CHARACTERIZATION OF A NOVEL LRRK2 BINDING PARTNER AND THE PHYSIOLOGICAL FUNCTION OF LRRK2

by

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ABSTRACT

Parkinson disease (PD) is the most common movement disorder and the second most common age-related progressive neurodegenerative disease after Alzheimer's disease. PD-associated hypokinesia results from the loss of dopaminergic neurons in the substantia nigra pars compacta, a brain region that normally regulates the initiation and control of movement. Over the last two decades, the identification of several mutated genes causative of PD has enabled the exploration of molecular pathways underlying PD pathophysiology. Although mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene are the most common genetic cause of both familial and sporadic PD, our understanding of the physiological function of LRRK2 remains incomplete. LRRK2 is a large multidomain protein with both a kinase and a GTPase domain. PD-associated mutations span the entire LRRK2 protein, yet research has mainly focused on a mutation in the kinase domain that enhances LRRK2 kinase activity and that is correlated with neuronal vulnerability. To date, the relationship between the structural domains of LRRK2 and their corresponding physiological functions remains poorly defined, as most domains remain understudied. In the first part of this doctoral thesis we present evidence of a novel interacting partner, clathrin-light chain, that interacts directly with the GTPase domain of LRRK2 on early endosomes to regulate Rac1 activity and thereby alter actin dynamics and cell morphology. Further understanding LRRK2's cellular function is essential to unravel the pathophysiology of LRRK2-associated PD. In the second part of this thesis we establish a human induced pluripotent stem cell line containing endogenously Flag-tagged LRRK2 for future physiological studies in dopaminergic neurons. These findings advance our fundamental understanding of the molecular function of LRRK2, and provide a novel tool to study LRRK2 in human dopaminergic neurons in the future.

RÉSUMÉ

La maladie de Parkinson (MP) est le trouble du mouvement le plus fréquent et la deuxième maladie neurodégénérative progressive liée à l'âge la plus fréquente, après la maladie d'Alzheimer. L'hypokinésie associée à la MP provient de la perte des neurones dopaminergiques dans la substantia nigra pars compacta, une région du cerveau qui régule l'initiation et le contrôle du mouvement. Un intérêt croissant pour l'étude des voies moléculaires sous-jacentes de la physiopathologie de la MP a vu le jour au cours des deux dernières décennies, avec l'identification de plusieurs gènes mutés dans la MP. Des mutations dans le gène *leucine-rich repeat kinase 2* (*LRRK2*) sont la cause génétique la plus commune de la MP d'origine familiale et sporadique, mais notre compréhension de la fonction physiologique de LRRK2 demeure insuffisante. LRRK2 est une grande protéine constituée de plusieurs domaines incluant un domaine kinase et un domaine GTPase. Les mutations associées à la MP se situent dans l'ensemble de la protéine LRRK2, mais jusqu'alors, les recherches ont porté principalement sur une mutation dans le domaine kinase qui augmente l'activité kinase de LRRK2, et est associée à la vulnérabilité neuronale. À ce jour, la relation entre les domaines structuraux de LRRK2 et leurs fonctions physiologiques respectives reste mal définie, et la plupart des domaines de LRRK2 restent peu étudiés. Dans la première partie de cette thèse de doctorat, nous montrons que la chaîne légère de clathrine interagit directement avec le domaine GTPase de LRRK2 au niveau des endosomes précoces, régulant l'activité de Rac1 et modifiant ainsi la dynamique de l'actine et la morphologie cellulaire. Une meilleure compréhension de la fonction cellulaire de LRRK2 est essentielle pour élucider la physiopathologie de la MP associée à la protéine. Dans la deuxième partie de cette thèse nous établissons une lignée de cellules souches humaines pluripotentes induites exprimant la protéine endogène LRRK2 étiquetée (Flag-LRRK2) qui servira à de futures études physiologiques dans les neurones dopaminergiques. L'ensemble de ces résultats fait avancer notre compréhension fondamentale de la fonction moléculaire de LRRK2, et fournit un nouvel outil d'importance pour étudier la fonction physiologique de LRRK2 dans les neurones dopaminergiques humain.

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AUTHOR CONTRIBUTIONS

Work presented in Chapter 1 contains part of an unpublished review of scientific literature relevant to this thesis, written by A.M.A. Schreij.

The published research presented in Chapter 2 was conceived and written by A.M.A. Schreij, with guidance from the principal investigators Dr. Edward A. Fon and Dr. Peter S. McPherson. Experiments were designed by: A.M.A. Schreij, Dr. Edward A. Fon, Dr. Peter S. McPherson, Dr. Mathilde Chaineau, Dr. Wenjing Ruan, and Dr. Philip A. Barker. All experiments were performed by A.M.A. Schreij with the exception of the following: Dr. Mathilde Chaineau contributed to Figures 2C,E; 3G,F; S1, Dr. Wenjing Ruan contributed to Figures 4; S6; S7, and Susan Lin contributed to Figures 4; S7.

The unpublished research presented in Chapter 3 was conceived and written by A.M.A. Schreij, with the guidance from the principal investigators Dr. Edward A. Fon and Dr. Peter S. McPherson. Experiments were designed by: A.M.A. Schreij, Dr. Carol X-Q Chen, Dr. Edward A. Fon, and Dr. Peter S. McPherson. Research was performed by A.M.A. Schreij with supporting contribution from Dr. Carol X-Q Chen for hiPSCs cell culture. Data was analyzed and figures were generated by A.M.A. Schreij. The unpublished manuscript was written by A.M.A. Schreij.

Work presented in Chapter 4 is a synthesis of conclusions drawn from Chapters 2 and 3, placed in the context of the literature, written by A.M.A. Schreij.

LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
ANK	ankyrin repeats
Cas9	caspase-9
CCPs	clathrin-coated pits
CCVs	clathrin-coated vesicles
CHC	clathrin-heavy chain
Clc	*Drosophila* clathrin-light chain
CLCs	clathrin-light chains
CME	clathrin-mediated endocytosis
co-IP	co-immunoprecipitate
COR	C-terminus of ROC
DA	dopamine
DAergic	dopaminergic
DAT	dopamine transporter
dlrrk	*Drosophila* LRRK2
dlrrk-3KD	kinase-dead dlrrk mutant
EB	embryoid body
EEA1	early endosome antigen 1
EGFR	EGF receptor
eIF4G1	Eukaryotic translation initiation factor 4-gamma 1
FBXO7	F-box protein 7
Fgf-8	fibroblast growth factor 8
GAP	GTPase activating proteins
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors
GIGYF2	Grb10-interacting GYF protein-2
GMR	glass multiple reporter
gRNA	guide RNA
GTP	guanosine triphosphate
HSPs	hereditary spastic paraplegias
HiP1R	huntingtin-interacting protein 1-related
hiPSC	human induced pluripotent stem cells
HMW	high molecular weight
irk3	inward rectifying potassium channel
KD	knock down
LBs	Lewy bodies
LRR	leucine-rich repeats
LRRK2	leucine-rich repeat kinase 2
MAPT	microtubule-associated protein tau
mDAergic	midbrain dopaminergic
MAO B	monoamine oxidase B
MAPKKK	mitogen-activated protein kinase kinase kinase domain
MPR	mannose 6-phosphate receptor
MVBs	multivesicular bodies

NPCs	neuronal precursors
PAK-CRIB	p21-activated protein kinase Cdc42/Rac1 interactive binding domain
PINK1	PTEN-induced putative kinase 1
PD	Parkinson disease
ROC	Ras of complex proteins
Shh	sonic hedgehog
SNc	substantial nigra pars compacta
SV	synaptic vesicle
TGN	trans-Golgi network
TH	tyrosine hydroxilase
Tuj1	βIII-tubulin
VAMP4	vesicle-associated membrane protein 4
WD40	WD40 repeats

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CHAPTER 1

INTRODUCTION

1.1 Neurodegenerative disorders

Neurodegenerative diseases are among the most devastating and feared of human afflictions. The economic impact of these diseases is massive, and with a rapidly aging population, the challenges they pose will only increase. Hundreds to perhaps thousands of genetic loci form the basis of risk factors for the genetic architecture of neurodegenerative diseases (1). Importantly, the protein products of these loci can often be placed into discrete cell biological machineries or pathways, providing clues regarding the pathophysiological underpinnings of the disorders. One such pathway emerging from these genetic studies is membrane trafficking in the endosomal/lysosomal system. These trafficking pathways play critical roles in controlling the localization and levels of a myriad of proteins and it is now clear that alterations in these pathways contribute to numerous neurodegenerative disorders.

