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

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Abstract

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common cause of dominant-inherited Parkinson's disease (PD), and yet we do not fully understand the physiological function(s) of LRRK2. Various components of the clathrin machinery have been recently found mutated in familial forms of PD. Here, we provide molecular insight into the association of LRRK2 with the clathrin machinery. We report that through its GTPase domain, LRRK2 binds directly to clathrin-light chains (CLCs). Using genome-edited HA-LRRK2 cells, we localize LRRK2 to endosomes on the degradative pathway, where it partially co-localizes with CLCs. Knockdown of CLCs and/or LRRK2 enhances the activation of the small GTPase Rac1, leading to alterations in cell morphology, including the disruption of neuronal dendritic spines. In *Drosphila*, a minimal rough eye phenotype caused by overexpression of Rac1, is dramatically enhanced by loss of function of CLC and LRRK2 homologues, confirming the importance of this pathway *in vivo*. Our data identify a new pathway in which CLCs function with LRRK2 to control Rac1 activation on endosomes, providing a new link between the clathrin machinery, the cytoskeleton and PD.

Keywords clathrin; *Drosophila melanogaster*; endosomes; Parkinson's disease; Rac1

Subject Categories Membrane & Intracellular Transport; Molecular Biology of Disease; Neuroscience

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Introduction

Parkinson's disease (PD) is the second most common age-related progressive neurodegenerative disorder, and mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene are the most common genetic cause of both familial and sporadic PD [1]. LRRK2 is a large molecular weight multi-domain protein that includes a kinase domain and a Ras of complex proteins (ROC) GTPase domain [1]. LRRK2 functions in at least four different processes related to membrane trafficking; (i) synaptic vesicle (SV) exo/endocytosis [2], (ii) trafficking of the mannose 6-phosphate receptor (MPR) and associated lysosomal hydrolases between the trans-Golgi network (TGN) and the endo-lysosomal system [3,4], (iii) control of epidermal growth factor receptor (EGFR) trafficking and degradation [5], and (iv) regulation of actin dynamics [6–8]. The relationship between the structural domains of LRRK2 and the protein's functional activity remains poorly defined.

Clathrin-mediated membrane trafficking provides a major mechanism for protein transport in cells including endocytosis of protein cargo [9], reformation of SVs [10], and trafficking of the MPR [11]. Intriguingly, mutations in key components of the clathrin machinery are rare variants in familial forms of PD [12–14]. Clathrin coats are assembled from triskelia, composed of three linked clathrin-heavy chain (CHC) proteins and associated clathrin-light chains (CLCs) [15]. There are four forms of CLCs, CLCa and b, which are functionally interchangeable and expressed in all tissues, and neuronal CLCa and b (nCLCa/b), which have short splice inserts and are expressed exclusively in neurons [15]. CLCs bind to huntingtin-interacting protein 1-related (HIP1R) [16] recruiting it to clathrin coats [17]. HIP1R binds actin [18] and functions as a negative regulator of actin assembly [19], and knockdown (KD) of either HIP1R or CLCs causes overly abundant actin assembly in the vicinity of clathrin coats [17, 19]. CLCs are also components of bilayered clathrin coats on early endosomes that recruit the "endosomal sorting complexes required for transport" machinery, which drives sorting and inward invagination of endocytic cargo, such as EGFR, allowing for formation of multivesicular bodies (MVBs) and degradation of cargo in lysosomes [20–22].

Here, we set out to better define the role of LRRK2 and discovered an interaction of its ROC domain with CLCs. We demonstrate that CLCs and LRRK2 interact biochemically and functionally to negatively regulate Rac1 activation and that disruption of this pathway leads to Rac1 activation and altered function, both in cells and in the *Drosophila* eye *in vivo*.

Results and Discussion

CLCs bind directly to the ROC domain of LRRK2

To better understand the cell physiological functions of LRRK2, we screened for ROC domain binding partners. GST-ROC was incubated

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with soluble rat brain extracts with affinity-selected proteins identified by mass spectrometry. nCLCa/b were detected in this analysis and their binding to GST-ROC was confirmed by blot (Fig 1A). nCLCa/b are functionally interchangeable [23] and their binding to GST-ROC is similar (Fig 1A). Endogenous LRRK2 from brain extracts binds to GST-nCLCb (Fig 1B), and purified full-length nCLCb (residues 1–228) binds robustly to GST-ROC (Fig 1C), demonstrating that the interaction is direct.

CLCb and nCLCb bind GST-ROC equally indicating that the neuronal-specific splice insert is not required for binding, whereas a CLC construct encoding residues 1-165 [16] does not bind, indicating that the binding site is between residues 166–228 (Supplementary Fig S1). We thus generated C-terminal deletion constructs; whereas purified nCLCb 1-215 and 1-205 bind GST-ROC equivalent to full-length, a 1–195 construct has no binding (Fig 1C). However, all constructs bind equally well to CHC (Fig 1D), indicating that loss of ROC binding is not due to a major alteration in folding. Thus, the LRRK2-binding site is between residues 195 and 205. This region of nCLCb is identical in all four CLC isoforms, is conserved across species with only one amino acid substitution in *Drosophila* and has no ascribed binding partner or functional role [24].

