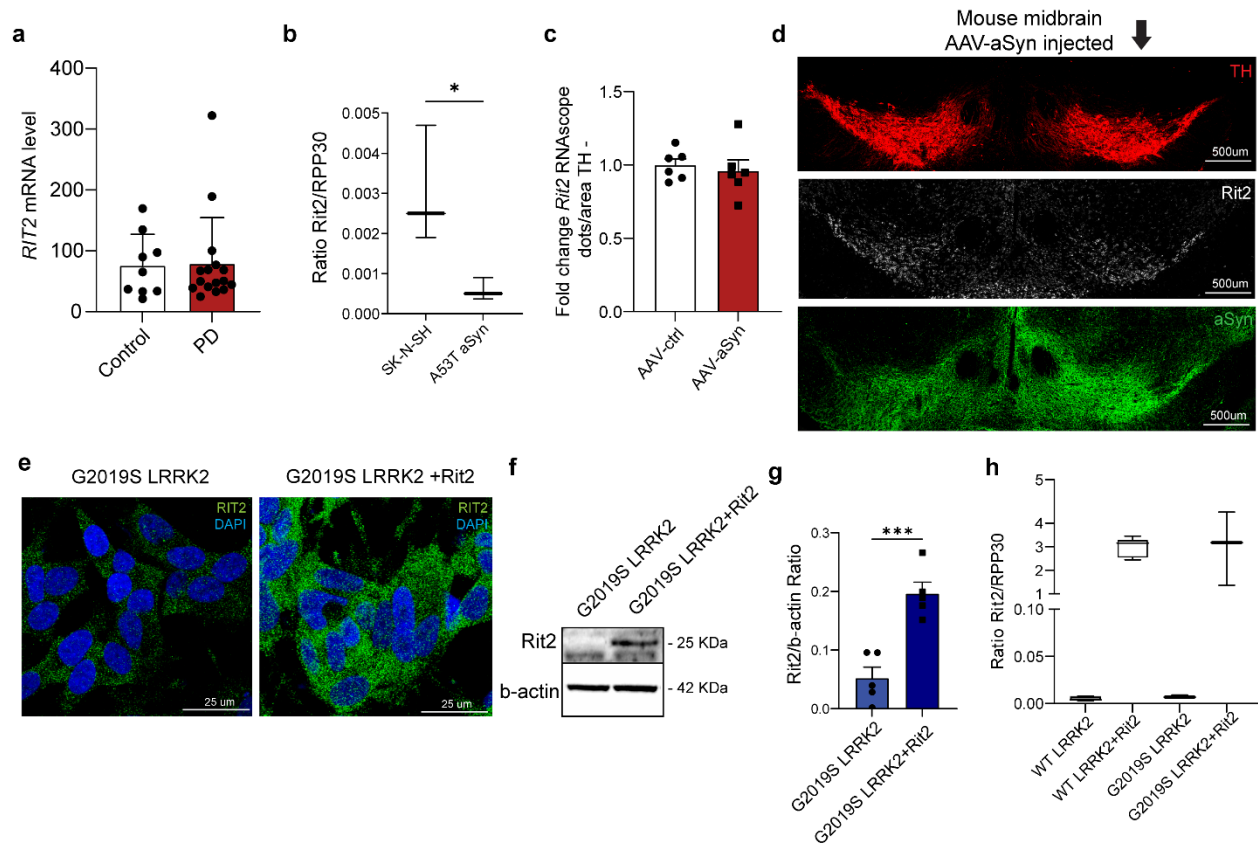


Supplementary Information

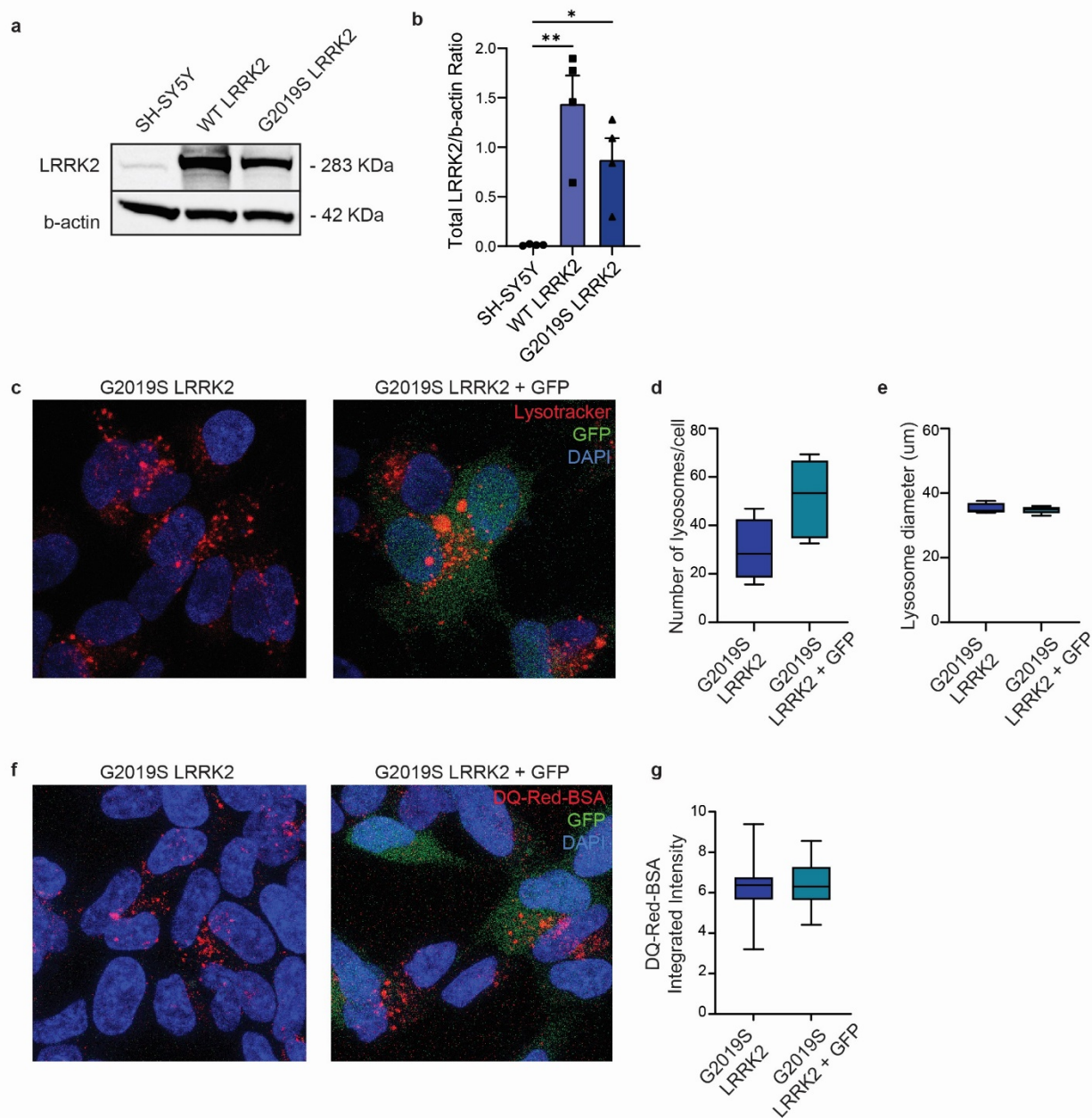
Supplementary Figures



Supplementary Figure 1: *RIT2* gene expression in brain tissue and neuroblastoma cell lines. a) *RIT2* mRNA levels are not altered in brain tissue of sporadic PD patients, when compared to controls (GSE7621, controls=9, PD=16). b) RT-qPCR was carried out to assess *RIT2* mRNA levels in recombinant neuroblastoma cell lines overexpressing A53T-aSyn. Fold change of *RIT2* mRNA is reduced in A53T-aSyn overexpressing cells (n=3). c) Rit2 RNAscope analysis in TH-areas of the midbrain are not different with aSyn viral expression. d) Low magnification overview of the AAV-aSyn injected midbrain used for the RNAscope experiment. Rit2 is visualized as mRNA, whereas TH and aSyn are stained using antibodies against the respective proteins. e) Nucleofection was used to efficiently express Rit2 in G2019S-LRRK2 cells and ICC for Rit2 was used to control for nucleofection efficiency. f) Western blot of Rit2 levels in

G2019S-LRRK2 cells with and without Rit2 nucleofection. g) Quantification of Rit2 protein levels in G2019S-LRRK2 cells. Rit2 overexpression significantly increases Rit2 levels (n=4). h) Droplet Digital PCR was carried out to assess *RIT2* mRNA levels in recombinant neuroblastoma cell lines with or without Rit2 overexpression. Ratio of *RIT2* mRNA, normalized to RPP30 is increased after Rit2 nucleofection. Data are represented as median, boxes show the IQ and whiskers show min-max or means \pm SEM.

*p<0.05, ***p<0.001 two-tailed Student's t-test.

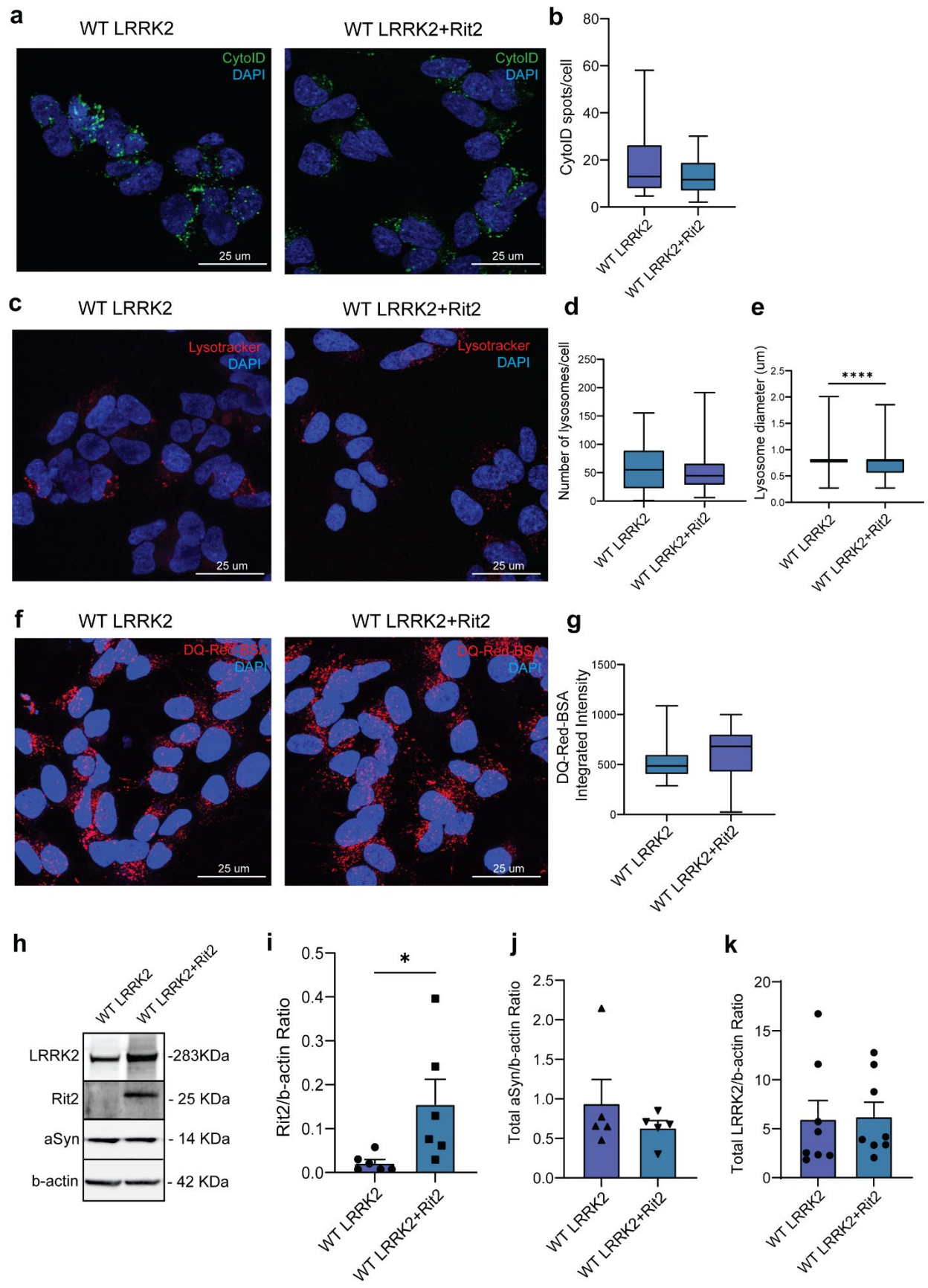


Supplementary Figure 2: *Control GFP overexpression does not modify lysosome morphology or activity.* a) Recombinant stable expression of LRRK2 in SH-SY5Y lines is confirmed by Western blotting, compared to naïve neuroblastoma SH-SY5Y cells. b) Quantification of LRRK2 protein levels in neuroblastoma SH-SY5Y cell lines stably expressing WT- or G2019S-LRRK2, compared to naïve SH-SY5Y cells (n=3). c) The Lysotracker Red dye was used to visualize cellular lysosomes in G2019S-LRRK2 cells with or without GFP nucleofection. d) The number of

lysosomes was determined and was not significantly affected by control GFP expression (n=4). e) Assessment of the diameter of lysosomes revealed that control GFP expression does not modify the size of the lysosomes in G2019S-LRRK2 cells (n=4). f) The DQ-Red-BSA assay was employed to determine the proteolytic activity of lysosomes in G2019S-LRRK2 expressing cells with and without GFP expression. g) The integrated intensity of the DQ-Red-BSA signal, that is proportional to lysosomal proteolysis, is not affected by control GFP expression (n=4).

Data are represented as median, boxes show the IQ and whiskers show min-max or means \pm SEM.

*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni's post-hoc test.



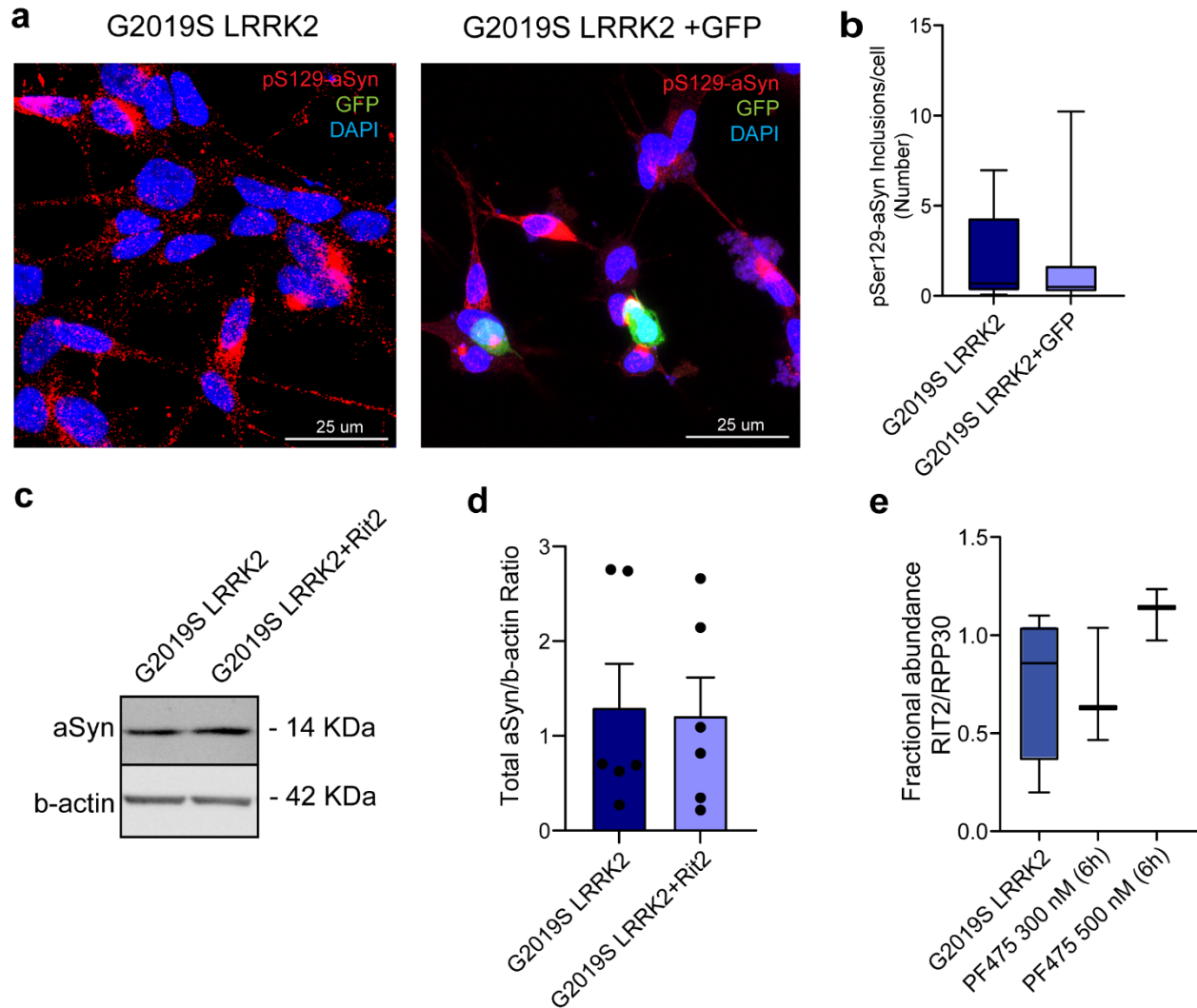
Supplementary Figure 3: *Rit2* overexpression in WT-LRRK2 cells does not alter ALP phenotypes.

a) CytoID assay was employed to visualize autophagosome and autolysosome distribution. b) Quantification of CytoID-positive puncta revealed no difference in WT-LRRK2 cells, when *Rit2* was overexpressed (n=4). c) Cell processing with the LysoTracker Red dye was performed to visualize lysosomes in WT-LRRK2 and WT-LRRK2+ *Rit2* cells. d) The number of lysosomes per cell was quantified and revealed no difference, when *Rit2* was transfected (n=4). e) The average size of lysosomes was assessed, and a significant decrease of the diameter was measured when *Rit2* was transfected to G2019S-LRRK2 cells (n=4). f) The DQ-Red-BSA assay was employed to assess the proteolytic activity of lysosomes. g) Quantification of DQ-Red-BSA fluorescent spots revealed no significant difference in WT-LRRK2 cells, with *Rit2* overexpression (n=4). h) Western blot analysis of total LRRK2, aSyn and *Rit2* in WT-LRRK2 with or without *Rit2* expression. i) *Rit2* protein levels are increased after nucleofection (n=6). j) Analysis demonstrated that *Rit2* overexpression does not alter total aSyn levels (n=5). k) Analysis demonstrated that *Rit2* overexpression does not alter total LRRK2 levels (n=6).

In imaging experiments analysis was conducted on 700-1000 cells per group in each experiment.

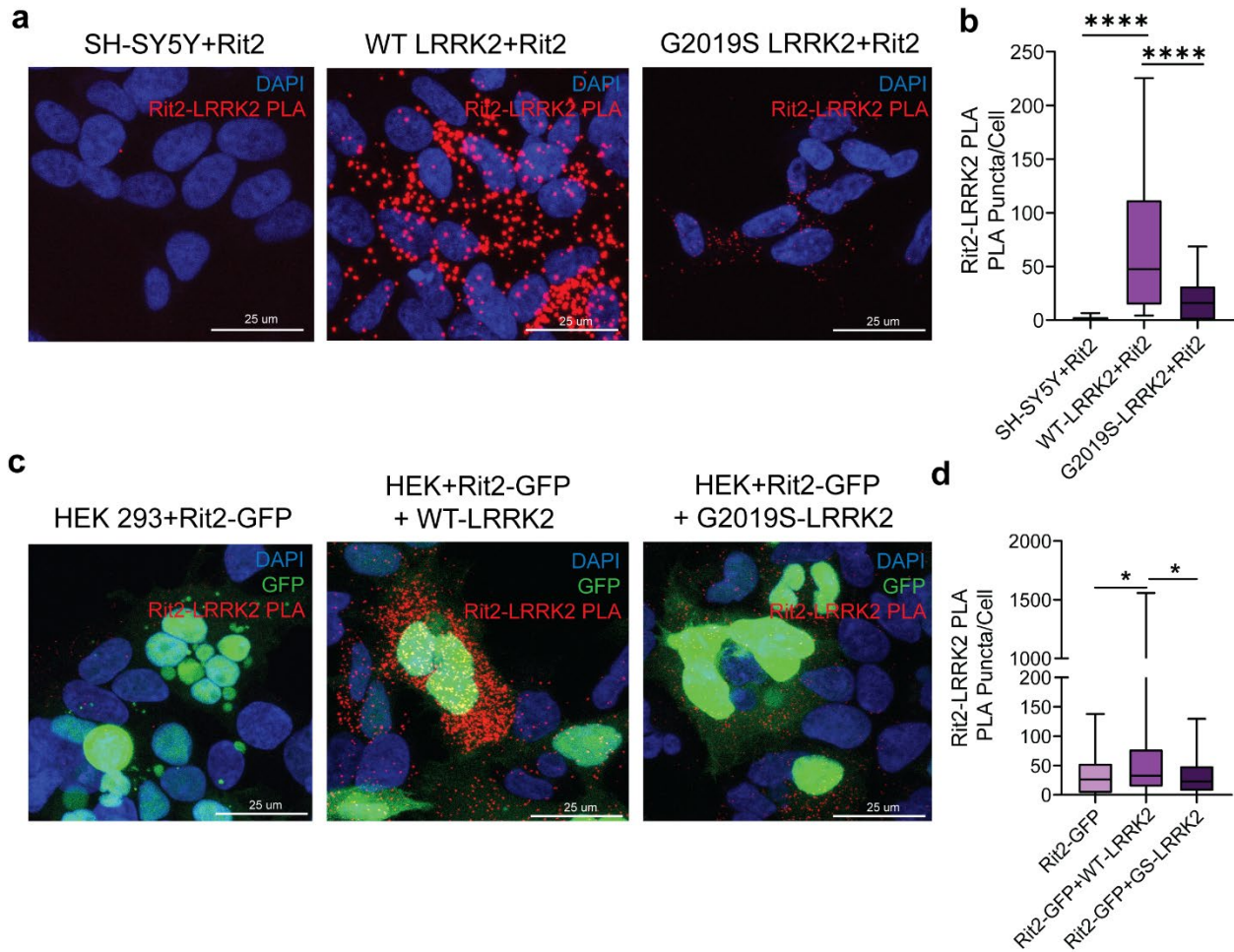
Data are represented as median, boxes show the IQ and whiskers show min-max or means±SEM.

*p<0.05. ****p<0.0001, two-tailed Student's t-test



Supplementary Figure 4: Nucleofection of *Rit2* and GFP constructs in G2019S LRRK2 cells a) Representative images for pS129-aSyn immunostaining and GFP expression in GFP-transfected G2019S-LRRK2 cells. b) Quantification of pS129-aSyn inclusions. Expression of GFP does not alter pS129-aSyn inclusion number in G2019S-LRRK2 cells. c) Western blot of total aSyn in G2019S LRRK2 cells. d) Protein levels are not changed with *Rit2* overexpression in G2019S LRRK2 cells. e) *Rit2* mRNA levels were measured using ddPCR and are presented as fractional abundance of gene of interest (*RIT2*) over housekeeping gene (*RPP30*) (n=3). In imaging

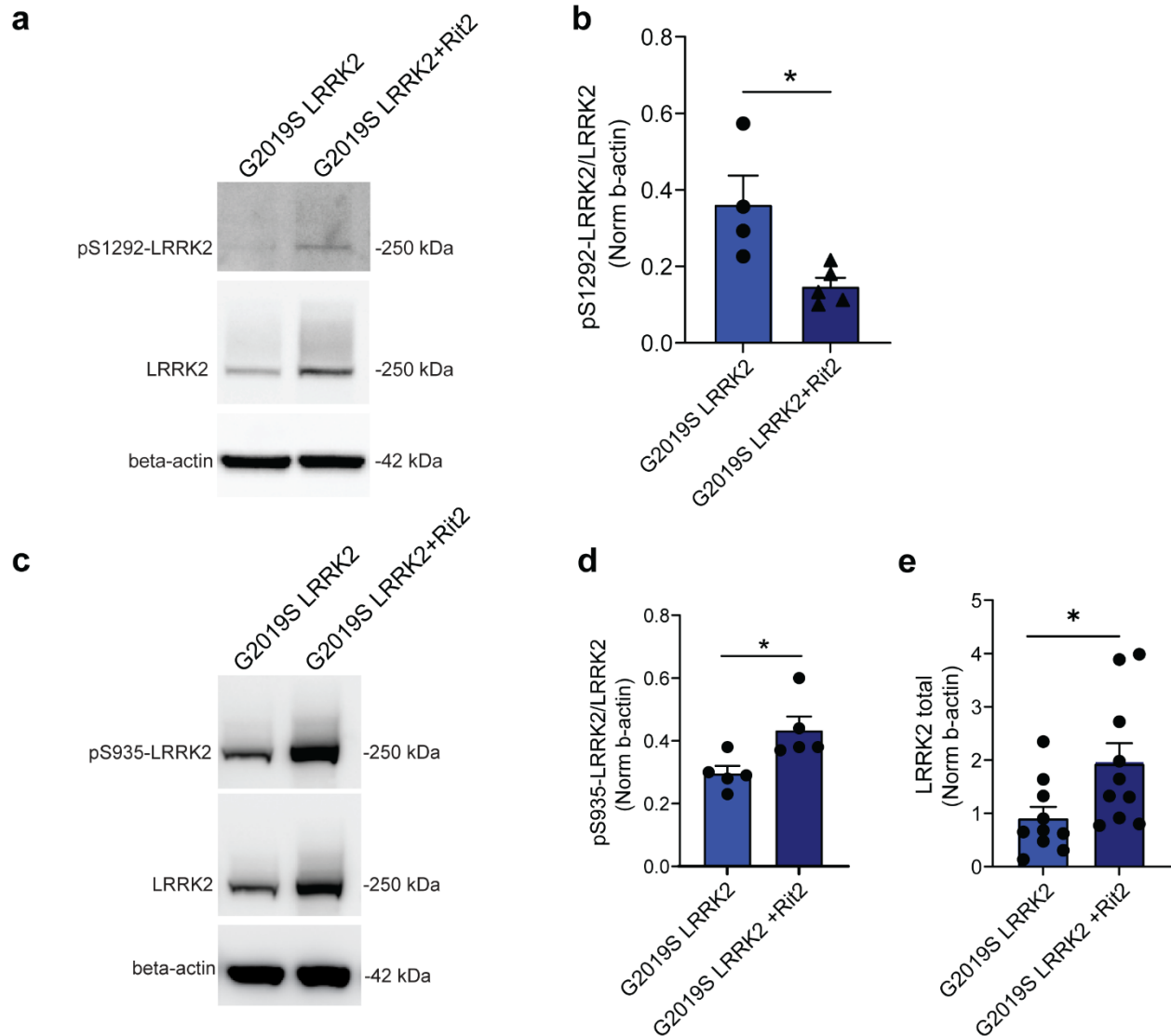
experiments analysis was conducted on 700-1000 cells per group in each experiment. Data are represented as median, boxes show the IQ and whiskers show min-max or means \pm SEM.



Supplementary Figure 5: *LRRK2* and *Rit2* are in close proximity. a) PLA for *Rit2* and *LRRK2* in neuroblastoma cells. b) Quantification of PLA puncta shows an increase of PLA signal in WT- and G2019S-*LRRK2*, when compared to naïve SH-SY5Y cells and a decrease when G2019S-*LRRK2* cells are compared to WT *LRRK2* cells. c) PLA for *Rit2* and *LRRK2* in HEK293 cells. d) *Rit2* and (WT or mutant) *LRRK2* protein were overexpressed and PLA signal quantified. The G2019S-*LRRK2* mutation leads to a decreased proximity of *Rit2* and *LRRK2*.

In imaging experiments analysis was conducted on 700-1000 cells per group in each experiment. Data are represented as median, boxes show the IQ and whiskers show min-max.

* $p < 0.05$, **** $p < 0.0001$, one-way ANOVA followed by Bonferroni's post-hoc test.



