Supplemental information

LRRK2 localizes to endosomes and interacts with clathrin-light chains to limit Rac1 activation

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Materials and Methods

Antibodies and DNA constructs

Polyclonal antibody recognizing CLCa/CLCb, and affinity-purified rabbit polyclonal antibody recognizing CHC were generated as described (*1, 2*). Rabbit monoclonal antibodies were from the Michael J Fox Foundation (LRRK2, c41-2) (Epitomics) and Cell Signalling (EEA1). Rabbit polyclonal antibodies were from the indicated sources; Cdc42 (Santa Cruz), synapsin (*3*), GFP (Invitrogen), dlrrk (*4*). Mouse monoclonal antibodies recognizing the following proteins were from the indicated commercial source; synaptophysin (clone SVP-38, Sigma-Aldrich), Ras and Rac1 (BD Transduction Laboratories), myc epitope tag (clone 9E10, Millipore), HA (6E2, Cell Signalling), 24B10/Chaoptin (Developmental Studies Hybridoma Bank), Cy3 (Jackson ImmunoResearch), and PSD-95 (Upstate). AffiPure goat anti-mouse IgG (Jackson ImmunoResearch) was used as a tertiary antibody. Alexa 488-conjugated donkey anti rabbit IgG, Alexa 568-conjugated donkey anti goat IgG, and Alexa 647-conjugated Phalloidin were from Invitrogen Inc.

The 2xmyc-LRRK2-WT plasmid (*5*) denoted here as myc-LRRK2 was from Addgene (#25361). GST-CLCb (1-211), GST-nCLCb (1-228), GST-nCLCb (1-165) were previously described (*6*). The following GST-nCLCb constructs were created by PCR amplification from full-length human nCLCb cDNA with the appropriate primers (see Supplemental Table I) with subsequent subcloning into pGEX-4T1 (Clontech); GST-nCLCb (1-215), GST-nCLCb (1-205), GST-nCLCb (1-195). GST-ROC (1333-1516) was PCR amplified from a human LRRK2 cDNA using appropriate primers (Supplemental Table I) and subcloned into pGEX-6P1 (Clontech). All constructs were confirmed by sequencing and transformed into *E. coli* BL21 for protein expression.

Immunofluorescence and immunoblotting

For standard immunofluorescence, COS-7 cells grown on poly-L-lysine-coated coverslips were washed in phosphate-buffered saline (PBS) and then fixed for 20 min in 4% paraformaldehyde. After fixation, cells were permeabilized with 0.2% Triton X-100 in PBS for 3 min, and processed for immunofluorescence with the appropriate primary and secondary antibodies in PBS + 0.01% BSA. NSC-23766 (Santa Cruz Biotechnology) at 100 µM in DMSO or DMSO alone was added to COS-7 cells 24 h prior to fixation. For actin staining, cells were incubated with Alexa 647-conjugated Phalloidin concomitantly with secondary antibodies.

We increased detection sensitivity using a tertiary antibody in our staining protocol to detect 3xHA-LRRK2 expressed from its endogenous locus using our clone E1 cells, as we were unable to detect a signal using conventional immunofluorescence (*7*). Thus, after incubation with the mouse anti-HA primary antibody we used unlabeled goat anti-mouse IgG followed by Alexa 568-conjugated donkey anti-goat IgG. Blocking and antibody incubations were performed in PBS + 0.1% Triton X-100 + 5% donkey serum. For EGF uptake, 3xHA-LRRK2 cells (clone E1) were starved for 2 h in DMEM without serum, and then incubated 20 min in the same medium with 488-EGF (100 ng/ml). The cells were then washed 3 times with ice-cold PBS and fixed with 4% paraformaldehyde at room temperature for 20 min.

For immunoblotting, samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk, and incubated overnight with primary antibodies. Membranes were washed and incubated with peroxidase-conjugated secondary antibodies (Jackson, West Grove, Pensylvania) for ~1 h. After subsequent washing, membranes were exposed with Enhanced Chemiluminescence Substrate (PerkinElmer). Densitometric quantification was performed using NIH ImageJ software and normalized to starting material.

Cell culture, transfection, and pharmacological treatments

COS-7 cells and clone E1 were cultured at 37°C with 5% CO_2 in DMEM supplemented with 10% bovine calf serum, 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. For siRNA-mediated protein KD, cells were transfected using Lipofectamine RNAiMAX (Invitrogen, Inc.) according to the manufacturer's instructions. Transfections used 10 nM siGENOME LRRK2 SMARTpool or its deconvolved siRNA components LRRK2-1 and LRRK2-2 (Dharmacon), and previously described and verified CLCa and CLCb siRNAs (*8*). Non-targeting control siRNA was from Dharmacon. Myc-LRRK2 was transfected for 48 h using Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions.