Although inherently different diseases, there are various commonalities that exist between, for instance, Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), and hereditary spastic paraplegias (HSPs). For example, they all involve altered motor function, have age as a risk factor, have early onset progressive forms, and by the end stage their neuropathology has spread beyond the selective population and brain area of primary affected neurons (2-7). Moreover, they share neuropathological hallmarks such as cytoplasmic inclusion bodies containing protein aggregates (4,5,7). Finally, all three diseases have both genetic and sporadic forms. Interestingly, the affected genes never appear to be expressed exclusively in their

respective vulnerable cellular populations, and in fact, these genes often have general cell biological functions, emphasizing the complexity of the underlying molecular mechanisms driving disease. To date it remains unknown why certain neuronal cellular populations are more vulnerable to death than others.

1.2 The pathology of Parkinson disease

Parkinson's disease (PD) is the second most common age-related progressive neurodegenerative disease of unknown etiology, affecting around 1% of the population at the age of 65, and rising to 4-5% at the age of 85 (8). With aging as the largest independent risk factor to develop PD (5), we will almost certainly experience a dramatic rise in the incidence of PD in the gradually aging western world (9), emphasizing the importance of developing a cure swiftly.

The central neuropathological features of PD are the loss of a subpopulation of midbrain neurons, called dopaminergic (DAergic) neurons, residing in the substantia nigra pars compacta (SNc) (8), and the presence of intraneuronal proteinacious cytoplasmic inclusions called Lewy bodies (LBs) in the remaining DAergic neurons as well as the brain stem (10). In addition, abnormal hyperphosphorylation of the microtubule-associated protein tau (MAPT or tau) results in self-aggregation observed as neurofibrillary tangles, neuropil threads, and dystrophic neurites, collectively termed tauopathy. All of these features have been observed in postmortem tissue from PD patients (11,12).

The DAergic neurons of the SNc form the dopaminergic nigrostriatal fiber system, and damage or degeneration results in the depletion of the striatal neurotransmitter dopamine (DA), with parkinsonism as its outcome (8). DAergic nigrostriatal neurons contain high levels of

neuromelanin, producing a dark pigmentation that can be observed in the SNc, which is lost in *post mortem* tissue of PD patients (13). Projections of these SNc DAergic neurons primarily synapse onto the striatal putamen in the basal ganglia which facilitates the initiation and control of movement. Loss of this control results in the manifestation of PD motor features such as: asymmetric tremor at rest and/or bradykinesia, muscle rigidity (14), postural instability, and gait abnormalities including festination and freezing (15). While these motor symptoms are apparent, the earliest clinical signs of PD include mainly non-motor symptoms that are more difficult to identify. Some of these early non-motor PD symptoms are: sleep disorders (16), loss of sense of smell (hyposmia) (17), and gastrointestinal dysfunction (18), all of which have been reported to affect life quality in PD patients before their disease had been diagnosed (19). However, the lack of systematic evaluation and large-scale placebo-controlled randomized trials of these early non-motor PD symptoms makes it difficult to identify them reliably (20).

At the visible onset of PD-related motor symptoms, the patient has already lost approximately 60% of SNc DAergic neurons, resulting in a roughly 80% decrease of DA in the putamen (21). This late manifestation of discernible symptoms renders the detection of PD at early stages quite difficult. The delayed clinical motor phenotype of PD has been referred to as a "dying-back" process. The dying-back hypothesis considers that neurites of DAergic neurons are the primary target of degeneration, leaving the cell body intact at first, after which the degeneration progresses to the cell body, and finally elimination of the "affected" cell takes place (22). As PD progresses, depression and dementia are also more commonly observed in patients (8). To date, no treatment exists to halt the progression of PD. Motor symptoms can only be symptomatically treated using dopamine replacement therapy with L-dopa (a metabolic precursor drug of dopamine) or dopamine agonist, nonetheless the disease remains incurable

(23). Further insight into the molecular mechanisms underlying PD is required to successfully develop a cure in the near future (24).

1.2.1 Parkinson disease genes and their general cellular function

Although to date the majority of PD incidence is sporadic, approximately 5-15% is caused by familial genetic alterations of a continuously growing list of genes (25). The protein function of genes causative of PD can be broadly associated with various cellular pathways, such as mitochondrial and endo-lysosomal function (Fig. 1.1).

Several genes causative of autosomal recessive early-onset forms of PD have been linked to selective engulfment of mitochondria by autophagosomes, a process termed mitophagy. Mitochondrial kinase PTEN-induced putative kinase 1 (PINK1, encoded by *PARK6* (26)) recruits the cytosolic E3 ligase parkin (*PARK2* (27)) to mitochondria where it phosphorylates substrates and facilitates mitophagy. Additionally, *PARK15*, encoding the F-box protein 7 (FBXO7), causative of another recessive form of early-onset PD (28), is also involved in parkin-PINK1-mediated mitophagy via its interaction with and recruitment of parkin to mitochondria (29). Another autosomal recessive PD gene involved with mitochondrial function is *PARK7* (30). *PARK7* encodes for DJ-1, a multifunctional redox-responsive cytoprotective protein that is also located at the nucleus and mitochondria. DJ-1 is thought to be critical to decrease oxidative cellular stress levels, and facilitate mitochondrial maintenance (31). mRNA translation is also vital for mitochondrial homeostasis, maintenance, and survival. Eukaryotic translation initiation factor 4-gamma 1 (eIF4G1) is a core scaffold component of the translation initiation machinery regulating translation of mitochondrial mRNA and cell survival molecules upon cellular stresses

(32). Mutations in *EIF4G1*, encoding for eIF4G1, have been discovered to lead to autosomal dominant late-onset PD and mitochondrial dysfunction (32).