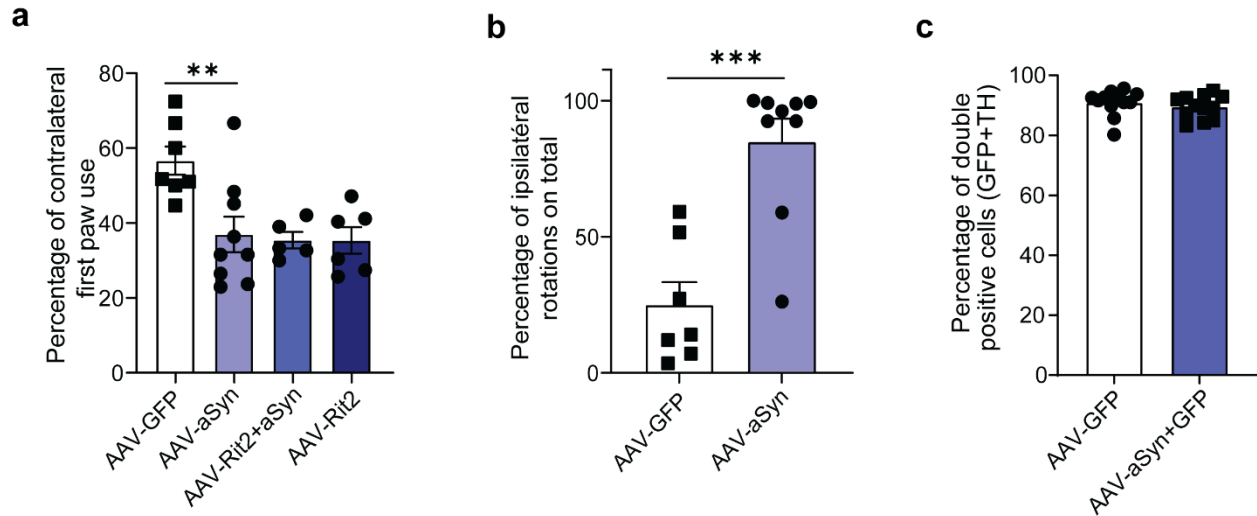
Supplementary Figure 6: *pS1292-LRRK2* levels are reduced and *pS935* levels are increased with the overexpression of *Rit2*.

a) Phosphorylation levels of S1292 in G2019S-LRRK2 and G2019S LRRK2+Rit2 were measured using WB for pS1292-LRRK2 and total LRRK2. b) pS1292 LRRK2 levels are reduced when Rit2 is overexpressed (normalized to total LRRK2) (n=5). c) Phosphorylation levels of S935 in G2019S-LRRK2 and G2019S LRRK2+Rit2 were measured using WB for pS935-LRRK2 and total LRRK2. d) pS935 LRRK2 levels are increased when Rit2 is overexpressed and when

normalized to total LRRK2 (n=10). e) Rit2 overexpression leads to increased total LRRK2 levels, when normalized to b-actin.

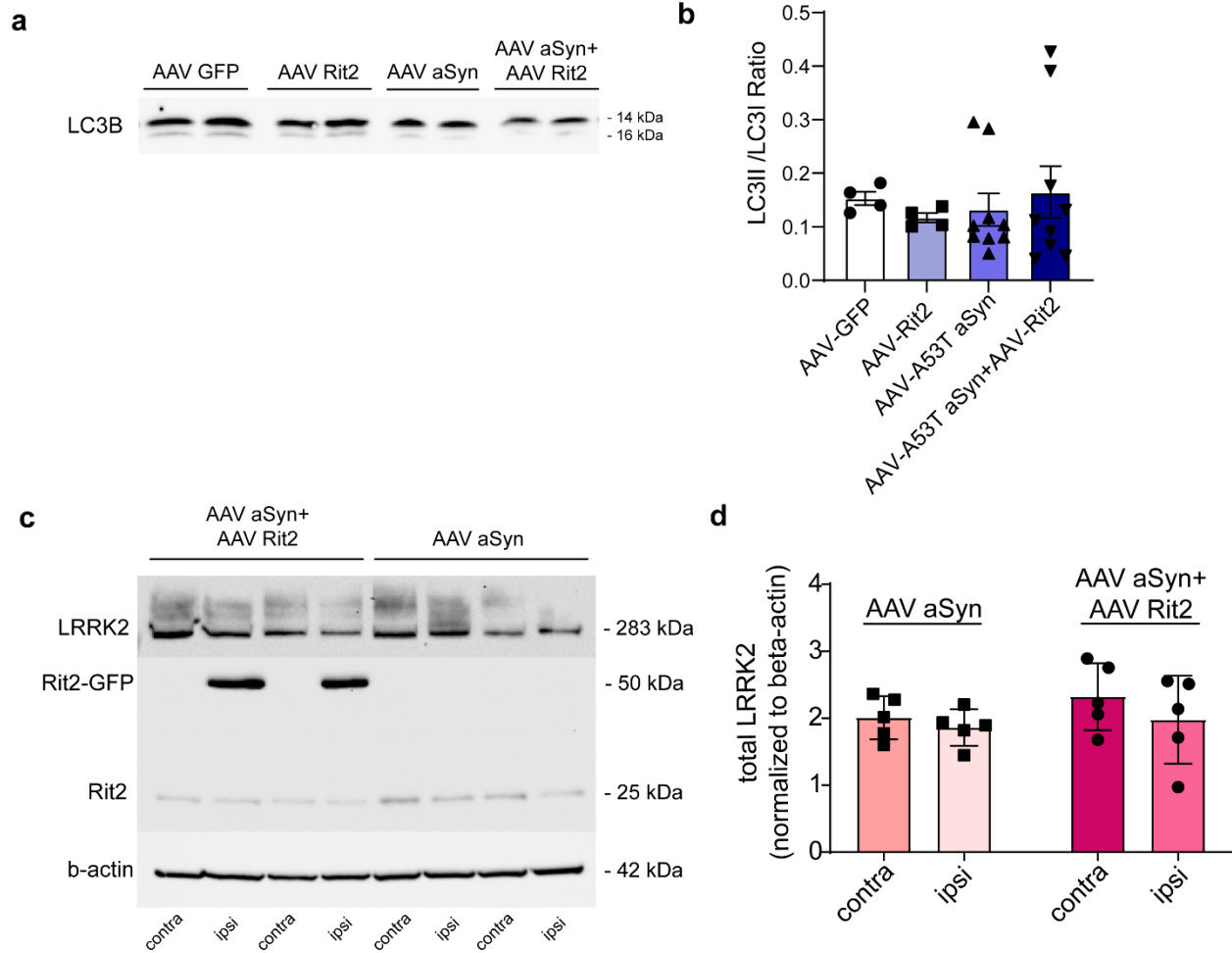
Data are means \pm SEM of 5-6 independent experiments for WB.

*p<0.05, unpaired two tailed Student's t-test.



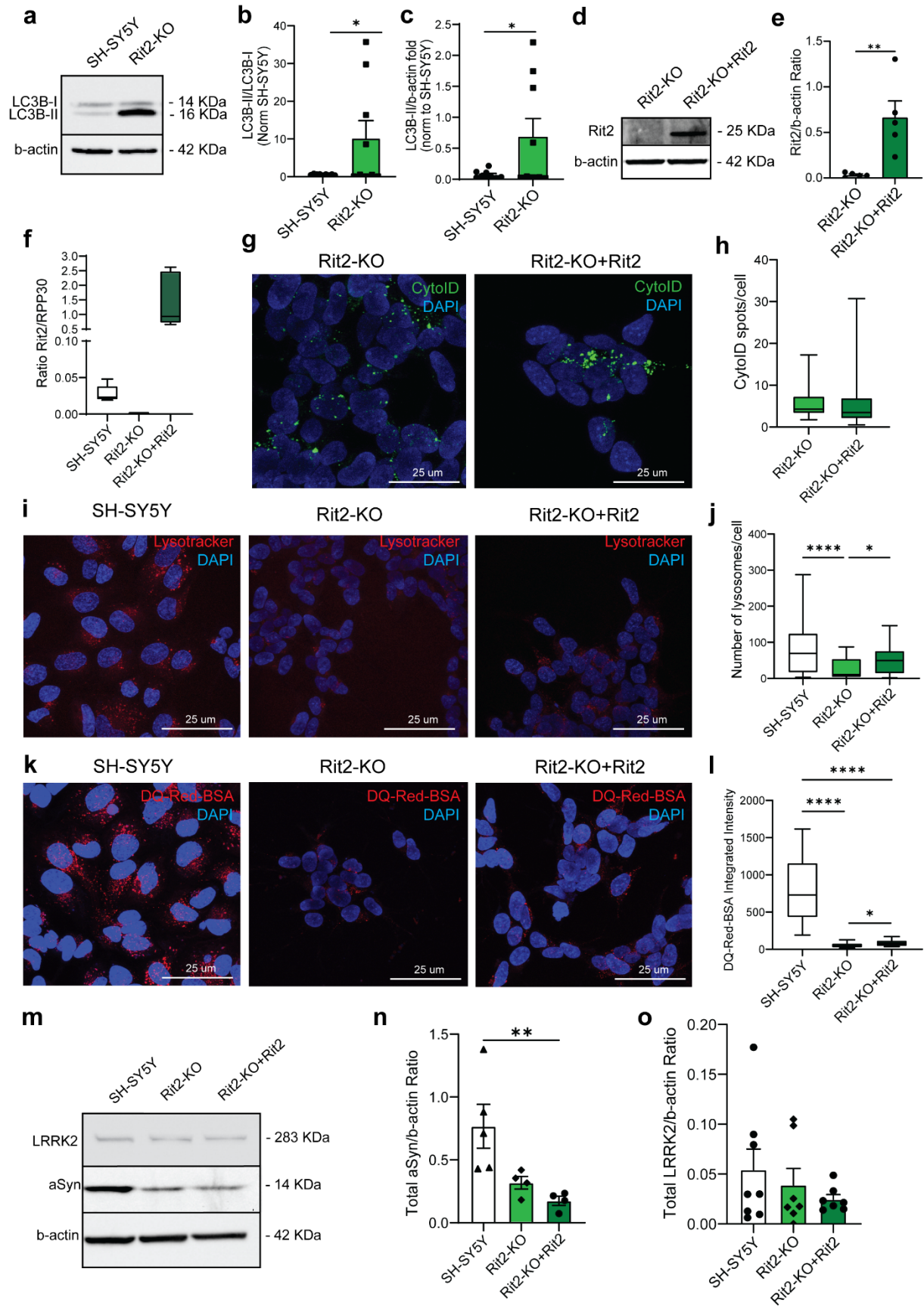
Supplementary Figure 7: Overexpression of aSyn increases ipsilateral rotations in the amphetamine test and doesn't affect expression level in DA neurons. a) Overexpression of A53T-aSyn reduces the percentage of contralateral forepaw use and co-injection with AAV-Flex-Rit2 has the same effect. b) aSyn overexpression induces a significant increase in ipsilateral rotations in the cylinder with amphetamine test (n: AAV-GFP=7, AAV-aSyn=9). c) Overexpression of aSyn in the SNc does not affect TH expression. Around 90% of the cells in the ipsilateral side of AAV-GFP alone or AAV-GFP+AAV-aSyn injected mice are double positive for GFP and TH (5 animals/group). Data represented as mean \pm SEM.

***p<0.001, unpaired two tailed Students's t-test. **p<0.01, one-way ANOVA followed by Bonferroni's post-hoc test.



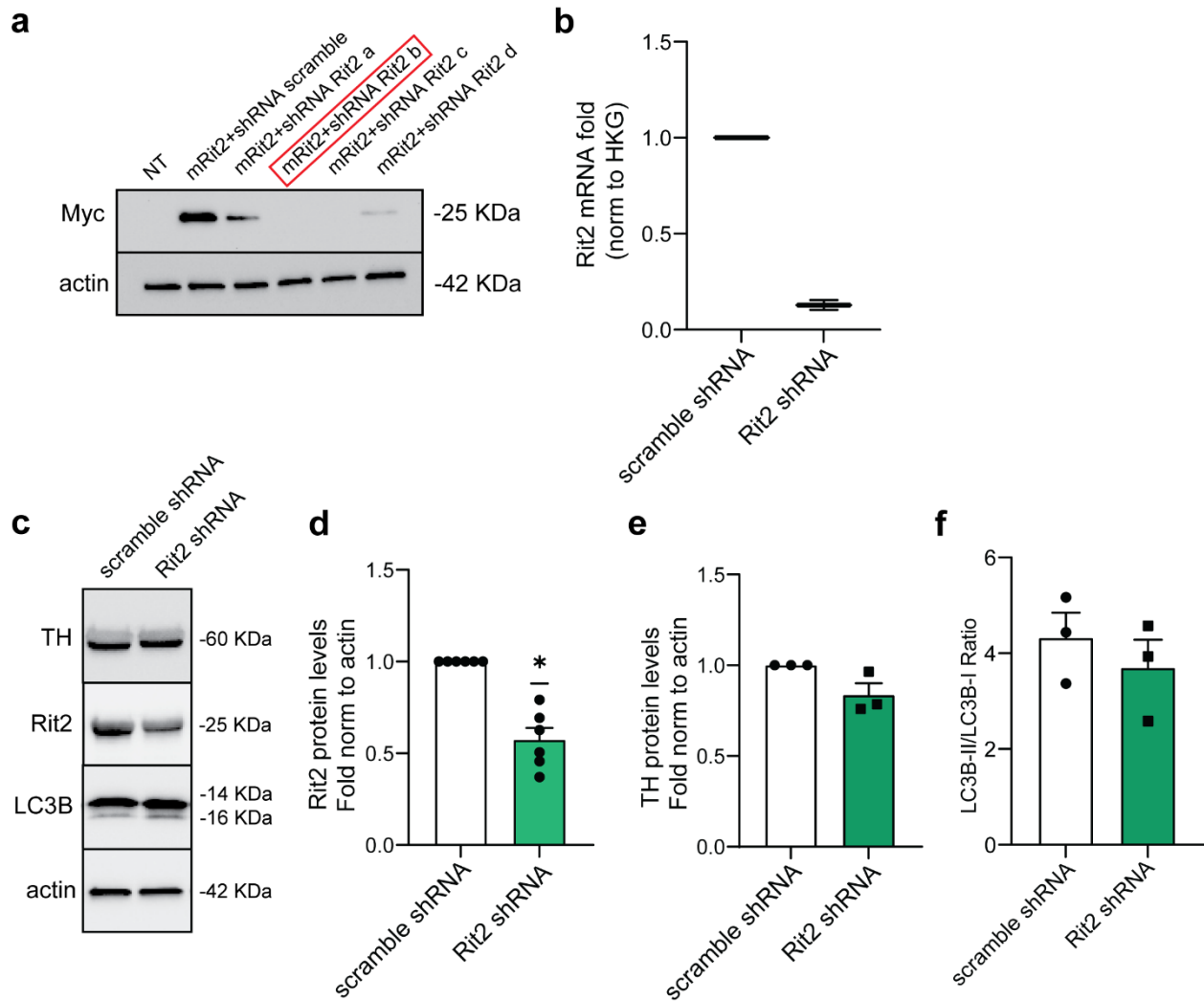
Supplementary Figure 8: *Total LRRK2 and LC3B levels are not altered by viral overexpression of aSyn and Rit2.* a) LC3BII and LC3BI levels were assessed by Western blot analysis in the different experimental groups (n=4). b) LC3BII/LC3BI ratio is not altered in the different experimental groups. c) Total LRRK2 and Rit2 levels were assessed by Western blot analysis in AAV-aSyn and AAV-aSyn+AAV- Rit2 injected mice (contra- and ipsilateral site). d) Total LRRK2 protein levels are not altered by viral overexpression of aSyn and Rit2 (normalized to b-actin) (n=5).

Data are means±SEM.



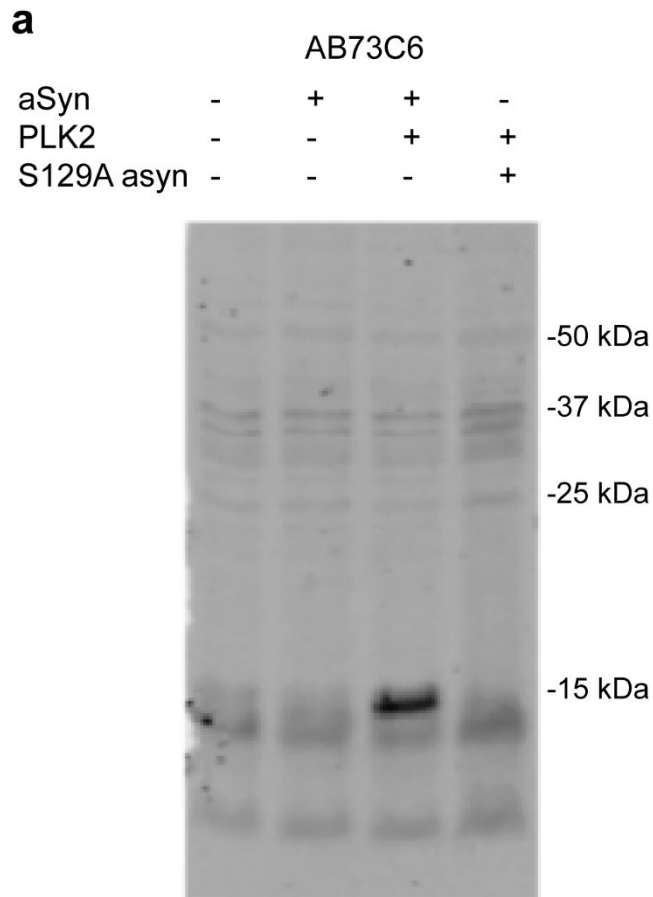
Supplementary Figure 9: *Rit2* rescues lysosome number and functionality in *Rit2*-KO cells. a) LC3BII and LC3BI levels were assessed by Western blot analysis in the different experimental groups. b) LC3BII/LC3BI ratio is increased in *Rit2*-KO cells. c) LC3BII/b-actin ratio is increase in *Rit2*-KO cells. d) Western blot of *Rit2* protein levels after *Rit2* nucleofection. e) *Rit2* protein levels are significantly increased after nucleofection. f) Droplet Digital PCR was carried out to assess *RIT2* mRNA levels in *Rit2*-KO cells with or without *Rit2* overexpression. Ratio of *RIT2* mRNA, normalized to RPP30 is increased after *Rit2* nucleofection (n=3). g) CytoID assay was employed to visualize autophagosome and autolysosome distribution. h) Quantification of CytoID-positive puncta revealed no difference in *Rit2*-KO cells, when *Rit2* was overexpressed (n=4). i) Cell processing with the LysoTracker Red dye was performed to visualize lysosomes in SH-SY5Y, *Rit2*-KO and *Rit2*-KO+ *Rit2* cells. j) The number of lysosomes per cell was quantified and revealed a rescue of the number of lysosomes when *Rit2* was transfected into *Rit2*-KO cells (n=4). k) The DQ-Red-BSA assay was employed to assess the proteolytic activity of lysosomes. l) Quantification of DQ-Red-BSA fluorescent spots an increase of the DQ-Red-BSA intensity in *Rit2*-KO cells, with *Rit2* overexpression (n=4). m) Western blot analysis of total LRRK2 and aSyn in *Rit2*-KO cells with or without *Rit2* expression. n) Analysis demonstrated that *Rit2* overexpression does not alter total aSyn levels in *Rit2*-KO cells, but total aSyn levels are lower than in SH-SY5Y cells. (n=5). o) Analysis demonstrated that *Rit2* overexpression does not alter total LRRK2 levels in *Rit2*-KO cells (n=6).

In imaging experiments analysis was conducted on 700-1000 cells per group in each experiment. Data are represented as median, boxes show the IQ and whiskers show min-max or means \pm SEM. *p<0.05, unpaired two tailed Students's t-test *p<0.05, **p<0.01 ****p<0.0001, one-way ANOVA followed by Bonferroni's post-hoc test.



Supplementary Figure 10: *Rit2* knock-down in midbrain cultures does not alter *TH* or *LC3B* levels. a) Four different shRNA constructs were tested in NIH-3T3 cells with the overexpression of *Rit2*. shRNA *Rit2 b* was chosen for further experiments. b) *Rit2* mRNA levels in primary dopaminergic neurons were reduced of about 90% after 7 days of shRNA expression as measured in qPCR (n=2). c) Western blot analysis of scramble and *Rit2* shRNA infected midbrain cultures. d) *Rit2* protein levels are decreased when midbrain cultures are infected with *Rit2* shRNA (n=6). e) *TH* protein levels are not altered with scramble or *Rit2* shRNA infection (n=3). f) *LC3BII/LC3BI* ratio is not altered with scramble or *Rit2* shRNA infection (n=3). Data are

represented as median, boxes show the IQ and whiskers show min-max or means±SEM. One sample Wilcoxon test, *p<0.05.

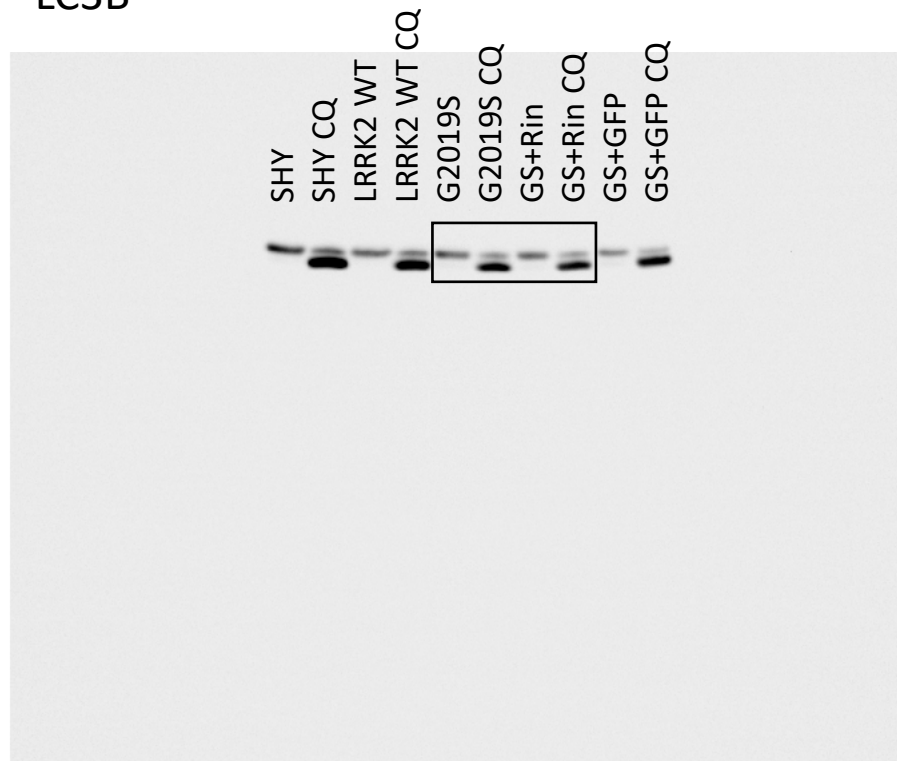


Supplementary Figure 11: *Custom antibody 73C6 is specific for pS129-aSyn.* a) HEK293 cells transfected with aSyn in combination with the PLK2 kinase, which is known to phosphorylate aSyn, results in a strong band at 15 kDa (lane 3). Cells transfected with PLK2 and a mutated version of aSyn that cannot be phosphorylated (S129A), no band is observed at 15 kDa (lane 4), indicating that the antibody recognizes specifically the phosphorylated form of aSyn. No detectable signal is observed in naïve HEK293 cells or when transfected with aSyn alone (lanes 1 and 2).

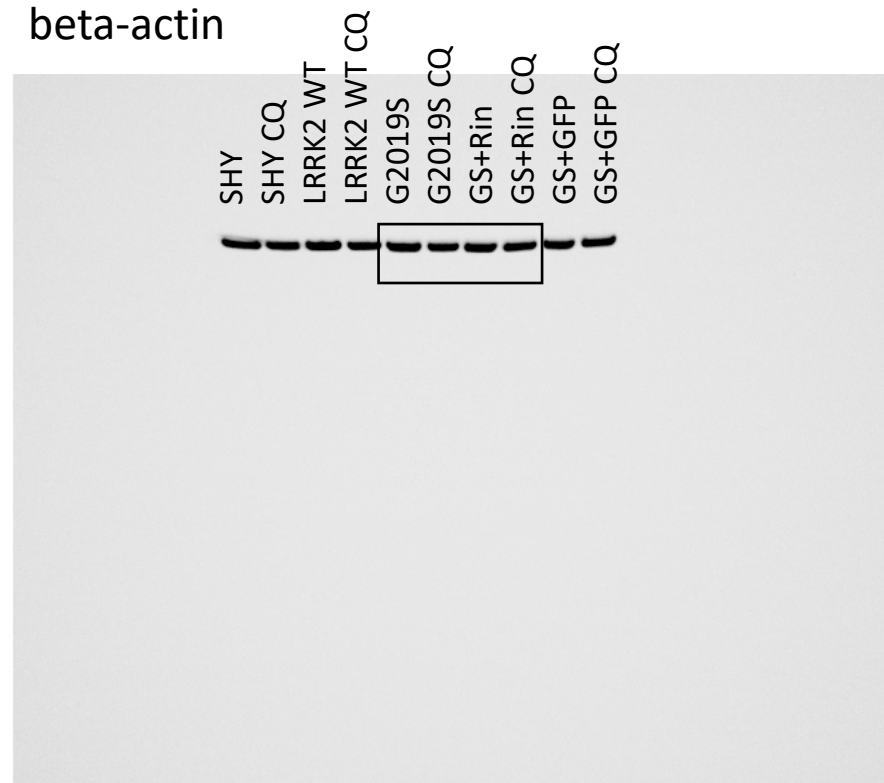
WB for LC3B (Fig 2 A-C)

N1

LC3B



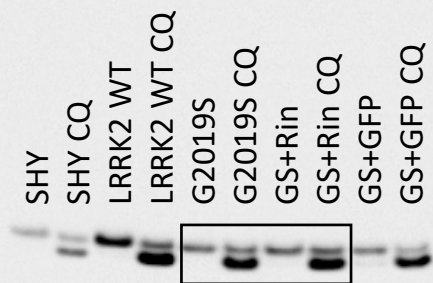
beta-actin



WB for LC3B (Fig 2 A-C)