RNA guided, Cas9-mediated engineering of the endogenous LRRK2 locus

COS-7 cells were transfected with pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (*9*) here denoted as plasmid encoding human optimized Cas9 from Addgene plasmids (#42230) and a guideRNA (gRNA) selective for LRRK2 flanking the start codon based on the protocol in Petit *et al*. (*10*). In short, the following sequence with a BbsI cleavage site: 5′-CACCGATGGCTAGTGGCAGCTGTC-3′ (LRRK2 start codon underlined) was cloned into the gRNA expression vector to direct Cas9 nuclease activity toward the first coding exon of LRRK2. The cells were co-transfected with a large oligonucleotide encoding a triple-HA tag (Supplemental Table 1), flanked on either side by DNA sequence homologous to the LRRK2 gene. This protocol leads to cleavage of the genomic DNA of the LRRK2 gene with insertion of the oligonucleotide by homologous recombination, generating a triple HA tag between residues 1 and 2 of LRRK2, driven by the endogenous LRRK2 promoter. Recombinants were selected by immunofluorescence with an HA antibody and confirmation of the recombination was based on PCR screening with both internal primers that detected the triple-HA insert and primers that detected endogenous LRRK2 (Supplemental Table 1). Our final E1 clone is a mix of both

unedited (endogeneous LRRK2) and CRISPR/Cas9 system edited (endogenous 3xHA-LRRK2) cells.

Affinity selection assays with brain extracts and purified proteins

To prepare brain extracts for affinity selection assays, frozen adult rat brain was homogenized in buffer A (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, supplemented with protease inhibitors: 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin) and centrifuged at 800 × *g* for 10 min, the supernatant was collected, and Triton X-100 was added to 1% final concentration. The samples were incubated for 15 min at 4°C, and then centrifuged at 205,000 × *g* for 30 min. The supernatant was adjusted to a final concentration of 2 mg/ml in buffer A with 1% Triton X-100 and $CaCl_2$ was added to 5 mM final. To prepare triskelia for affinity selection assays, CCVs were purified from adult rat brain and stripped in 0.5 M Tris (*11*) and buffer exchanged into buffer A at a final concentration of 20 μg/ml with 1% Triton X-100. Aliquots of 1 ml of the Triton-soluble brain extract or purified triskelia were incubated with GST fusion proteins pre-coupled to glutathione–Sepharose beads. Samples were incubated for ∼30 min at room temperature and washed 3 times with buffer A containing 5 mM $CaCl_2$ and 1% Triton X-100. Samples were eluted in SDS–PAGE sample buffer, resolved by SDS–PAGE, and processed for Western blotting. For purified proteins, GST fusion proteins were expressed in *E. coli* BL21. Bacterial lysates were incubated with gluthatione-Sepharose beads, and, after washing, the beads were incubated o/n with thrombin (Sigma-Aldrich) in thrombin cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 1 mM DTT) to cleave off the GST tag, which remains bound to the beads. The thrombin was cleared by incubation with benzamidine-Sepharose beads for 30 min at 4°C, followed by buffer exchange to buffer A. Purified proteins were incubated for ~30 min at room temperature with GST fusion proteins pre-coupled to glutathione-Sepharose beads and the beads were washed 3 times with buffer A containing 5 mM $CaCl_2$ and 1% Triton X-100. Samples

were eluted in SDS–PAGE sample buffer, resolved by SDS–PAGE, and processed for Western blotting.

Rac1 activation assays

For Rac1 activation assays, 96 h post-transfection with siRNA, COS-7 cells were washed with ice cold PBS, scraped into buffer B (2.5 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, and 1% NP-40) supplemented with protease inhibitors, and sonicated. The lysates were centrifuged at 21,000 × *g* for 15 min at 4°C. In parallel, GST-PAK-CRIB domain fusion proteins were expressed in *E. coli* BL21. After coupling to glutathione-Sepharose beads, GST-PAK-CRIB was washed in buffer B and kept at 4°C. Aliquots of 1 ml of cell lysates (1.5 mg/ml) were incubated for 40-50 min at 4°C with ~28 µg pre-coupled GST-PAK-CRIB fusion proteins, washed 3 times with ice-cold buffer B, and samples were eluted in SDS–PAGE sample buffer, resolved by SDS–PAGE, and processed for Western blotting.

Mass spectrometry analysis

Proteins from Triton soluble rat brain extracts affinity selected with GST-ROC were eluted by SDS-PAGE sample buffer, resolved by SDS-PAGE and stained with colloidal coomassie. Protein bands exclusively present in GST-ROC incubated with tissue extracts and not with GST alone incubated with extracts or GST-ROC incubated with buffer alone were excised and processed for mass spectrometry as previously described (*12*).

***Drosophila* stocks, crosses and analysis**

GMR-GAL4, longGMR-GAL4, UAS-Rac1W, *UAS-GFP-Clc*, and *dlrrk*^{e03680} lines were obtained from the Bloomington *Drosophil*a Stock Center. *Drosophila* socks of *UAS-dLRRK-RNAi* (GD11670^{V22139} and GD11670^{v22140}), *UAS-Clc-RNAi* (GD12083^{V22318} and KK107357^{V106632}), *UAS-slipper-RNAi* (GD9771^{V33516}), a *Drosophila* homologue of the mammalian mixed lineage kinase

family that suppresses Rac1 activation phenotypes and *UAS-irk3-RNAi* (KK107031^{V101174}), an inward rectifying potassium channel with no known relationship to Rac1, were obtained from the Vienna *Drosophila* RNAi Center. *UAS-dlrrk-WT, UAS-dlrrk-3KD,* and the dlrrk RNAi line *UAS-IR-Lu* were obained from Dr. Binwei Lu (*4*). Flies were raised on standard fly food containing yeast, corn syrup and cornmeal at 25°C. Fly eye pictures were acquired with a Canon EOS 1000D DSLR (rebel XS) camera mounted on a Zeiss Axioskop 40 microscope with 10x objective (0.25=N.A). In order to obtain pictures of the entire eye in focus, we took a series of pictures at different focal plans of the eye (10-35 pictures depending on the eye shape). Focus stacking was performed on the picture stack with the use of the Helicon Focus software (HeliconSoft) to generate the final picture with extended depth of field.