Clathrin-light chains interact with CHC as part of triskelia (Fig 1E). CLCs are on the outer surface of the triskelia, facing the cytosol in an assembled clathrin coat such that resides 196–205 are accessible to cytosolic proteins [25] (Fig 1E). We thus tested whether LRRK2 interacts with CLCs as part of triskelia. GST-ROC was incubated with triskelia stripped from purified CCVs [26] and both CLCs and CHC are detected in the pull down (Fig 1F), indicating that CLCs bound to CHC are still accessible to LRRK2. Despite extensive efforts, we were unable to co-immunoprecipitate (co-IP) the two proteins. Like many large multidomain proteins, LRRK2 is predominantly insoluble when generating lysates from cultured cells [27] or tissue [28], and similarly, clathrin triskelia form massive protein complexes when incorporated into coats. Thus, if LRRK2 associates selectively with CLC assembled in coats, this would hinder co-IP. However, we cannot exclude that a transient or low-affinity interaction hampers the ability to observe LRRK2/CLC co-IP. Nevertheless, our discovery that LRRK2 binds directly to CLCs indicates that CLCs have a dual

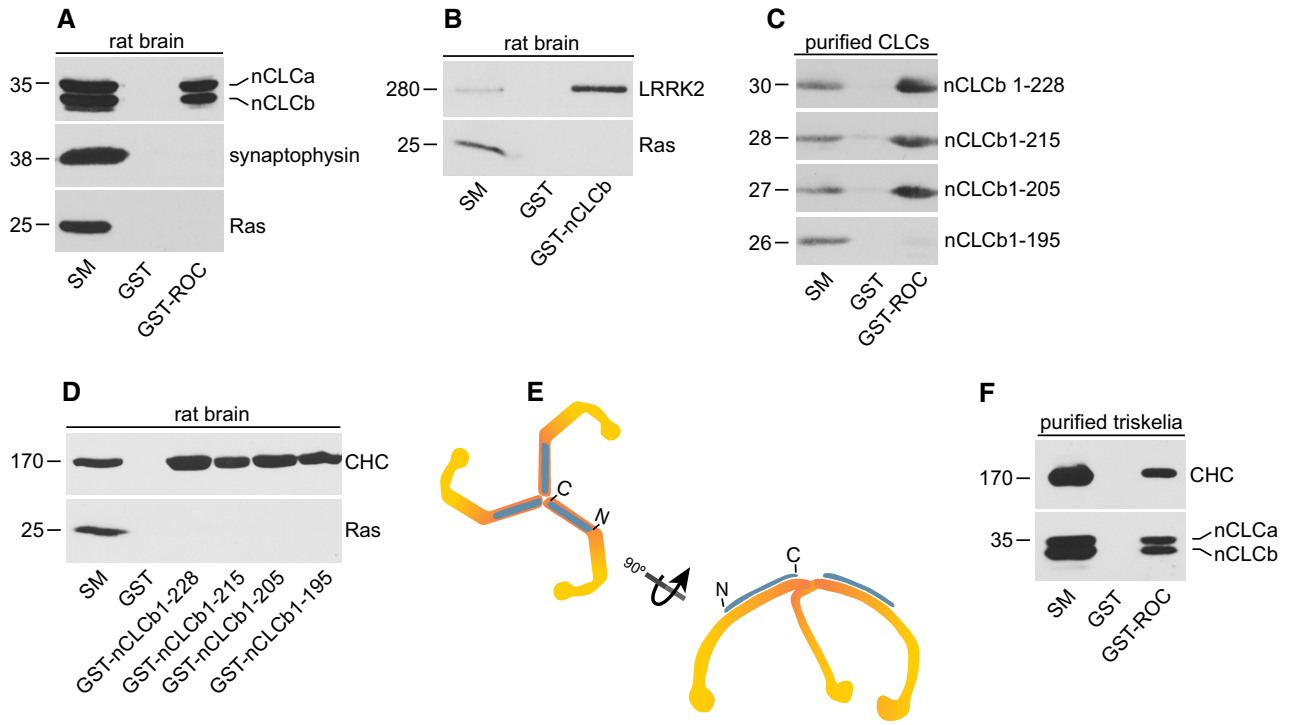

Figure 1. Identification of CLCs as LRRK2-binding partners.

A Equal aliquots (2 mg) of a Triton X-100-solubilized lysate from rat brain were incubated with GST or GST-ROC. Specifically bound proteins were detected by Western blot with antibodies recognizing the indicated proteins.

B Brain lysate as in (A) was incubated with GST or GST-nCLCb. Specifically bound proteins were detected by Western blot with antibodies recognizing the indicated proteins.