N2

LC3B



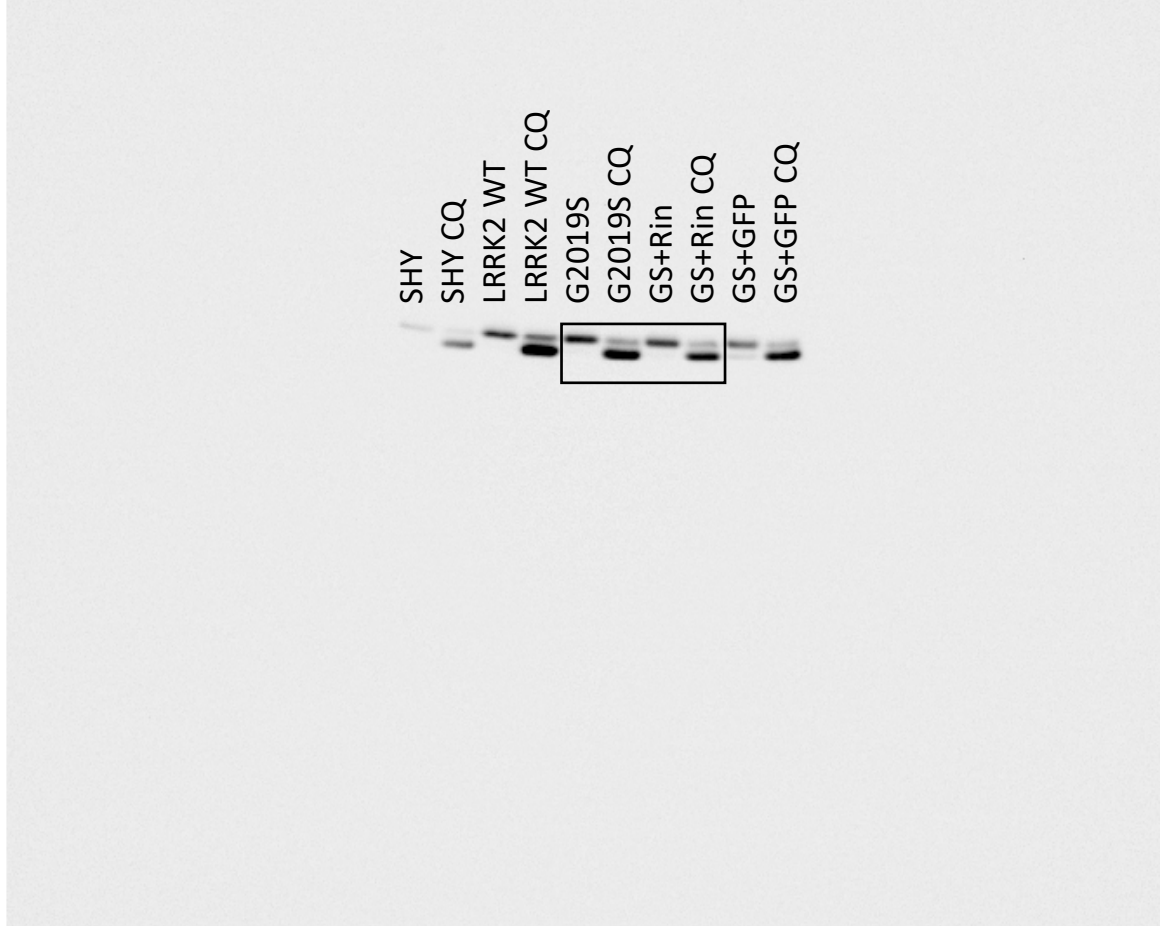
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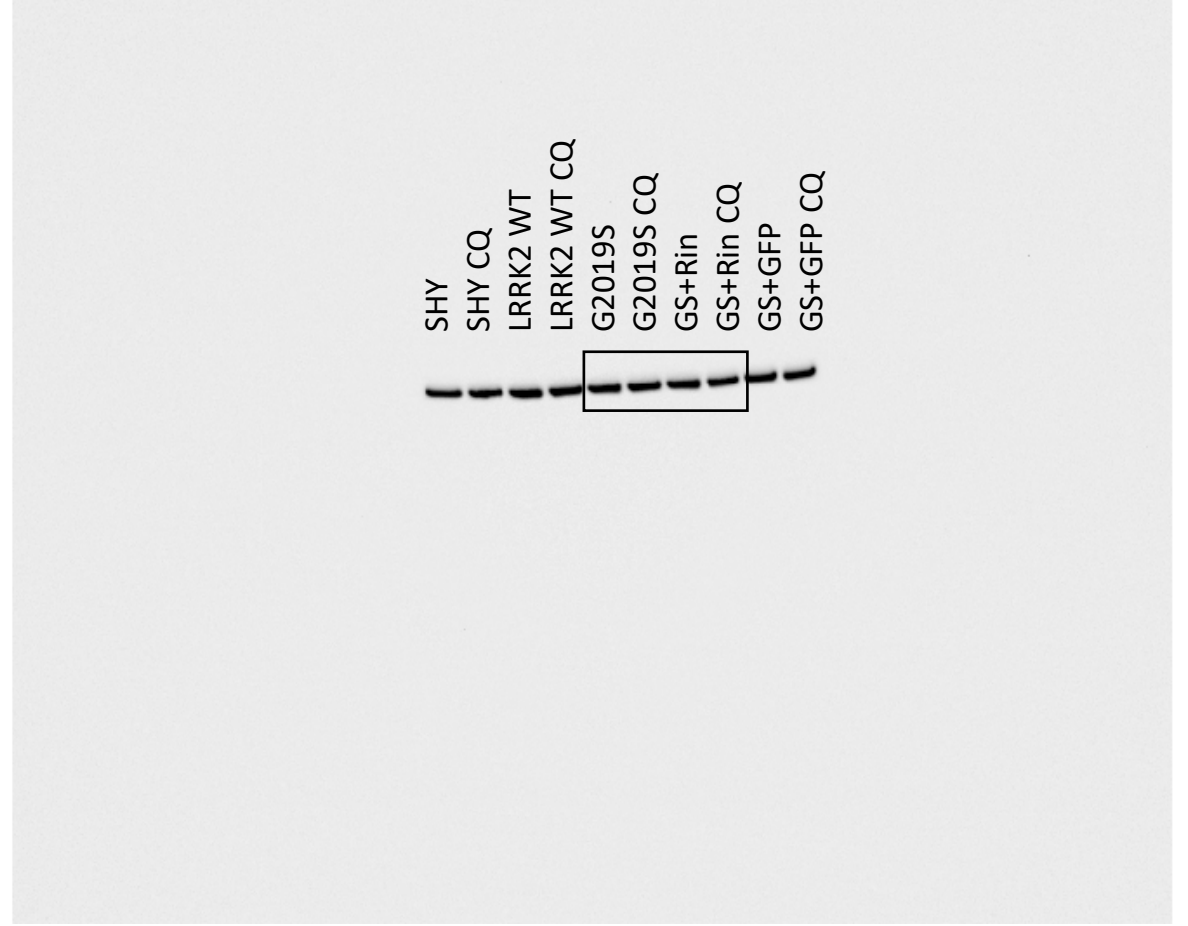
WB for LC3B (Fig 2 A-C)

N3

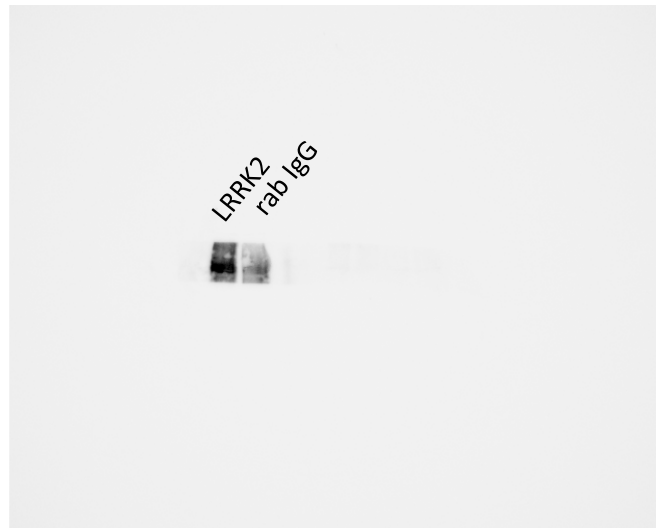
LC3B



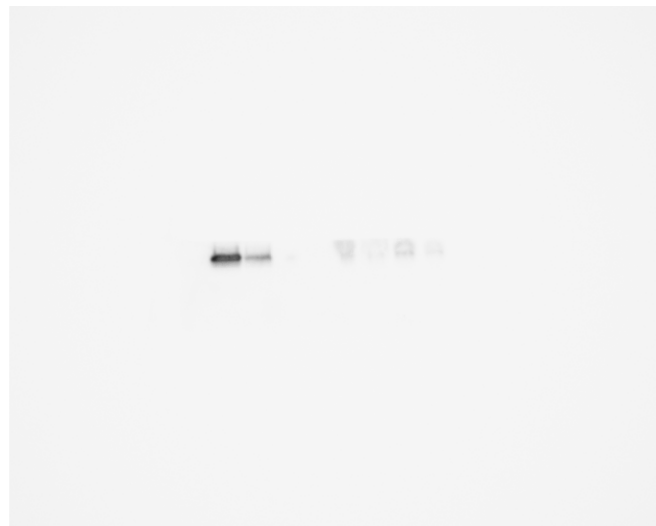
beta-actin



CO IP (Fig 4)



LRRK2 CO IP

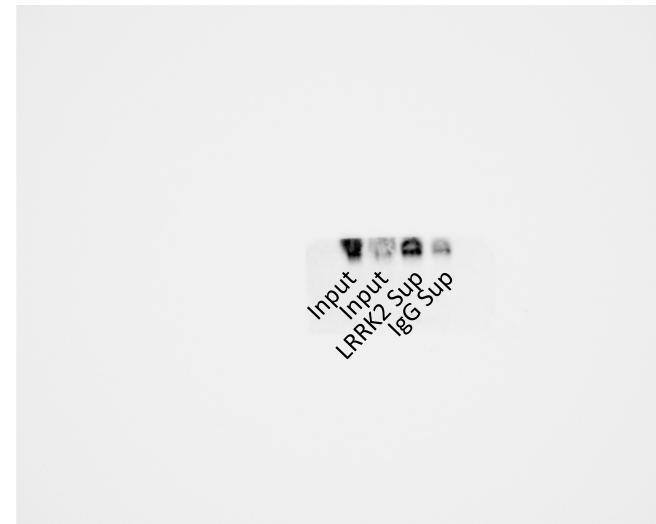


Rit2 CO IP

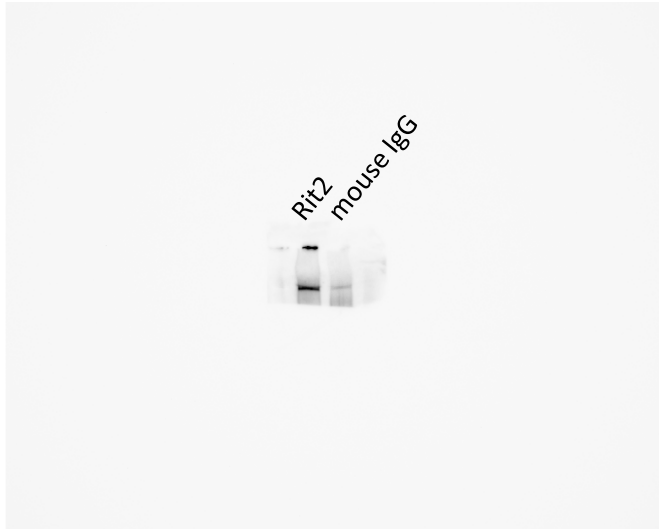
LRRK2



Rit2



CO IP (Fig 4)



LRRK2 CO IP

LRRK2



Rit2

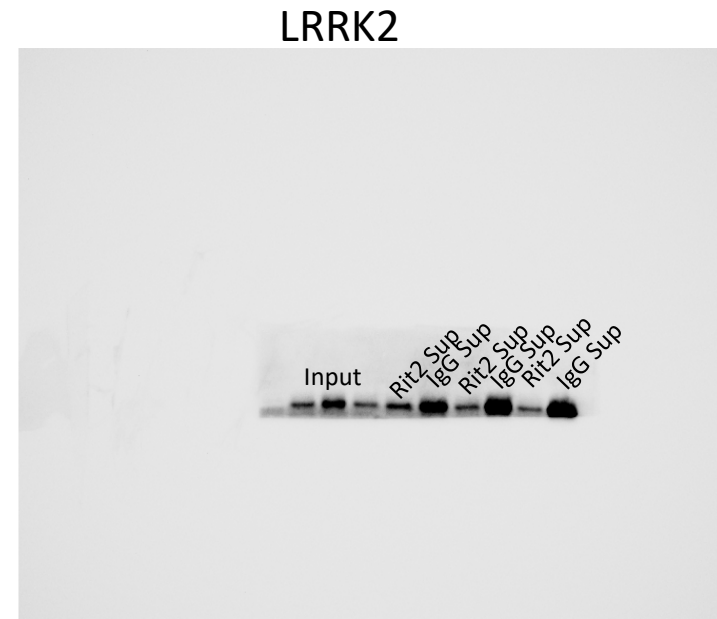


Rit2 CO IP

CO IP (Fig 4)



LRRK2 CO IP

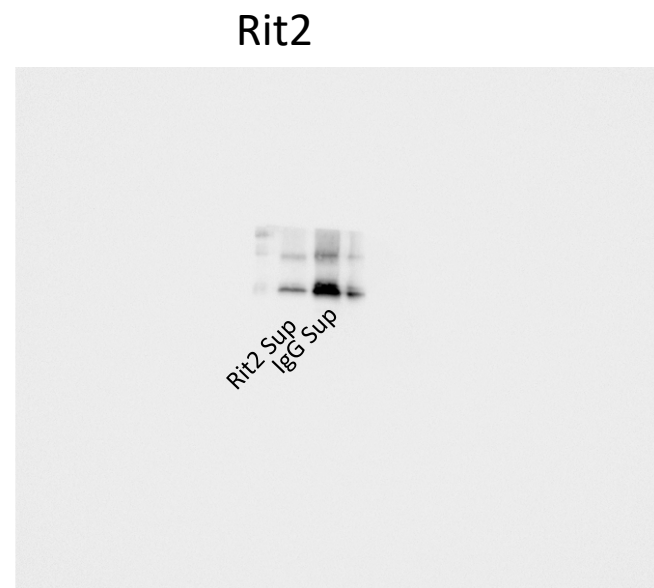


Rit2 CO IP

CO IP Fig 4

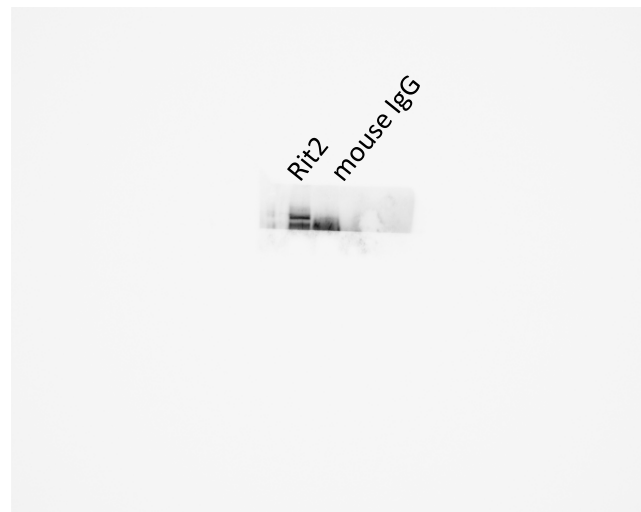


LRRK2 CO IP



Rit2 CO IP

CO IP Fig 4

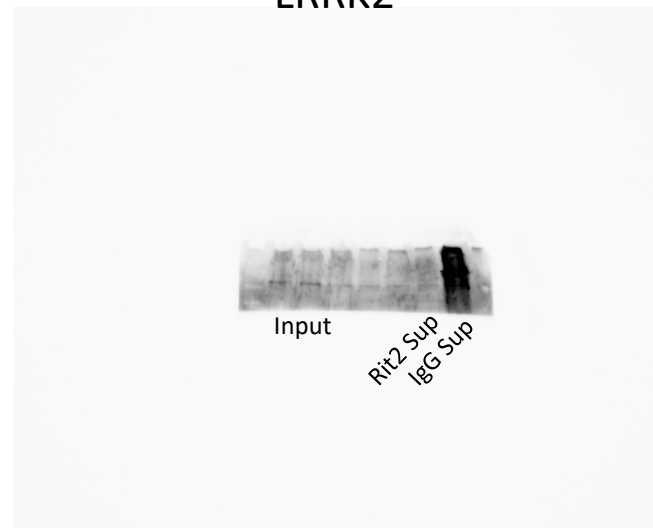


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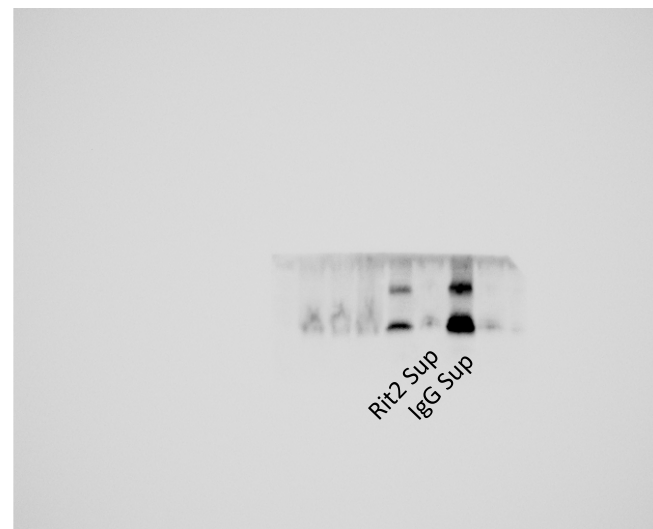


Rit2 CO IP

LRRK2



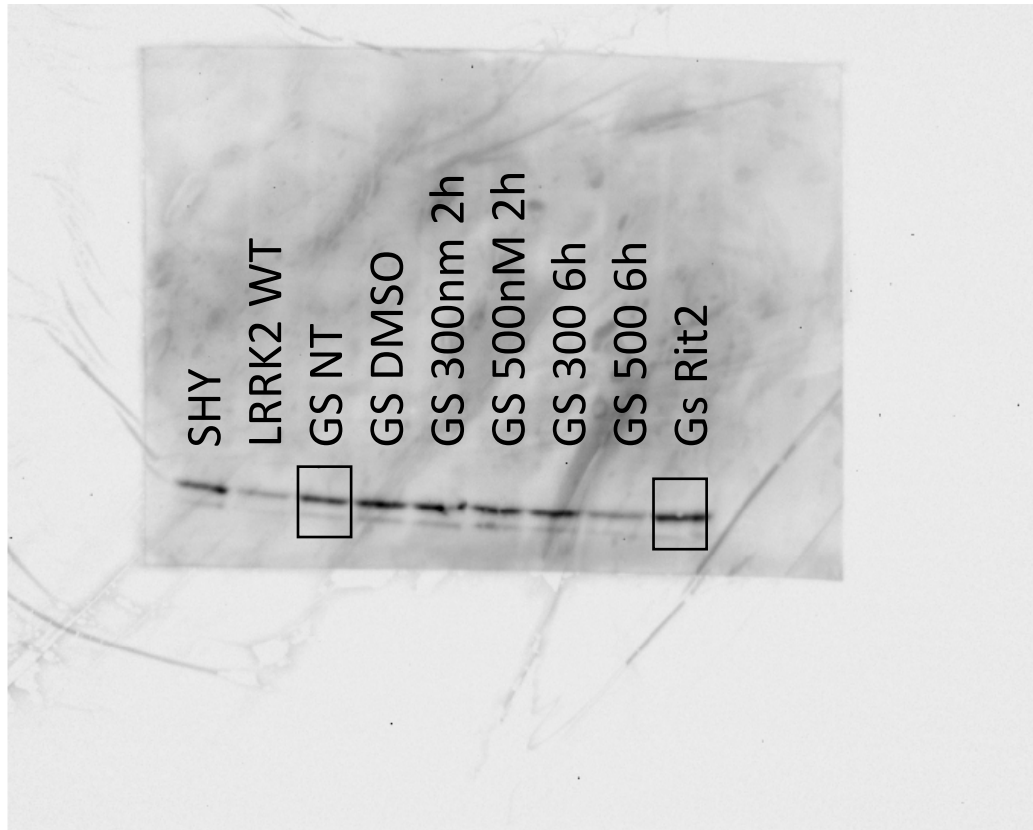
Rit2



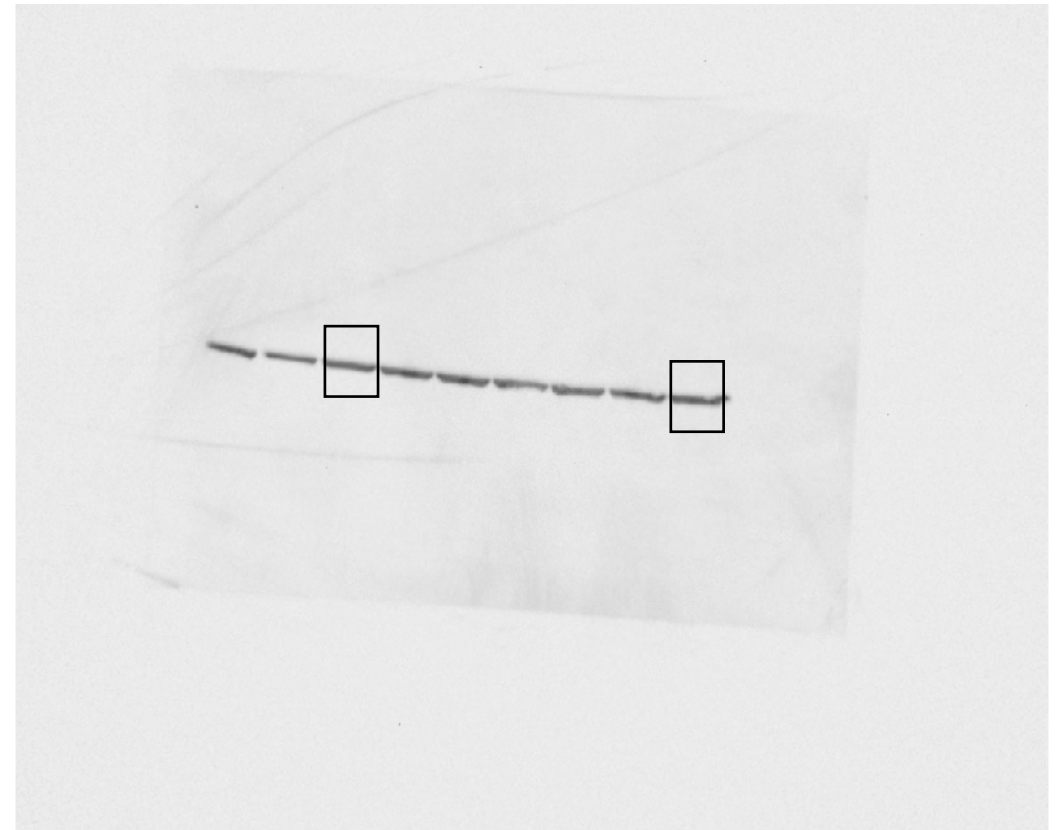
WB for total aSyn (Fig S4)

N1

aSyn



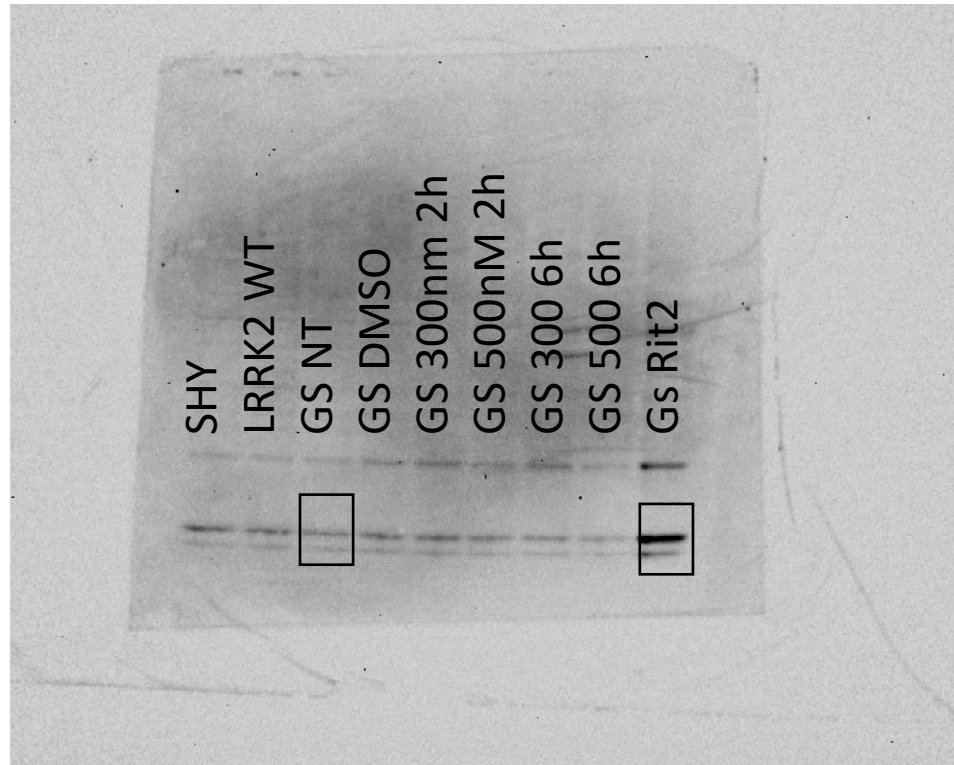
actin



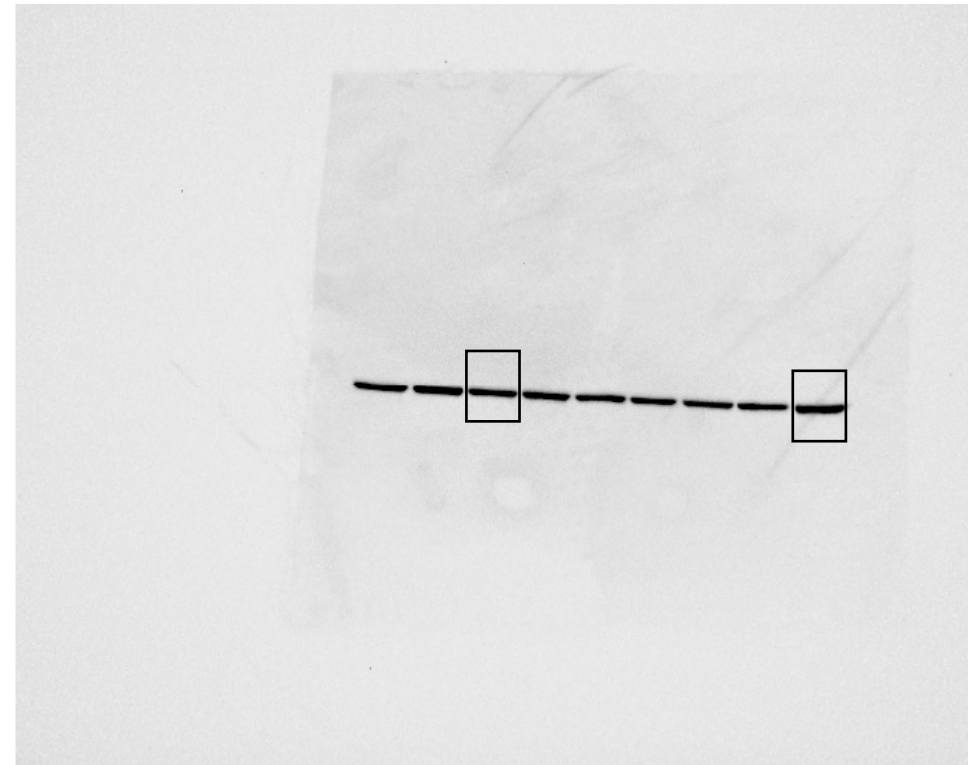
WB for total aSyn (Fig S4)

N2

aSyn



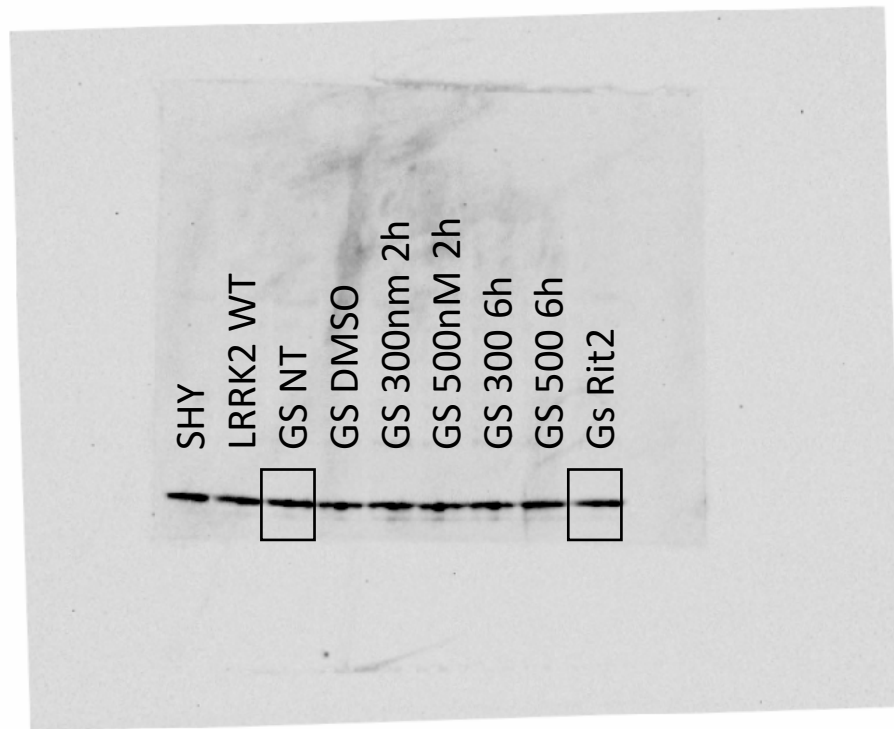
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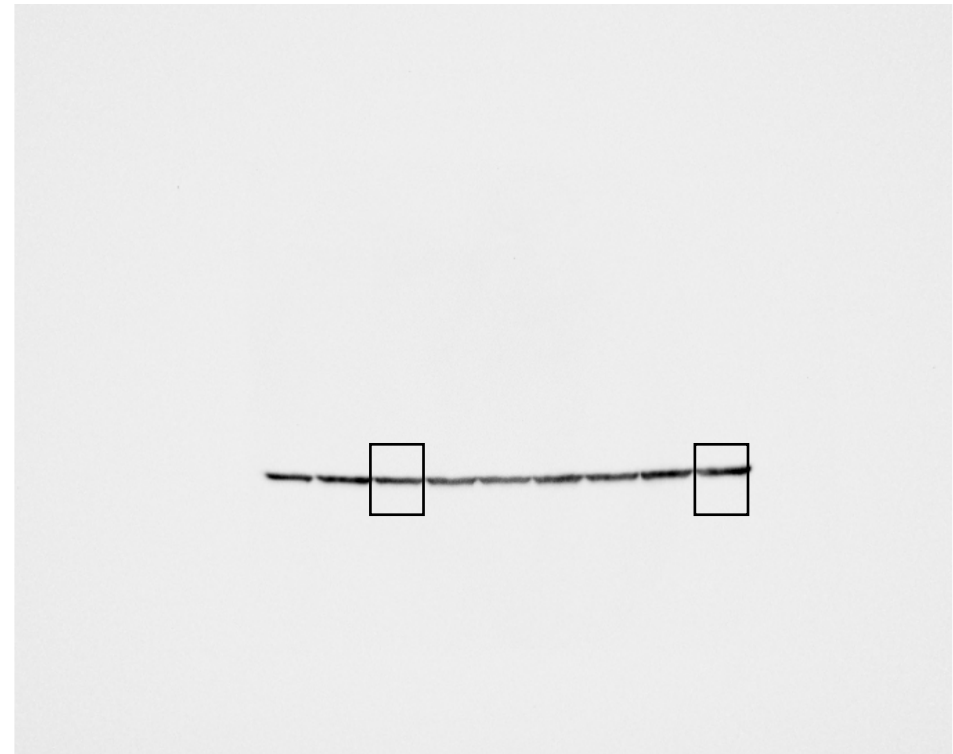
WB for total aSyn (Fig S4)