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Supplemental Figures Legends

Supplemental Figure 1. GST-ROC has similar binding to nCLCb and CLCb.

Full-length GST-CLCb or GST-nCLCb, as well as a truncated construct (GST-nCLCb 1-165) were purified from bacteria and cleaved with thrombin to remove the GST tag. Aliquots of the purified proteins as indicated were incubated with GST-ROC. Specifically bound proteins were detected by Western blot with antibody recognizing CLCs (top panel). Aliquots of the purified proteins as indicated were run in parallel (starting material, SM). The ponceau shows equal amounts of GST and GST-ROC between conditions, as well as the purified proteins indicated with an asterisk.

Supplemental Figure 2. LRRK2 rescues Rac1-related phenotypes mediated by CLC KD.

A. NP-40 soluble lysates were prepared from COS-7 cells transfected with the indicated siRNA. At 48 h prior to the assay myc-LRRK2 was transfected in both CLCs and LRRK2 siRNA treated cells, as indicated and equal protein aliquots (1.5 mg) were incubated with GST-PAK-CRIB domain. Specifically bound proteins were detected by Western blot with antibody recognizing Rac1. An aliquot of the starting material equal to 5% of the cell lysate added to GST-PAK-CRIB was analyzed in parallel.

B. Band intensities of blots as in A are presented as % control siRNA. Bars represent mean ± sem. One-way ANOVA, Dunnet *post hoc* test, $*p < 0.05$, $**p < 0.01$, N=3.

C. COS-7 cells were transfected with siRNA as indicated. At 48 h, some of the cells were transfected with myc-LRRK2, as indicated, and the cells were fixed 48 h later and processed for immunofluorescence using Phalloidin-647 and antibody recognizing CLCs. Scale bar = 10 μm.

D. Perimeter and area were measured and a perimeter:area ratio was plotted as a % of the control siRNA treated cells. Bars represent mean ± sem. One-way ANOVA, Bonferroni's *post hoc* test, ***$p < 0.001$, N=3.

Supplemental Figure 3. LRRK2 and CLC KD do not influence Cdc42 activation.

A. NP-40 soluble lysates were prepared from COS-7 cells transfected with the indicated siRNAs and equal protein aliquots (1.5 mg) were incubated with GST-PAK-CRIB domain. Specifically bound proteins were detected by Western blot with an antibody recognizing Cdc42.

B. Band intensities of blots as in A were measured using ImageJ and presented as % control siRNA. Bars equal mean ± sem. One-way ANOVA, Bonferroni's *post hoc* test, N=3.

Supplemental Figure 4. Deconvolving the LRRK2 smartpool.

A. Lysates from COS-7 cells transfected with control siRNA, smartpool siRNAs for LRRK2, or two of the four individual siRNAs from the smartpool, as indicated were processed for Western blot with antibody recognizing LRRK2.

B. NP-40 soluble lysates were prepared from cells treated as in A and equal protein aliquots (1.5 mg) were incubated with GST-PAK-CRIB domain. Specifically bound proteins were detected by Western blot with an antibody recognizing Rac1. An aliquot of the starting material equal to 5% of the cell lysate added to the beads was analyzed in parallel.

Supplemental Figure 5. Altered cell morphology following LRRK2 KD is partially reversed by a Rac1 inhibitor.

A. COS-7 were transfected with control siRNA or LRRK2-2 siRNA, as indicated, and at 48 h, DMSO or 100 μM NSC-23766 (Rac1 inhibitor) in DMSO was added to the media. Following additional 24 h incubation, cells were fixed and processed for fluorescence staining with Phalloidin-647.

B. Percentage of total cells with elongated morphology, with or without NSC (short for NSC-23766) treatment in control and LRRK2 KD COS-7 cells. One-way ANOVA, Bonferroni's *post hoc* test, ***$p < 0.001$, N=17-20 images. Scale bar = 50 μm.

Supplemental Figure 6. KD efficiency of *Drosophila* RNAi lines in the eye disc.

A. Immunofluorescence on the eye disc of *GMR>UAS-GFP-Clc+v101174* as control siRNA (top row) shows normal GFP signal and 24B10 (Chaoptin protein) to stain photoreceptors. *GMR>UAS-GFP-Clc+v106632* to show KD of Clc (bottom row) shows significantly reduced GFP signal, but normal 24B10 staining. Scale bar = 10 μm.

B. Immunofluorescence on the eye disc of *longGMR>UAS-dlrrk-WT+v101174* as control siRNA (top row), shows normal dlrrk staining and 24B10 to stain photoreceptors. *longGMR>UAS-dlrrk-WT+IR-Lu, v22140* or *v22139* to show KD of dlrrk (bottom three rows), show significantly reduced dlrrk but normal 24B10 staining. Scale bar = 10 μm.

