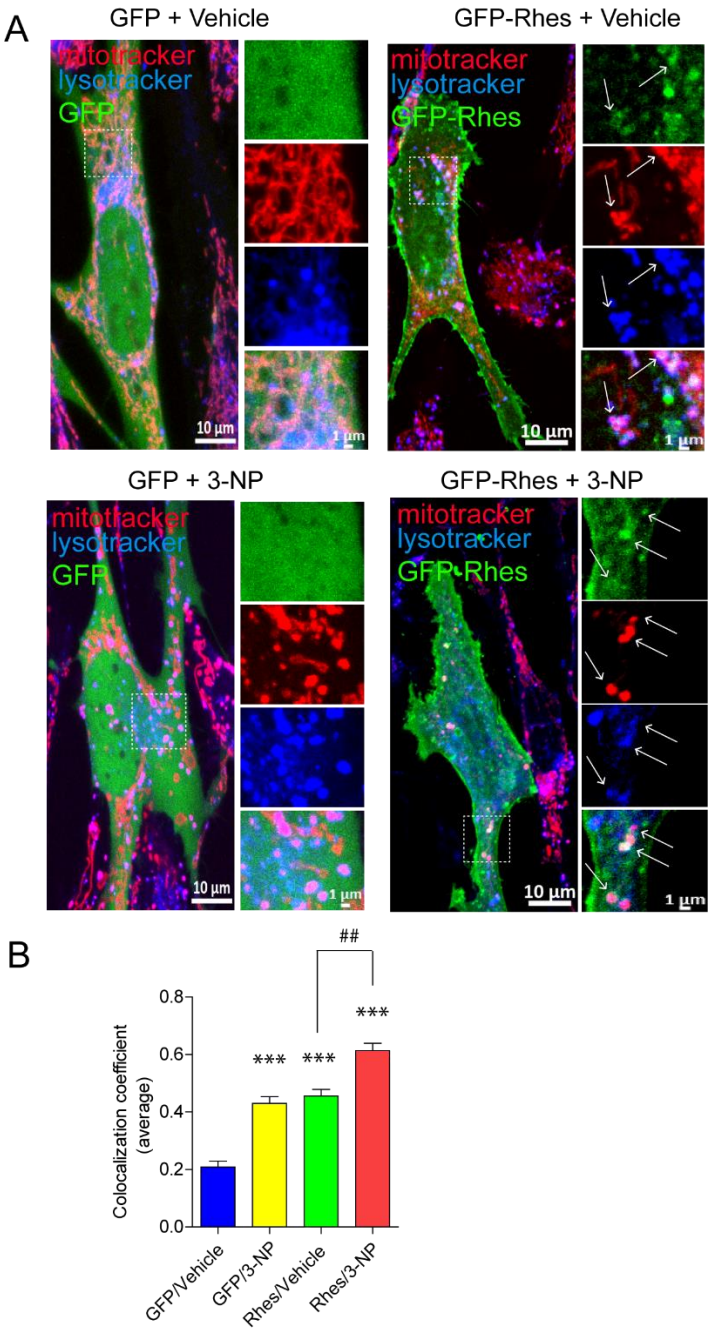

Fig. S11. 3-NP promotes lysosomal enrichment of mitochondria in both GFP or GFP-Rhes transfected cells. (A) Representative confocal image of striatal neuronal cells transfected with GFP or GFP-Rhes, treated with vehicle or 3-NP (10 mM for 2 hr). Cells were stained with mitotracker (Red) and lysotracker (Blue). Inset shows the magnified region from selected area and arrows represents the globular mitochondria positive for GFP-Rhes and lysotracker. (B) Bar graph shows the average of Pearson's coefficient of colocalization between mitotracker and lysotracker in GFP or GFP-Rhes transfected cells (n=21-28 cells per group, One-way ANOVA vs. GFP/vehicle; ***p<0.001. ##p<0.01 between Rhes/vehicle vs. Rhes/3-NP using Student's t-test. Data mean ± SEM.