N3

aSyn



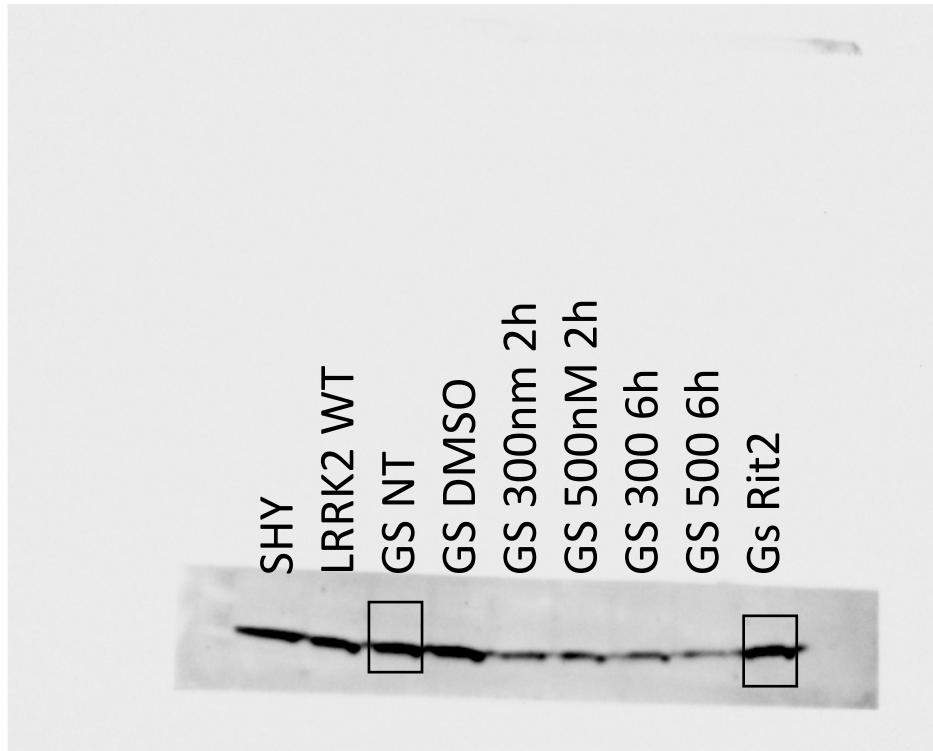
actin



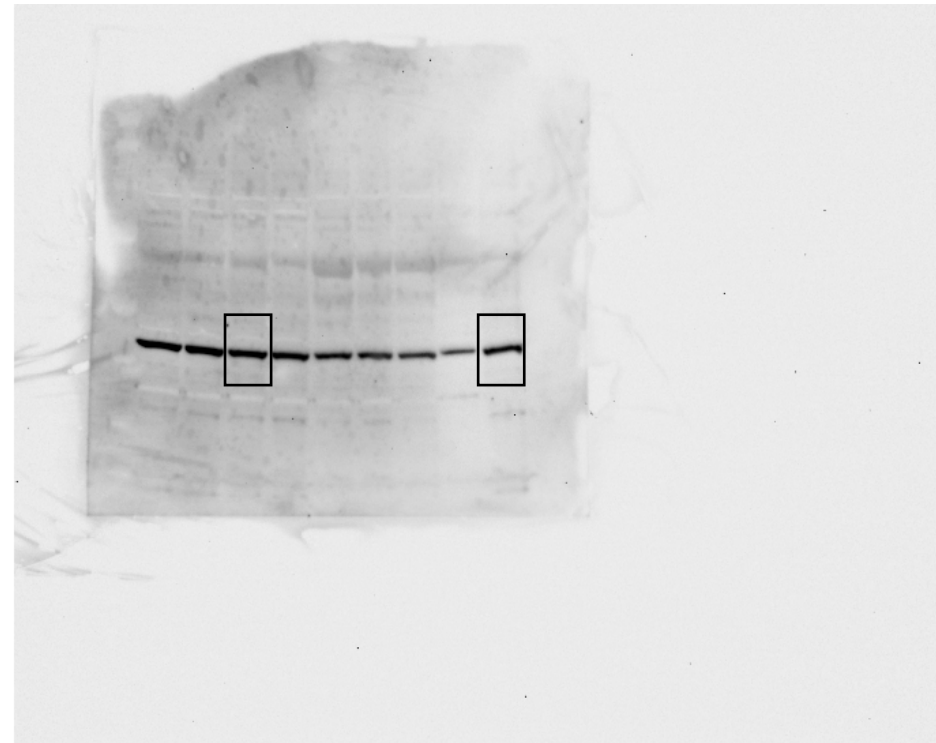
WB for total aSyn (Fig S4)

N4

aSyn



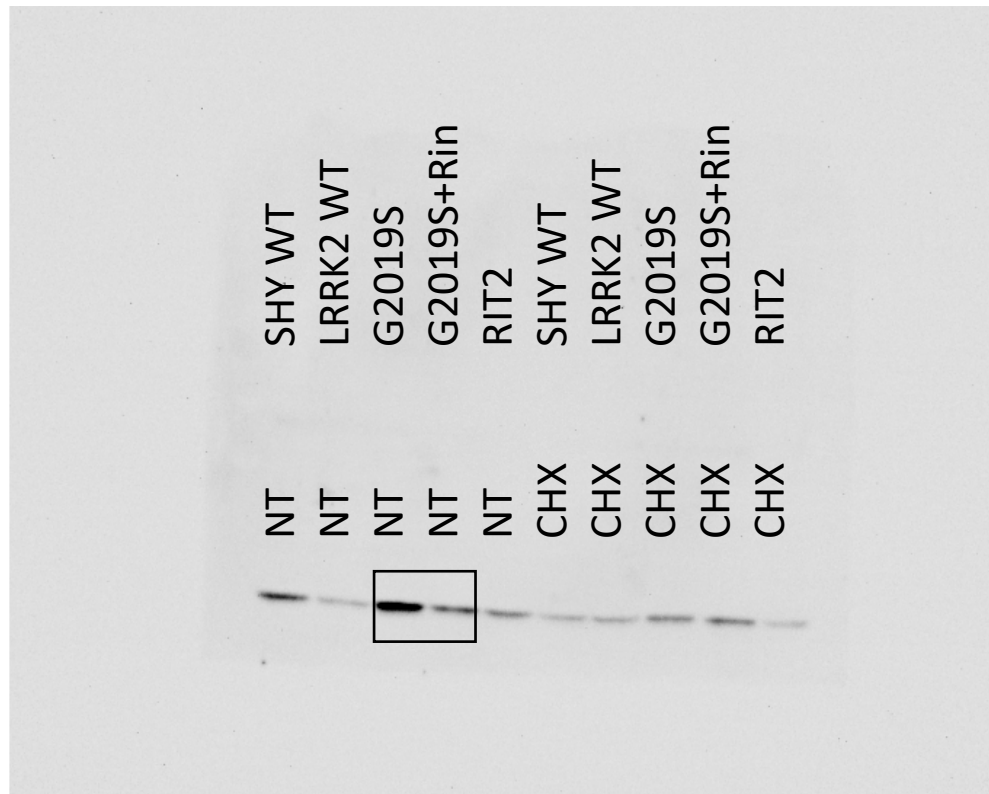
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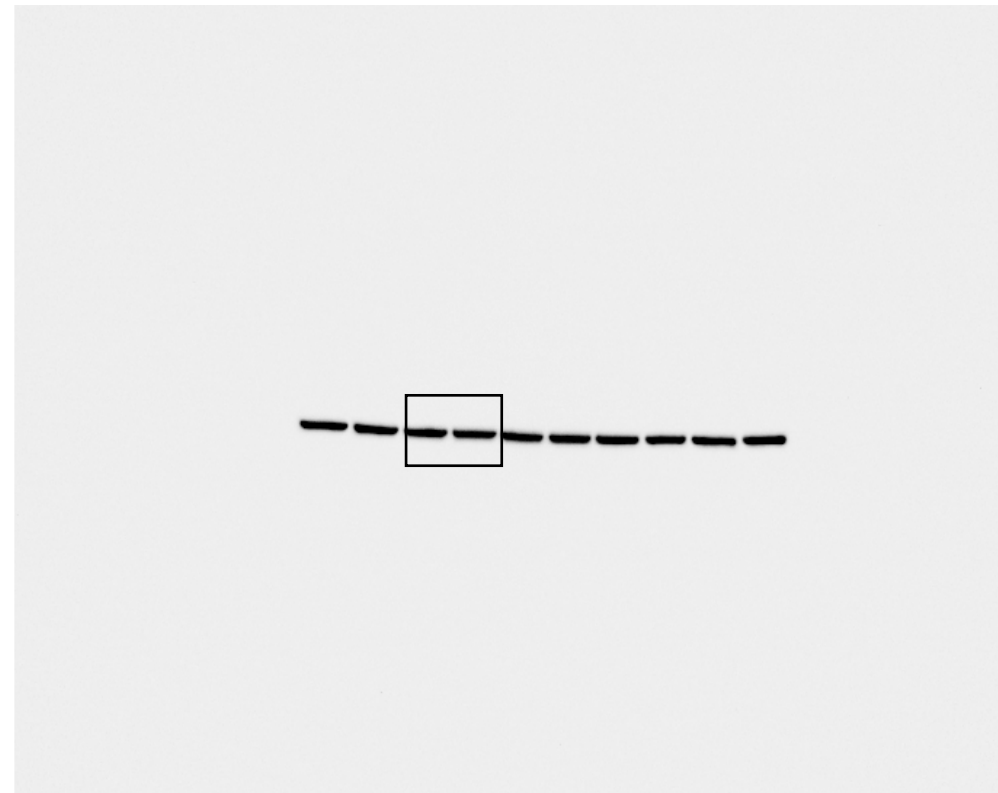
WB for total aSyn (Fig S4)

N5

aSyn



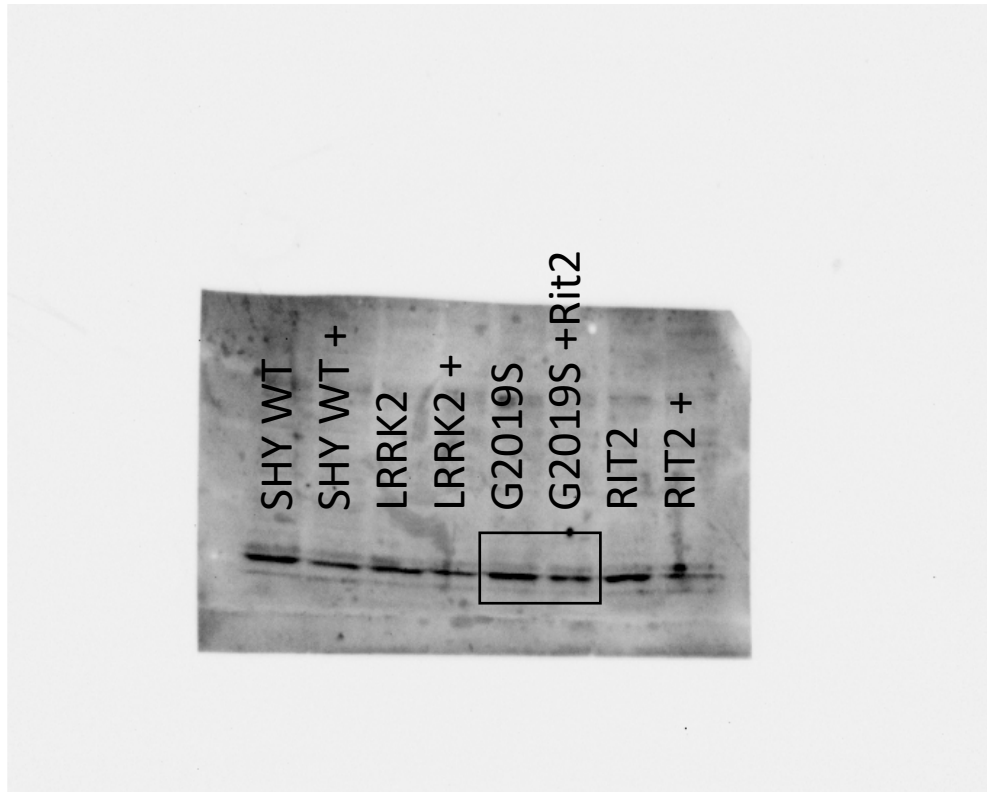
actin



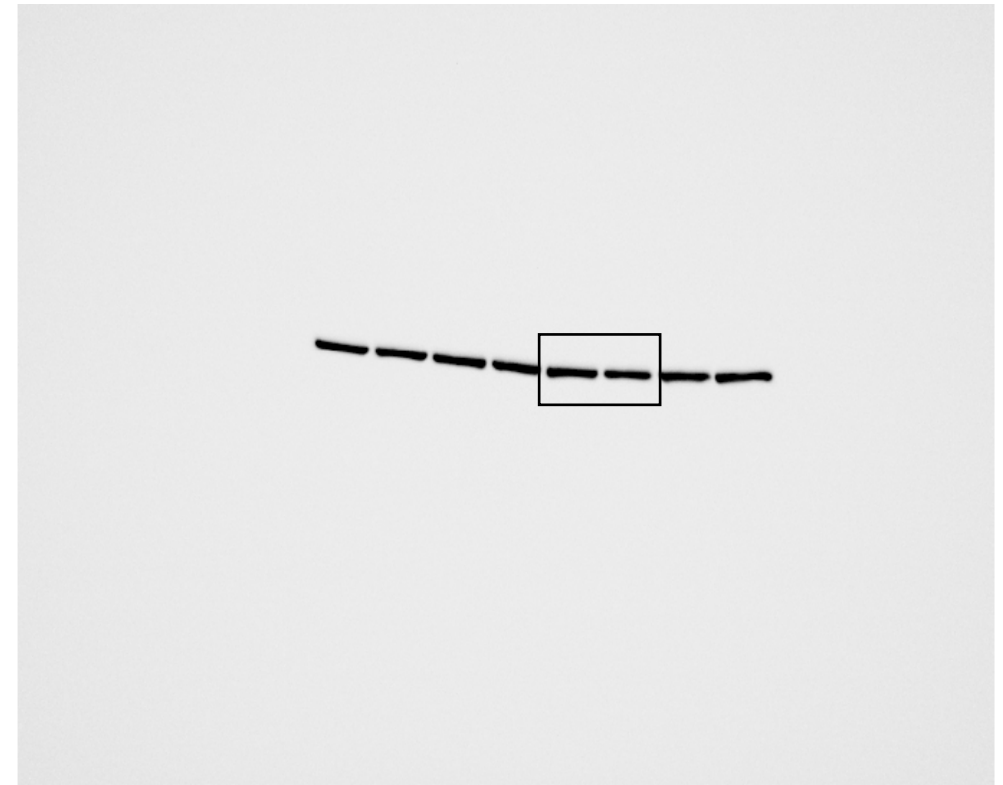
WB for total aSyn (Fig S4)

N6

aSyn



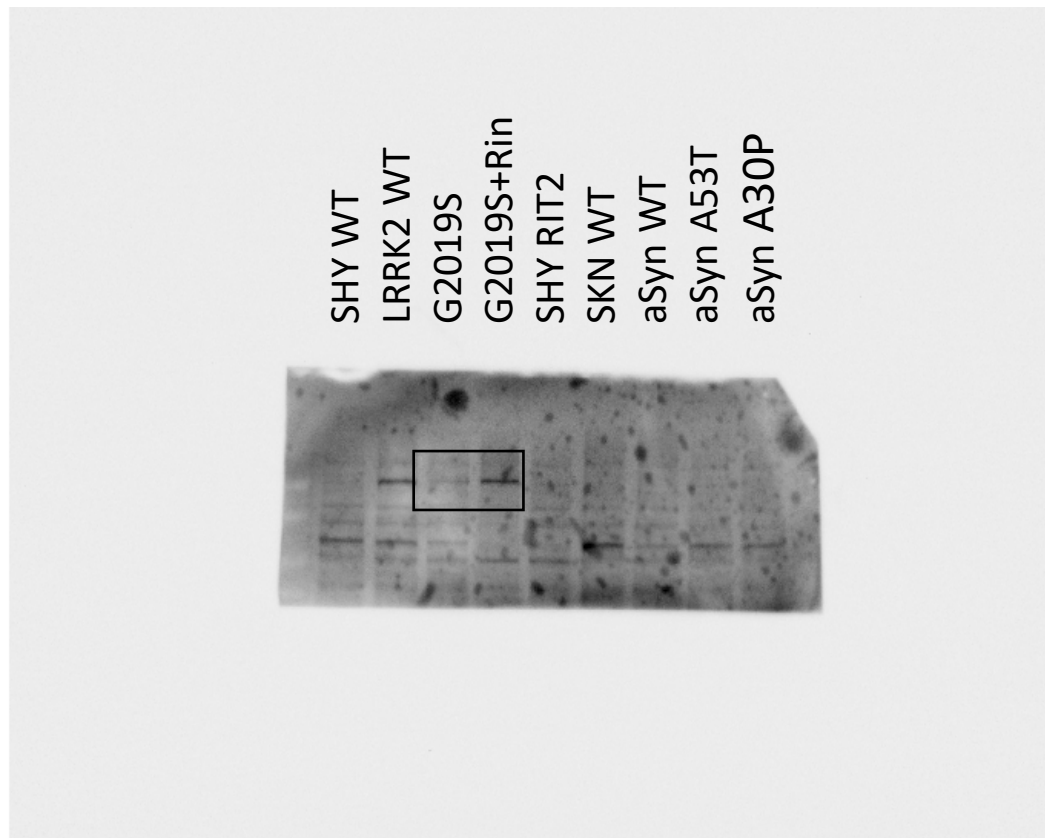
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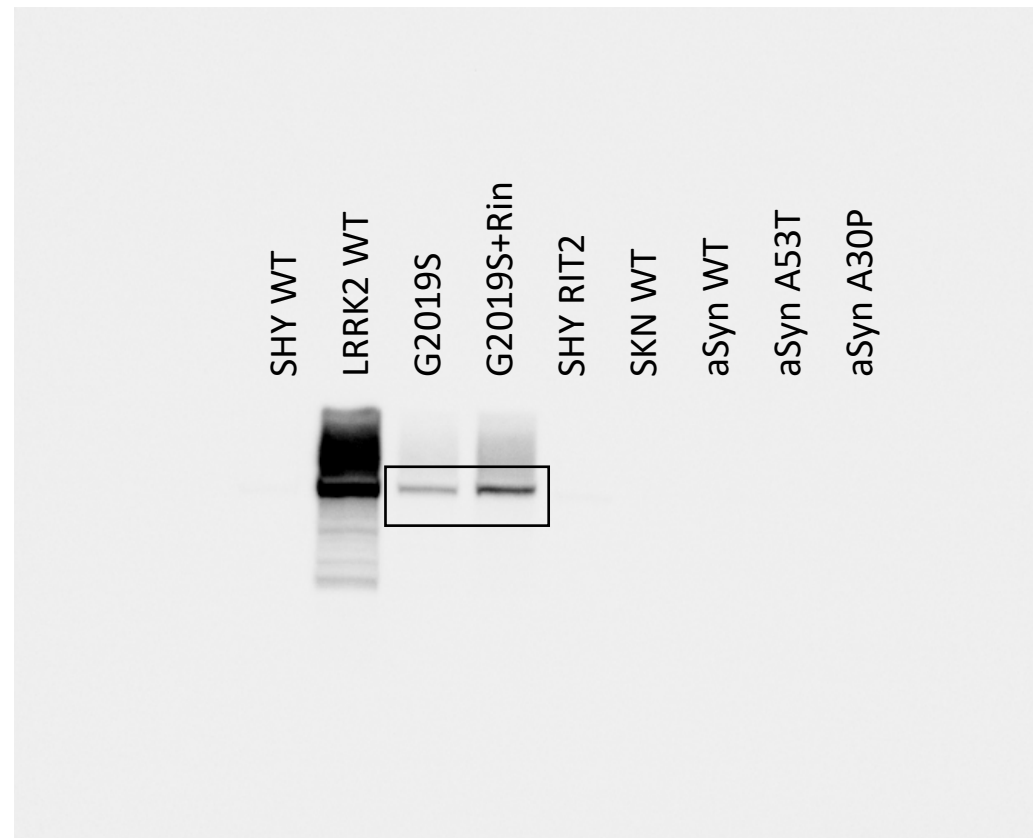
WB for pS1292-LRRK2 (Fig S6)

N1

pSer1292- LRRK2



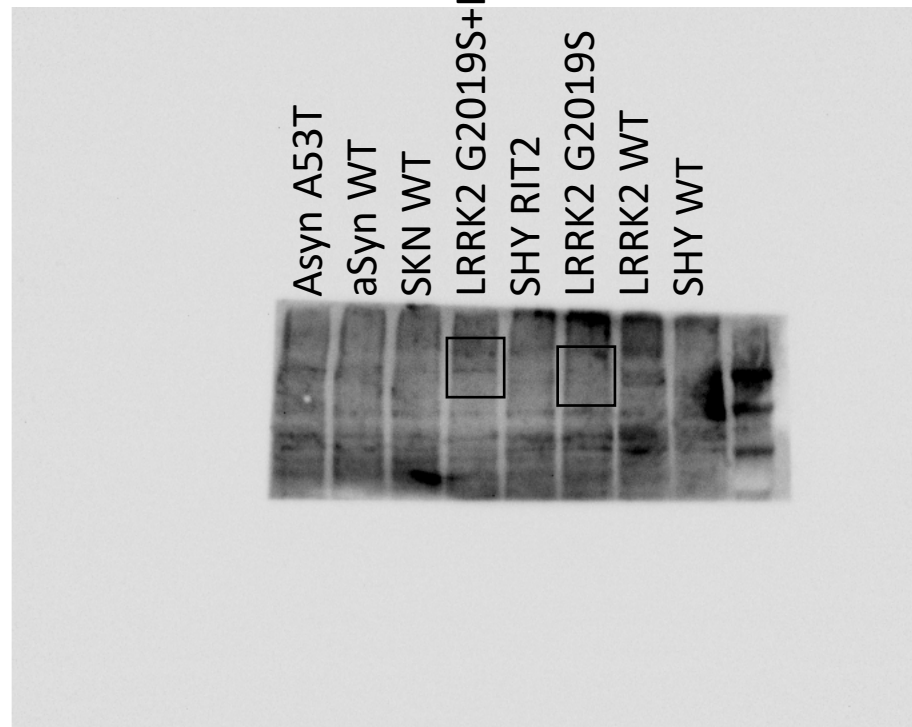
LRRK2



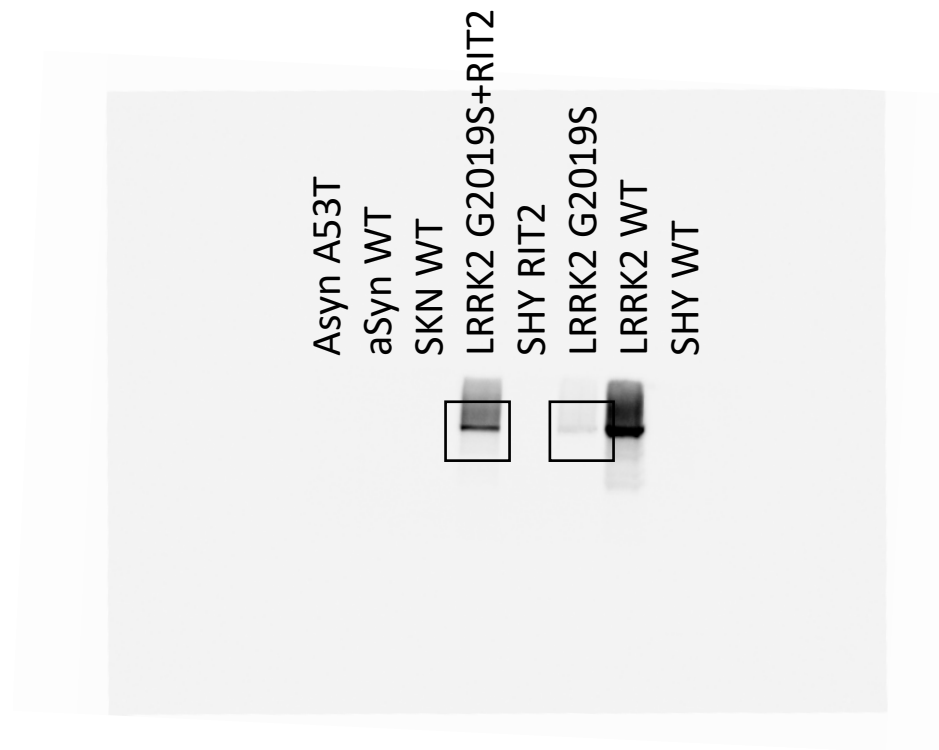
WB for pS1292-LRRK2 (Fig S6)

N2

pSer1292- LRRK2



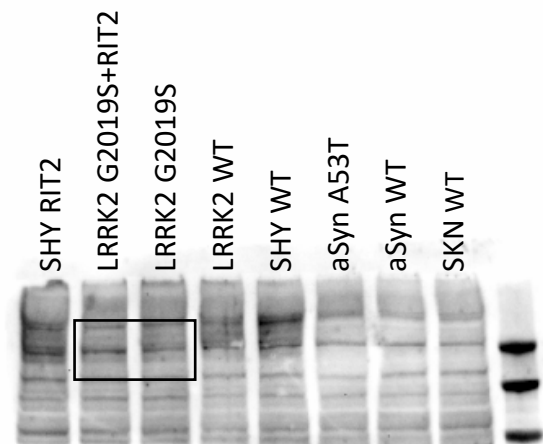
LRRK2



WB for pS1292-LRRK2 (Fig S6)

N3

pSer1292- LRRK2



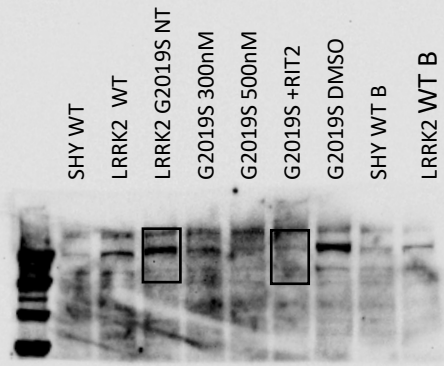
LRRK2



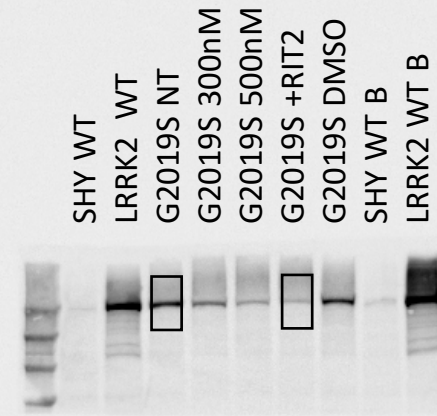
WB for pS1292-LRRK2 (Fig S6)