C Full-length GST-nCLCb (1-228) and various truncation mutants were purified from bacteria and the GST tag was removed by thrombin cleavage. Equal aliquots of purified protein were incubated with GST or GST-ROC. Specifically bound proteins were detected by Western blot with antibody against CLCs.

D Brain lysate as in (A) was incubated with GST or GST-nCLCb proteins with the indicated boundaries. Specifically bound proteins were detected by Western blot with antibody against CLCs.

E Representation of a triskelia composed of three CHCs (orange) with three associated CLCs (blue).

F Highly enriched triskelia were stripped from purified CCVs. The triskelia were incubated with GST or GST-ROC. Specifically bound proteins were detected by Western blot with antibodies recognizing the indicated proteins.

Data information: For (A-D, F), aliquots of the starting material (SM) equal to 10% of that added to the beads was analyzed in parallel.

scaffolding function, recruiting LRRK2 and HIP1R via C-terminal and N-terminal regions, respectively.

Endogenous genome-edited LRRK2 localizes to endosomes

A recent systematic analysis of known LRRK2 antibodies shows they are problematic in their recognition of endogenous LRRK2 by immunofluorescence [28]. Thus, to assess the localization of endogenous LRRK2, we used CRISPR/Cas9 technology to genome edit LRRK2 in COS-7 cells, adding a triple HA tag between amino acids 1 and 2, downstream of the endogenous promoter (Fig 2A and B). Remarkably, LRRK2 co-localizes with internalized EGF (Fig 2C), indicating that a significant fraction of the protein is present on membranes of the endosomal system, specifically on the degradative pathway. We also detect partial co-localization with CLCs (Fig 2D), likely reflecting bilayered clathrin coats on early endosomes involved in the formation of MVBs during protein degradation [20–22]. Consistently, HA-LRRK2 partially co-localizes with the early endosomal marker EEA1 (Fig 2E). LRRK2 functions in EGFR trafficking from early endosomes to MVBs and lysosomes, while PD-LRRK2 mutants delay EGFR degradation by trapping the receptor in endosomes [5]. Thus, CLCs likely function as a scaffold to recruit LRRK2 to bilayered clathrin coats on early endosomes.

KD of CLCs or LRRK2 activates Rac1 altering cell morphology

Knockdown of CLCs leads to over assembly of actin, [17] and actin was identified in a screen for LRRK2-binding partners [6]. Moreover, LRRK2 binds directly to the small GTPase Rac1, which regulates actin assembly [7]. Interestingly, Rac1 activation occurs on early endosomes [29]. We thus tested whether LRRK2 and CLCs regulate Rac1 activity. We used previously characterized siRNAs for CLCa/b [17] and a smartpool of four LRRK2 siRNAs to efficiently knock down the proteins (Fig 3A and B). To measure Rac1 activity, we performed affinity-selection assays with the p21-activated protein kinase Cdc42/Rac1 interactive binding domain (GST-PAK-CRIB), which binds preferentially to the GTP-bound form of Rac1 [30]. Interestingly, KD of CLCs or LRRK2 causes a > twofold and > threefold activation of Rac1, respectively, compared to control siRNA (Fig 3C and D). The simultaneous KD of both does not further increase Rac1 activity, suggesting that CLCs and LRRK2 are on the same pathway for Rac1 regulation. Consistently, expression of myc-LRRK2 rescues the enhanced activation of Rac1 seen upon LRRK2 and CLC KD (Supplementary Fig S2A and B). Activity of the related GTPase Cdc42 is not influenced by CLCs/LRRK2 KD; thus, activation of Rac1 is selective (Supplementary Fig S3). Deconvolution of the LRRK2 smartpool siRNA sequences reveals that two distinct sequences that KD LRRK2 lead to Rac1 activation (Supplementary Fig S4). Thus, CLCs and LRRK2 function to limit Rac1 activity.

Consistent with the Rac1 activation phenotype, KD of either CLCa/b or LRRK2 leads to alterations in cell morphology, with cells appearing more irregularly shaped with variable protrusions (Fig 3E). These changes were quantified as an increase in the perimeter:area ratio (Fig 3F). KD of CLCs, LRRK2, or both led to quantitatively similar phenotypes, suggesting that the proteins function in a common pathway. Consistently, the enhanced perimeter:area resulting from CLC KD is rescued by myc-LRRK2 expression (Supplementary Fig S2C and D). Moreover, the Rac1 inhibitor NSC-23766 [31] significantly decreases changes in cell morphology resulting from LRRK2 KD, indicating that they result from Rac1 activation (Supplementary Fig S5).