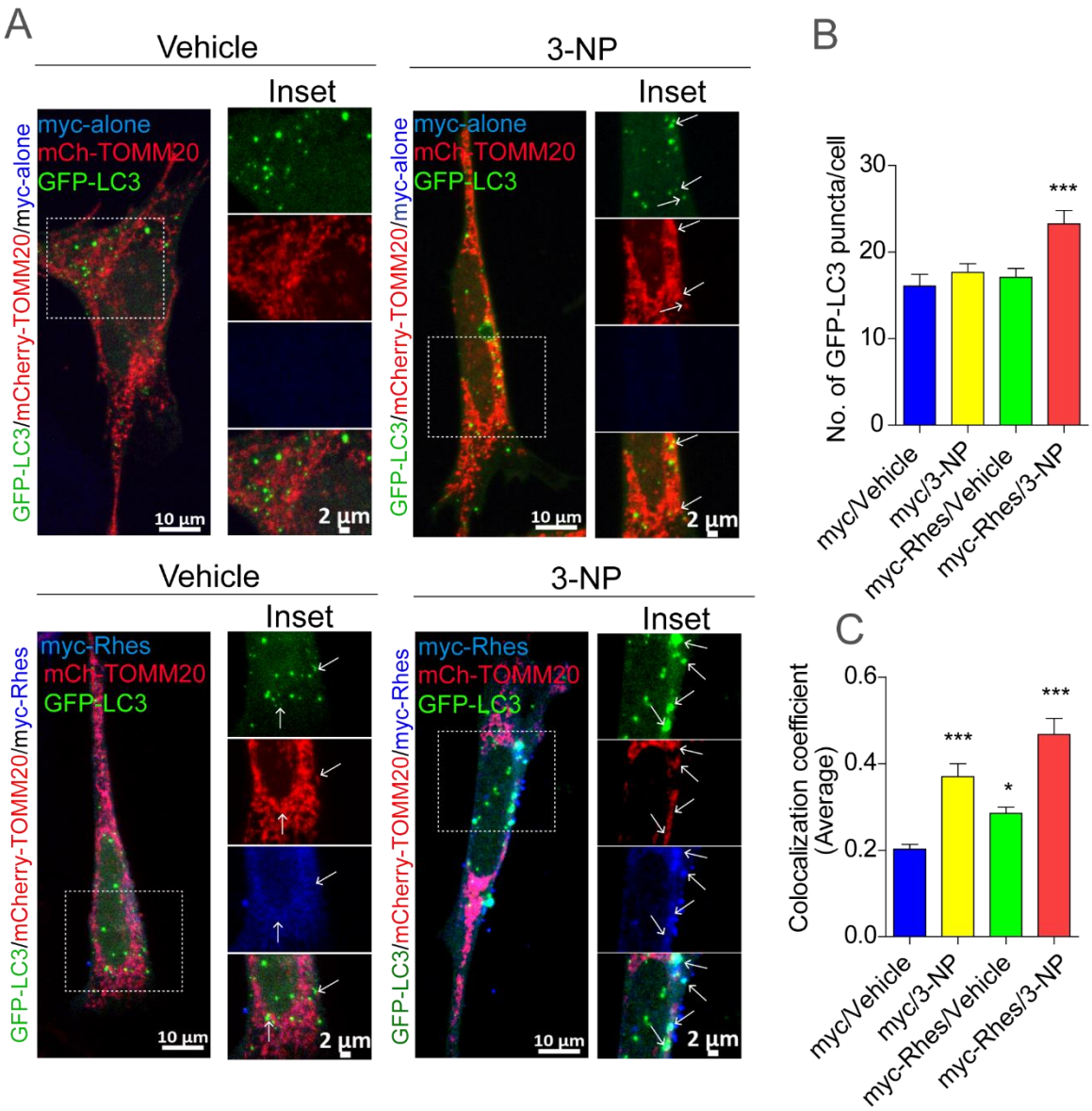

Fig. S12. Rhes induces autophagy and TOMM20 aggregation in 3-NP treated cells. (A) Representative confocal images of striatal neuronal cells co-transfected with GFP-LC3 and mCherry-TOMM20 along with either myc-alone or myc-Rhes. Cells were treated with Vehicle or 3-NP (10 mM for 2 hr). Cells were fixed and stained for myc antibody (blue). Corresponding inset shows the magnified area from selected region. Arrows show the colocalization between GFP-LC3 and mCherry-TOMM20 in indicated groups. (B-C) Bar graph shows (B) the quantification of GFP-LC3 puncta per cell and (C) average of Pearson's coefficient of colocalization between GFP-LC3 and mCherry-TOMM20 in indicated groups. n=21-24 cells per group; One-way ANOVA, *p<0.05 ***p<0.001. Data mean ± SEM.

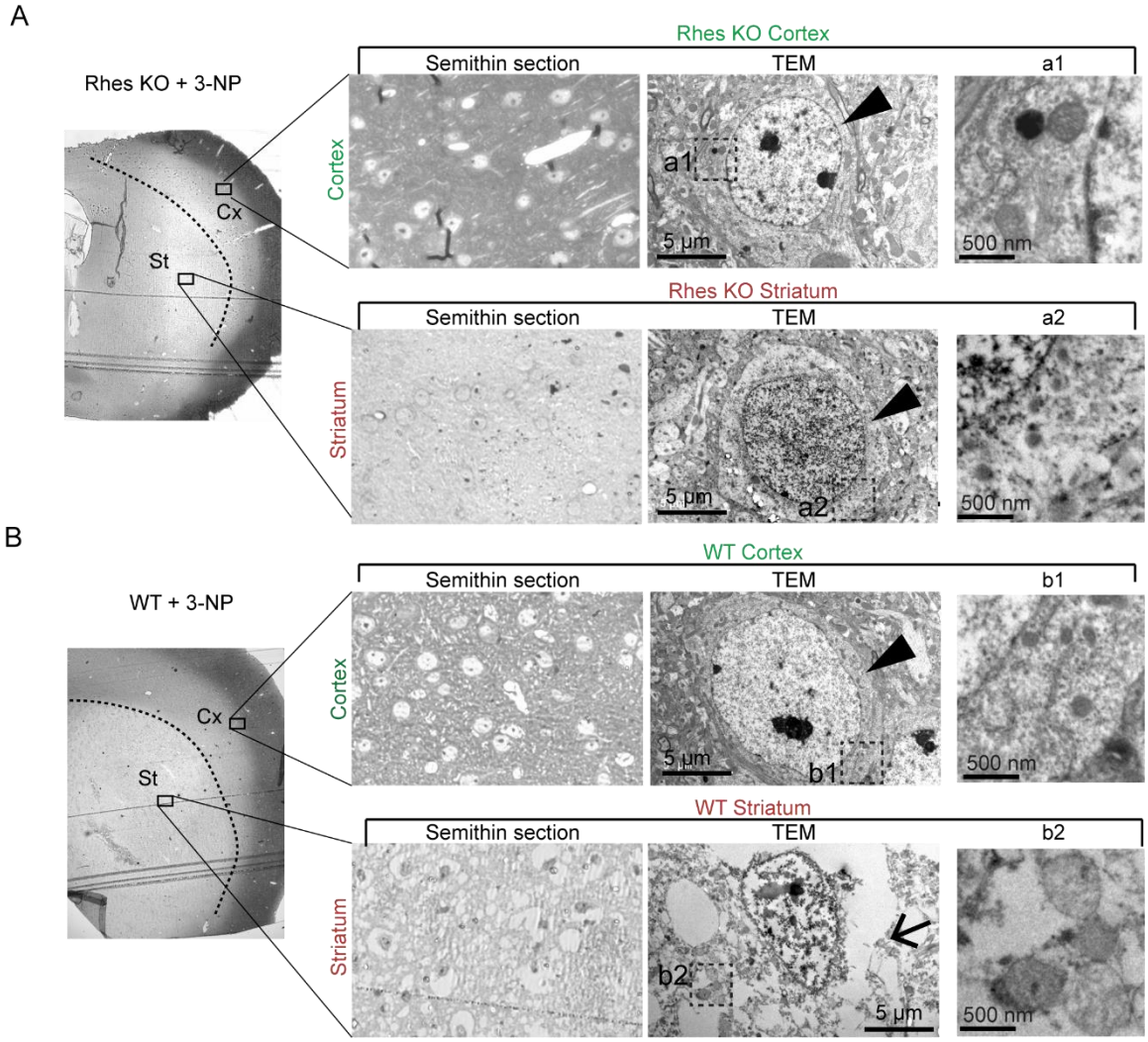

Fig. S13. 3-NP does not promote mitophagy or lesion in Cortex. (A, B) Representative electron micrographs from Cortex and striatum of Rhes KO (A) and WT mice (B) after 3 days of 3-NP treatment. Arrowhead indicates intact neuronal morphology; arrow indicates loss of nuclear integrity and abnormal shrunken nuclei. Insets a1, a2, b1 and b2 indicates mitochondrial morphology in cortical and striatal areas of Rhes KO and WT mice brain.