C. Lysates from adult heads of the indicated genotypes were processed for Western blot with antibodies recognizing the indicated proteins to show KD efficiency of the RNAi lines.

Supplemental Figure 7. A rough eye phenotype resulting from Rac1 overexpression is rescued by *slipper* KD.

Ai-iii. Lateral view of the adult fly head. Scale bar: 100 μm.

i. Eye morphology of one-week old fly of *longGMR-GAL4, UAS-Rac1W/TM3, Sb*.

ii. G*MR-GAL4/+;UAS-slipper-RNAi(v33516)/TM6B, Hu,Tb*, as a control.

iii. *UAS-slipper-RNAi(v33516)/longGMR-GAL4,UAS-Rac1W*, rescued eye morphology.

Supplemental Table 1: Oligonucleotides used to generate constructs for this study

Oligonuleotide name	Sequence
ROC aa1333 S-BamHI	GCGGGATCCAACCGAATGAAACTTATGATTGTGG
ROC aa1516 AS-NotI	GCGGCGGCCGCTCACTGATCTCGGATCTTGAAATTAAGG
nCLCb aa1 S-BamHI	GCGGGATCCATGGCTGATGACTTTGG
nCLCb aa195 AS-XhoI	GCGCTCGAGTCACACCTTCTCCCACTC
nCLCb aa205 AS-XhoI	GCGCTCGAGTCAGCTCTTGGGGTTG
nCLCb aa215 AS-XhoI	GCGCTCGAGTCACAGGCGGGACACATC
3xHA-CRISPR_AS	CAACTACCGCTGGCGGATC
LRRK2-CRISPR_S	GCAGCGGACGTTCGTGCT
LRRK2-CRISPR_AS	CGTGCTCGGAGTACGTGAAC

Macaque LRRK2-3xHA Ultramer oligonucleotide from Integrated DNA Technologies:

CGTGCTGGGAGGGCGGCGGGTTGGAAGCAGGGGCCACCATGTACCCATACGATGTTC
CAGATTACGCTGGCTATCCCTATGACGTCCCGGACTATGCAGGATATCCATATGACG
TTCCAGATTACGCTGGTGGCGGAGGATCCGCCAGCGGTAGTTGCCAGGGGTGCGAGG
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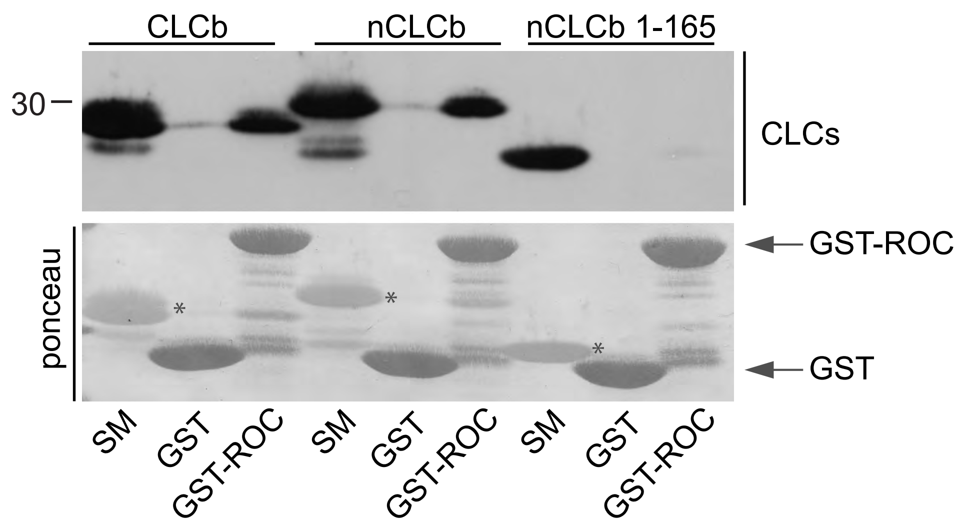

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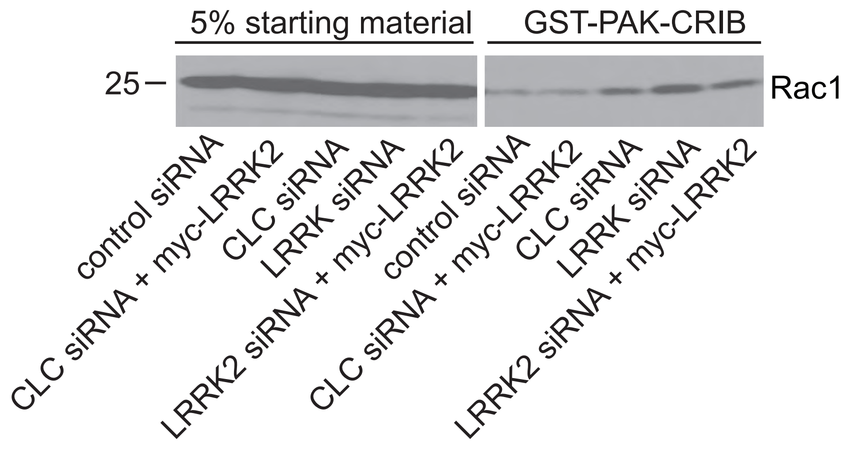