Other genes causing both autosomal recessive and dominant PD can be related to the uptake of protein and lipid cargo from the plasma membrane by clathrin-mediated endocytosis (CME, the most prominent form of endocytosis), and subsequent cargo sorting at endosomes followed by degradation at lysosomes. Among the autosomal recessive genes are: *SYNJ1* encoding for synaptojanin (33,34), and *DNAJC6* encoding for auxilin (35), both enriched in the nervous system and in particular at presynaptic nerve terminals, where they function in recycling of synaptic vesicles (SVs) via CME by uncoating SVs (36,37). Retromer dysfunction at endosomes, which normally facilitates retrograde cargo trafficking from endosomal membrane tubules to the trans-Golgi network (TGN), can also be related to PD. Alterations in *VPS35*, encoding for Vps35 which is a core component of the retromer, and *DNAJC13* encoding for RME-8 a DNAJ protein that interacts with the retromer and the WASH complex, lead to autosomal dominant late-onset PD (38-40). Additionally, another autosomal dominant PD gene *PARK8* encodes leucine-rich repeat kinase 2 (LRRK2) (41,42). LRRK2 is a kinase which appears to play a role in both retrograde trafficking and endo-lysosomal function. Furthermore, V-ATPase driven acidification of intracellular endo/lysosomal vesicles is crucial for endo-lysosomal maturation. ATP6AP2/(pro)renin receptor is a vital accessory protein of the V-ATPase complex and mutations in ATP6AP2 were discovered as causative of X-linked Parkinsonism (43). The ATP6AP2 mutations cause altered splicing, giving rise to overexpression of a minor splice isoform with reduced ability to activate the V-ATPase (43). Another autosomal recessive PD gene in this pathway is *ATP13A2/PARK9* (44). The product of the *ATP13A2/PARK9* gene is ATP13A2, a P-type ATPase transporter involved in α-synuclein

clearance (45,46) and lysosomal function (47). Accumulation and intracellular aggregation of the multifunctional cytosolic protein α-synuclein is a pathological hallmark of PD. Gene multiplications as well as missense mutations of *SNCA*, which encodes for α-synuclein, are associated with early-onset autosomal dominant PD (48-50), and aberrant degradation of α-synuclein has been ascribed to reduced endosomal and autophagosome function. Glucocerebrosidase (GBA) is a lysosomal enzyme responsible for the conversion of the glycolipid glucosylceramide into glucose and ceramide. *GBA* loss-of-function mutations are causative of Gaucher disease with parkinsonism, the most common lysosomal storage disease (51). *GBA* mutations have since been ascribed as a risk factor for PD (52-54). One of the last steps in the endo-lysosomal pathway is autophagy, a vital process for the elimination of pathogens, ubiquitinated damaged mitochondria (mitophagy), and misfolded aggregated proteins driven by their engulfment into an autophagosome that merges with the lysosome for its cargo degradation. Grb10-interacting GYF protein-2 (GIGYF2) was recently identified to regulate autophagy in *Drosophila* (55). Alterations in *PARK11*, encoding for GIGYF2, are also associated with autosomal dominant PD (56).

Thus far, the majority of PD cases are classified as idiopathic. With the rapid evolution of genetic screening, expanded banking of patient samples and worldwide collaborations, it is likely that many more genetic causes of PD will be identified in the coming years. Future discoveries of such genetic causes of PD may identify additional proteins linked to mitochondrial and endo-lysosomal function, help unravel the pathophysiological changes occurring in PD neurodegeneration, and assist in designing targeted novel therapeutics.

1.3 LRRK2

Mutations in the *LRRK2* gene are the most common genetic cause of both autosomal dominant (6 - 8 %) and sporadic (2%) PD (57), with a higher prevalence in Ashkenazi Jews (18%) and North Africa (42%) (57-59). Intriguingly, the clinical presentations of *LRRK2*-linked PD are indistinguishable from typical idiopathic PD. Though the cause of *LRRK2*-linked PD has been researched at length, our understanding of LRRK2 function remains incomplete. Thus, studying both LRRK2's physiological function as well as disease mutants may provide insight into the pathophysiology of PD.

1.3.1 LRRK2 multidomain structure

LRRK2 is a large molecular weight (predicted 280kDa), multi-domain protein that belongs to the family of ROCO proteins (Fig. 1.2). These proteins are characterized by the presence of a 200-250 amino acid Ras of complex proteins (ROC) GTPase domain, succeeded by a 300-400 amino acid domain called C-terminus of ROC (COR) (60). The characteristic feature of the ROC-COR structure may be considered as a "supradomain" distinguishing ROCO family members from other proteins (61). Domain architecture of most ROCO proteins depicts that leucine-rich repeats (LRR) and/or ankyrin repeats (ANK) are common at the N-terminus, followed by their ROC-COR domains and a mitogen-activated protein kinase kinase kinase domain (MAPKKK), while WD40 repeats (WD40) can be found at the C-terminus (62). LRRK2 contains all of these domains from N to C terminus: ANK, LRR, ROC, COR, MAPKKK-like, and WD40. Additionally, PD-associated missense mutations have been found in each domain (25,63) underlining the complexity of LRRK2 function. The most prominently studied mutations

are: I1371V and R1441C/G/H in the ROC domain, Y1699C in the COR domain, and G2019S and I2020T in the catalytic site of the kinase domain (25,63). The latter both enhance LRRK2 kinase activity, which is believed to cause neuronal toxicity (64) (Fig. 1.2). Despite its low penetrance of approximately 25-35% (65), LRRK2 G2019S is the most frequent mutation found in PD, accounting for 4% of autosomal dominant and 1% of sporadic PD (66).

1.3.1.1 LRRK2 GTPase function, structure and dimerization

The ROC (GTPase) domain of LRRK2 has sequence similarity to the small GTPase Ras (67). In general, all small GTPases behave as molecular switches that control diverse cellular processes, such as proliferation, cytoskeletal rearrangement, and vesicular trafficking (68). GTPases cycle between active, guanosine triphosphate (GTP)-bound, and inactive guanosine diphosphate (GDP)-bound states to exercise their functions. In their active state, and sometimes their inactive state, small GTPases can interact with specific proteins, called effectors. In turn, this interaction may set off cascades that regulate all the different cellular processes in which GTPases are involved (69). Additionally, guanine nucleotide exchange factors (GEFs) catalyze the disturbance of Mg^{2+} (an essential cofactor that forms an ion bond with the GTPase and GTP or GDP) allowing the GEF to stabilize an intermediate nucleotide-free GTPase state (70). As GTP exists in a higher molar ratio in the cell than GDP (71), the nucleotide-free GTPase will bind GTP and become activated. Conversely, the GTPase activating proteins (GAPs) promote GTP hydrolysis and consequently render the GTPase inactive (69).

As expected from a GTPase, the ROC domain of LRRK2 can bind both GTP and GDP (72) as well as catalyze intrinsic GTP hydrolysis (73,74). To date, controversy remains over the

rate of LRRK2's GTP hydrolysis, with some studies reporting similar (73,75,76), and others relatively low GTP hydrolysis rates compared to other small GTPases (77,78). Variation in experimental conditions between studies may account for these differences. For example, overexpression of full-length LRRK2 vs. the isolated ROC domain, and myc-tagged vs. flag-tagged constructs, as well as different host cell lines. Further inconsistency exists regarding the effect of the pathogenic LRRK2 mutations in the ROC and COR domain on GTP-binding and its intrinsic GTP hydrolysis. Of interest is the discovery that the pathogenic LRRK2 mutants R1441C/G and Y1699C appear to disrupt GTP hydrolysis (75,78-81), although one study reports no difference (73). Furthermore, β-PIX (ARHGEF7) and ArfGAP1 have been identified as a GEF and GAP for LRRK2 respectively (82-85). Intriguingly, it appears that pathogenic mutants in the ROC and COR modulate the interaction between LRRK2 and its GEF and GAP (83,85). Future research is required to reveal any implications of these modifications for the pathophysiology of PD.

Dimerization of LRRK2 has also been attributed to mediate its intrinsic GTP hydrolysis (86). Furthermore, LRRK2 dimerization appears to be required for autophosphorylation by its intrinsic kinase activity (87,88), which is not surprising as homodimerization has been previously shown to regulate kinase activity of other kinases (89). LRRK2 dimerization occurs via the ROC-COR domains, which although not exclusively involved in dimerization, favor intra-molecular interactions (79,81,90). Dimerization is also observed in the crystal structure of the ROC-COR domains, where these two domains are packaged twice into one unit of the crystal, hence indicating dimerization (91,92). Pathogenic mutations (R1441C/G/H and Y1699C) in either domain weaken the dimerization of LRRK2 (79,81,90,93). It has been suggested that as the pathogenic mutations are situated in the intra-molecular interface they may strengthen the

ROC-COR interaction, which may locally weaken the dimerization of LRRK2 and lead to reduced GTPase activity (79,81). Furthermore, it is postulated that LRRK2 dimerization controls autophosphorylation and prompts subsequent substrate phosphorylation (91,92).