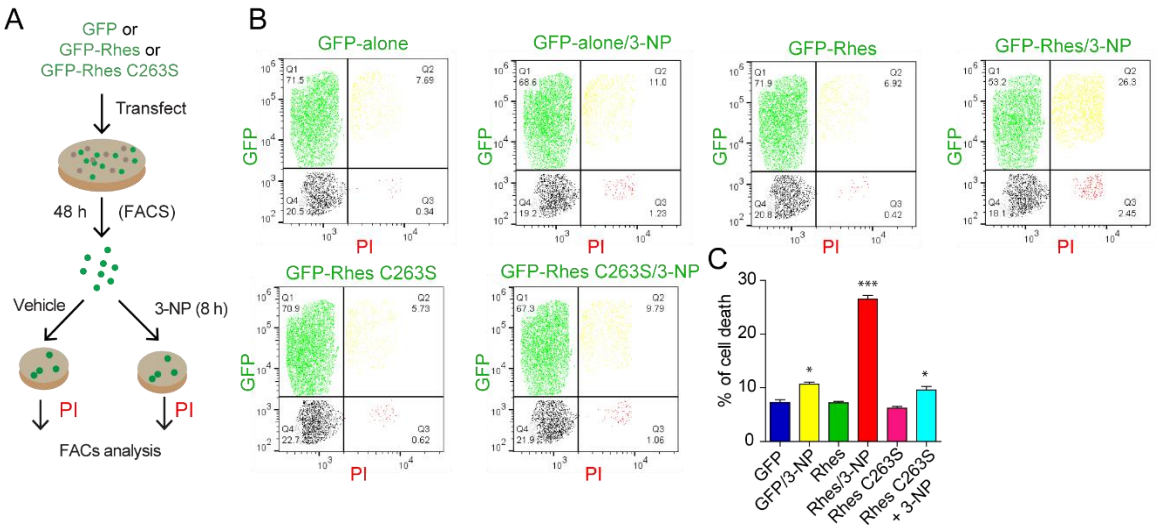

Fig. S14. Rhes positives cells are highly susceptible for 3-NP induced cell death. (A) Experimental design for panel B. (B) Flow cytometry plot of FACs sorted striatal neuronal cells positive for GFP or GFP-Rhes or GFP-Rhes C263S treated with vehicle or 3-NP (10 mM and for 8 hr) and stained for propidium Iodide (PI). (C) Bar graph shows the % of cell death in indicated group. *$p < 0.05$, ***$p < 0.001$ vs. GFP/Vehicle sample ($n = 3$ per group; Data mean ± SEM; One-way ANOVA followed by Tukey post hoc test).

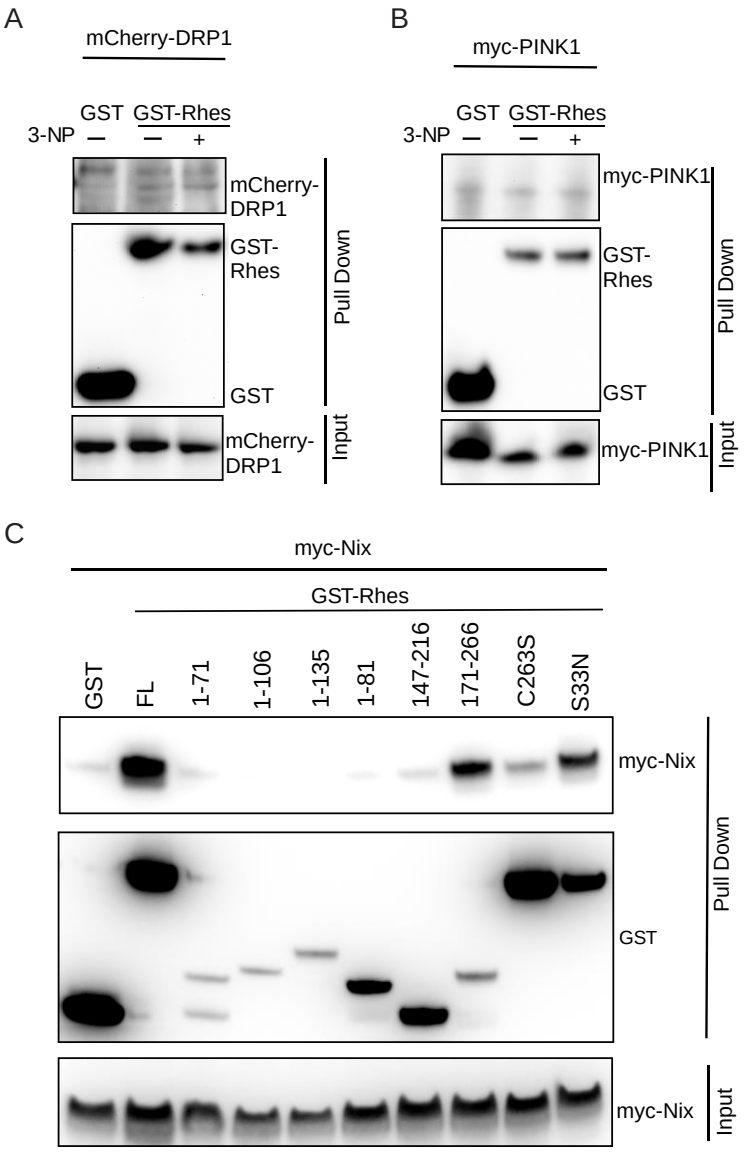

Fig. S15. A. Rhes does not interact with DRP1. (A) HEK 293 cells were co-transfected with GST or GST-Rhes and mCherry-DRP1. Cells were treated with vehicle or 3-NP (10 mM) for 2 hr, lysed and protein lysate was pull down using GST-sepharose beads and processed for immunoblotting. Blots were probed for mCherry and GST antibody to detect mCherry-DRP1 and GST or GST-Rhes respectively in pulled down samples. Input was loaded as 5% of total lysate. (B). Rhes does not interact with PINK1. HEK 293 cells were co-transfected with GST or GST-Rhes and myc-Pink1. Cells were treated with vehicle or 3-NP for 2 hr and processed as describe above. Blots were probed for myc and GST antibody to detect myc-Pink1 and GST or GST-Rhes respectively in pulled down samples. Input was loaded as 5% of total lysate. (C). Rhes binds to Nix via SUMO E3 like and CAAX domain. HEK 293 cells were co-transfected with myc-Nix and GST or GST-Rhes or its domain and mutants as indicated. After 48 hr of transfection cells were lysed and protein lysate was pull down using GST-sepharose beads and processed for immunoblotting. Blots were probed for myc and GST antibody to detect myc-Nix and GST or GST-Rhes or Rhes domain and mutants in pulled down samples. Input was loaded as 5% of total lysate.