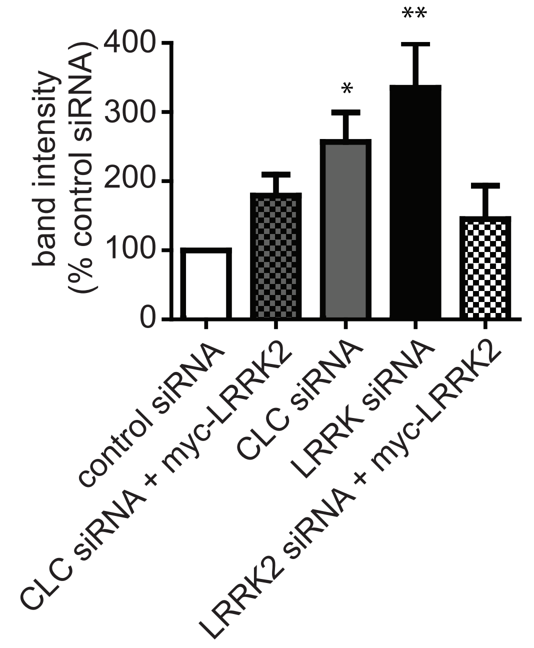

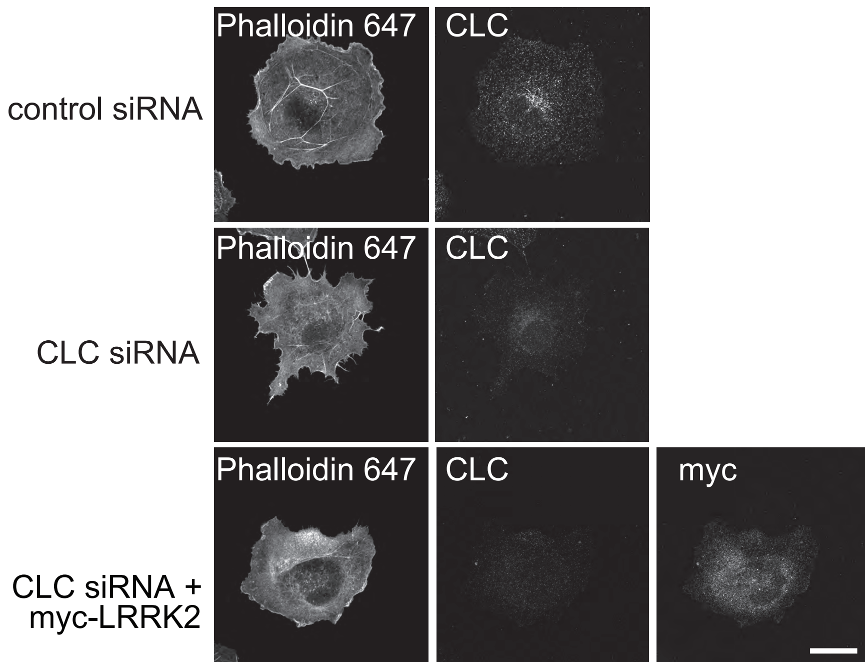

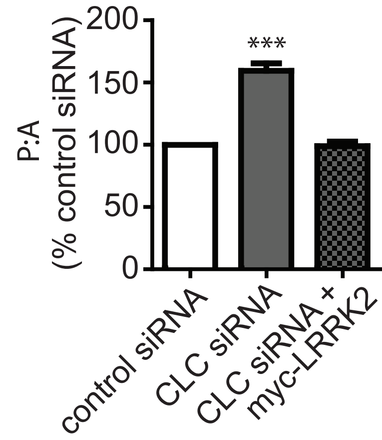

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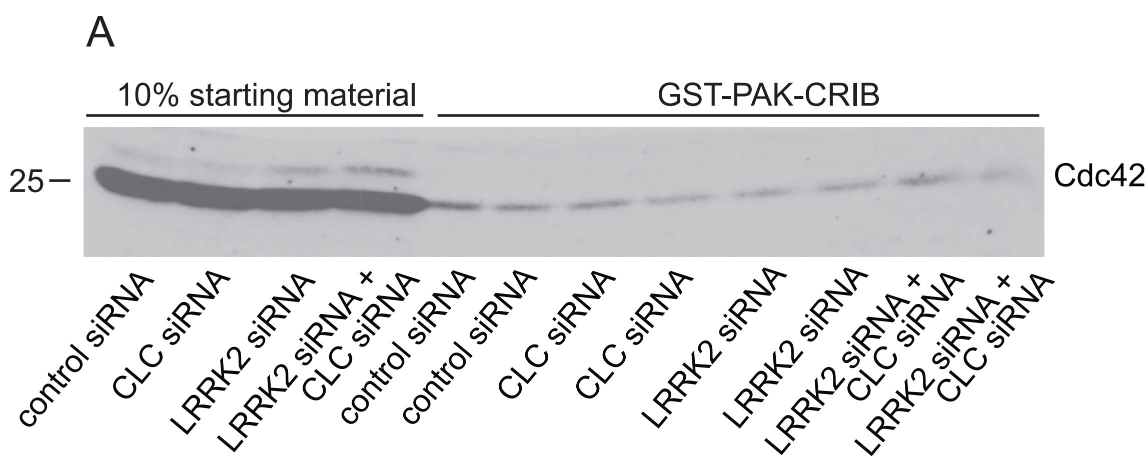