1.3.1.2 LRRK2 kinase activity, autophosphorylation and substrates

To date, it is believed that enhanced kinase activity − resulting from mutations in LRRK2 − is the fundamental cause mediating LRRK2 toxicity in PD (64), rendering it a gain-of-function disease. Although LRRK2-PD presents as a gain-of-function disease, *in vivo* findings in *Drosophila* demonstrate that both overexpression of a kinase mutant and knock down (KD) of LRRK2 result in similar dysregulation of endocytosis (94), supporting a loss-of-function model to study the role of LRRK2-PD. Nonetheless, the LRRK2 field has focused primarily on the activity and regulation of its kinase domain. LRRK2 kinase inhibitors have been developed, of which the most prominently used (LRRK2-IN-1 (95-98)) recently showed many off-target effects in a LRRK2 knock out background (99). The fact that the most prominently used LRRK2 kinase inhibitor has similar effects in LRRK2 knock out cells as wild-type warns for caution when interpreting LRRK2 kinase inhibitor data.

For a long time, the focus of studying mechanisms underlying LRRK2-associated PD has mainly been on its intrinsic kinase activity. To date, it has been shown that a link exists between GTPase activity and kinase activation in LRRK2. More specifically, the addition of GTP, but not GDP, to an autophosphorylation assay of LRRK2 significantly enhances its autophosphorylation (72). GTP-binding dead mutants of LRRK2 (K1347A, T1348N) show neither autophosphorylation (72,77,100) or MBP phosphorylation (73,77), supporting that GTP binding

in the ROC is required for LRRK2 kinase activation. Moreover, kinase activity not only seems activated by GTP-binding in the ROC, but is also stimulated upon its binding, indicative of an intramolecular regulation in LRRK2 (72,73). It has further been reported that in addition to GTP-binding to the ROC, the functional and intact ROC domain itself is essential for kinase activation (101). This may be related to the fact that the ROC domain contains residues that are autophosphorylated. Indeed, LRRK2 lacking phosphorylation sites in its ROC domain is deficient in autophosphorylation (101). To date, several groups have mapped autophosphorylation residues to the ROC domain (102-105). Moreover, the crystal structure of the ROC-COR depicts that a few autophospho-residues are in a region affected by the pathogenic mutations R1441C/G/H, indicating that autophosphorylation of the ROC may be changed in PD (104).

A growing list of LRRK2 phosphorylation substrates are implicated in various cellular functions, including cytoskeletal rearrangement and endosomal trafficking. The LRRK2 phosphorylation consensus motif F/Y-x-T-x-R/K has been identified (106), yet not all LRRK2 phosphorylation substrates appear to bear this motif (e.g. β-PIX), suggesting that it is not exclusively required for LRRK2 phosphorylation. Putative LRRK2 substrates are the ERM proteins ezrin, radixin and moesin. These are activated by LRRK2 phosphorylation, which allows for their association with F-actin and leads to cytoskeletal rearrangements (107,108). Both microtubule associated proteins β-tubulin and Futsch (a *Drosophila* homolog of human MAP1B) are phosphorylated by LRRK2 (109,110), as is the eukaryotic initiation factor 4E-binding protein which negatively regulates protein translation (111). Furthermore, LRRK2 phosphorylation of its GEF β-PIX, also a known GEF for Rac1 (112), and of the GAP ArfGAP1, may regulate their activity. However, the precise physiological changes and effects of such phosphorylation

remains unclear (83,84). Of interest is the connection between LRRK2 phosphorylation and membrane trafficking in the endosomal system – an emerging pathway in neurodegenerative disorders. To this end, LRRK2 phosphorylates the SV exo-endocytic machinery proteins Snapin (113) and Endophilin A (94,114,115), respectively, thereby regulating neurotransmitter release and CME. Furthermore, LRRK2 also phosphorylates Rab5 on early endosomes, mediating their maturation (116). Together, these endocytic substrates reveal a strong association between LRRK2 phosphorylation and membrane trafficking in the endosomal system.

1.3.2 LRRK2 protein localization

The expression of *LRRK2* mRNA in human *post mortem* tissue and cultured cells depicts a wide expression pattern including: liver, heart, brain, SH-SY5Y cells, as well as oligodendroglia, astrocytes and microglia (117). Furthermore, *LRRK2* mRNA expression in rodent and human brain has been observed at high levels in dopamine-innervated areas like the striatum (caudate-putamen), cortex and olfactory tubercle, while it appears devoid in dopamine-synthesizing neurons of the SNc (118-121). Intriguingly, the lack of *LRRK2* mRNA in SNc neurons is in contrast with the mRNA expression pattern of other PD-linked genes (e.g. *α-synuclein*, *parkin* (122) *DJ-1* (123), and *PINK1* (124)). This suggests that LRRK2-linked PD may arise from LRRK2-dependent alterations in the target structures of dopaminergic innervation, particularly in the striatum (118). Furthermore, the molecular mechanisms underlying LRRK2-linked PD are likely independent of broad changes in basal gene expression, as pathogenic LRRK2 mutations do not appear to alter gene expression in cultured cells or human brain tissue (125).

In contrast to the mRNA levels of LRRK2, LRRK2 protein can be found abundantly in most regions of the rodent brain including low to moderate levels in the SNc (120,126,127). The highest levels of LRRK2 protein are observed in the striatum, consistent with the presence of high LRRK2 mRNA levels (126,127). However, the antibodies used to detect LRRK2 protein in these studies show various degrees of nonspecific cross-reactivity, as additional lower bands are detected on immunoblots from cell lines and LRRK2 transgenic mice. Additionally, a recent systematic analysis of known LRRK2 antibodies shows that they are problematic in their recognition of LRRK2 by immunofluorescence (128).

At a sub-cellular level, in addition to cytosolic localization, LRRK2 also localizes to and associates with membranes. Sub-cellular fractionation of whole rodent brain tissue shows LRRK2 enrichment on membrane fractions as well as its presence in synaptic terminals (129,130). Studies using (light) electron microscopy ((l)EM) of rodent SNc neurons and cell lines demonstrate the presence of LRRK2 on vesicular and membranous structures, as well as on microtubules, mitochondria, microvilli (filopodia), and other membrane-bound structures (130,131). Furthermore, in primate striatum, the majority (40-60%) of LRRK2 labeling is found in dendritic processes and axon terminals (10-30%), which is in agreement with the observation that LRRK2 is associated with vesicular and membranous structures (132).

Moreover, characterization of a high molecular weight (HMW) pool of LRRK2 suggests that LRRK2 exists in dimeric (HMW pool) as well as monomeric forms. These LRRK2 dimers appear enriched at membranes, while the majority of LRRK2 is cytosolic and generally remains monomeric. Intriguingly, LRRK2 dimers associated with membranes are thought to represent an active state of LRRK2 equipped to phosphorylate substrates. LRRK2-linked pathogenic mutations, however, seem to exert no effect on the sub-cellular localization or oligomeric state of

LRRK2. Together, these findings suggest that LRRK2 activity may be regulated by membrane translocation and/or association (133). Supporting this idea is the finding that LRRK2 not only localizes to membranes but more specifically associates with lipid rafts in different cell lines (129), further suggesting a specific function or regulation of LRRK2 activity at the membrane. Most studies show that overexpression of PD-associated LRRK2 mutants in cell lines does not alter LRRK2 localization; however it does lead to the formation of inclusions (134) and microtubule associated filaments (135), implying that aberrant LRRK2 function rather than mislocalization leads to PD (86,129,136).