Our results are consistent with a previous study reporting an increased perimeter:area following LRRK2 KD in NIH3T3 cells [6]. Moreover, LRRK2 loss of function leads to neurite over-branching phenotypes at the neuromuscular junction in *Drosophila* [32], and in primary neuronal culture [8]. Additionally, there is a decrease in the number of mature dendritic spines in LRRK2$^{-/-}$ neurons, which is accompanied by altered synaptic transmission [33]. We therefore examined whether KD of CLCs through lentivirus-driven expression of shRNAmiRs [34] would result in a similar actin-dependent phenotype. KD of nCLCa/b in cultured neurons was confirmed by Western blot (Fig 3G), and we observe a loss of mature dendritic spines (Fig 3H). Although the underlying molecular mechanisms for these various alterations in cell morphology remain undefined, they may result from hyper activation of Rac1 at early endosomes. Once active on endosomes, Rac1 recycles back to the plasma membrane to regulate actin cytoskeleton dynamics [29]. Thus, it appears that CLCs interact with LRRK2 at endosomes to limit actin assembly by inhibiting the activation of Rac1. Our finding that LRRK2 KD activates Rac1 seems contrary to Chan *et al* [7], who observe that LRRK2 overexpression activates Rac1 resulting in decreased neurite outgrowth. However, Matta *et al* [2] report that both overexpression and KD of LRRK2 negatively affects SV endocytosis. Thus, it appears that a fine balance in the level of LRRK2 is required for normal function, which could explain the apparent discrepancy between our results and those of Chan and colleagues [7].

Disruption of *Clc* or *dlrrk* enhances morphogenetic eye phenotypes resulting from Rac1 overexpression

Drosophila melanogaster provides a powerful model system to examine cell physiological pathways *in vivo*. We therefore adopted *Drosophila* to demonstrate that the LRRK2/CLC interaction is physiologically relevant and phylogenetically conserved. Overexpression of Rac1 under control of the glass multiple reporter (*GMR-GAL4*) promoter, which is expressed predominantly in the eye, leads to rough eye phenotypes [35]. We thus tested for *Clc* (*Drosophila* CLC) and *dLRRK* (*Drosophila* LRRK2)-dependent regulation of Rac1 *in vivo*. We generated a *UAS-Rac1* line driven by *longGMR-GAL4*, an eye-specific promoter, and observed a mild rough eye phenotype of several fused ommatidia patches (Fig 4Ai, arrows). When this line is crossed with two separate *UAS-RNAi* lines for *dLRRK* and two *UAS-RNAi* lines for *Clc*, there is a dramatically enhanced rough eye phenotype with most ommatidia fused and smaller overall eye size (Fig 4Aii-v). KD efficiency was verified by immunofluorescence on eye disks and Western blot of protein levels on adult head with a previously validated dlrrk antibody [36] (Supplementary Fig S6). Additionally, we used a previously established dlrrk UAS-RNAi line from Imai and colleagues [36] to show similar KD efficiency (Supplementary Fig S6B and C). No phenotype is seen with the four *UAS-RNAi* lines alone, even when driven off of the strong *GMR-GAL4* promoter (Fig 4Avii-x). Moreover, the mild rough eye phenotype with Rac1 overexpression