N4

pSer1292- LRRK2



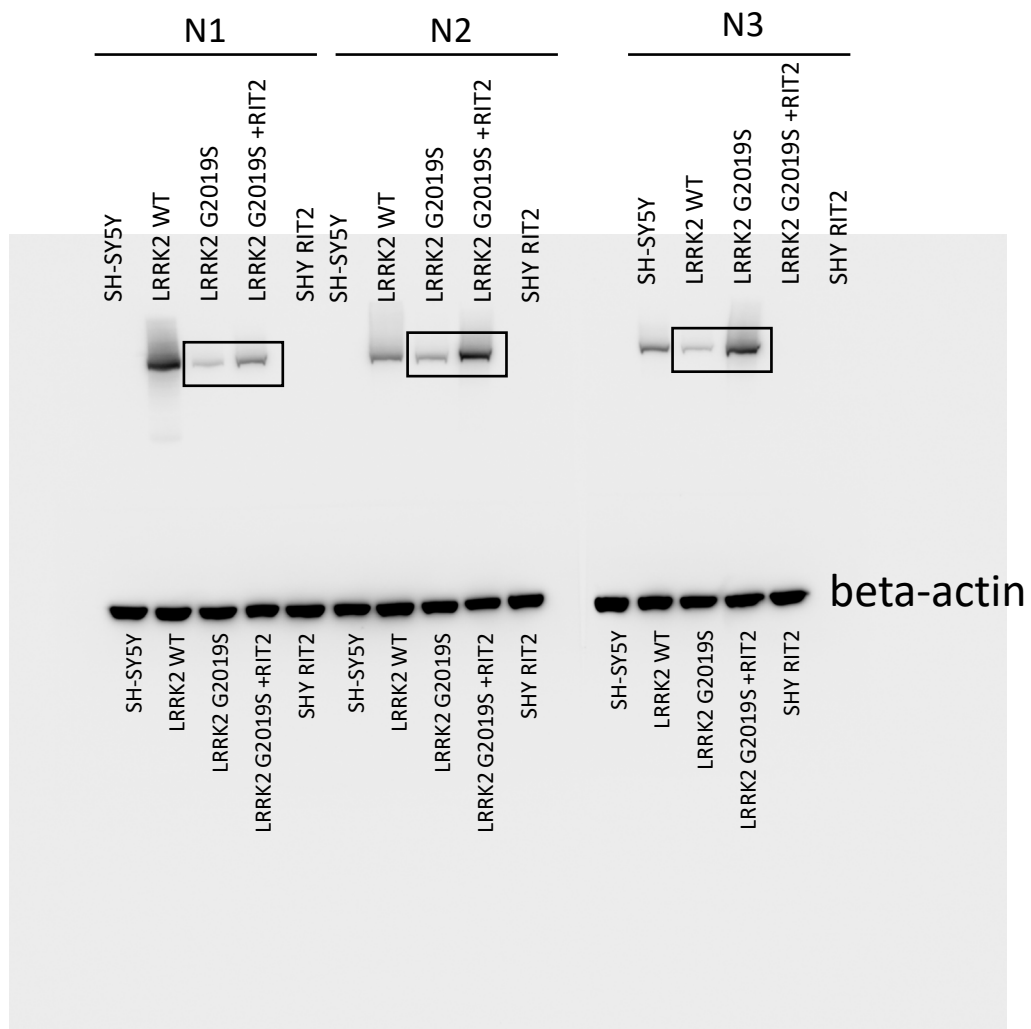
LRRK2



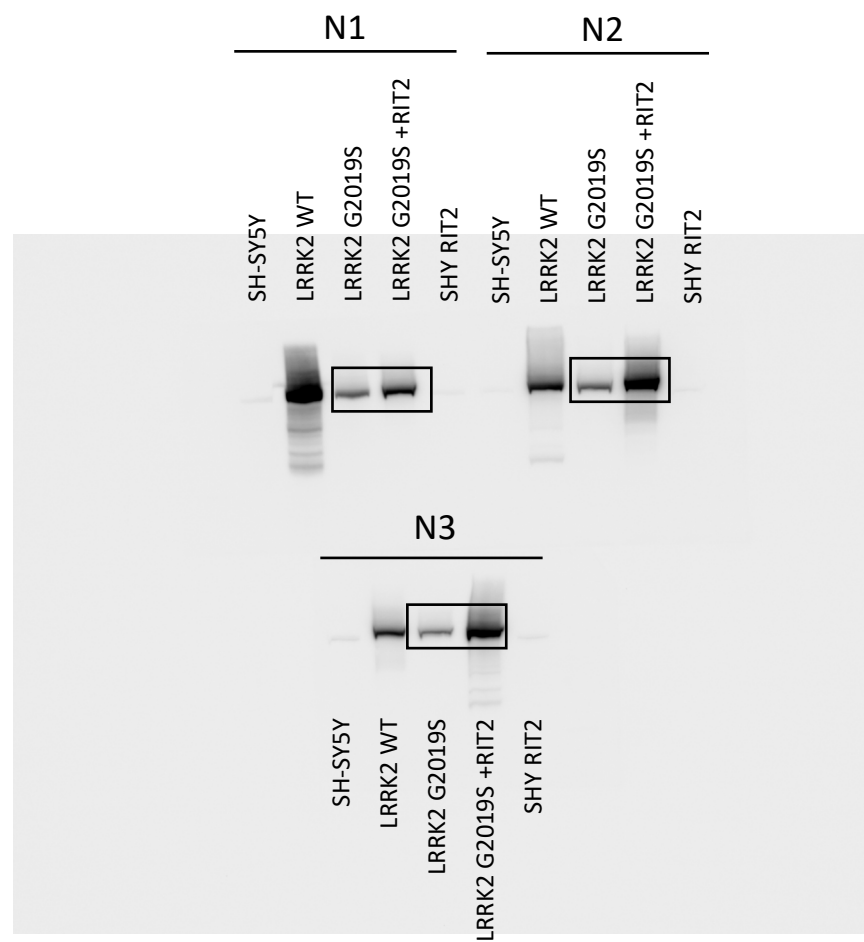
WB for pS935-LRRK2 (Fig S6)

N1, N2, N3

pSer935- LRRK2



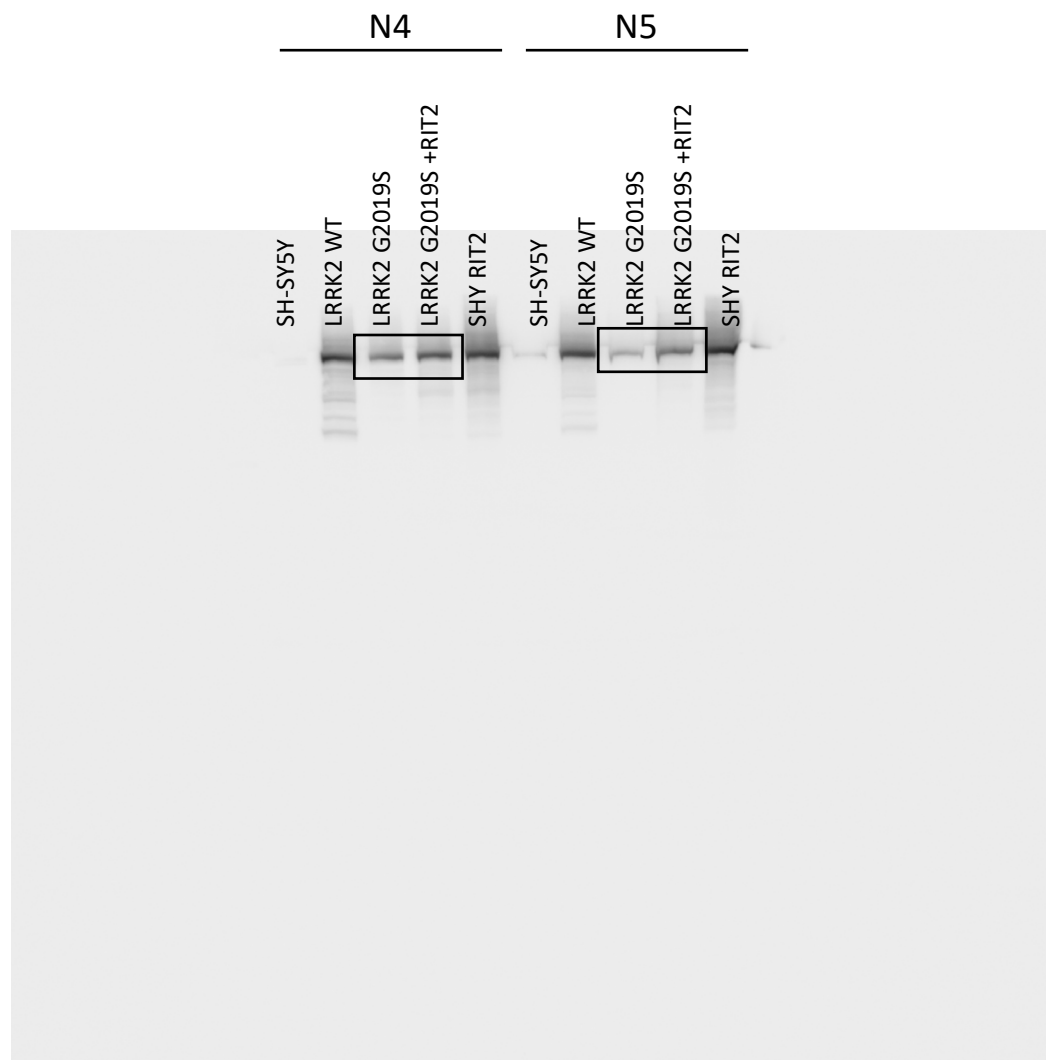
LRRK2



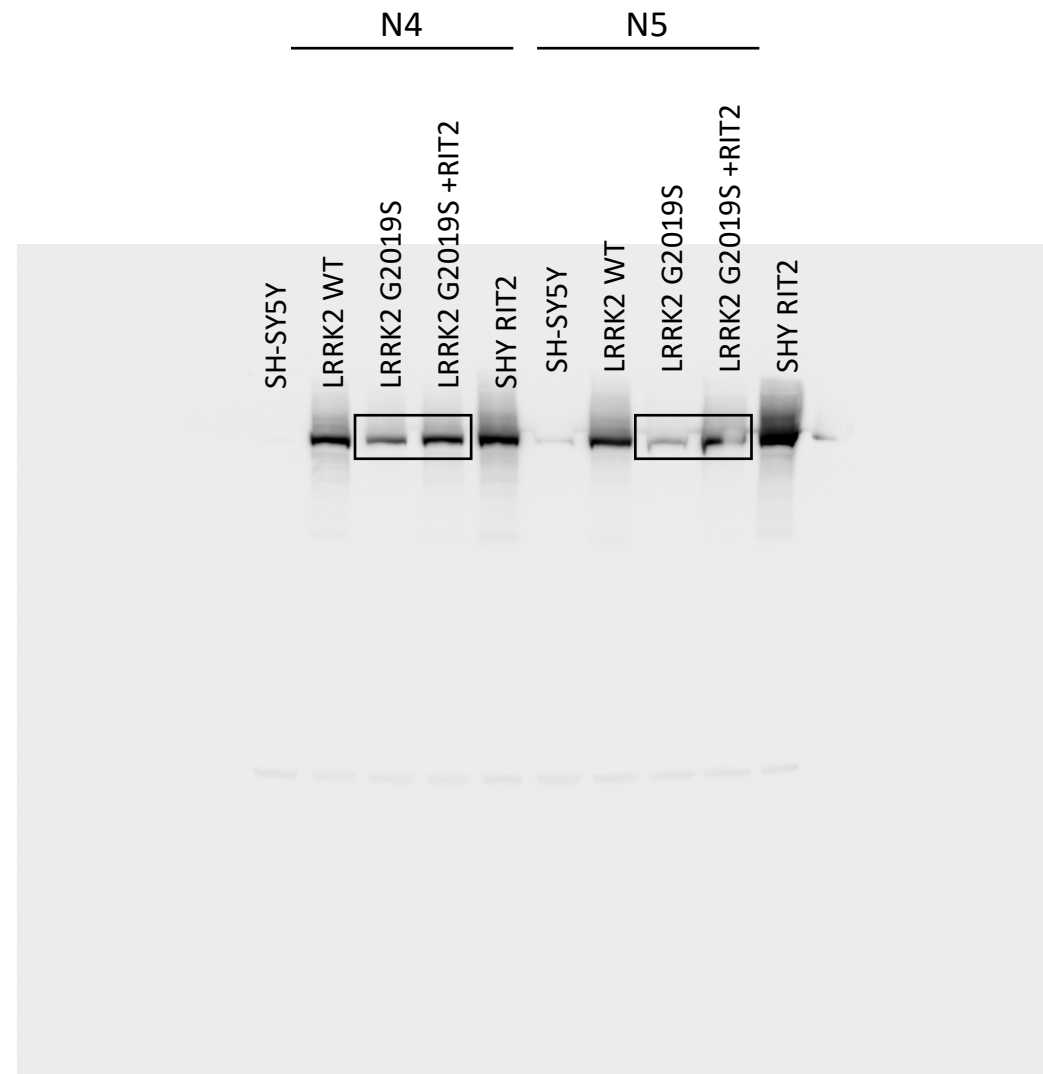
WB for pS935-LRRK2 (Fig S6)

N4, N5

pSer935- LRRK2



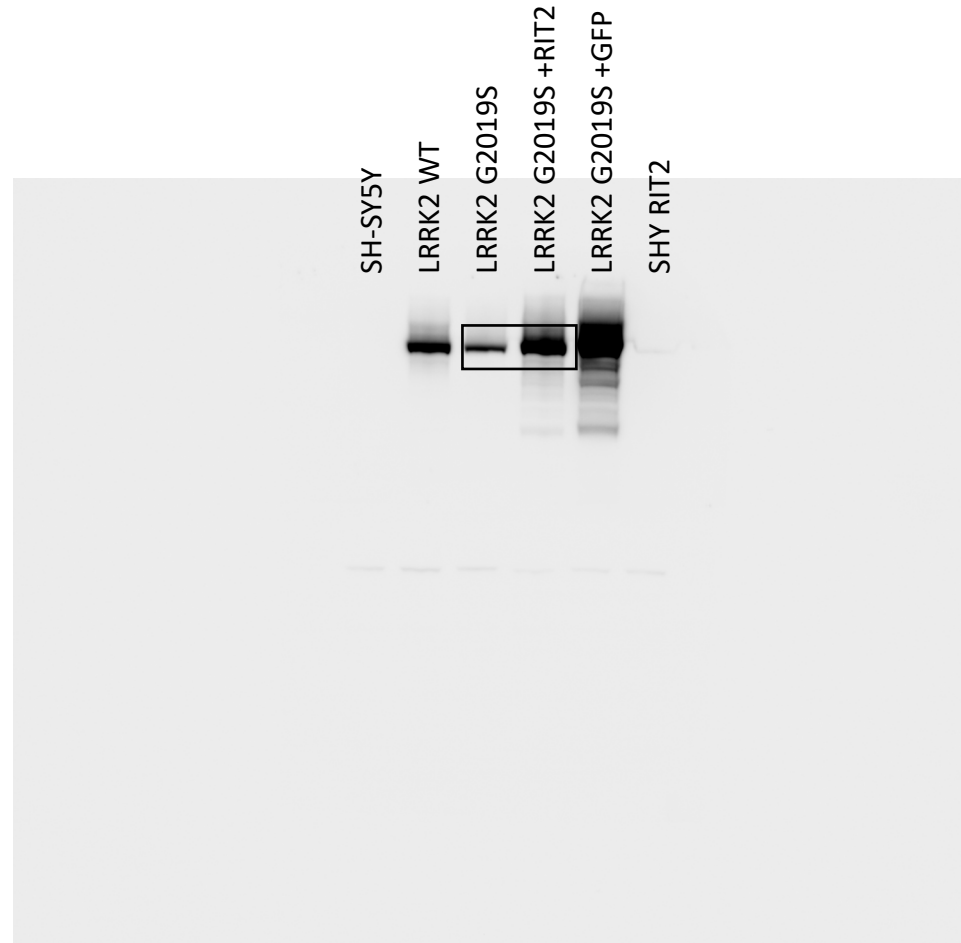
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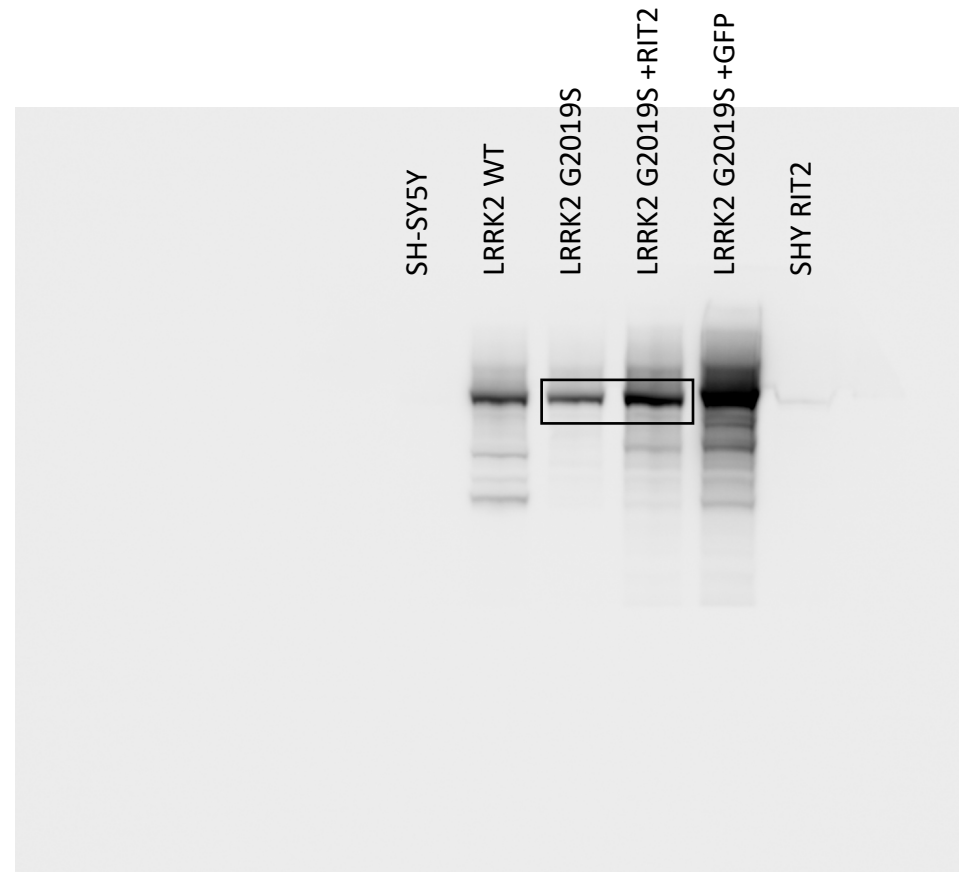
WB for pS935-LRRK2 (Fig S6)

N6

pSer935- LRRK2



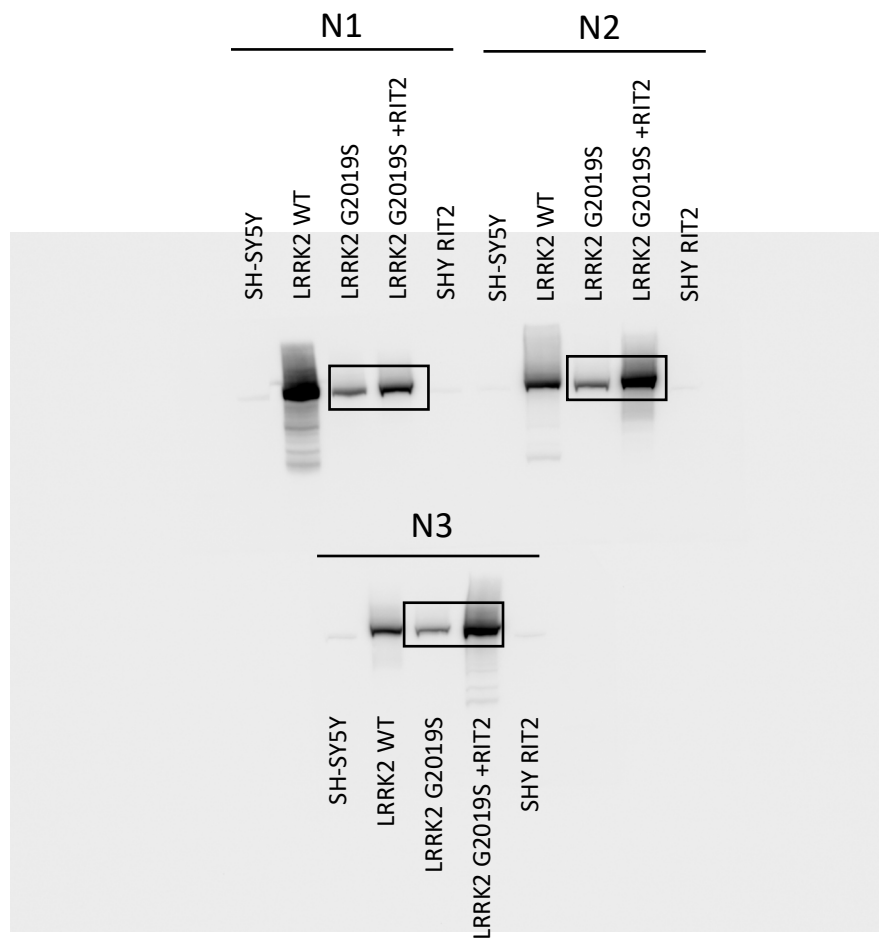
LRRK2



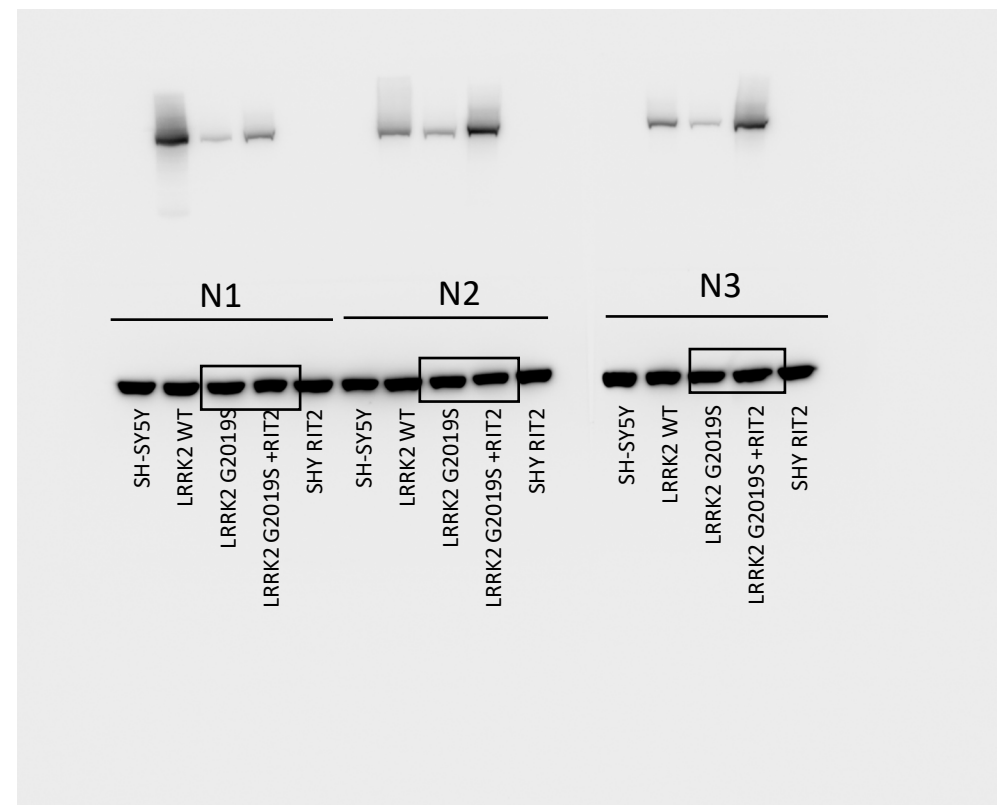
WB for total LRRK2 (Fig S6)