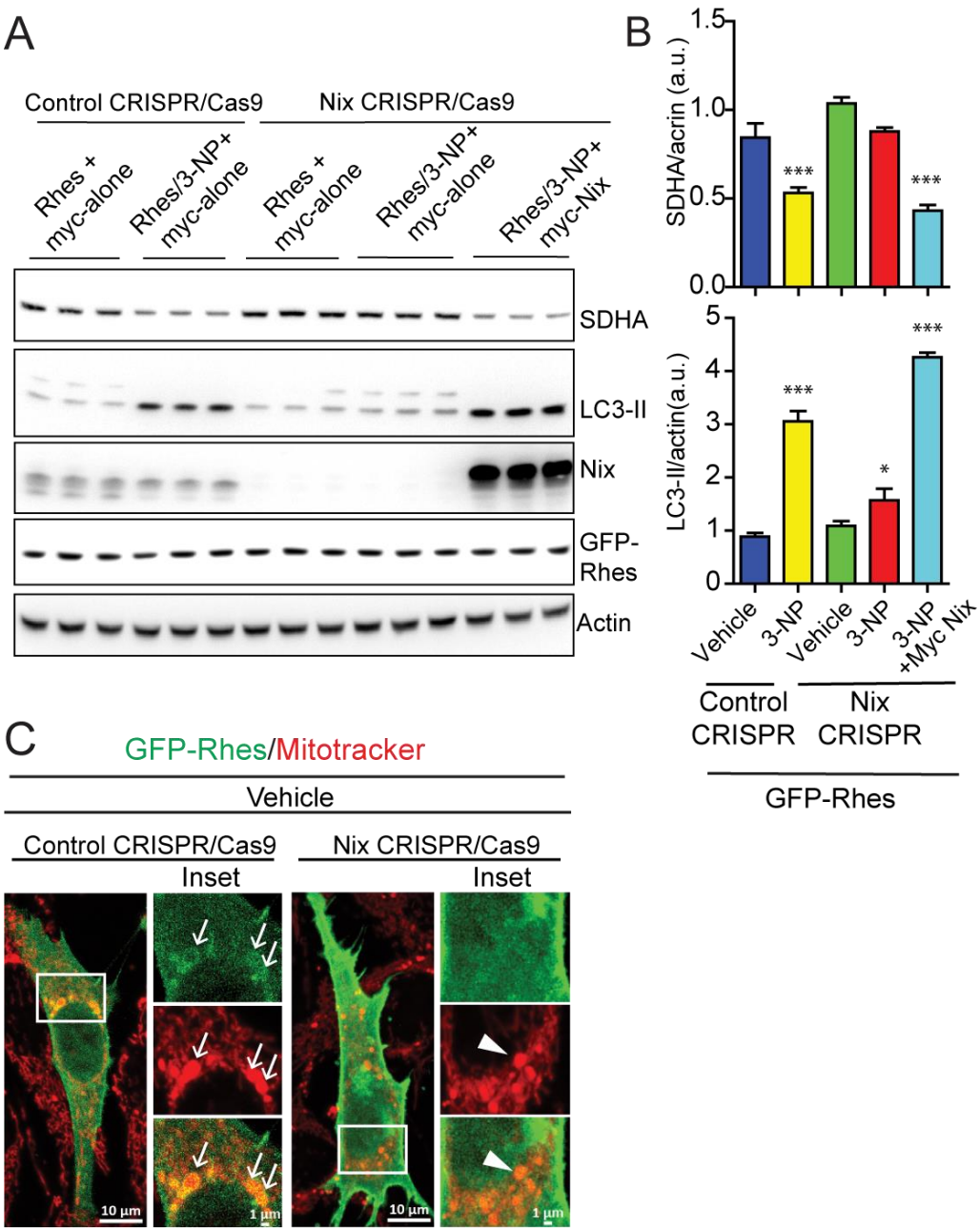

Fig. S16. Rhes promotes mitophagy via Nix. (A) Representative Western blots of indicated proteins from control CRISPR or NIX CRISPR/Cas9 striatal cells transfected with GFP-Rhes and myc-alone or myc-Nix constructs that are treated with vehicle or 3-NP (10 mM for 2 hr). (B) Bar graph shows normalized SDHA and LC3-II protein levels. *$p < 0.05$; ***$p < 0.001$ vs. control CRISPR/vehicle (n=4, Data mean ± SEM; One-way ANOVA followed by Tukey post hoc test). Indicated antibody are used to detect myc-Nix and GST or GST-Rhes or Rhes domain and mutants in pulled down samples. Input was loaded as 5% of total lysate. (C) Representative confocal images and their insets of control CRISPR/Cas9 or Nix CRISPR/Cas9 striatal neuronal cells transfected with GFP-Rhes and co-stained for mitotracker (red) that were treated with vehicle (water). Arrows represents the globular mitochondria, positive for GFP-Rhes.