1.4 Emerging endo-lysosomal function for LRRK2

Pinpointing the normal function of disease-associated proteins can lead to a new understanding of the cellular machineries and pathways that are altered in the disease process. One such emerging pathway in PD is membrane trafficking in the endosomal system. Most current evidence points to a role for LRRK2 in four different processes all related to endosomal membrane trafficking: i) SV exo/endocytosis (80,94,110,113-115,137-139), ii) trafficking of the mannose 6-phosphate receptor (MPR) and associated lysosomal hydrolases between the TGN and the endo-lysosomal system (140,141), iii) regulation of EGF receptor (EGFR) trafficking and degradation (116,142,143), and iv) regulation of actin dynamics (108,144-149). However, the relationship between the structural domains of LRRK2 and its functional activity remains poorly defined.

1.4.1 The endo-lysosomal system

Endocytic entry of protein and lipid cargo can be considered the first step in endosomal trafficking (Fig. 1.3). The most prominent form of endocytosis, known as CME, is driven by the formation of clathrin-coated pits (CCPs) maturing into vesicles (CCVs) that in their final stage pinch off from the plasma membrane and traffic to the early endosome. CME is the major entry route for nutrient and signaling receptors, cell adhesion molecules, ion channels and transporters, and numerous pathogens co-opt CME to access cells (150). Additionally, specialized forms of CME drive the reformation of SVs following neurotransmitter release (151,152). Following CME, CCVs uncoat and fuse with early endosomes – the initial sorting station in the endocytic pathway, and a major sorting hub in the cell for internalized ligand-receptor complexes, proteins, and lipids (153). Cargo that is delivered to early endosomes has three main destinations: i) recycling to the plasma membrane (153,154), ii) retrograde trafficking to the TGN − a process mediated by the retromer, a membrane sculpting/protein-sorting complex (155), and iii) degradation in lysosomes (156). Clathrin is an important organizer at early endosome membranes for endocytosed cargoes destined for degradation. Clathrin forms a lattice consisting of a bilayered clathrin coat on the early endosome (157-159) that directs inward invagination of cargoes into the lumen of the endosome, leading to the formation of multivesicular bodies (MVBs)/late endosomes. This clathrin coat is important for sorting specific cargoes for degradation, and acts as a scaffold for recruitment of proteins that drive inward budding and other aspects of early endosome dynamics (160). The transition from early to late endosomes occurs on multiple levels and includes changes in organelle structure, protein, and lipid composition. A crucial step is the transition of Rab5-positive early endosomes to Rab7-positive late endosomes mediated by active Rab5. Rab5 recruits effectors that activate Rab7, initiating

maturation to late endosomes (161). Even as cargo is transported out of the early endosome, the endosomal membrane is maturing into MVBs/late endosomes (161). Eventually, late endosomes fuse with lysosomes for degradation of their lipid and protein content. Trafficking between the plasma membrane and the endo/lysosomal system controls the localization and levels of numerous proteins, and thus has a major influence on cell function. By extension, alterations in these pathways can lead to human disease, such as PD.

1.4.1.1 Clathrin-light chain function

Clathrin-light chains (CLCs) are involved in CME as a coat-component of CCVs. There are two functionally interchangeable forms of CLCs: CLCa and CLCb, collectively referred to as CLCs. Both isoforms are expressed in all tissues at varying levels (162). Additionally, two neuronal-specific CLC splice variants bearing 60% sequence identity – nCLCa and nCLCb – are exclusively found in the brain, and contain a 30 and 18 amino acid insert, respectively (163,164). Together with clathrin heavy chain (CHC), CLCs form the triskelia that construct the outer cage of CCVs (Fig. 1.4) (165,166). CLCs were originally thought to function exclusively as universal regulators of CCV formation in CME (167). In *Drosophila*, endogenous CLC does regulate SV endocytosis in this manner (168). However, recent studies provide insight into another, more specialized, function of CLCs in CME as actin assembly regulators in polarized cells, where the plasma membrane is under tension and CME is actin-dependent. Upon loss of CLCs function in these polarized cells, enhanced actin polymerization and disruption of actin dynamics results in impaired actin-dependent CME (169). Indeed, it has previously been shown that CLCs are important mediators of actin dynamics and can be viewed as scaffolds that recruit an actin

regulator called huntingtin-interacting protein 1-related (HiP1R), to inhibit actin assembly/polymerization (170). Through this mechanism, KD of CLCs results in the formation of abnormal actin structures due to over-assembly of actin (170,171). Furthermore, MPR, which normally facilitates trafficking of newly synthesized lysosomal hydrolases from the Golgi to endosomes (172), becomes disrupted upon CLCs KD, leading to a delay in the processing of lysosomal hydrolases and the clustering of MPR near the Golgi (170).

1.4.2 LRRK2 and the endo-lysosomal system

Alterations in synaptic transmission, such as defective dopamine transmission, are implicated in PD and robustly observed in most LRRK2-PD mutant rodents (173-179). This may represent an important pathogenic feature preceding DAergic cell death. LRRK2 KD in hippocampal cultures reduces CME of SVs, as monitored by a pH-sensitive GFP fused to either synaptobrevin-2 or synaptophysin (both presynaptic SV transmembrane proteins) (138). A key component of the SV endocytic machinery is Endophilin, which recruits synaptojanin to facilitate disassembly of the clathrin coat (180). In primary mammalian striatal neurons and *Drosophila*, LRRK2 phosphorylates Endophilin A at serine 75 (S75) (94,114,115). Expression of LRRK2 G2019S enhances this phosphorylation, which disrupts Endophilin A interaction with the membrane, whereas LRRK2 knock out leads to an overly stable association of Endophilin A with membranes (94,114). Both treatments disrupt CME of SVs (94,115). Taken together, LRRK2-mediated phosphorylation facilitates CME of SVs, and alterations in this process may contribute to the pathophysiological defects in dopamine transmission observed in PD and LRRK2-PD mutant rodents.

Subsequently, at the endosomal level, it was recently demonstrated that expression of LRRK2-G2019S delays EGFR degradation by decreasing Rab7 activity (142). Transition from Rab5-positive early endosomes to Rab7-positive late endosomes is mediated by active Rab5, which recruits effectors that activate Rab7, and subsequent late endosome maturation (161). Remarkably, LRRK2 phosphorylates Rab5, accelerating its GTPase activity (116). Thus, expression of LRRK2-G2019S, a mutant with enhanced kinase activity, should lead to decreased levels of active Rab5 (due to increased GTPase activity), and decreased transition of early endosomes to late endocomes. Consistent with this hypothesis, expression of LRRK2-G2019S in astrocytes diminishes the lysosomal capacity of the cells (143). It is thus possible that LRRK2 controls an early step in degradative membrane trafficking. Interestingly, using protein-protein interaction arrays, Rab7L1 was found to associate with LRRK2 (140). In primary neurons, depletion of Rab7L1 mimics phenotypes of lysosomal dysfunction seen with expression of LRRK2-G2019S, while Rab7L1 overexpression rescued the LRRK2-G2019S-induced phenotypes (140,181). Of interest is the involvement of the retromer. In particular, expression of the PD-associated retromer protein Vps35 (38,39) abolishes alterations in MPR trafficking seen with Rab7L1 depletion/LRRK2-G2019S overexpression (140), further placing LRRK2 in a common (retrograde) endosomal/TGN/lysosomal trafficking pathway.