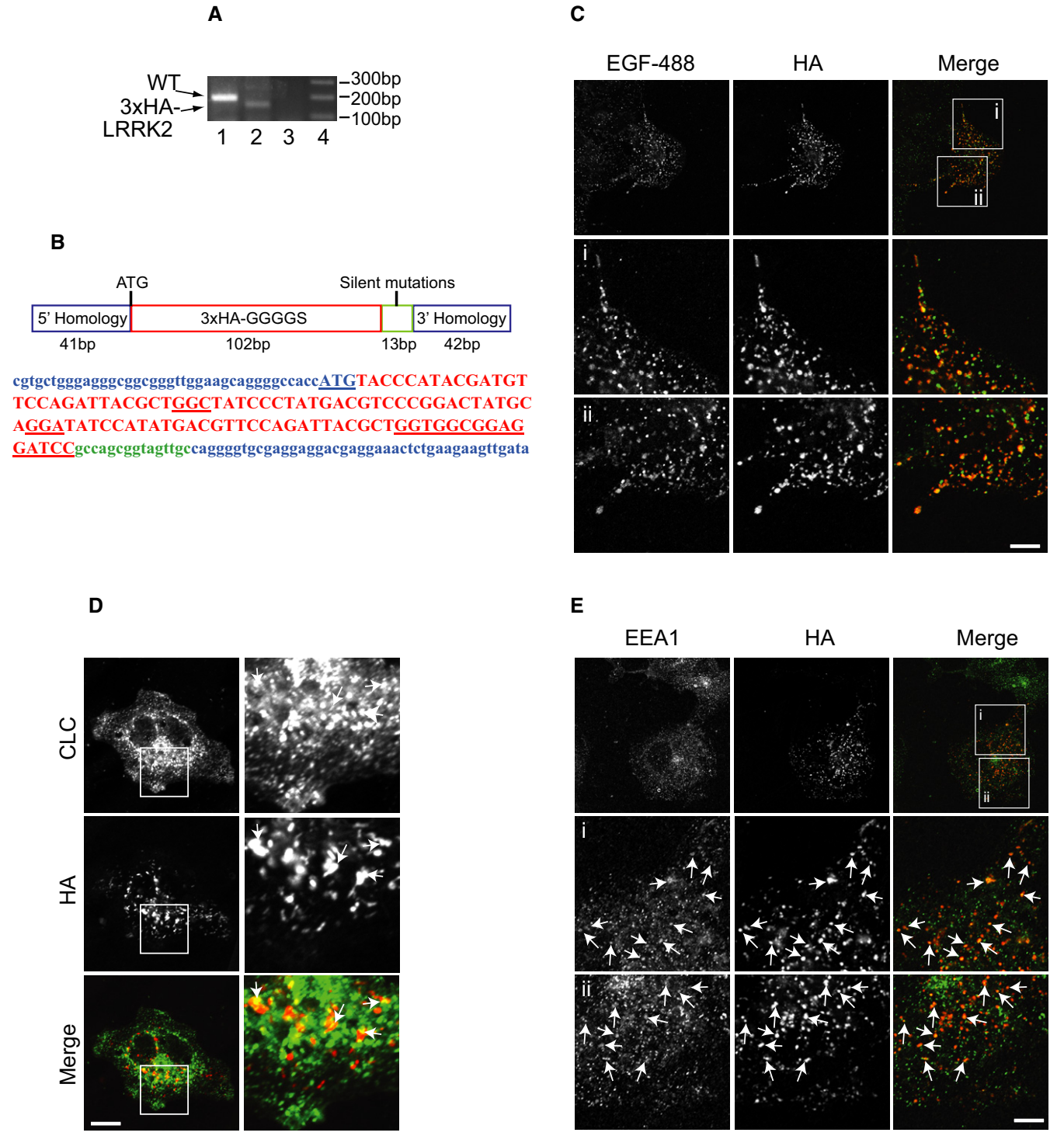

Figure 2. Endogenous genome-edited LRRK2 localizes to endosomes.

A PCR results of LRRK2-WT from clone E1 (1) using primers that detect endogenous LRRK2. Clone E1 is positive for 3× HA-LRRK2 (2) using a primer pair with the antisense in the 3× HA insert and the sense primer in endogenous LRRK2. (3) Control unedited COS-7 cells using the same primer combination as in (2). (4) 1 kb marker.

B Schematic diagram of the oligonucleotide used to direct insertion of the 3× HA tag into the 5′ end of the human LRRK2 coding sequence and the corresponding coding sequence in the same colors (the LRRK2 start codon is underlined, as is the GGGGS linker).

C COS-7 clone E1 cells were serum-starved followed by 20-min incubation with Alexa488-EGF, after which the cells were fixed and processed for immunofluorescence using HA antibody. Scale bar, 10 μm for bottom 6 panels and 25 μm for top 3 panels.

D, E COS-7 clone E1 cells were fixed and processed for immunofluorescence using HA and CLC (D) or HA and EEA1 (E) antibodies. Scale bar, 10 μm (D, bottom 6 panels in E) and 25 μm (top 3 panels in E).