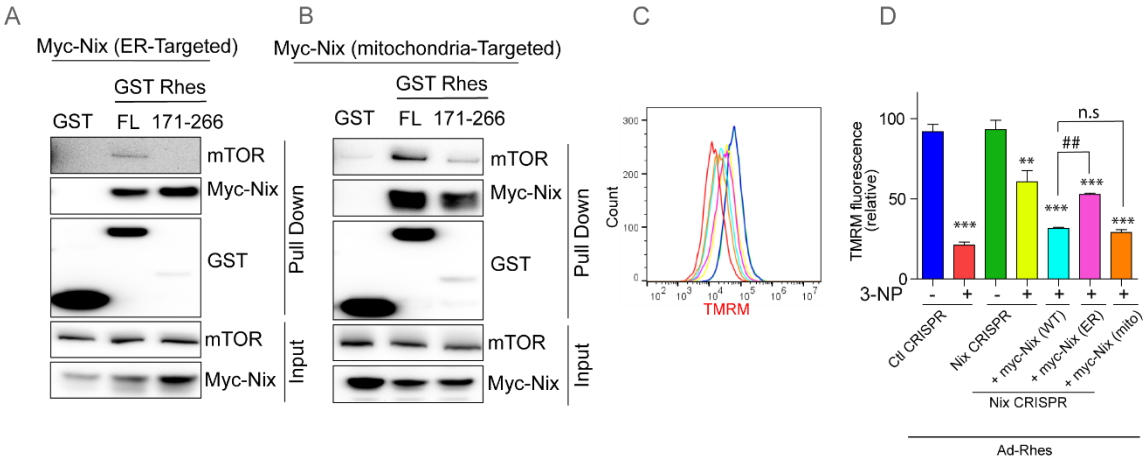

Fig. S17. Rhes promotes cell death and disrupts mitochondrial potential ($\Delta\Psi_m$) via mitochondrial targeted Nix. (A-B) Representative GST pull down and Western blots of HEK293 cells transfected with GST or GST-Rhes full length (FL) or GST-Rhes 171-266 plasmid together with either myc-Nix-ER (targeted to ER) (A) or myc-Nix-mitochondria (targeted to mitochondria) (B). Input (5%) of total lysate. Flow cytometry plot (C) and Bar graph (D) of striatal neuronal cells infected with Ad-Rhes and transfected with indicated vectors showing TMRM fluorescence signal in vehicle or 3-NP (10 mM for 2 hr) treated striatal neuronal cells (control or Nix depleted cells). n = 3/group, **p < 0.01, ***p < 0.001 vs Rhes vehicle control CRISPR (dark blue bar), $^{\#\#}$p < 0.01 between indicated groups, Data mean ± SEM; One-way ANOVA followed by Tukey post hoc test. Not significant (n.s).

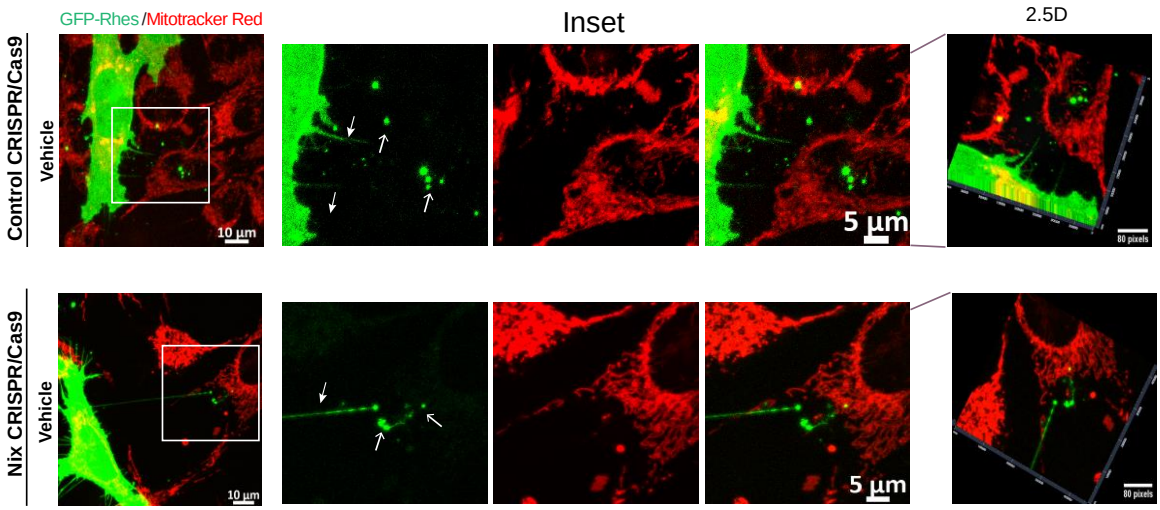

Fig. S18. Rhes transported to neighboring cell but does not interact with mitochondria in vehicle treated cells. Representative confocal images of FACS sorted GFP-Rhes expressing striatal neuronal cells co-cultured with either vehicle treated Control CRISPR cells or Nix CRISPR cells (Nix KO). For detail, see Main Figure 9A (experimental design). GFP-Rhes transported to adjacent cells but did not bind to mitochondria in both Control and Nix KO cells upon vehicle (water) treatment. White box shows the corresponding inset and their 2.5D rendered images. Closed arrow indicates Rhes induced TNT-like protrusions. Open arrow represents GFP-Rhes puncta in acceptor cell.