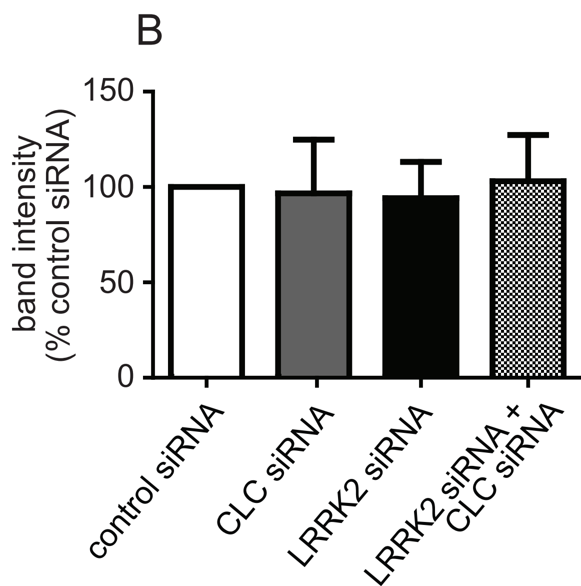

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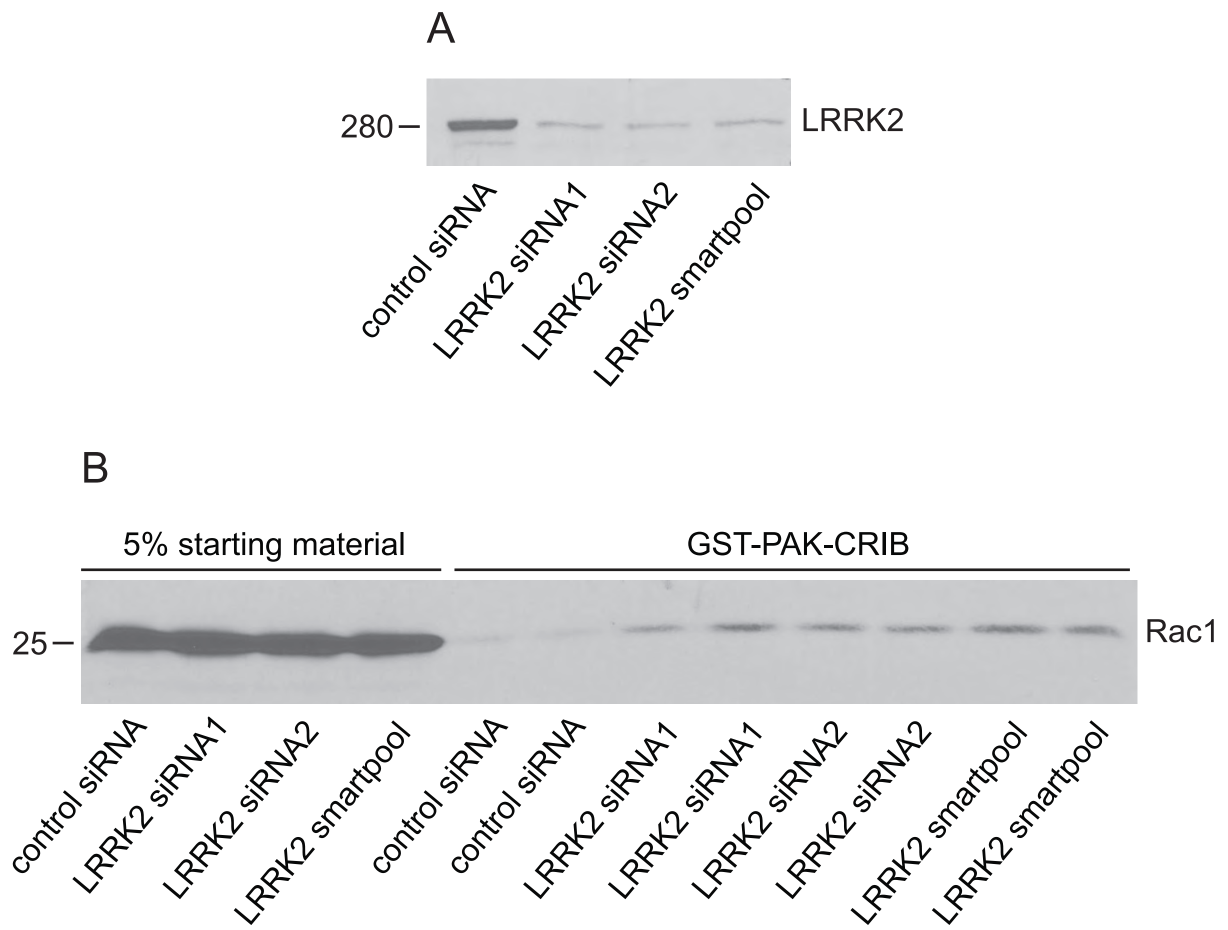