As described above, enhanced actin polymerization and disruption of actin dynamics causes impaired actin-dependent CME in polarized cells (169). Although no direct evidence exists for LRRK2 functioning in this actin-dependent form of CME, several reports describe interactions between LRRK2 and actin. In a screen for LRRK2-binding partners, Meixner *et al*. (145) identified actin and several actin-interacting proteins. Moreover, subcellular fractionation reveals that both LRRK2 and actin are enriched in the presynaptic synaptosomal membrane

(139), potentially indicating an actin-related regulatory role for LRRK2 at the synapse. Consistently, LRRK2 KD/knock out alters cell morphology in fibroblasts (145) and enhances neurite outgrowth, with extensive branching seen in cultured hippocampal and dopaminergic neurons (144,147,182). Conversely, the pathogenic mutants G2019S, I2020T, and Y1699C show reduced branching and increased neurite loss leading to neuronal toxicity (144,177,182-184). Together, these findings demonstrate that LRRK2 can regulate actin-mediated neurite process morphology, as well as neuronal survival. Furthermore, LRRK2 binds directly to the small GTPase Rac1, which is well known to control actin assembly (146). Interestingly, Rac1 activation occurs on early endosomes (185), where LRRK2 has been shown to phosphorylate Rab5 (116). Thus, LRRK2 may participate in actin remodeling at the synapse through regulation of Rac1.

Although the precise physiological role of LRRK2 remains poorly understood, LRRK2 function is associated with many steps in the endo-lysosomal trafficking pathway. In general, alterations in endo-lysosomal trafficking lead to dysfunctional lysosomes and accumulation of undegraded macromolecules toxic to the cell (186), which may, in part, contribute to the pathophysiology of LRRK2-associated PD.

1.5 Overall thesis rationale and research objectives

PD is a devastating and debilitating neurodegenerative motor neuron disease with no cure, that affects an estimated 7 to 10 million people worldwide (187). Thus far, the LRRK2 field has focused primarily on the activity and regulation of its kinase domain, as the PD mutation G2019S in the kinase domain is the most frequent among LRRK2-associated PD cases

(188). Given the lack of knowledge about LRRK2's physiological role we set out to better define LRRK2's endogenous function. As the ROC domain of LRRK2 is a functional GTPase, it is expected that specific effectors of the ROC exist. These effectors may regulate LRRK2 function and/or localization, and thereby may provide mechanistic insight into LRRK2-mediated physiological pathways and possibly even the pathology of LRRK2-associated PD.

Prior to work presented in Chapter 2 little was known regarding potential effectors of LRRK2's ROC domain. Such information is of essence to decipher the physiological role of LRRK2, and may be used for the design of therapeutics that effectively stop the progression of PD. Our research objectives for this project were to: (1) identify ROC binding partners, (2) determine the functional role of LRRK2 and ROC binding partner(s), (3) use multiple experimental approaches to address our objectives.

Extensive degeneration of DAergic neurons in the SNc is the hallmark of PD (8). So far, *in vitro* research has mainly relied on general cell culture models (e.g. 293T, SH-SY5Y, HeLa) and primary rodent neuronal cultures to investigate PD. Although great insights have come to light using these *in vitro* models, they do not encompass the complexity of human PD-associated genes that confer vulnerability to DAergic neurons. An emerging novel technology that allows for the conversion of human adult fibroblasts into human induced pluripotent stem cells (hiPSCs) which can subsequently be differentiated into numerous cell types, including DAergic neurons, has great potential to overcome the limitations of traditional PD models (189,190). To date, LRRK2 localization and its physiological function in human DAergic neurons remains undetermined, as such investigations are hindered by the lack of antibodies that reliably label LRRK2 by immunofluorescence (128). In the work presented in Chapter 3 we set out to determine LRRK2 localization and physiological function in human DAergic neurons. Our

research objectives for this project were to: (1) optimize current protocols for differentiation of hiPSCs into DAergic neurons, (2) genome-edit a Flag-tag under the endogenous promoter of LRRK2 for localization studies.

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1.7 FIGURE LEGENDS FOR CHAPTER 1

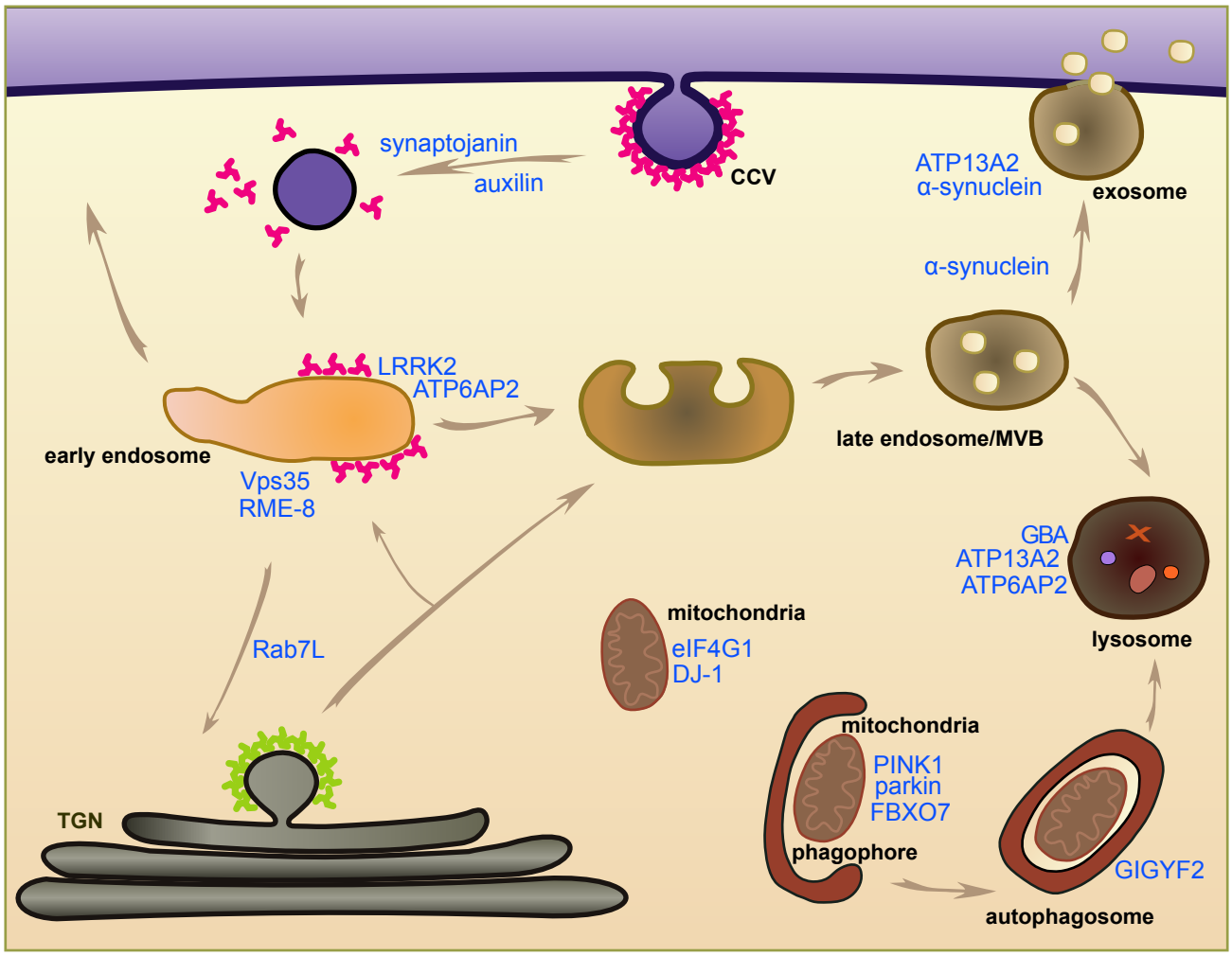
Figure 1.1: Model of PD genes and their general cellular function. Synaptojanin and auxilin are involved in CCV uncoating. LRRK2 regulates Rab5 activity and thereby the transition of early endosomes into late endosomes. ATP6AP2 is crucial for acidification. Vps35, RME-8, and Rab7L1 are involved in retrograde trafficking from the endosome to the TGN. ATP13A2 is involved in α-synuclein clearance and lysosomal function. α-Synuclein accumulation disrupts endosomal and autophagosome function. GBA regulates lysosomal function. GIGYF2 regulates autophagy. PINK1, Parkin, and FBXO7 regulate mitochondrial clearance. eIF4G1 and DJ-1 mediate mitochondrial maintenance.