N1, N2, N3

LRRK2

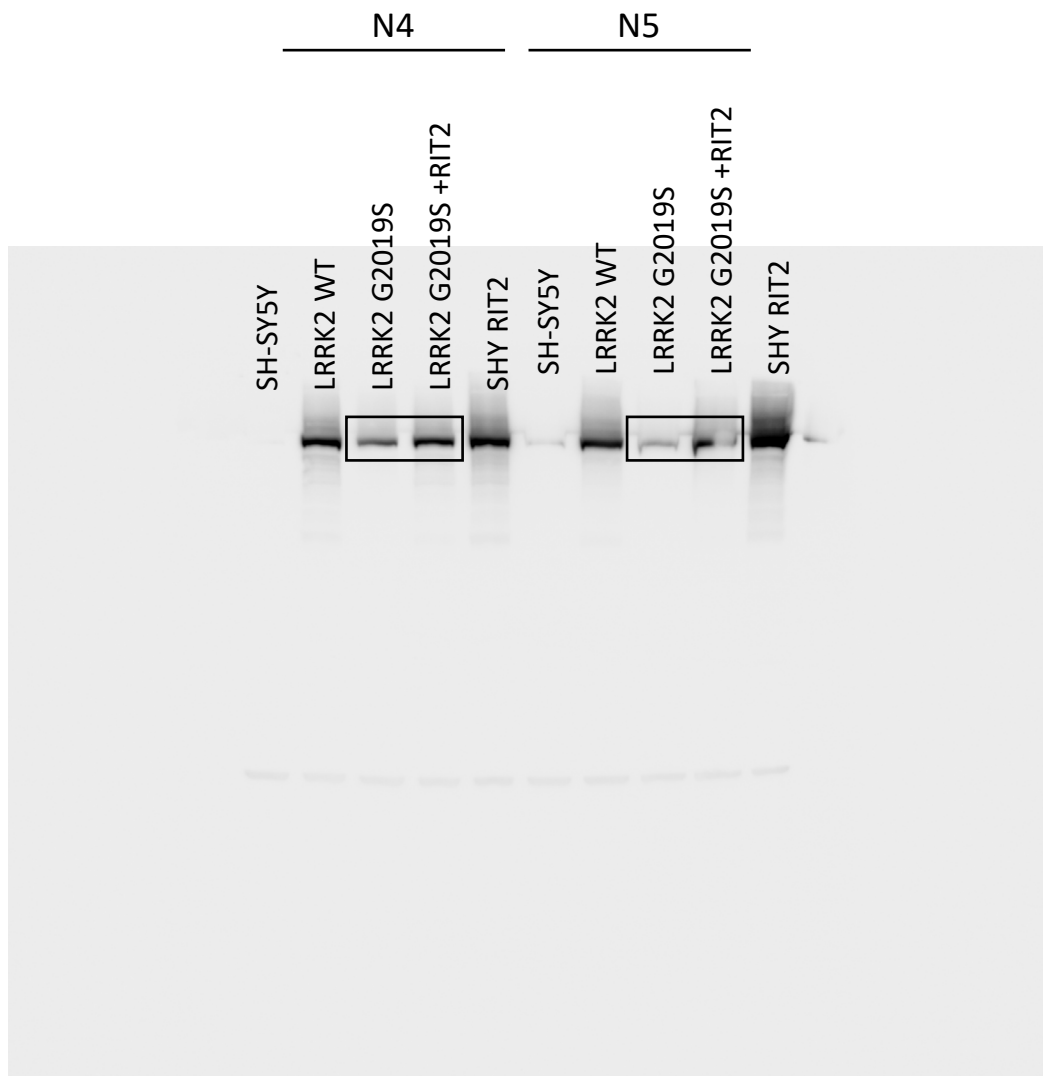


beta-actin



WB for total LRRK2 (Fig S6)
N4, N5

LRRK2



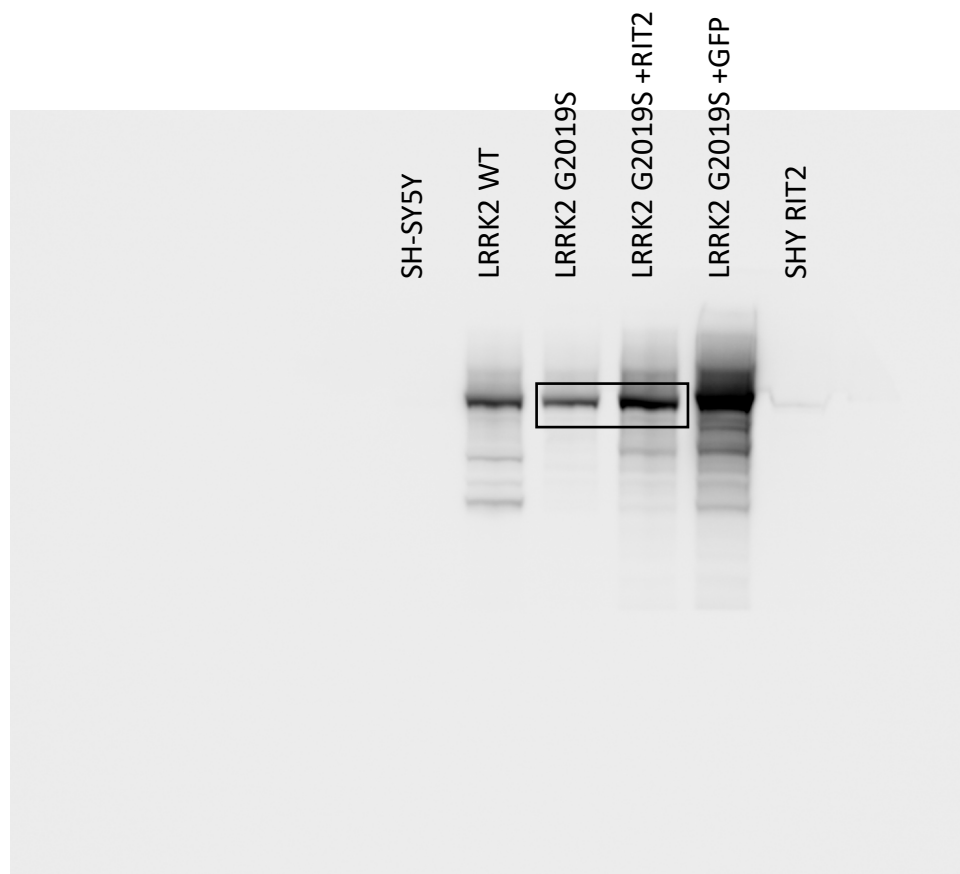
beta-actin



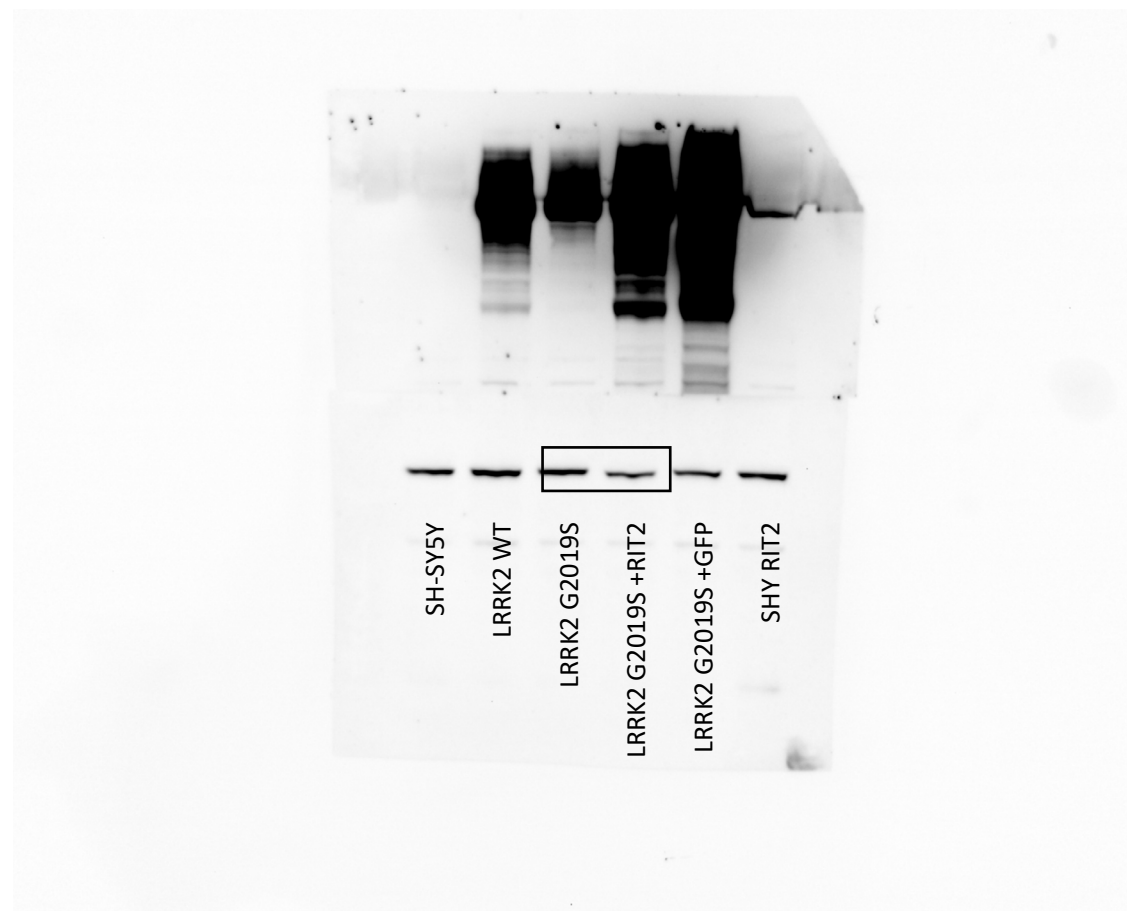
WB for total LRRK2 (Fig S6)

N6

LRRK2



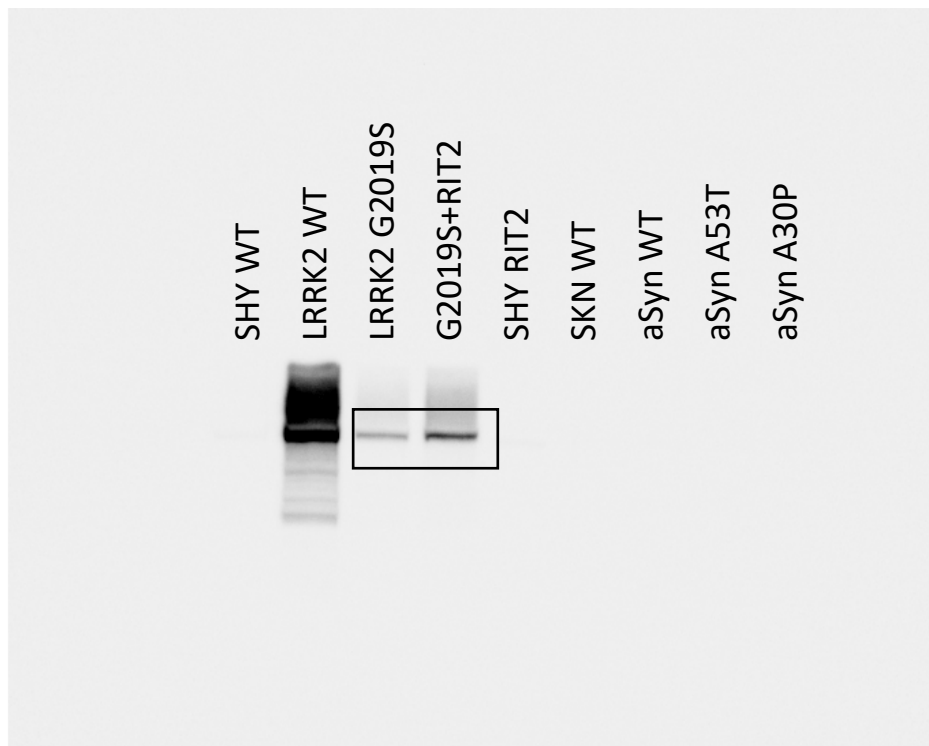
beta-actin



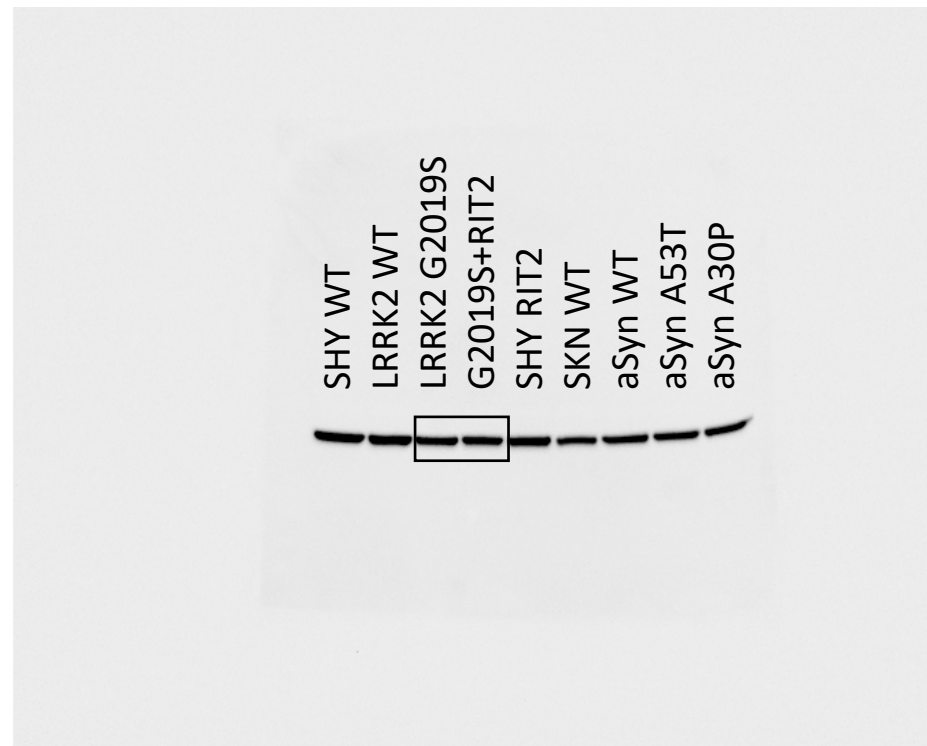
WB for total LRRK2 (Fig S6)

N7

LRRK2



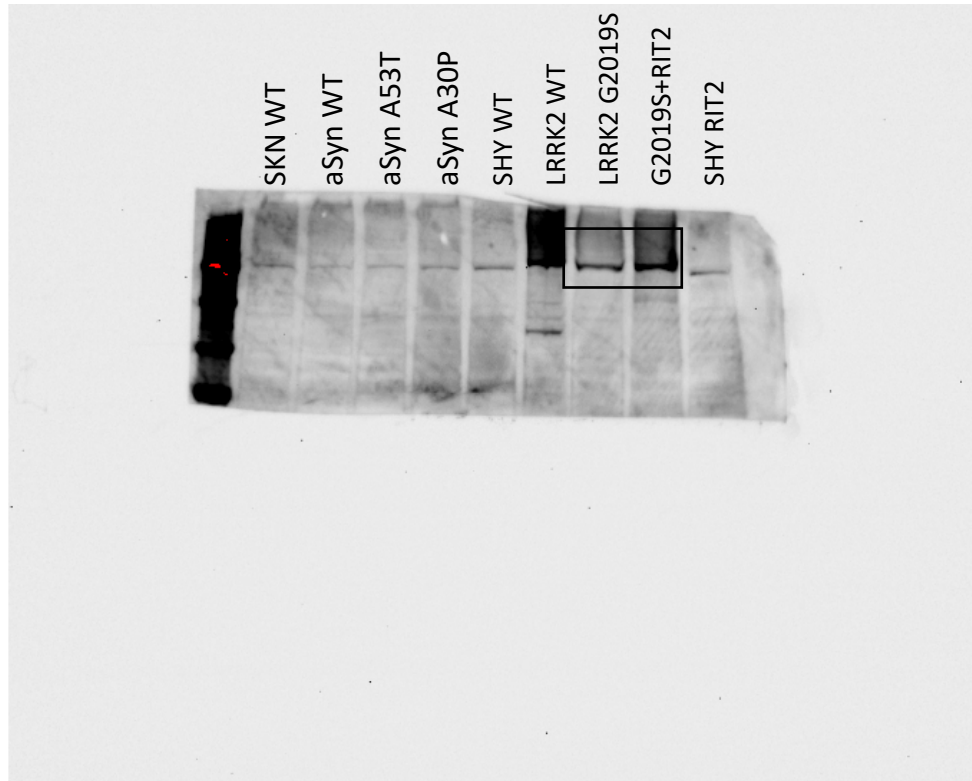
b-actin



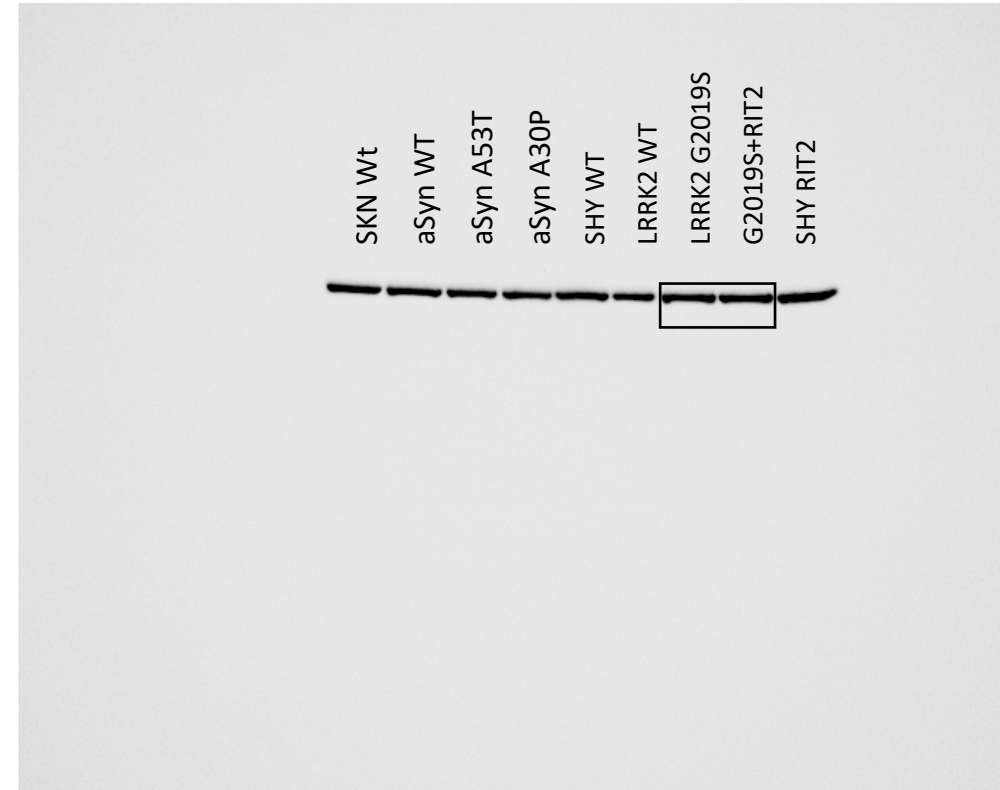
WB for total LRRK2 (Fig S6)

N8

LRRK2



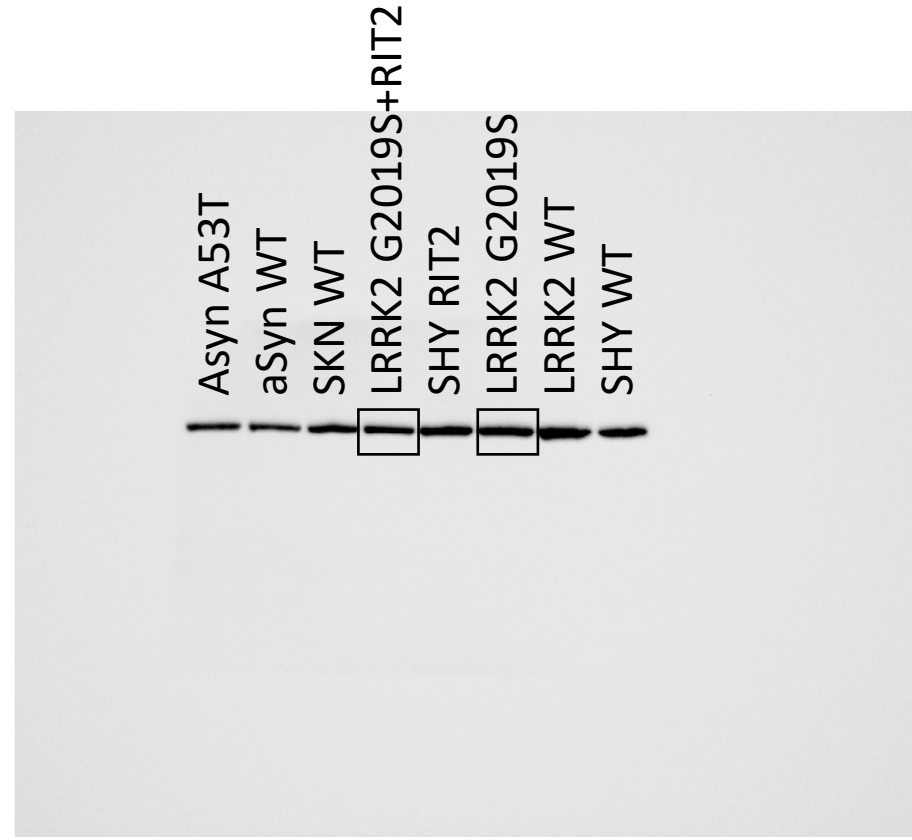
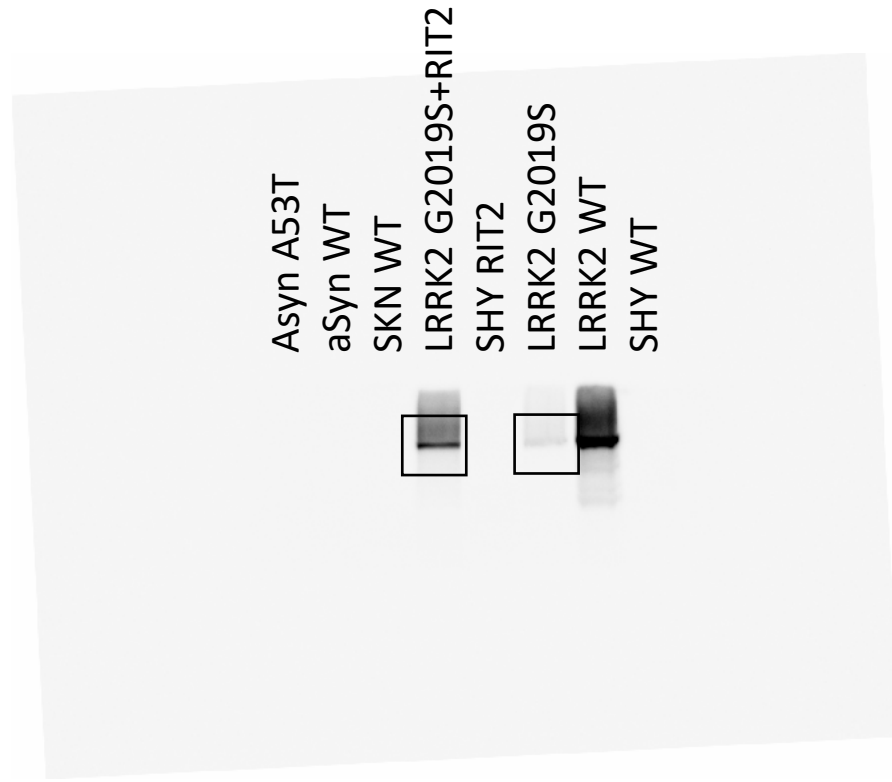
b-actin



WB for total LRRK2 (Fig S6)

N9

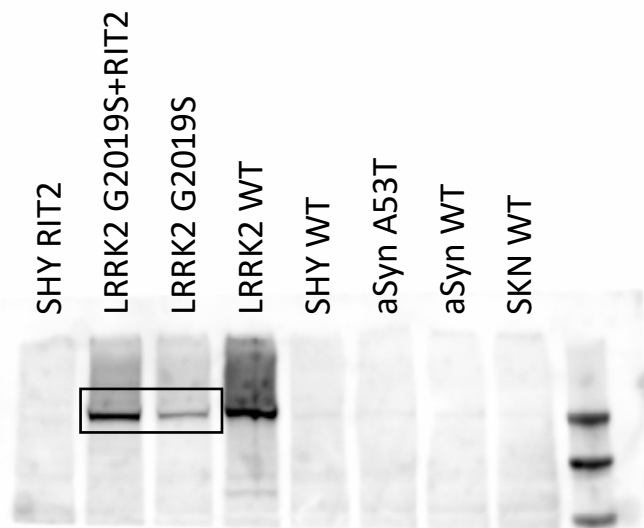
LRRK2



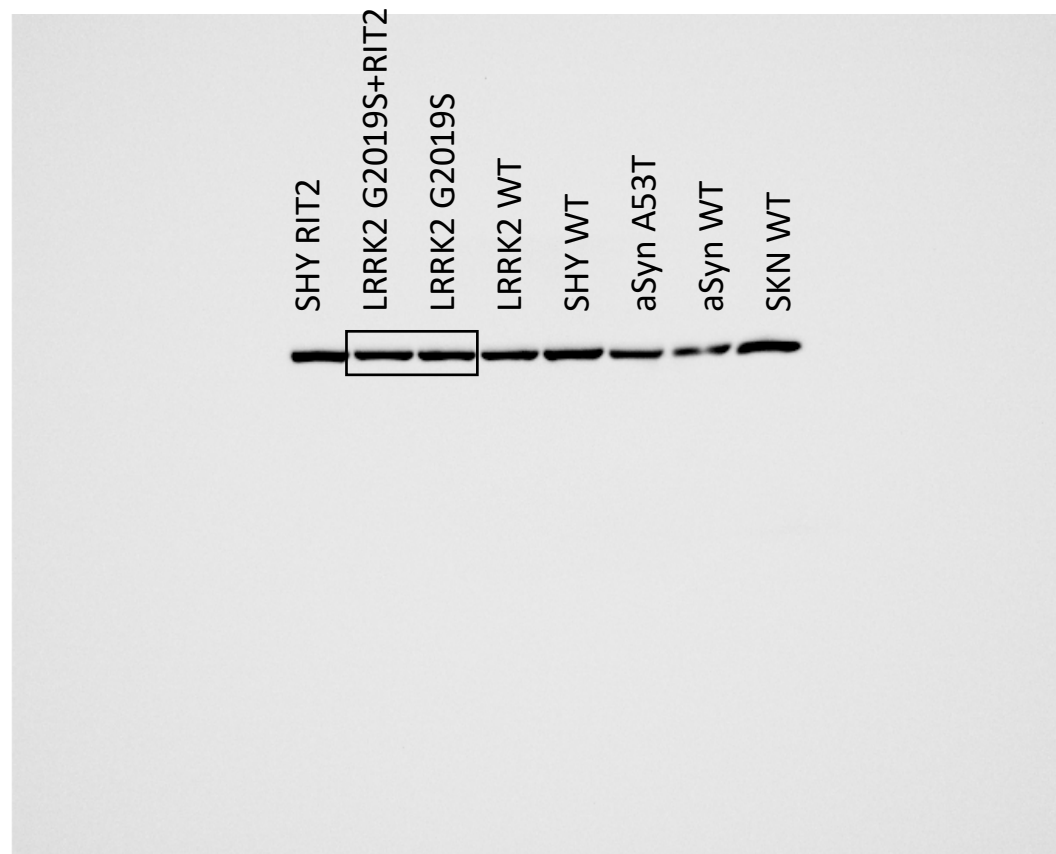
WB for total LRRK2 (Fig S6)

N10

LRRK2



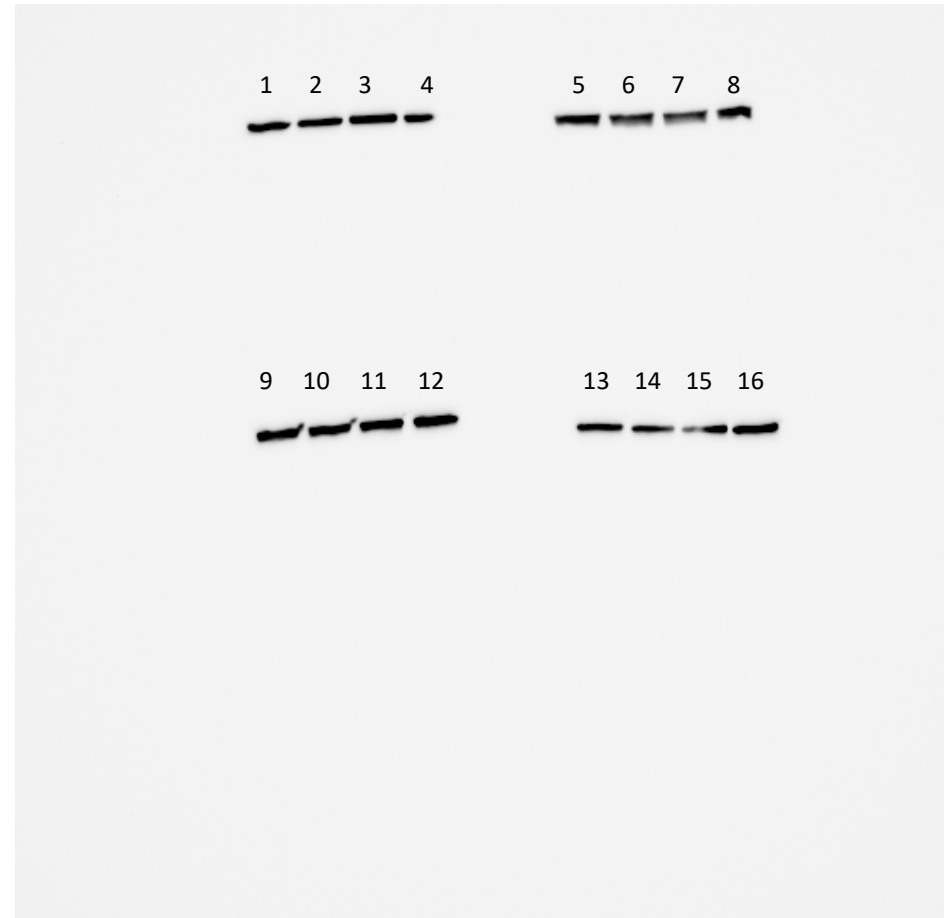
beta-actin



WB for aSyn in vivo (Fig 6)

aSyn

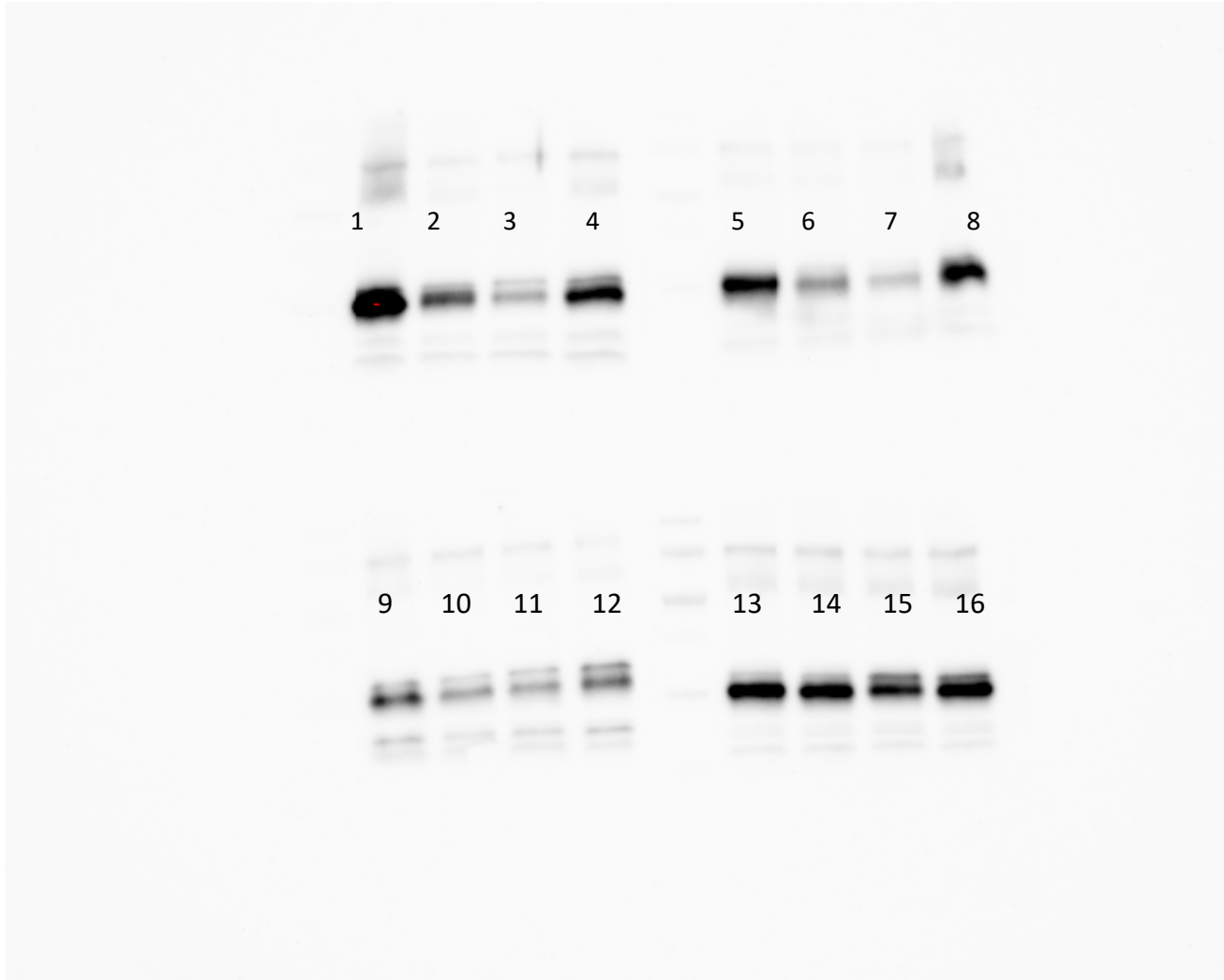
b-actin



	Mouse ID
AAV-GFP	1
	2
	3
	4
AAV-RIT2	5
	6
	7
	8
AAV-GFP+AAV-aSyn	9
	10
	11
	12
AAV-RIT2+AAV-aSyn	13
	14
	15
	16