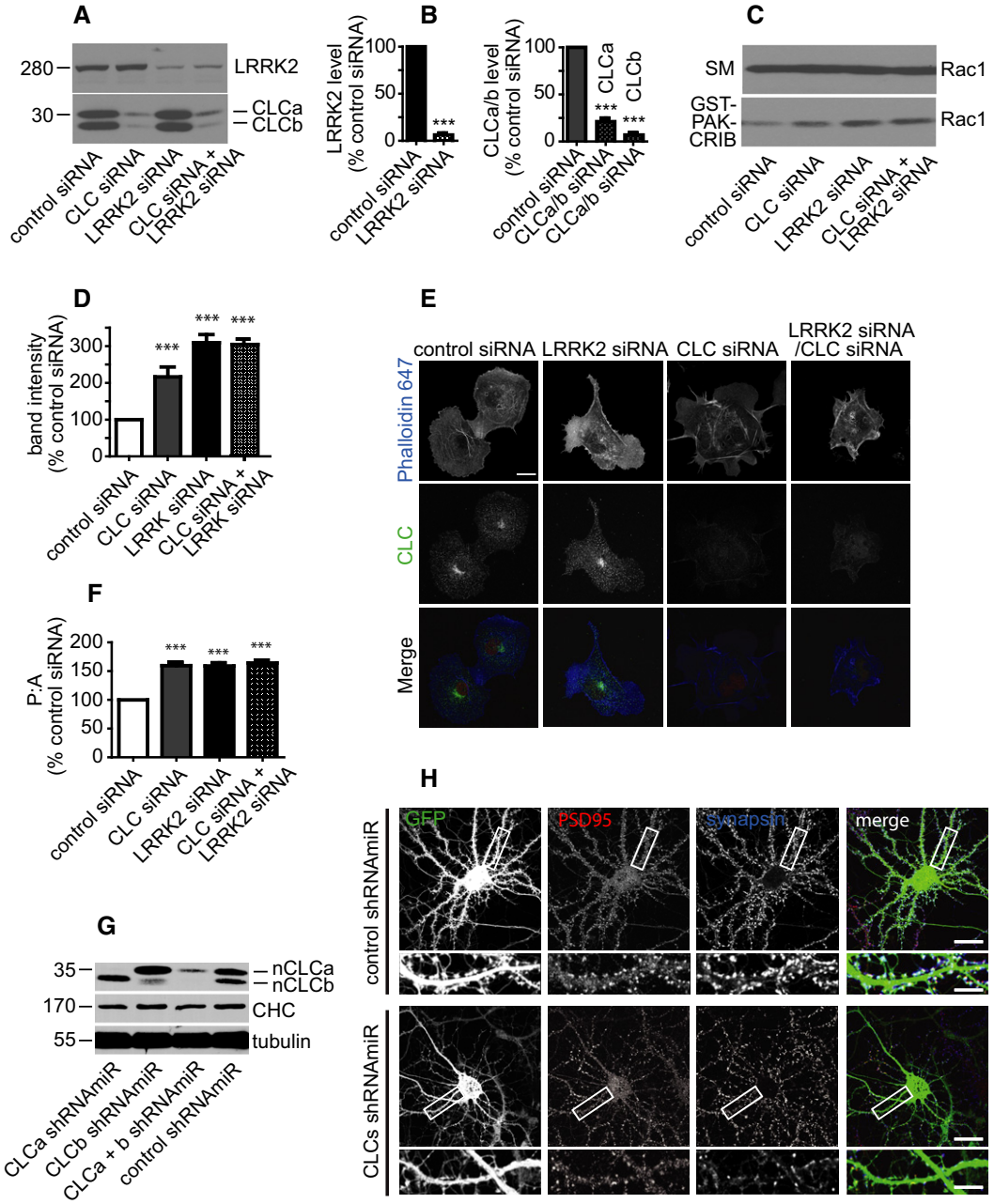

Figure 3. KD of LRRK2 and CLCs lead to Rac1 activation.

A, B Lysates from COS-7 cells transfected with siRNA as indicated were processed for Western blot with antibodies recognizing the indicated proteins (A). Band intensities are presented in (B) as % control siRNA. Two-tailed Student's *t*-test, Mann–Whitney *post hoc* test, ***$P < 0.001$, $N = 3$.

C, D NP-40 soluble lysates were prepared from COS-7 cells transfected with siRNA 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 (C). An aliquot of the starting material (SM) equal to 5% of that added to GST-PAK-CRIB was analyzed in parallel. Band intensities are presented in (D) as % control siRNA. Bars represent mean ± s.e.m. One-way ANOVA, Bonferroni's *post hoc* test, ***$P < 0.001$, $N = 3$.

E, F COS-7 cells transfected with siRNA as indicated were fixed and processed for immunofluorescence using Phalloidin-647 and antibody recognizing CLCs (E). Scale bar, 10 μm. Perimeter and area of cells were measured (F), and a perimeter:area ratio was plotted as a % of the control siRNA-treated cells. One-way ANOVA, Bonferroni's *post hoc* test, ***$P < 0.001$, $N = 3$.

G Lysates from 21-day *in vitro* cultured hippocampal neurons transduced with lentivirus (at 7 days *in vitro*) driving expression of control shRNAmiR or shRNAmiRs specific for CLCa, CLCb, or both viruses in combination. The lysates were processed for Western blot with antibody that recognizes all forms of CLCs.

H Hippocampal neurons transduced as in (G) were fixed at 21 days *in vitro* and processed for immunofluorescence with polyclonal antibody specific for the pre-synaptic protein synapsin and monoclonal antibody specific for the post-synaptic protein PSD95. The lentivirus drives expression of GFP. The areas indicated by white boxes in the low power images (squares) are shown enlarged immediately below. The scale bars represents 10 μm for the low magnification and 2.5 μm for the high magnification.

is not enhanced with KD of an inward rectifying potassium channel (*UAS-irk3-RNAi*) as a control (compare Fig 4Avi with Ai), but is largely rescued with KD of Slipper (Supplementary Fig S7), a *Drosophila* homologue of the mammalian mixed lineage kinase family shown to rescue the rough eye phenotype resulting from Rac1 overexpression [37]. To further verify that these findings were not due to off-target effects, we used a null allele line for *dLRRK* (*dLRRK*^{e03680}), which when crossed with the Rac1 overexpressing line showed a similar enhanced eye phenotype as the KD lines (compare Fig 4Bi/ii with Aii/iii).