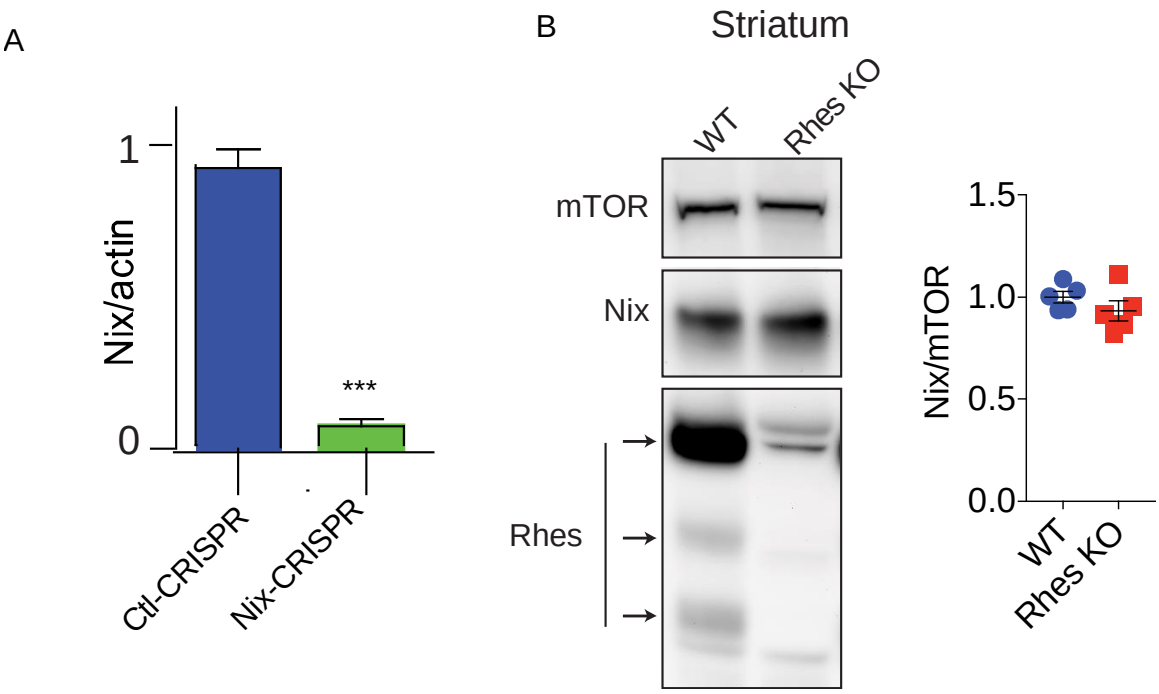

Fig. S19. Nix levels in Nix CIRSPR cells and WT and Rhes KO mice striatum. (A) Efficient depletion of Nix using CRISPR/Cas-9 tool. Bar graph shows the quantification for Nix protein normalized to Actin in CRISPR/Cas9 mediated control or Nix depleted striatal neuronal cells. ***$p < 0.001$ vs. control CRISPR, Student *t*-test. (B). Nix levels are unaltered in the striatum of Rhes KO mice. Protein lysates from WT or Rhes KO mice striatum were probed for mTOR, Nix and Rhes protein. Bar graph shows the Nix protein levels (normalized to mTOR) in WT or Rhes KO striatum (n=5 mice per group).

Supplementary discussion

Is it possible to exploit Rhes-mitophagy link for therapeutics interferences? Diseases that affect striatum showing an apparent deficit in mitophagy remains unknown. The mHTT, which promotes rapid striatal loss in HD, shown to affect mitochondria via multiple mechanisms, involving PGC-1, DRP1, or blocking mitochondrial transport (1). Moreover, the exact role of mHTT in modulating mitophagy also remains less clear. Some studies suggest mHTT block mitophagy via mechanisms involving inhibition of GAPDH, leading to cell death (2). Another research indicates valosin-containing protein (VCP) and mHTT interaction promotes excessive mitophagy and causes cell death (3). It has been demonstrated that the mHTT can also block complex-II to promote cell death, thus in this case, mHTT behaves like 3-NP (4). mHTT is also shown to inhibit general autophagy process (5, 6). The latter finding is interesting because Rhes binds Beclin-1 and promote starvation-induced autophagy which is blocked by mHTT (7). Thus, the exact details by which Rhes and mHTT combination would affect mitophagy in HD remains unknown. Blocking Rhes and mHTT interaction, however, may afford benefits in HD by reducing or increasing mitophagy. Consistent with the latter notion, increasing mitophagy provides neuroprotection in fly and cellular model of HD (8). Therefore, identifying Rhes-mediated mitophagy mechanisms in genetic models of HD may help in developing mitophagy-based therapies for HD.

Supplementary movie legends

Movie S1. Striatal neuronal cells were transfected with GFP-Rhes (green) and stained for mitotracker orange (red) and lysotracker (blue). Movie shows time-lapse video of green, red and blue and its magnified area from selected rectangle region. Arrows shows the GFP-Rhes enrichment to globular mitochondria (also positive for lysotracker staining) in time dependent fashion. A total of six Z-stacks (planes) were acquired and presented as maximum intensity projection. Total time of movie file is 38 minutes. Twenty-eight cycles were collected at a rate of 1 minute 24 seconds per cycle. Movie was rendered slow motion with iMovie software.

Supplementary Materials and methods:

Primary neuron culture

Primary neuron culture was prepared as described before (9). In brief, animals were cared in accordance to the guidelines set forth by the National Institutes of Health regarding the proper treatment and use of laboratory animals and with the approval of Institutional Animal Care and Use Committee of The Scripps Research Institute. Striata of postnatal C57BL/6 mice (P1) were removed and digested at 37°C for 15 min in a final concentration of 0.25% papain and resuspended in neuronal plating media (Neurobasal-A media, Thermo Fisher Scientific), with 5% FBS, 0.5 mM glutamax and 1% penicillin-streptomycin. Tissues were dissociated by trituration with a pipette. Further, cells plated in 35 mm glass bottom dishes (Matsunami D11140H) coated with 100 µg/ml poly-D lysine at the density of $2X10^5$ cells per dish. Dishes were maintained in a 37°C, 5% CO2 incubator. After the cells adhered (1–3 h after plating), plating media was replaced with growth media (Neurobasal-A media, 2% B27, 0.5 mM glutamax and 1% penicillin-streptomycin).

Antibodies, chemicals and treatments of cells

Lamp1 antibody was purchased from DSHB (AB 528127). GFP (sc-9996), VDAC1 (sc-390996), Myc (sc-40), GST (sc-138 HRP) and actin monoclonal antibody (sc-47778) were obtained from Santacruz Biotechnology. mTOR (2983), SDHA (11998), LC3B (3868), Nix (12396) and Bip (3177) antibodies were from Cell Signaling Technologies. Rasd2 (Rhes) antibody (RHES-101AP) was obtained from FabGennix. mCherry antibody (NBP2-25157) was purchased from Novus Biologicals. Alexa 568 anti-mouse antibody was purchased from Thermo Fisher Scientific. Cycloheximide (CHX) (C1988), Chloroquine (C6628), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (C2920) and 3-NP (N5636) were purchased from Sigma-Aldrich. CHX was dissolved in 95% ethanol and used at 100 µM for indicated time point. To assess autophagy flux, cells were pretreated with chloroquine (50 µM) for 4 hr, then proceeded for either vehicle or 3-NP treatment. 3-NP was dissolved in water, pH was adjusted to 7.4 and used at 10 mM for indicated time point. To assess mitophagy process in CHX chase experiment, cells were transfected with GFP or GFP-Rhes for 48 hr. Cells were treated with CHX 100 µM for indicated time point. Vehicle or 3-NP (10 mM) was added to culture for 1 hr and samples were harvested for western blotting. FCCP was dissolved in DMSO and used at 1 µM concentration for 6 hr. For virus infection, Striatal neuronal cells were infected with either adenovirus-CMV-null (abmgood) or adenovirus-CMV-Rhes (1×10^{10} opu/µl) at 1 MOI for 32-36 hr in complete

media. Mitotracker orange (M7511), mitotracker green (M7514) and Lysotracker (L12492) were obtained from Thermo Fisher Scientific. For mitochondrial staining, mitotracker was dissolved in DMSO and used at 200 nM. Lysotracker was used at 100 nM concentration. Mitotracker and/or Lysotracker was added for 30 mins after 48 h of GFP or GFP-Rhes transfection; cells were washed with D-PBS and live cell imaging was performed using live cell imaging solution (Thermo Scientific). TMRM (M20036) and propidium iodide (P3566) were obtained from Thermo Scientific and used as per manufacturer's recommendation.