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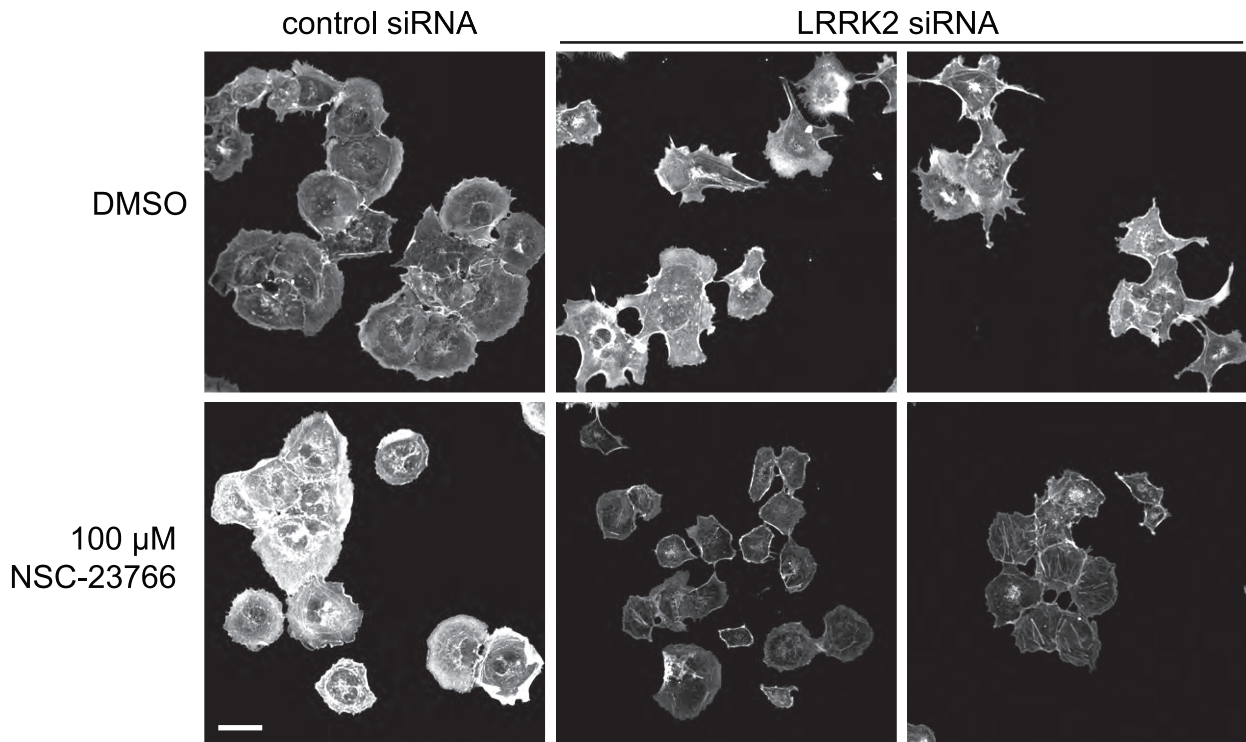

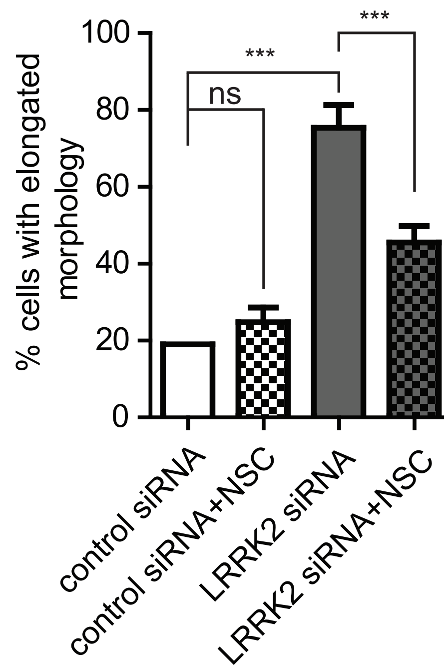

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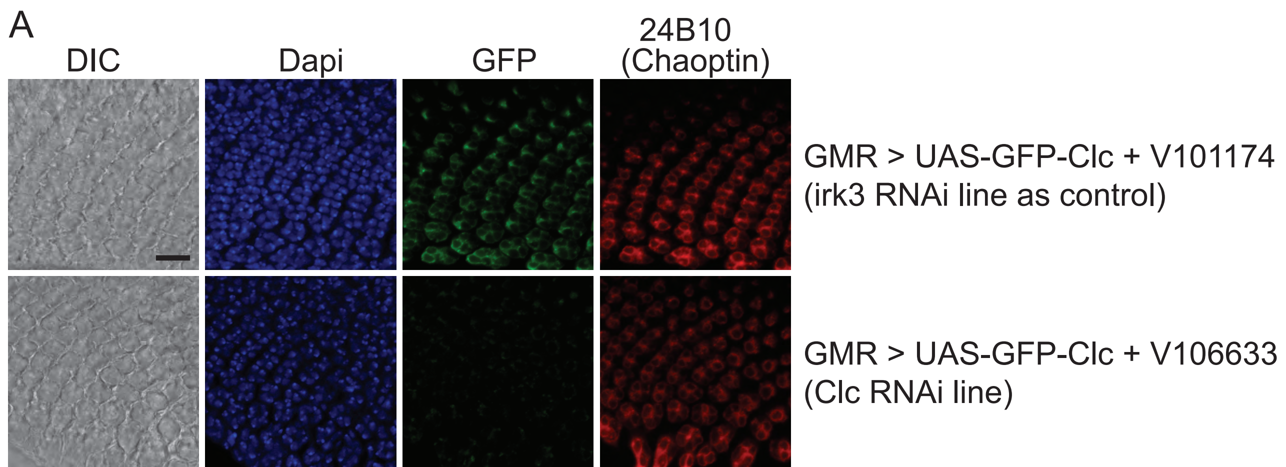