Figure 1.2: Schematic representation of LRRK2. LRRK2 bears six different domains of which two are enzymatic. Ankyrin repeats (ANK), leucine-rich repeats (LRR), and the WD40 are all domains believed to provide a versatile framework to mediate protein-protein interactions. The ROC domain has GTPase activity and together with the COR domain mediates dimerization of LRRK2. The kinase domain regulates both autophosphorylation and substrate phosphorylation. The pathogenic LRRK2-associated PD mutations discussed in this thesis are indicated at the top.

Figure 1.3: Model of CME and endo-lysosomal trafficking. Cargo at the plasma membrane is engulfed by CCVs that pinch of and uncoat before fusing with the early endosome to deliver their cargo. At the early endosome, cargo is sorted in one of three pathways: i) recycling back to the plasma membrane via recycling endosomes, ii) retrograde trafficking to the TGN, or iii) degradation in lysosomes which requires the maturation of early endosomes into late endosomes.

Figure 1.4: Triskelion structure and assembly. Through weak charge interactions, primarily between CHC proximal leg segments, clathrin triskelia assemble into clathrin cages. The triskelia top view shows three copies of CHC linked at their C-termini in association with three CLCs. The triskelia side view demonstrates that triskelia are curved and that CLCs are facing the cytosol where they function as a platform to recruit proteins to the CCV.

Fig. 1.1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α-synuclein
exosome
α-synuclein
LRRK2
ATP6AP2
early endosome
Vps35
RME-8
late endosome/MVB
GBA
ATP13A2
ATP6AP2
lysosome
mitochondria
eIF4G1
DJ-1
Rab7L
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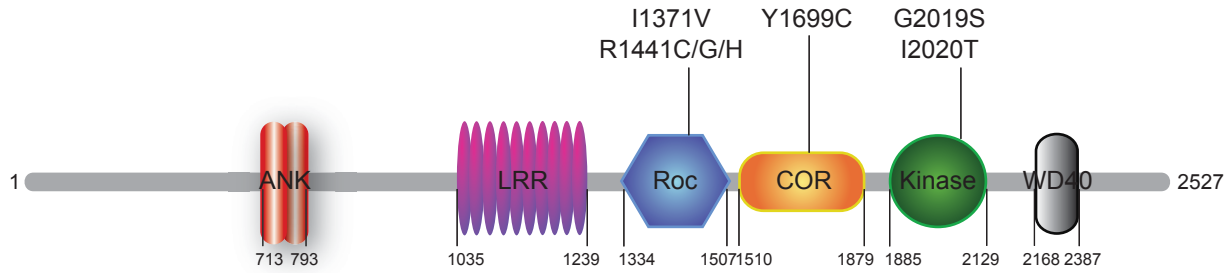
PINK1
parkin
FBXO7
phagophore
GIGYF2
autophagosome
TGN

Fig. 1.2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ANK
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Fig. 1.3

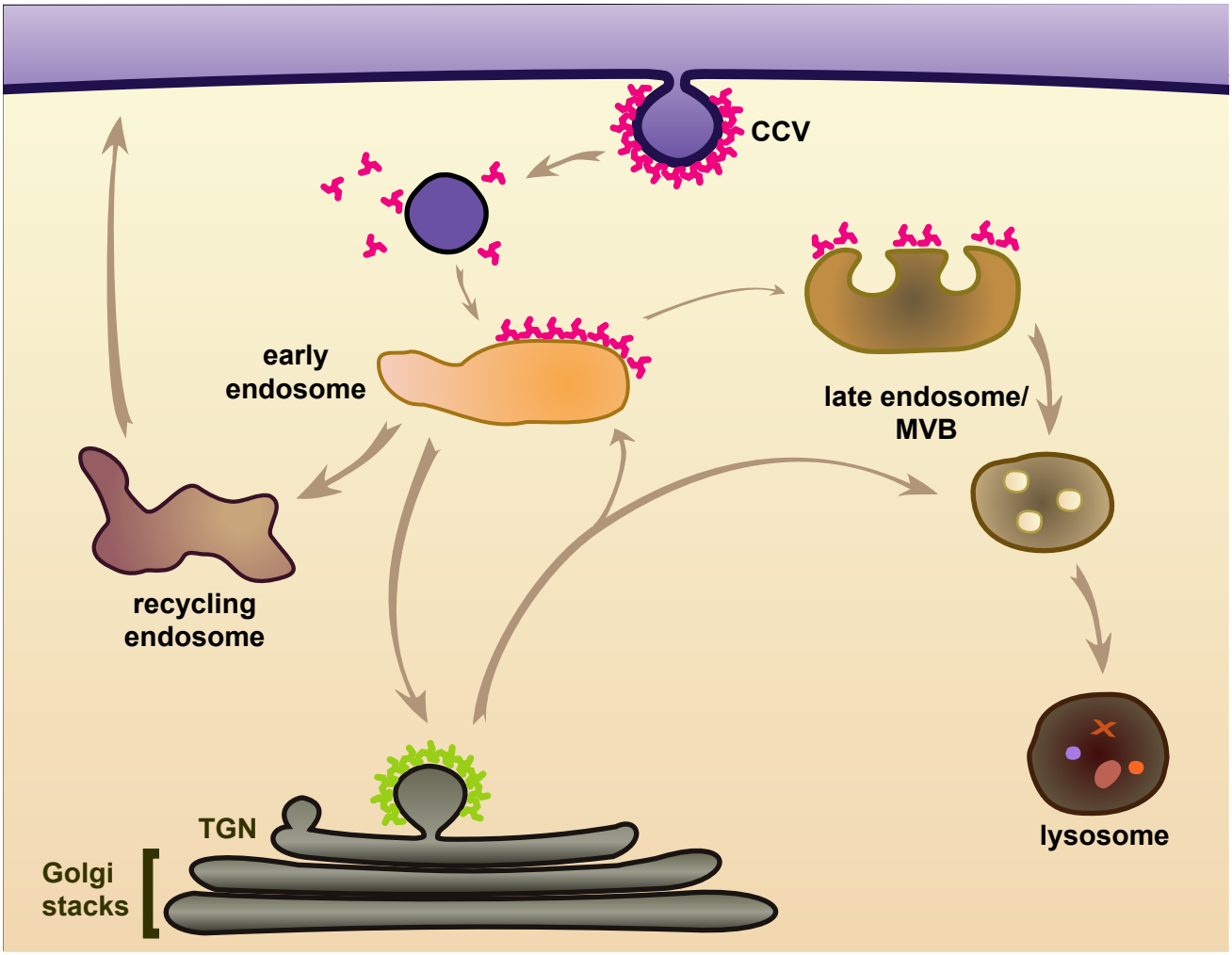
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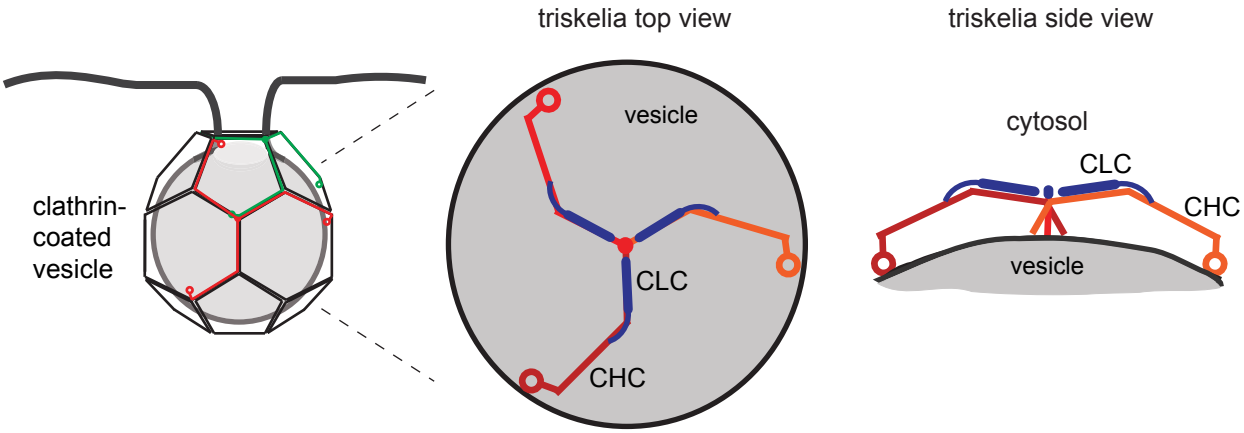
Fig. 1.4