Mitochondrial respiration analysis (Seahorse assay)

Striatal neuronal cells were plated in Sea Horse V7 culture plates and infected with Ad-null or Ad-Rhes (1 MOI for 32 hr) treated with vehicle (water) or 3-NP (10 mM for 2 hr). To assess mitochondrial respiratory function, the growth media was replaced with XF media (unbuffered DMEM, pH 7.4, Agilent Technologies, Santa Clara, CA, USA; catalog # 103334-100) with added 10mM pyruvate and 2.5mM glucose. The plate was equilibrated for 30 minutes at 37^0C in a CO_2-free incubator before being loaded in the XF-96 analyzer according to the manufacturer's instructions. Once in the analyzer, the plate was combined with the pre-calibrated XF cartridge containing the individual well ports A, B and C loaded with 10x stocks of the following chemicals, respectively: A. Oligomycin (20µg/ml); B. FCCP (20 µM); C. Rotenone and Antimycin A (20 µM each). Oxygen consumption rate was monitored in real-time at 37^0C under basal conditions and following the sequential delivery of the compounds in the cartridge ports, achieving their final 1x working concentrations. Non-mitochondrial rotenone/antimycin-insensitive OCR was subtracted from all other OCR measurements.

Plasmids and Transfection

For GFP-Rhes and GFP-Rhes C263S, we amplified their respective cDNA from pCMV-Myc-Rhes (10) and cloned it in EGFP-C1 vector. For GST-Rhes and GST-Rhes domains or mutants, their respective cDNAs were amplified from pCMV-Myc-Rhes (10) and cloned in pCMV-GST vector. The following plasmids were obtained from Addgene (Table 1). Striatal neuronal cells seeded in 35 mm glass bottom dishes or other plates were transfected 24 h later with cDNA constructs using lipofectamine 2000 (Invitrogen) in the ratio of 1:2 (DNA: Lipofectamine) and for Primary neurons, we used 1:3 (DNA:

Lipofectamine). For 10 cm dish, we transfected ~8 µg of DNA, and for 35 mm dishes ranging from 1 to 2 µg total DNA.

Table-1

Plasmids	**Gift from**	**Addgene ID**
mCherry-TOMM20-N-10	Michael Davidson, The Florida State University, Florida	55146
pBMN-mCherry-Parkin	Richard J. Youle, NINDS, Maryland	59419
Myc-Nix	Joseph W. Gordon, University of Manitoba, Canada	100795
pCMVTNT PINK1 C-myc	Mark R Cookson, National Institute on Aging, Maryland	13314
mCh-Drp1	Gia K. Voeltz, University of Colorado Boulder, Colorado	49152
Myc-Nix-CytoB5	Joseph W. Gordon, University of Manitoba, Canada	100756
Myc-Nix-MaoB	Joseph W. Gordon, University of Manitoba, Canada	100757
pDEST-CMV GFP-LC3B	Robin Ketteler, Medical research counsil, United Kingdom	123114

Protein expression and Western blots

Mitophagy process was assessed by Western blotting, cells were seeded in 6 well plate and next day transfected with 2 µg of DNA (GFP or GFP-Rhes) or infected with Ad-null or Ad-Rhes at 1 MOI for 48 hr. Cells were treated with indicated drugs or chemicles and washed 3 times with PBS. Protein lysates were prepared and loaded 40 µg of protein on polyacrylamide gels. For protein-protein interaction experiments, GST or GST-Rhes or GST-Rhes fragments or Myc-Nix or Myc-Pink1 or mCherry Parkin or mCherry DRP1 (4 µg each) were transfected in HEK293 cells (10 cm dish), and, after 48 hr, cells were lysed in lysis/binding buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1.0% NP-40) with a protease inhibitor cocktail (Roche) and phosphatase inhibitor II (Sigma). Glutathione beads were added, and the lysates were then rotated in a cold room for at least 5 hours. The beads were washed 3 times in binding buffer without a protease inhibitor cocktail, the proteins were eluted with 2X LDS-loading buffer.

Proteins were separated on 4-12% Bis-Tris Gel (Invitrogen), and then transferred to PVDF membranes and probed with the indicated antibodies. HRP-conjugated secondary antibodies (Jackson Immuno Research, Inc.) were probed to detect bound primary IgG with a chemiluminescence imager (Alpha Innotech) using enhanced chemiluminescence (ECL), from WesternBright Quantum (Advansta). Indicated band intensities were quantified with ImageJ software.

Immunofluorescence

All the images stained for mitotracker and/or Lysotracker are acquired on live cells. For SDHA staining in GFP-Rhes transfected striatal neuronal cells following protocol was performed. At the indicated times after the post-transfection, cells were washed in D-PBS and fixed for 20 min in 4% PFA (Electron Microscopy Sciences). The cells were permeabilized with methanol and labeled with rabbit anti-SDHA antibody; (1:100, for 18 hours at 4°C). For Myc-Rhes (1:100, for 18 hr at 4°C) staining, we used similar protocol as described above. The Alexa Fluor® 568 secondary antibodies were purchased from Thermo Fisher Scientific and used at 1:500 for 1 hour at room temperature.

Image processing and colocalization coefficient quantification

All the fluorescent confocal images were taken in Zeiss 880 microscope using 20X or 63X oil immersion Plan- apochromat objective (1.4 NA). Excitation was via a 405 or 561 or 633 nm diode-pumped solid-state laser and the 488 nm line of an argon ion laser. Time-lapse acquisitions were performed using a 63X oil-immersion lens (1.4 NA). Images of striatal neuronal cells used for 2.5D or 3D reconstruction were acquired with an optimal Z-step of 0.27 µm covering the whole cellular volume. Colocalization processing and analysis was performed with Zen software black/blue edition 2012. Three-dimensional analyses and remodeling were done by Zen 2012 black edition software. Rhes mediated regulation of mitophagy in neighboring cell was performed as following. Striatal neuronal cells were transfected with GFP-Rhes and sorted through flow cytometry sorter to enrich GFP-Rhes expressing cell population. These sorted cells were co-cultured with striatal neuronal cells (control or Nix depleted) which were pretreated with vehicle or 3-NP (10 mM for 6 hr). Before coculture, vehicle or 3-NP added media was exchanged with fresh media so that complex II inhibition could be restricted to Rhes negative cells (pretreated cells). After 18 hr of co-culture, cells were stained for mitotracker orange for 30 min in serum free media. After that Media was changed to live cell imaging solution and images were acquired in

confocal microscope. GFP-Rhes puncta, migrated to neighboring cells were selected and their colocalization with mitotracker was analyzed in Zen software.