We next examined whether overexpression of dLRRK or Clc could rescue the Rac1-mediated rough eye phenotype. We co-expressed Rac1 and either dLRRK or Clc in the *Drosophila* eye and saw a dramatic improvement in the rough eye phenotype resulting

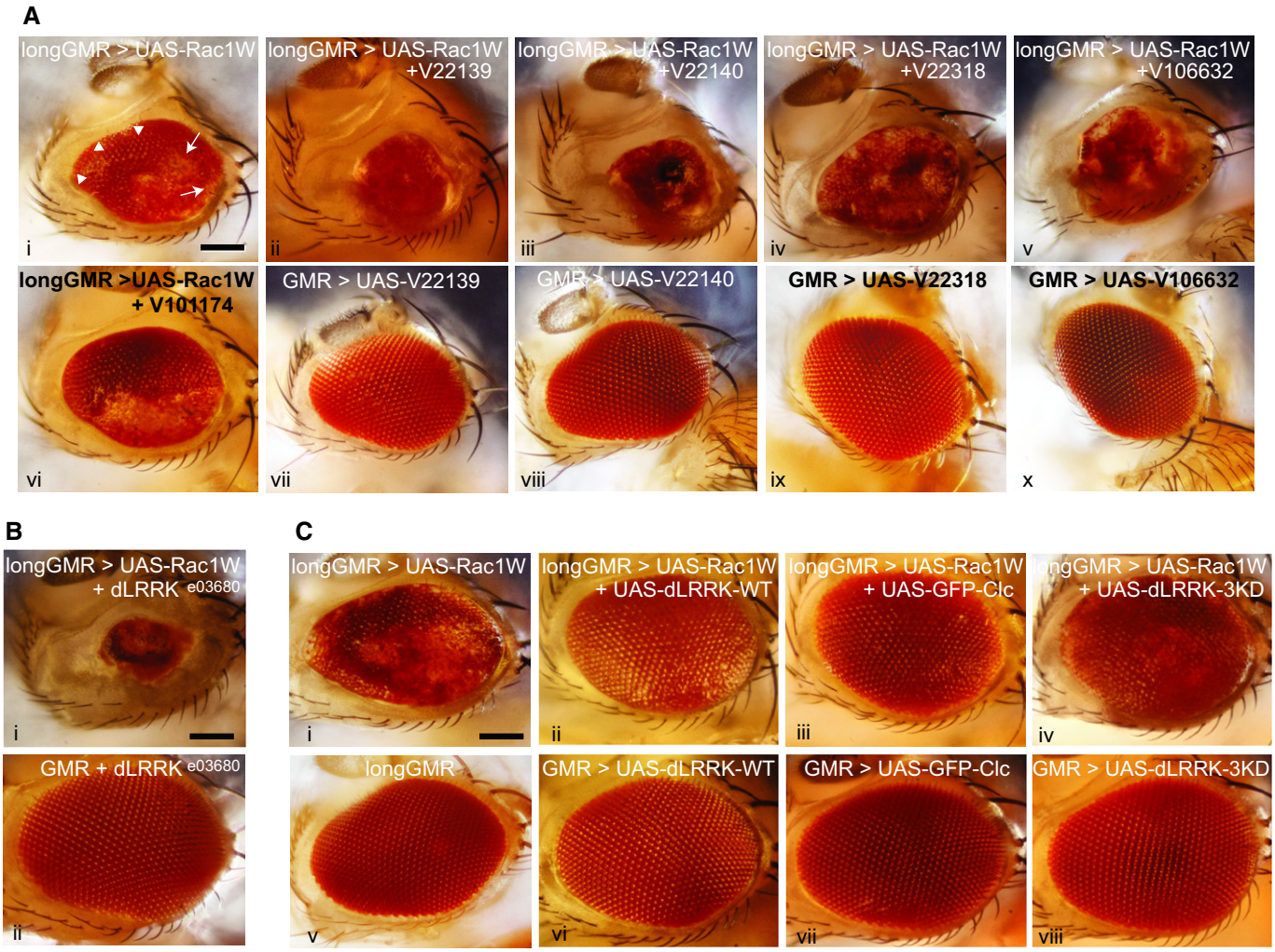

Figure 4. KD of *dLRRK* or *Clc* enhances eye morphogenetic defects caused by Rac1 overexpression in the *Drosophila* eye.

Ai-x Lateral view of the adult fly head. Scale bar, 100 μm. (i) *longGMR,UAS-Rac1W/TM6B, Hu, Tb* causes a mild rough eye phenotype with fused (arrows) and disorganized but still separated ommatidia (arrowheads). (ii, iii) Rac1 overexpression with KD of *dLRRK* [*UAS-dLRRK-RNAi (υ22139* or *υ22140)/longGMR-GAL4, UAS-Rac1W*], results in smaller eyes where most ommatidia are fused. (iv, v) Rac1 overexpression with KD of Clc [*UAS-Clc-RNAi (υ22318* or *υ106632)/+;longGMR-GAL4, UAS-Rac1W/+*], results in smaller eyes with some ommatidia fused. (vi) Rac1 overexpression with KD of the inwardly rectifying potassium channel 3 (*irk3*), a non-relevant RNAi control [*UAS-irk3-RNAi(υ101174)/+; longGMR-GAL4,UAS-Rac1W/+*], has no effect. (vii, viii) KD of *dLRRK* with *GMR-GAL4/+; UAS-dLRRK-RNAi(υ22139* or *22140)/+*, respectively as control have no effect on eye morphology. (ix, x) KD of Clc with *GMR-GAL4/UAS-Clc-RNAi* (*υ22318* or *υ106632*), respectively, as control has no effect on eye morphology.