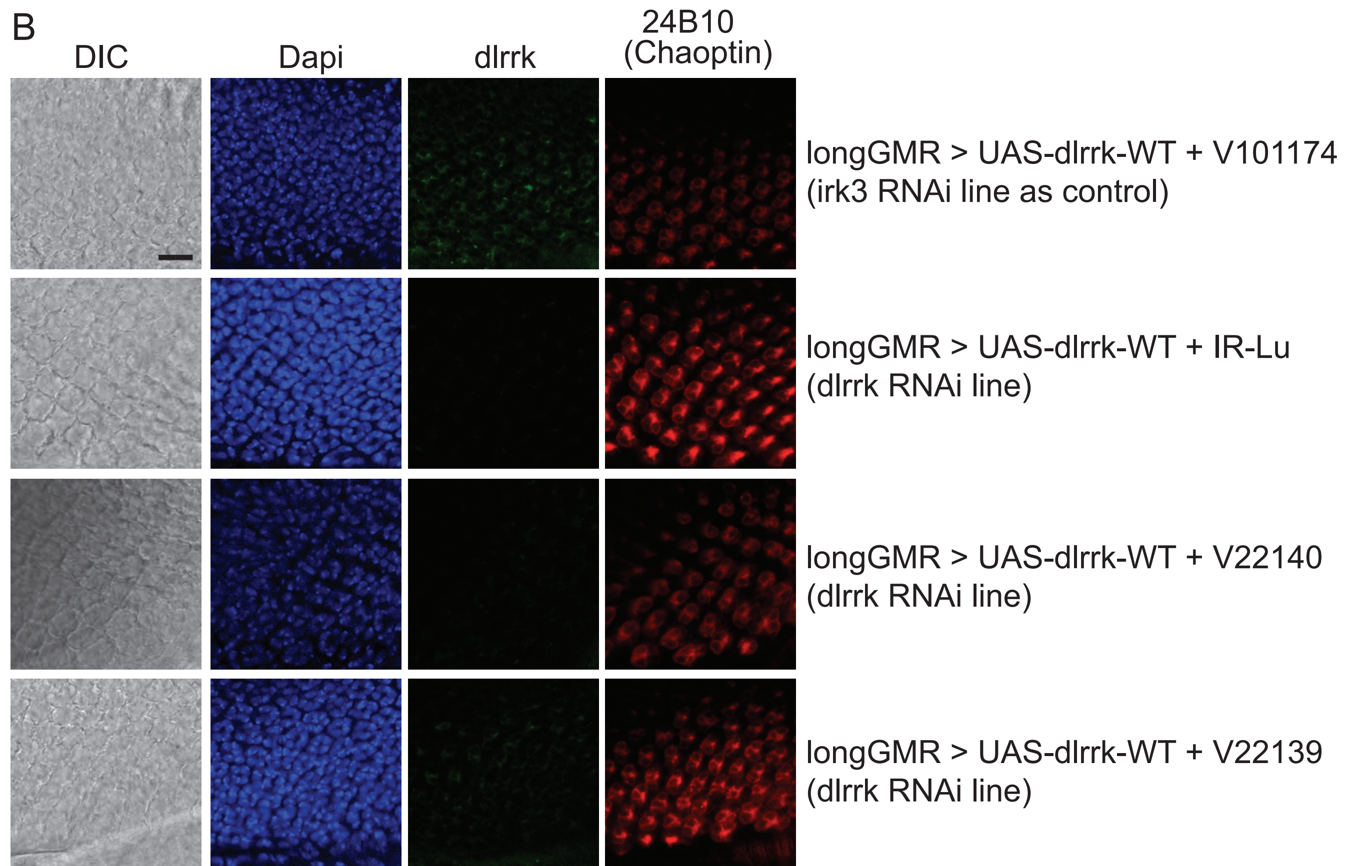

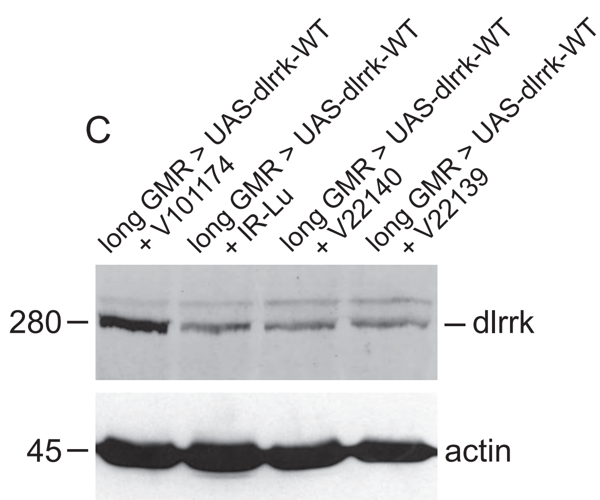

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