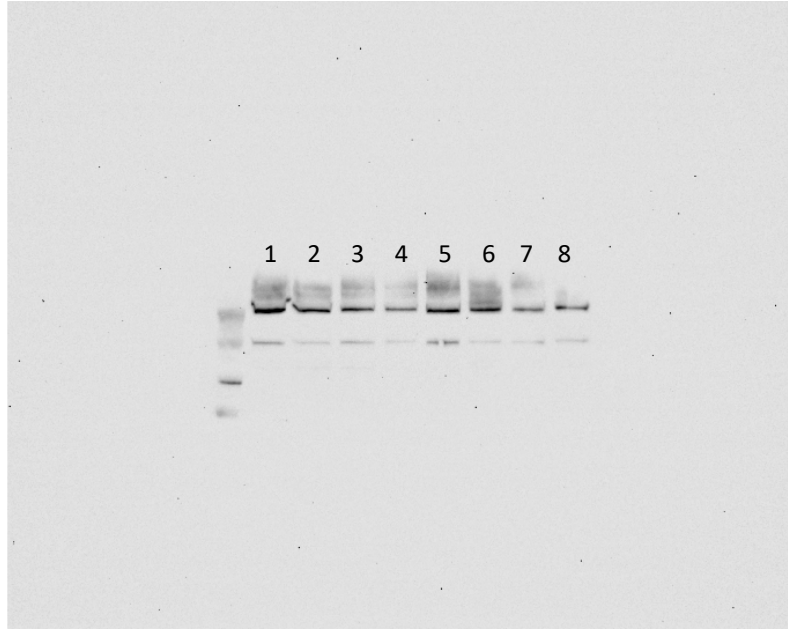
WB for aSyn in vivo (Fig 6)

pS129-aSyn

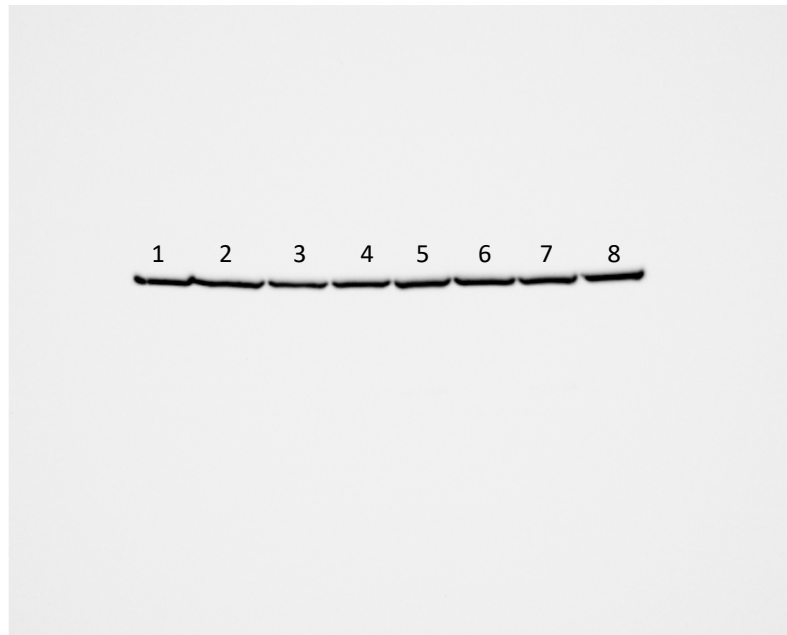


WB for LRRK2, Rit2 in vivo (Fig S8)

LRRK2



b-actin



Rit2



LC3

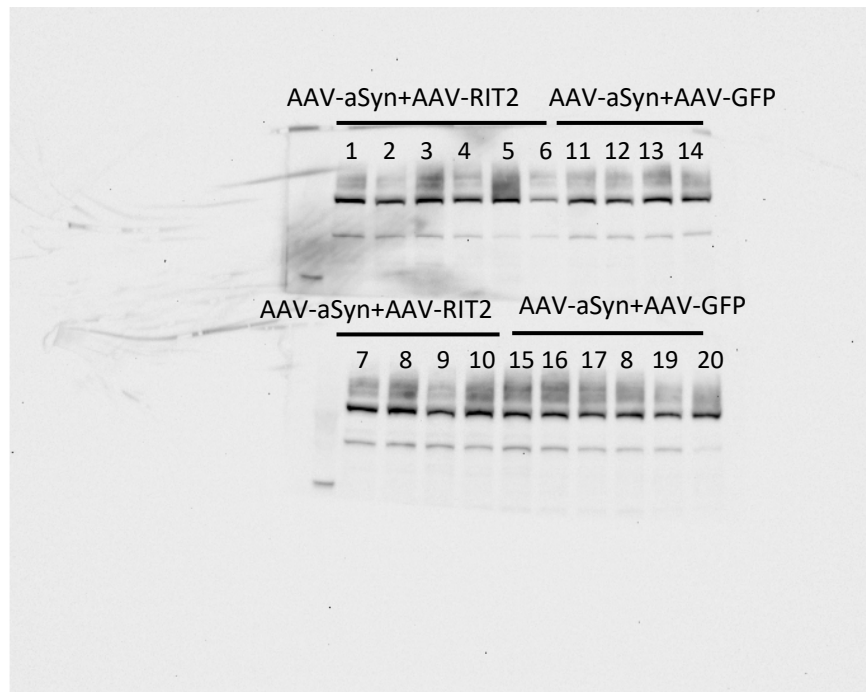


1	aSyn+RIT2	244 G
2		244 D
3		240 G
4		240 D
5	aSyn+GFP	245 G
6		245 D
7		189 D
8		189 G

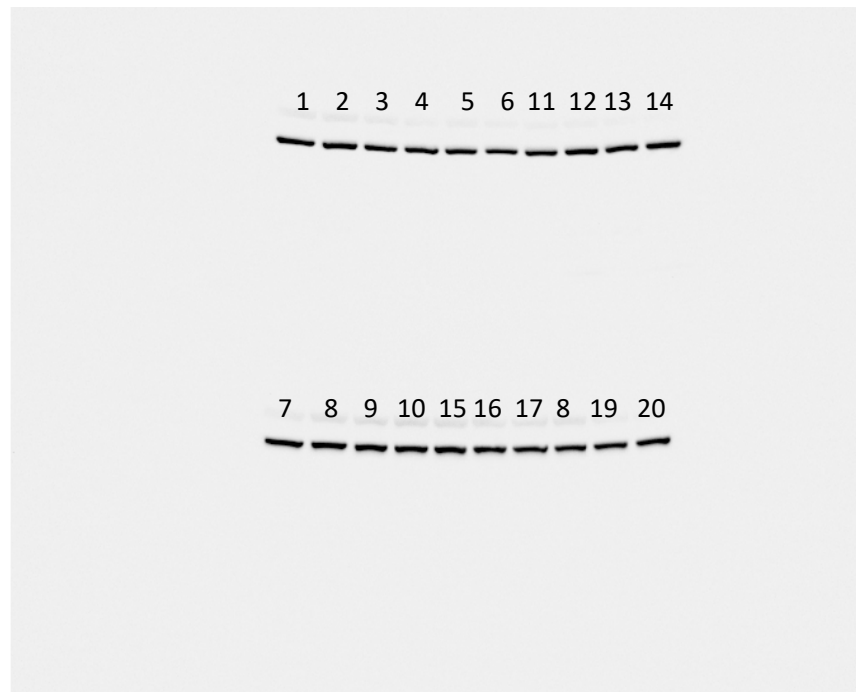
G=contralateral
D= ipsilateral

WB for LRRK2 in vivo (Fig S8)

LRRK2



b-actin



aSyn+RIT2

19244G	1
19244D	2
19240G	3
19240D	4
19191G	5
19191D	6
19192G	7
19192D	8
19241G	9
19241D	10

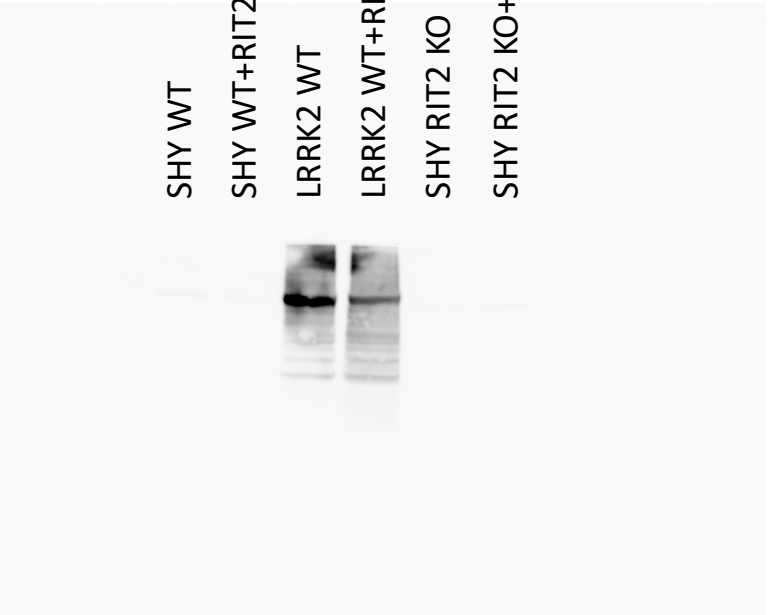
aSyn+GFP

19190G	11
19190D	12
19246G	13
19246D	14
19245G	15
19245D	16
19189G	17
19189D	18
19248G	19
19248D	20

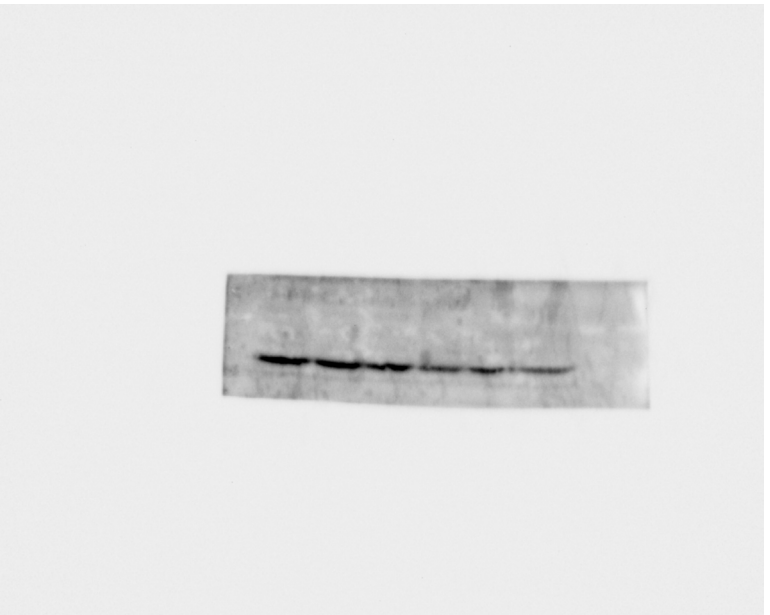
G=contralateral
D= ipsilateral

SHY WT
SHY WT+RIT2
LRRK2 WT
LRRK2 WT+RIT2
SHY RIT2 KO
SHY RIT2 KO+RIT2

Total LRRK2



Total aSyn



Beta-actin

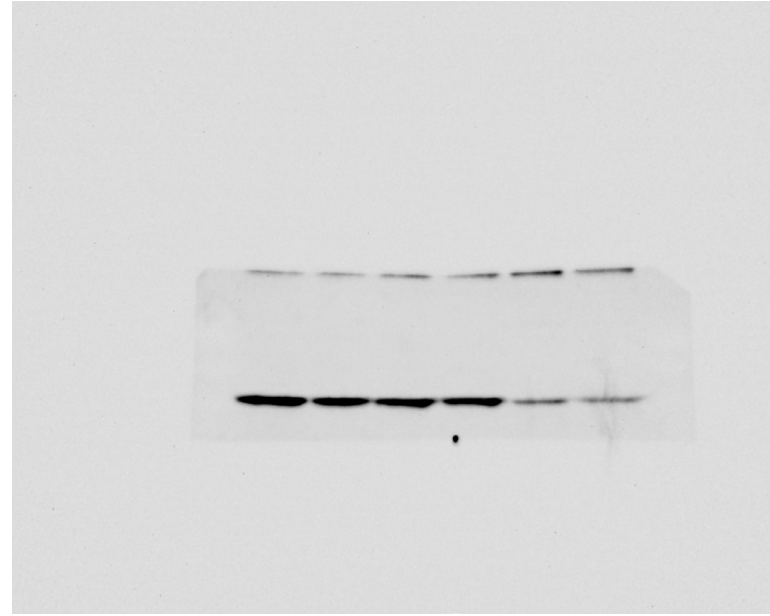


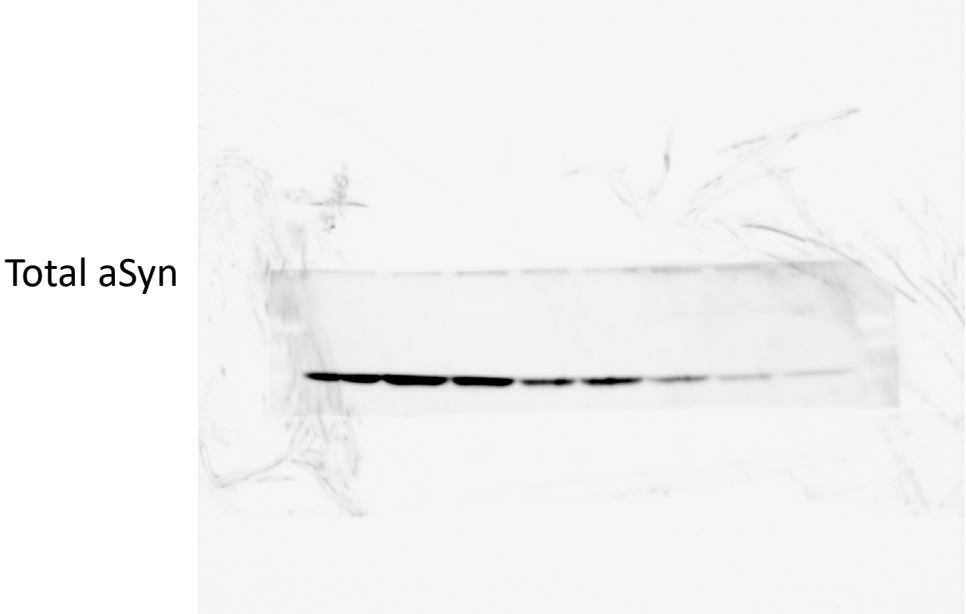
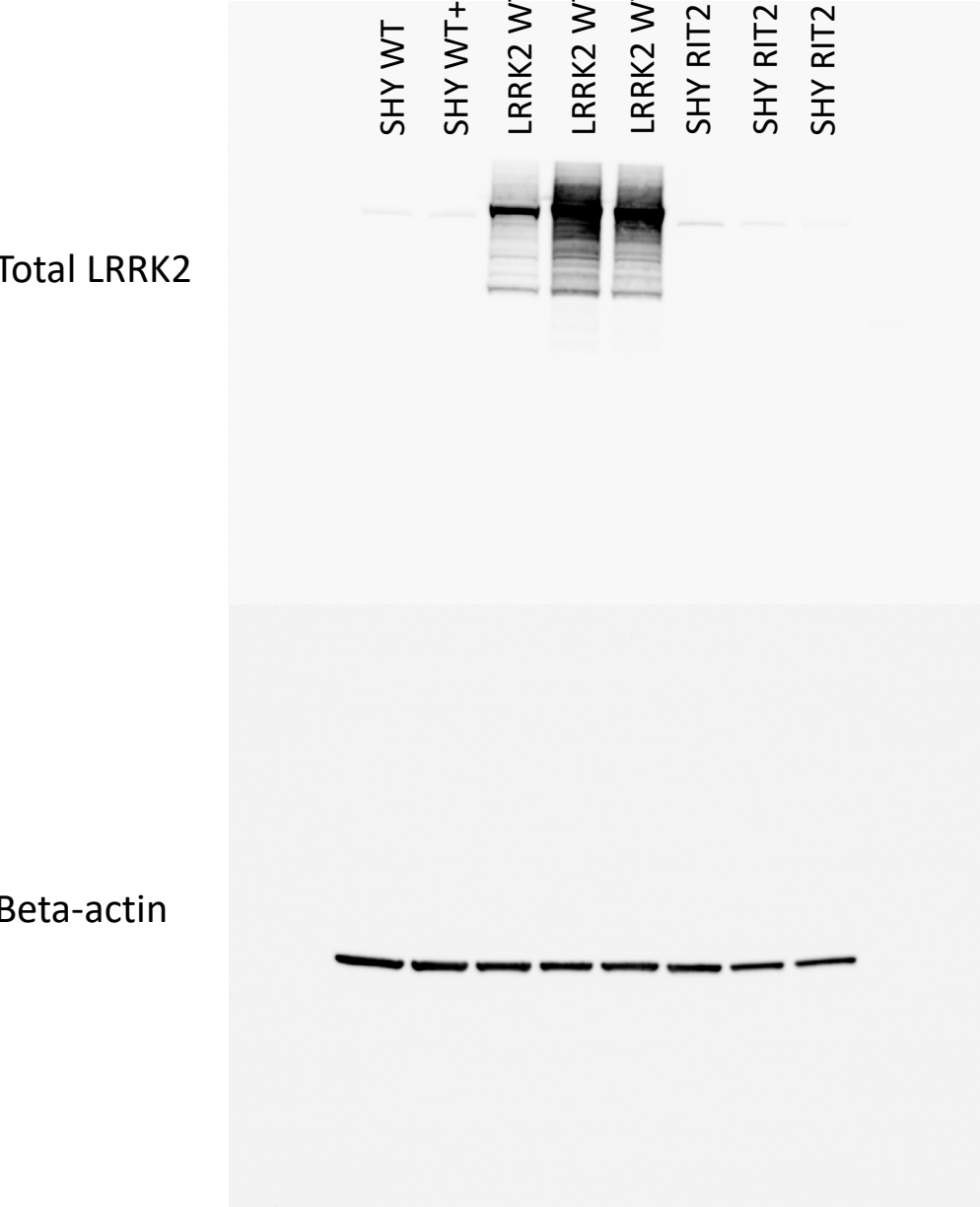
WB total LRRK2, total aSyn in WT LRRK2 cells (Fig S3) and Rit2-KO cells (Fig S9)

Total LRRK2



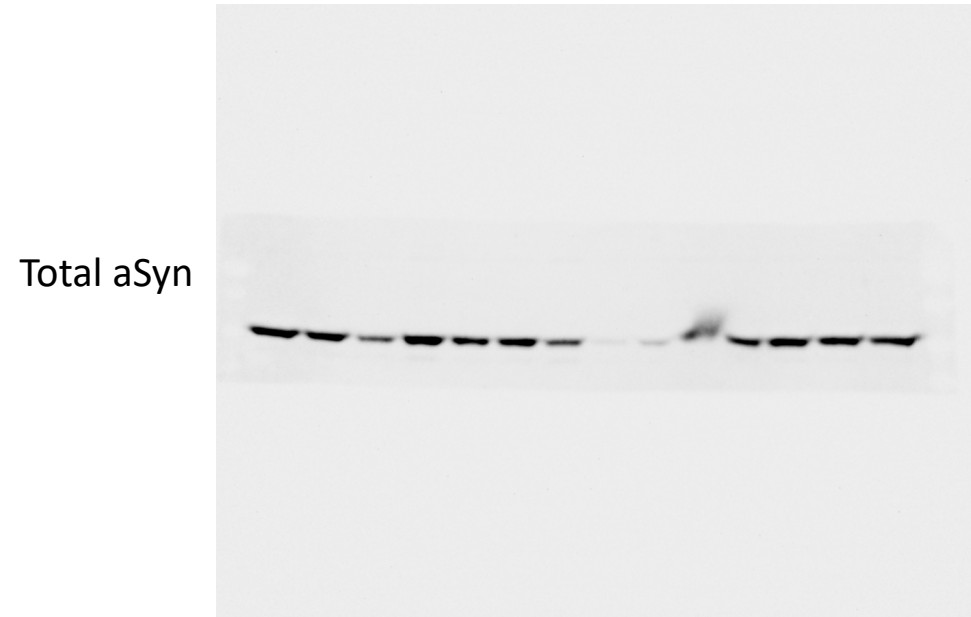
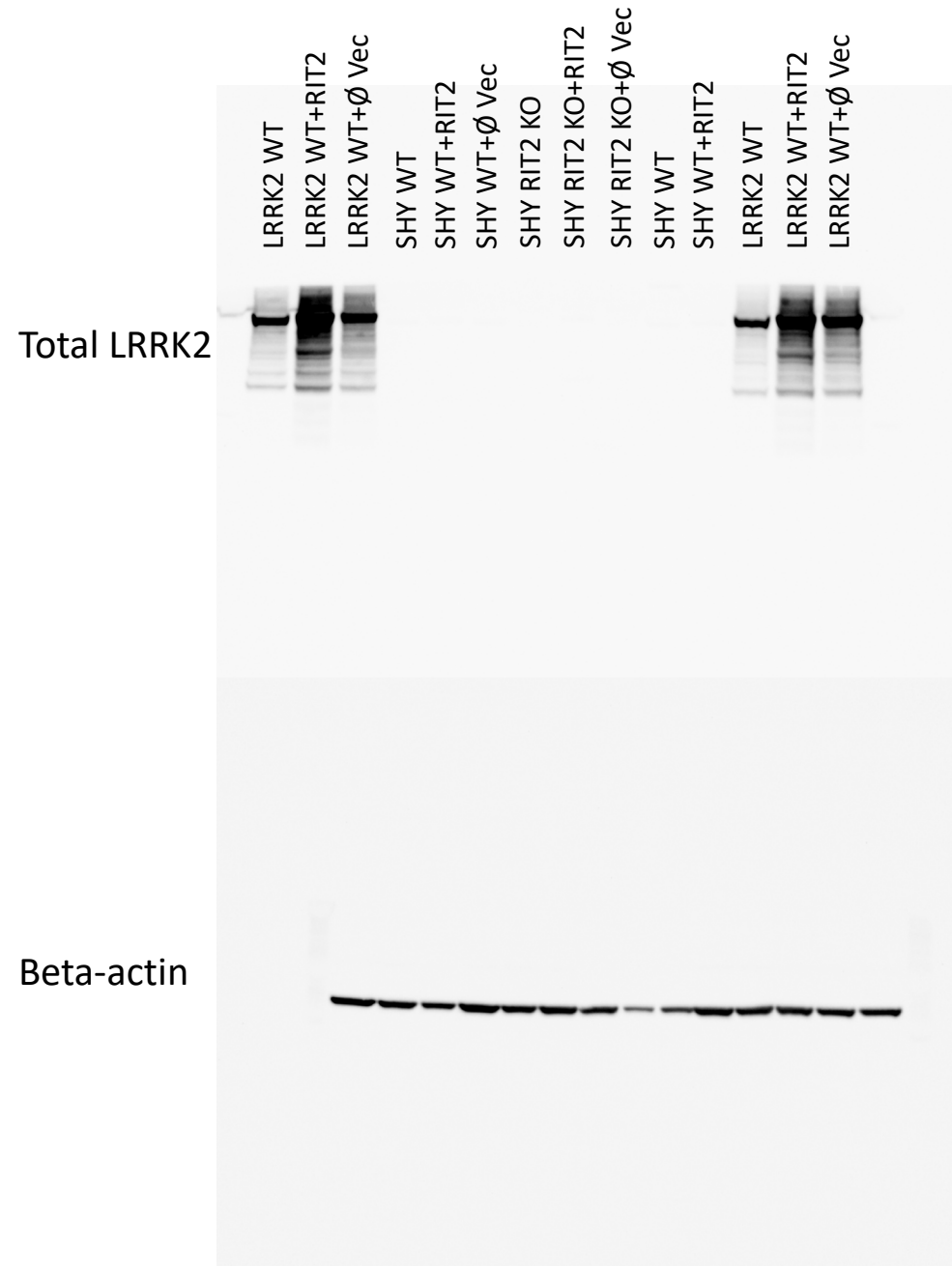
Total aSyn