Subcellular Fractionation

For subcellular fractionations, mice were euthanized and both striatum were dissected immediately and homogenized using a glass dounce homogenizer (5 loose and 5 tight strokes) in buffer A of mitochondria isolation buffer (Thermo Scientific 89874) and kept on ice for 2 minutes. Buffer C was added to each sample and mixed by inverting 5 times. The homogenates were centrifuged at low speed (700g) for 10 minutes to separate nuclei and tissue chunks. The supernatants were immediately loaded on top of 10-50% sucrose gradients and centrifuged at 40000 RPM (SW41Ti rotor) at 4^0C for 2 hours. The gradients were fractionated manually (11 X 1 ml fractions). Using methanol/chloroform, total protein of each fraction was precipitated. The protein pellets were resuspended in 2X LDS buffer and used for western blotting experiments. All the samples were proceeded at the same time. During developing the blots, all the groups were developed at the same time with corresponding antibody. For example, all the groups probed for SDHA, were developed together at the same time to get equal exposure for further analysis.

Behavior evaluation

Beam walk test

Before injections, the mice were trained to walk over a wood beam (0.6 cm x 100 cm), suspended ~50 cm from the benchtop with a safety box at one end. The training protocol was 4 days: 1st day traversing half the distance (50 cm) of the thickest beam (1.2 cm x 100 cm), and 2^{nd} and 3^{rd} days traversing the entirety of the beam (0.7 cm x 100 cm), and 4^{th} day traversing the skinniest beam (0.6 cm x 100 cm). Training sessions consisted of 10 min habituation in the safety box, followed immediately by 4 attempts to cross the bar, each separated by 2 min to allow the mice to rest. After training, the daily performance was measured after a 10 min habituation period with 4 crossing attempts per mouse, using the skinniest beam. Baseline performance was measured on day 0 before injections, and testing was performed each subsequent morning before the next injection for 3 days. Sessions were videotaped and reviewed later for time to cross the 100 cm beam, the number of foot slips below the bar (errors), and the number of extra-supports under the beam used for crossing.

Open Field

Total activity was measured using the open field test. Each mouse was placed in the center of each open-top box (50 x 50 cm), under bright light and recorded via ceiling-mounted video camera for 10 min. Locomotor activity was analyzed using Ethovision XT11.5 animal tracking software (Noldus), total activity was measured on day 0 before injections, and each subsequent morning before rotarod, beam walk, and the next injection for 3 days. Because total activity can be affected for the continuous exposition of the mice to the test, based on preliminary results, animals were trained for 2 days before injections.

Rotarod

Alteration in motor coordination was determined using the accelerating rotarod in wild type and Rhes KO mice. After placing the mice on the rotating rod (diameter of 5 cm), they were tested using the accelerated rotarod from 4 to 40 RPM (cut off time of 300 s). Each mouse was tested three times and the average of the latency to fall was used for group analysis. Before injections, all the mice were trained for three days. Rotarod evaluation was measured on day 0 before injections, and each subsequent morning before the next injections for 3 days.

Transmission electron microscopy and histology

After finishing the experiments, mice were fixed at 2 and 3 days of 3-NP treatment. For fixation, animals were anesthetized with tribromoethanol (1:50) in sterilized physiologic solution (NaCl 0.9%), and perfused transcardially with 15 ml of ice-cold 0.9% saline, followed by 20 ml of ice-cold 4% paraformaldehyde - 2.5% glutaraldehyde in 0.1 M Phosphate buffer, pH 7.4. The brain was removed, post-fixed in the same solution. Right hemispheres were used for transmission electron microscopy (TEM) and left hemispheres for hematoxylin-eosin staining. For staining, hemisphere was cryoprotected in sucrose gradients (up to 30%), and sagittal striatal sections (40 µm-thick) were obtained.

The right hemisphere was used for transmission electron microscopy, after perfusion the striatum area containing cortical tissue was delimited. Then the tissue was osmicated (1% osmium tetroxide in PBS), washed with phosphate buffer, dehydrated and embedded in epoxyresin. Semithin sections (500 nm) were obtained in an ultramicrotome (Reichert-Jung) and stained with Toluidine Blue for light microscopic observation, to identify the area of interest. Ultrathin sections were obtained and stained with Reynolds mixture (2% lead citrate and 2% uranyl acetate) and observed in a Jeol1200EXII transmission electron

microscope. Electron micrographs were obtained and analyzed using the GATAN software. Mitochondrial alterations were determined using the circularity index and mitochondrial area, which were quantified using the ImageJ software from at least 200 mitochondria from 4-5 mice. Organelle identification and quantification was made from around 40 neurons from 4-5 mice per group.

Flow cytometry

For TMRM staining, we seeded striatal neuronal cells in 6 well plate. Next day cells were transfected with 2 µg of DNA (GFP or GFP-Rhes) or infected with Ad-null or Ad-Rhes at 1 MOI for 48 hr. Cells were treated with vehicle or 3-NP for indicated time point. Cells were washed with PBS and trypsinized, centrifuged at 1800 RPM and resuspended in 1XPBS. The TMRM dye was added at 1 µl/ml for 30 min, incubated on dancing rocker and later washed 3 times with 1XPBS, filtered through a 40 µm nylon filter and resuspended in flow buffer [1xD-PBS (Ca^{++}/Mg^{++} free), 1 mM EDTA, 25mM HEPES pH 7.0, 2% FCS/FBS (Heat-Inactivated), 10 units/mL DNase]. Similar strategy was used to stain the cells with propidium iodide (1 µl/ml for 30 min). Cells were analyzed by flow cytometry (BD Biosciences LSR Fortessa cell analyzer). Each experiment was performed three times (in duplicate), and 10000-20000 cells were recorded for each sample. Data was compensated with single color controls and plotted using FlowJo software.

Statistical analysis

Unless otherwise noted all experiments were carried out in duplicates and repeated at least three times. The statistical comparison was carried out between groups using one-way ANOVA, two-way ANOVA followed by appropriate post hoc test and Student's-*t*-test as mentioned in legends. The significance values were set at $p<0.05$, using graph pad Prism7.

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