Bi-ii Lateral view of the adult fly head. Scale bar, 100 μm. (i) Rac1 overexpression with removing one copy of *dlrrk* (*dLRRK*^{e03680}*/longGMR-GAL4,UAS-Rac1W*) results in smaller eyes where most ommatidia are fused together. (ii) Removing one copy of *dLRRK*^{e03680} in *GMR-GAL4* background, as control has no effect on eye morphology (*GMR-GAL4/+;dLRRK*^{e03680}*/+*).

Ci-viii Lateral view of the adult fly head. Scale bar, 100 μm. (i) *longGMR,UAS-Rac1W/TM6B, Hu, Tb* causes a mild rough eye phenotype with fused and disorganized but still separated ommatidia (as explained in Ai). (ii) Rac1 co-expression with *dLRRK*-WT using *UAS-dLRRK-WT/+;longGMR-GAL4,UAS-Rac1W/+*, results in rescued eye morphology. (iii) Rac1 co-expression with Clc using *UAS-GFP-Clc/+;longGMR-GAL4,UAS-Rac1W/+*, results in rescued eye morphology. (iv) Rac1 co-expression with *dLRRK*-WT-3KD using *UAS-dLRRK-3KD/+;longGMR-GAL4,UAS-Rac1W/+*, results in rescued eye morphology. (v) A wild-type eye of the *longGMR-GAL4* promoter as a control. (vi–viii) Overexpression of *dLRRK*-WT, *dLRRK*-3KD, or Clc alone with *GMR-GAL4* as control has no effect on eye morphology.

from Rac1 overexpression (Fig 4Ci-iii). A kinase-dead dlrrk mutant (dlrrk-3KD) [36] yielded similar rescue (Fig 4Civ). No phenotype is seen with Clc or dLRRK, WT or 3KD alone (Fig 4Cv-viii). These observations demonstrate that dLRRK and Clc regulate Rac1 activity *in vivo*.

Enhanced intrinsic kinase activity of LRRK2 pathogenic mutants [38] is correlated to increased neurotoxicity [39], suggesting that aberrant enzymatic activity of LRRK2 underlies neuropathogenesis in LRRK2-PD. The LRRK2 field has thus focused on gain-of-function effects of its kinase activity, and the fundamental physiological role(s) of LRRK2 remain understudied. By investigating a basic biological function of LRRK2 in a loss-of-function paradigm, we have uncovered a novel CLC/LRRK2-dependent pathway regulating actin dynamics that functions both *in vitro* and *in vivo*. This newfound link between the clathrin machinery and a major PD gene, coupled with the observation that mutations in key components of the clathrin machinery are rare variants in PD [12–14], provides undeniable evidence of the importance of clathrin-mediated membrane trafficking in PD.

Materials and Methods

Affinity-selection and Rac1 activation assays

Brain extract or purified proteins were incubated for 30 min with GST fusion proteins pre-coupled to glutathione–Sepharose beads and washed three times with buffer A (20 mM HEPES, pH 7.4, 0.83 mM benzamidine, 0.23 mM PMSF, 0.5 μg/ml aprotinin/leupeptin) containing 5 mM $CaCl_2$ and 1% Triton X-100. For Rac1 activation assays, 96 h post-transfection with siRNA, COS-7 cells lysates were incubated for 40–50 min at 4°C with ~28 μg GST-PAK-CRIB fusion proteins pre-coupled to glutathione–Sepharose beads and washed 3 times with ice-cold buffer B (2.5 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1% NP-40, 0.5 μg/ml aprotinin/leupeptin). Samples were eluted, resolved by SDS–PAGE, and processed for Western blotting.

Microscopy

Cells were imaged on a Zeiss LSM710 confocal microscope using a plan-apochromat 63× oil objective and 20× objective (Zeiss). Perimeter:area ratio was determined using NIH ImageJ software. Fly eye pictures were acquired with a Canon EOS 1000D DSLR (rebel XS) camera mounted on a Zeiss Axioskop 40 microscope with 10× objective (0.25 = N.A).

Statistical analysis

All statistical analyses were performed by one-way, parametric analysis of variance (ANOVA) or two-tailed Student's *t*-tests, using GraphPad Prism 5 software. Error bars represent the mean ± s.e.m. Differences were considered significant if $P < 0.05$. For the NSC-23766 experiments, images were randomized and an independent, blinded observer counted normal and elongated cell morphology. Percentage of elongated cells was determined for each image and analyzed by one-way ANOVA.

Other materials and methods can be found in the Supplementary Information.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

AMAS, EAF, and PSM conceived the experiments. AMAS, EAF, PSM, MC, WR, and PAB designed the experiments. AMAS, MC, WR, and SL performed the experiments. AMAS, EAF, and PSM wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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