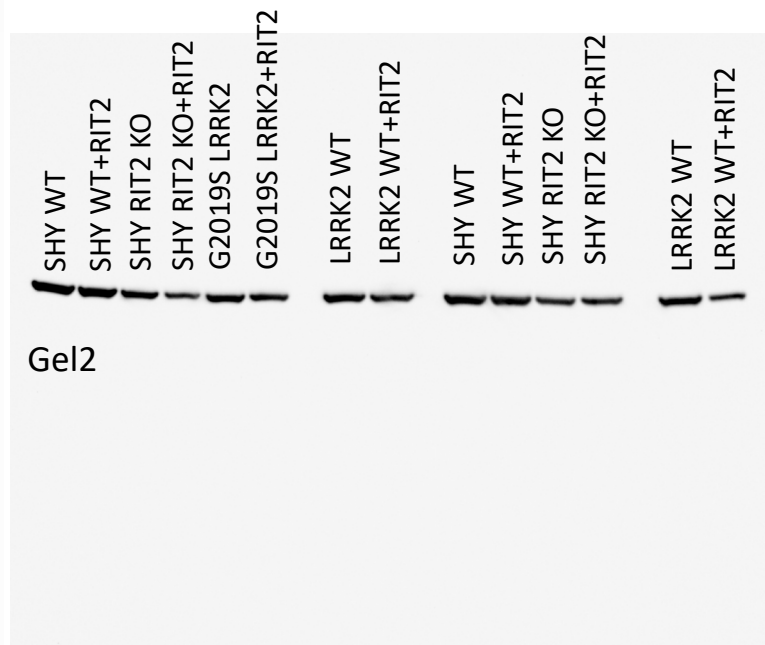




Beta-actin

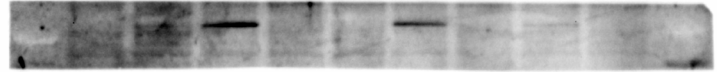
Total aSyn



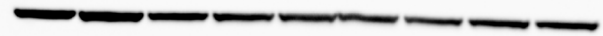


SHY WT
SHY WT+RIT2
LRRK2 WT+RIT2
LRRK2 WT
SHY RIT2 KO
SHY RIT2 KO+RIT2
G2019S LRRK2
G2019S LRRK2+RIT2
G2019S LRRK2+∅ Vec

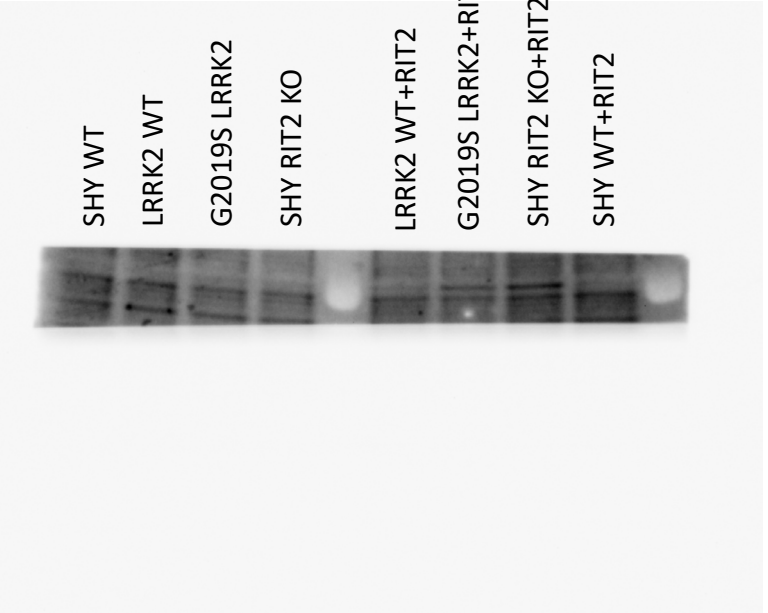
Rit2



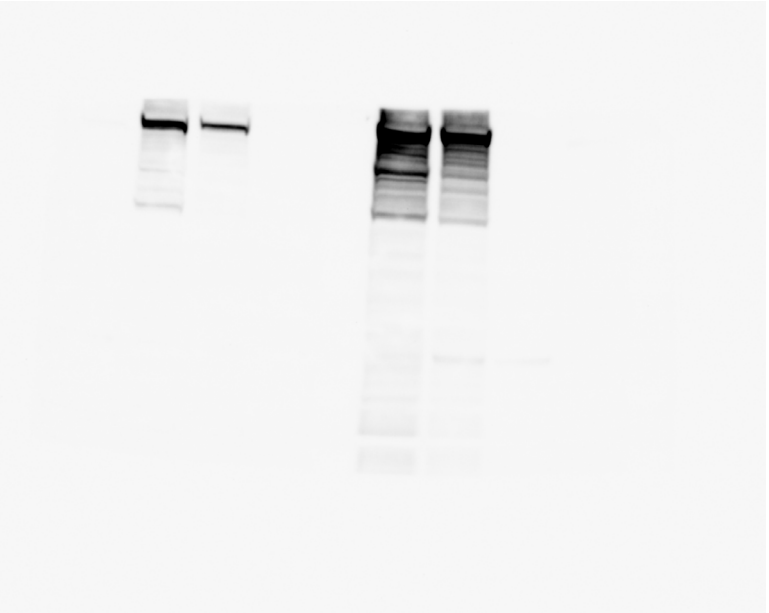
Beta-actin



Rit2

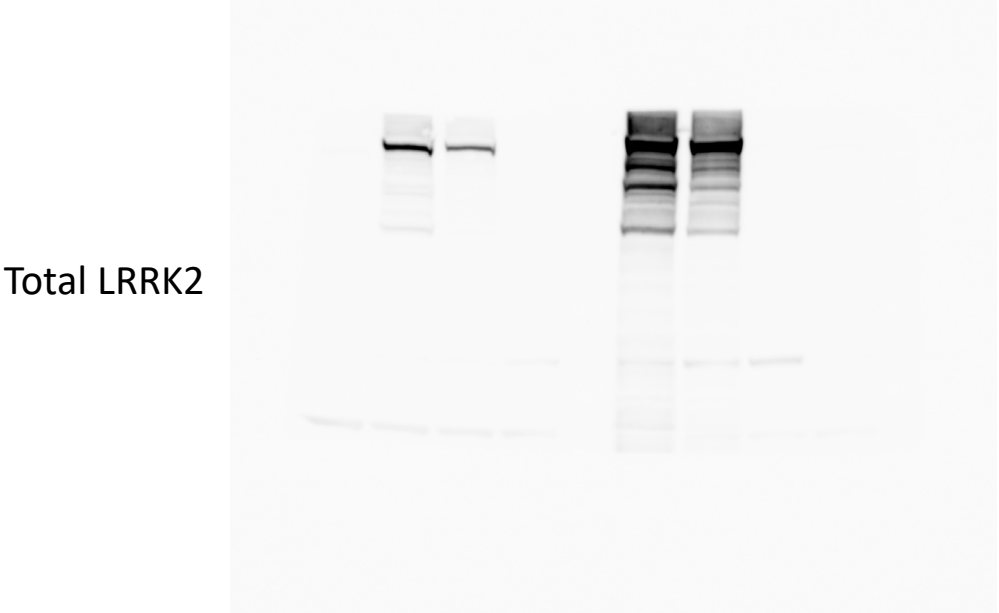
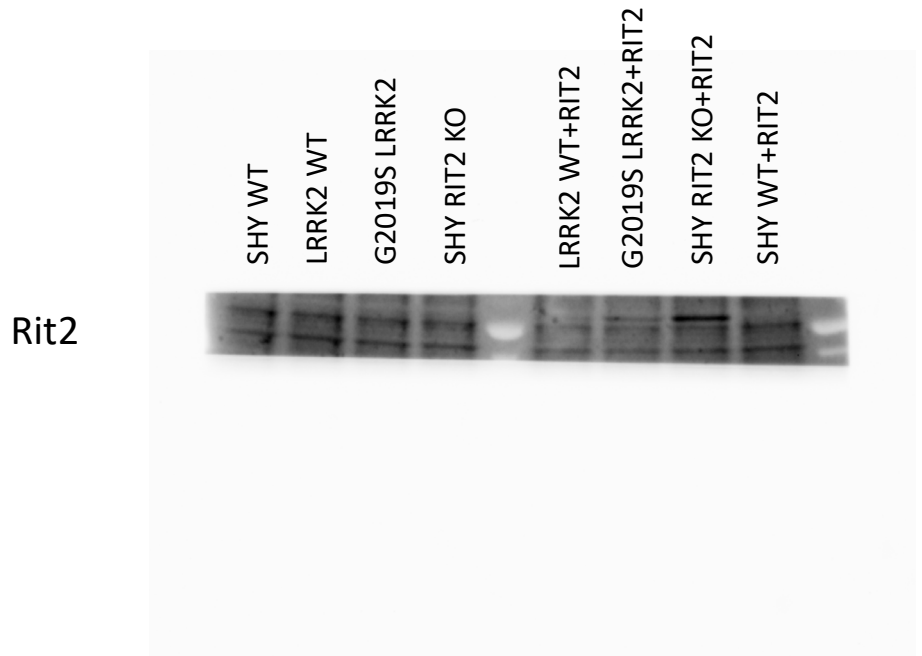


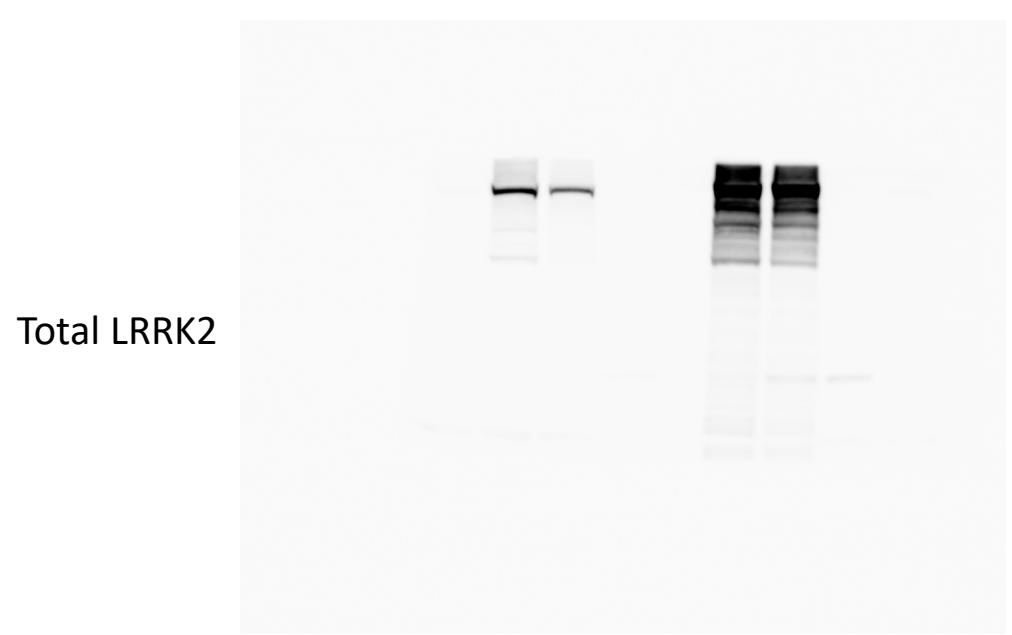
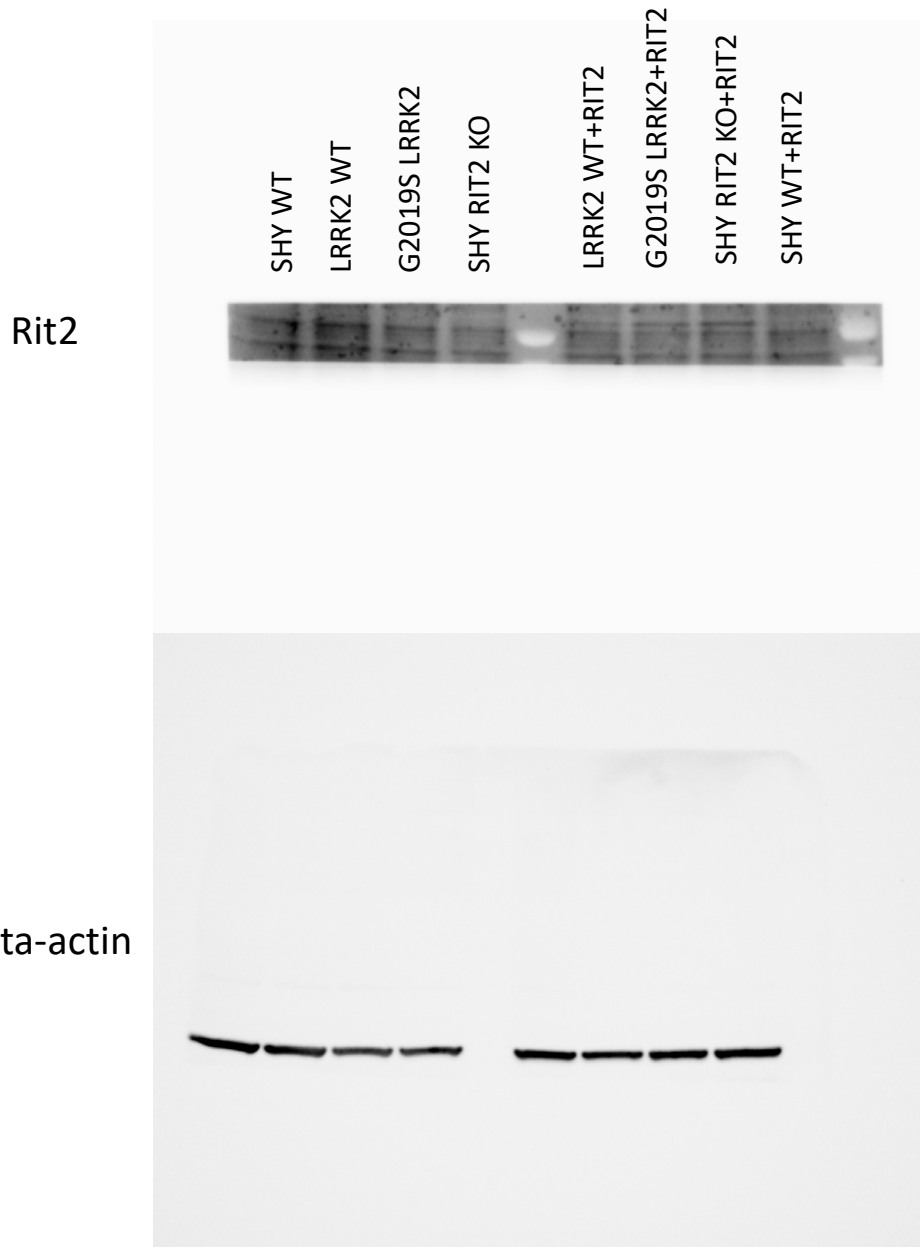
Total LRRK2



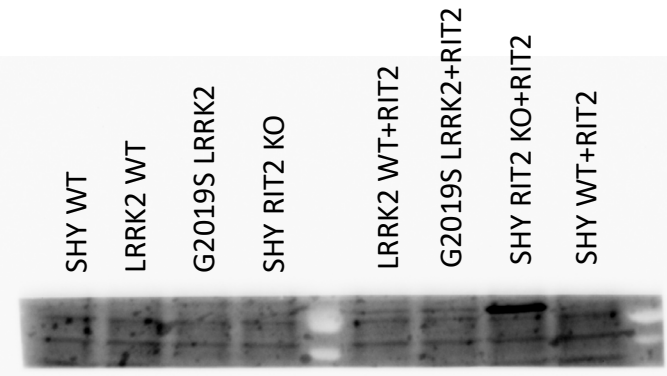
Beta-actin







Rit2



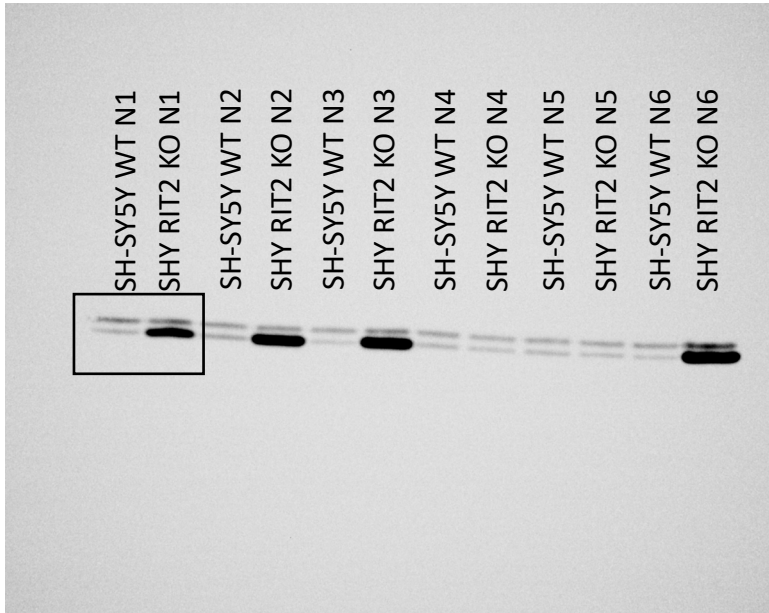
Total LRRK2



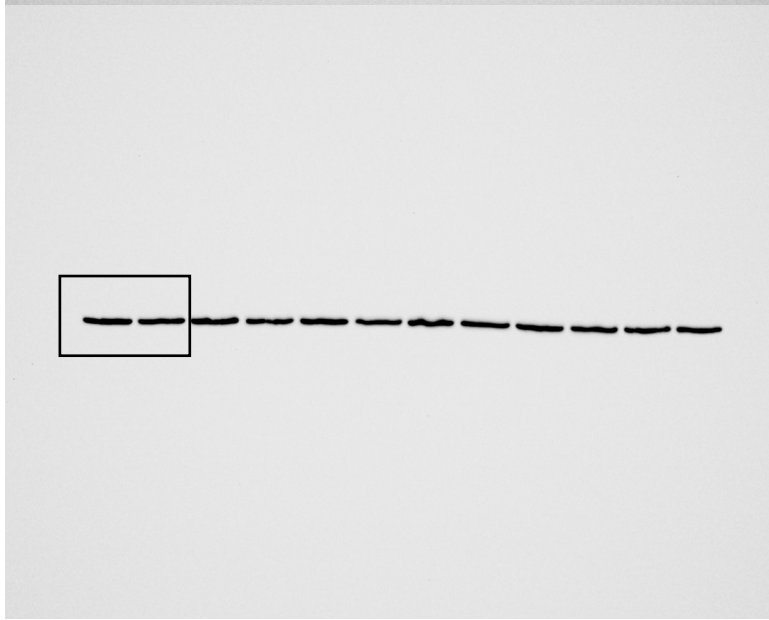
Beta-actin



LC3B

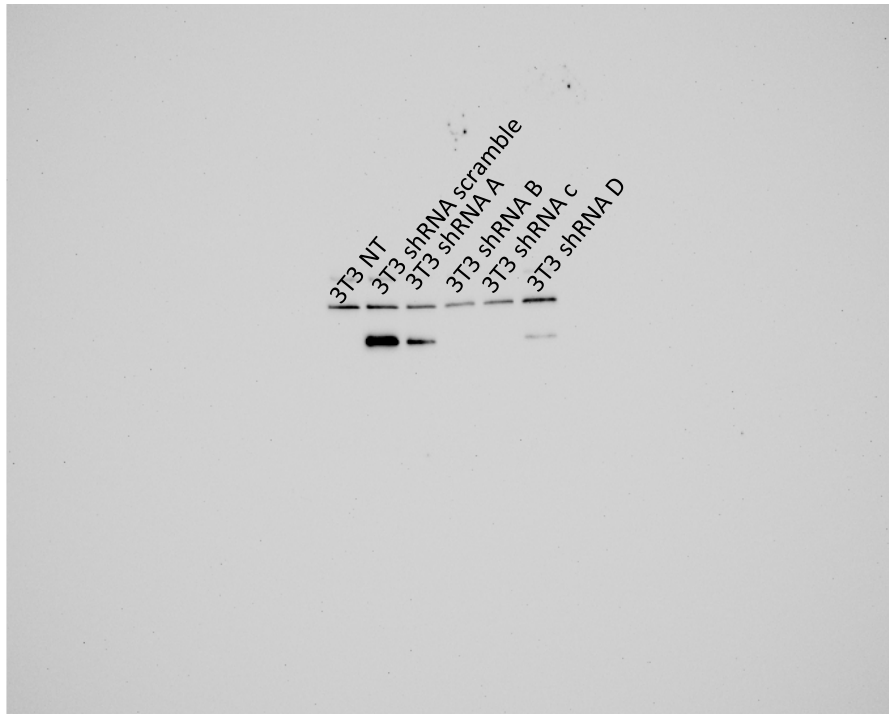


Beta-actin

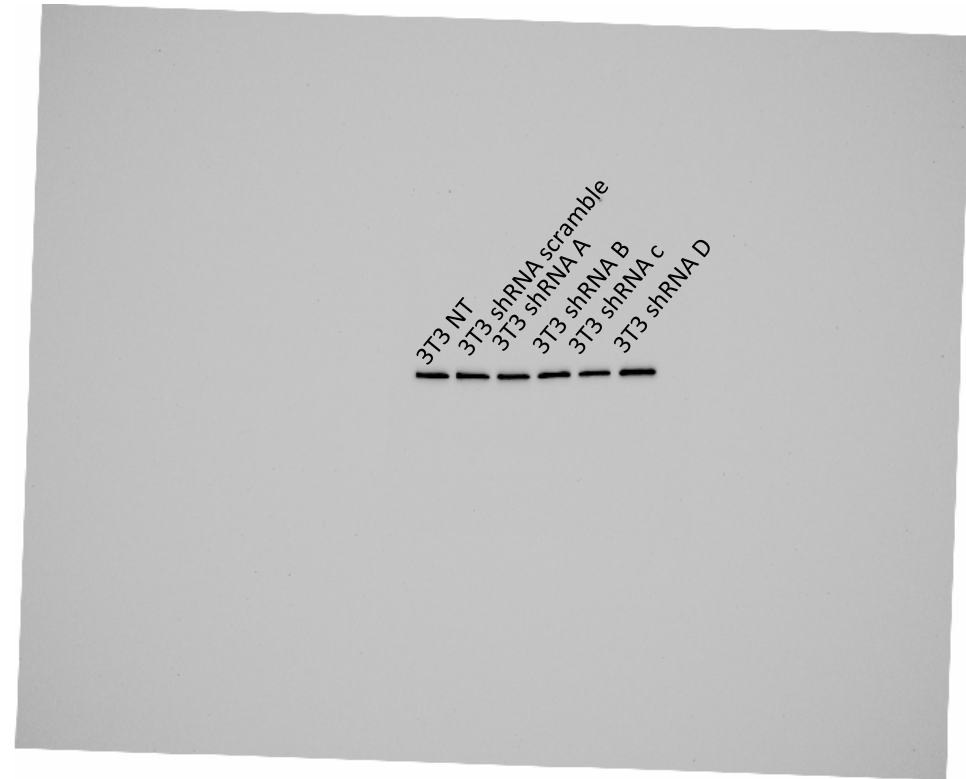


WB for Myc in NIH-3T3 cells (Fig S10)

Myc

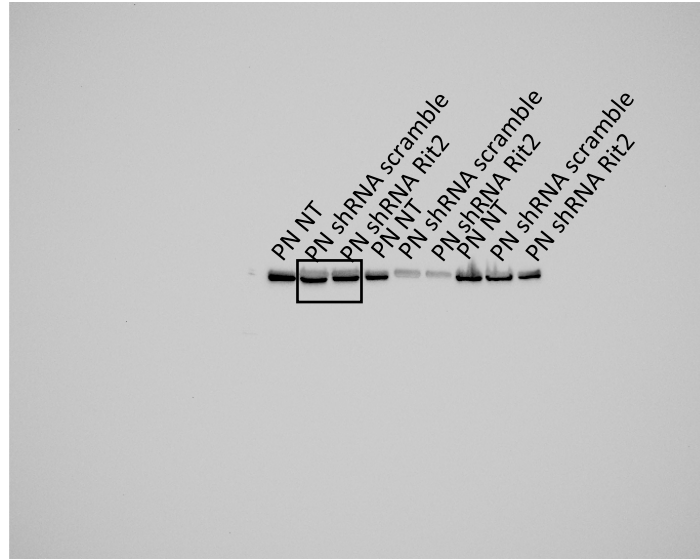


actin

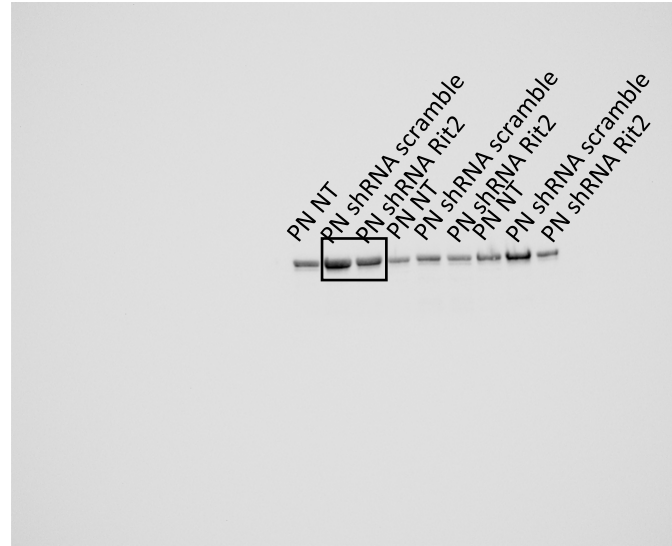


WB for LRRK2, Rit2, LC3B, TH in primary neurons (Fig S10)

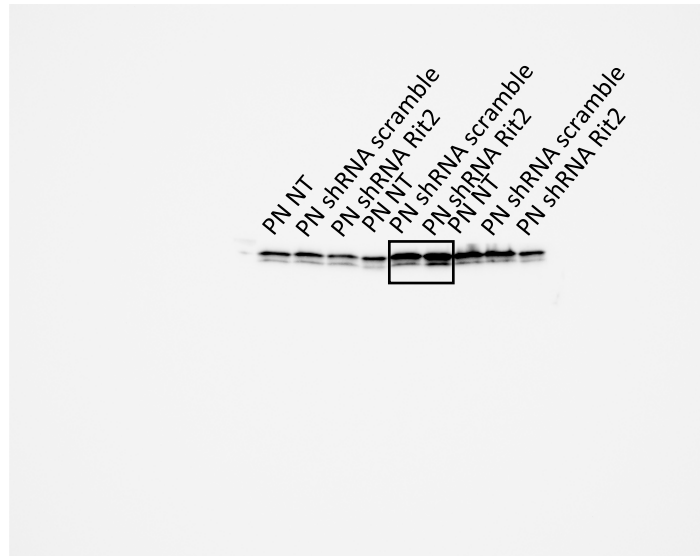
TH



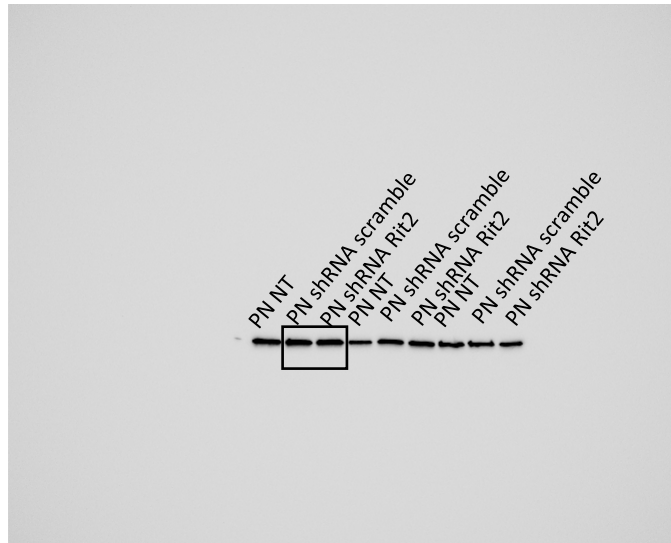
Rit2



LC3B

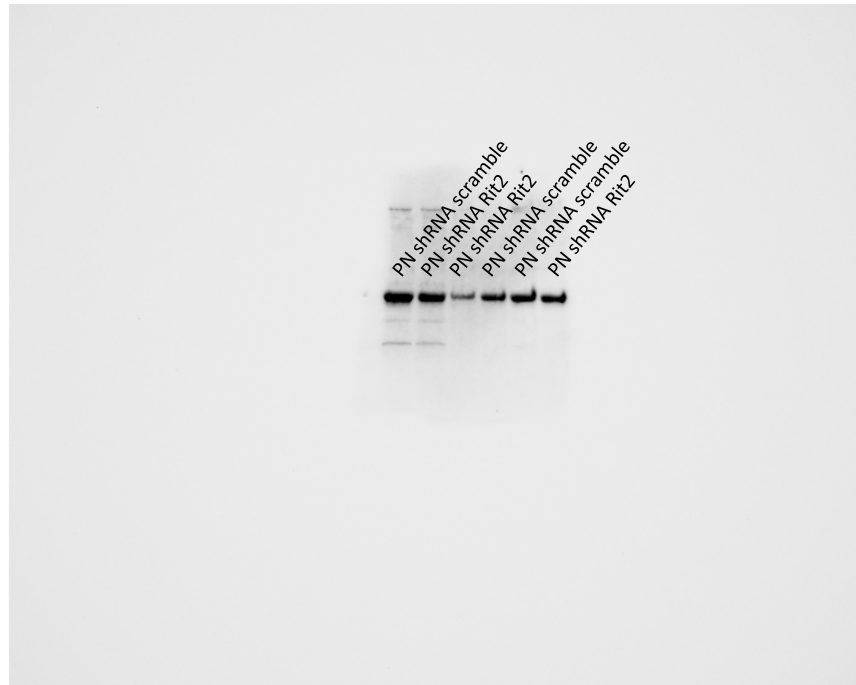


actin



WB for LRRK2, Rit2, LC3B, TH in primary neurons (Fig S10)

Rit2



actin