clathrin-
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CHC
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PREFACE TO CHAPTER 2

Although mutations in *leucine-rich repeat kinase 2* (LRRK2) are a major cause of Parkinson disease (PD) (*1*), relatively little is known about the physiological function(s) of LRRK2. So far, the largest body of evidence connects LRRK2 to the actin regulatory network (*2-4*), potentially as a direct or indirect regulator of actin polymerization. In particular, several groups have shown that LRRK2$^{-/-}$ neurons have a neurite overbranching phenotype both at the *Drosophila* NMJ (*5*), and in primary rodent neuronal cultures (*6, 7*). Additionally, a recent study shows a decrease of mature spines in LRRK2$^{-/-}$ neurons which is accompanied by altered synaptic transmission (*8*). The underlying molecular mechanisms, however, remain largely ill-defined as the role and interaction partners of the multiple domains of LRRK2 remain mostly uncharacterized.

In order to increase scientific understanding of the mechanisms associated with LRRK2 cell physiological function(s) we undertook the study presented in the following chapter. We used a combination of *in vitro* biochemical assays, genomic editing, and *in vivo* techniques to characterize the physiological interaction between LRRK2 and novel binding partner, clathrin-light chains (CLCs). The main resulting original contribution to the scientific literature is the identification of a new pathway that links together the clathrin machinery, the cytoskeleton and PD, as CLCs recruit LRRK2 to endosomes to limit Rac1 activation.

CHAPTER 2

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 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 activations in cell morphology, including the disruption of neuronal dendritic spines. In *Drosophila*, 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.

INTRODUCTION

Parkinson 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 four different processes related to membrane trafficking; i) synaptic vesicle (SV) exo/endocytosis (*9*), ii) trafficking of the mannose 6-phosphate receptor (MPR) and associated lysosomal hydrolases between the trans-Golgi network (TGN) and the endo-lysosomal system (*10, 11*), iii) control of epidermal growth factor receptor (EGFR) trafficking and degradation (*12*) and iv) regulation of actin dynamics (*2, 3, 7*). 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 (*13*), reformation of SVs (*14*), and trafficking of the MPR (*15*). Intriguingly, mutations in key components of the clathrin machinery are rare variants in familial forms of PD (*16-18*). Clathrin coats are assembled from triskelia, composed of three linked clathrin heavy chain (CHC) proteins and associated clathrin-light chains (CLCs) (*19*). 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 (*19*). CLCs bind to huntingtin-interacting protein 1-related (HIP1R) (*20*) recruiting it to clathrin coats (*21*). HIP1R binds actin (*22*) and functions as a negative regulator of actin assembly (*23*), and knock down (KD) of either

HIP1R or CLCs causes overly abundant actin assembly in the vicinity of clathrin coats (*21, 23*). 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 (*24-26*).

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 cell function, both in cell lines 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 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. 2.1A). nCLCa/b are functionally interchangeable (*27*) and their binding to GST-ROC is similar (Fig. 2.1A). Endogenous LRRK2 from brain extracts binds to GST-nCLCb (Fig. 2.1B) and purified full-length (residues 1-228) nCLCb binds robustly to GST-ROC (Fig. 2.1C), 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 (*20*) does not bind, indicating that the binding site is between 166-228 (Fig. S2.1). 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. 2.1C). However, all constructs bind equally well to CHC (Fig. 2.1D) indicating that loss of ROC binding is not due to a major alteration in folding. Thus, the LRRK2-binding site is between residues 195-205. 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 (*28*).

CLCs interact with CHC as part of triskelia (Fig. 2.1E) 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 (*29*) (Fig. 2.1E). We thus tested if LRRK2 interacts with CLCs as

part of triskelia. GST-ROC was incubated with triskelia stripped from purified CCVs (*30*) and both CLCs and CHC are detected in the pull down (Fig. 2.1F), 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 (*31*) or tissue (*32*), 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 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 (*32*). 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. 2.2A/B). Remarkably, LRRK2 co-localizes with internalized EGF (Fig. 2.2C), 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. 2.2D), likely reflecting bilayered clathrin coats on early endosomes involved in the formation of MVBs during protein degradation (*24-26*) and consistently, HA-

LRRK2 partially co-lcoalizes with the early endosomal marker EEA1 (Fig. 2.2E). 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 (*12*). 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

KD of CLCs leads to over assembly of actin (*21*) and actin was identified in a screen for LRRK2-binding partners (*2*). Moreover, LRRK2 binds directly to the small GTPase Rac1, which regulates actin assembly (*3*). Interestingly, Rac1 activation occurs on early endosomes (*33*). We thus tested if LRRK2 and CLCs regulate Rac1 activity. We used previously characterized siRNAs for CLCa/b (*21*) and a smartpool of four LRRK2 siRNAs to efficiently KD the proteins (Fig. 2.3A/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 (*34*). Interestingly, KD of CLCs or LRRK2 causes a > 2-fold and > 3-fold activation of Rac1, respectively, compared to control siRNA (Fig. 2.3C/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 (Fig. S2.2A/B). Activity of the related GTPase Cdc42 is not influenced by CLCs/LRRK2 KD, thus activation of Rac1 is selective (Fig. S2.3). Deconvolution of the LRRK2 smartpool siRNA sequences reveals that two distinct sequences that KD LRRK2 lead to Rac1 activation (Fig. S2.4). 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. 2.3E). These changes were quantified as an increase in the perimeter:area ratio (Fig. 2.3F). 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 (Fig. S2.2C/D). Moreover, the Rac1 inhibitor NSC-23766 (*35*) significantly decreases changes in cell morphology resulting from LRRK2 KD, indicating that they result from Rac1 activation (Fig. S2.5).

Our results are consistent with a previous study reporting an increased perimeter:area following LRRK2 KD in NIH3T3 cells (*2*). Moreover, LRRK2 loss of function leads to neurite over-branching phenotypes at the neuromuscular junction in *Drosophila* (*5*), and in primary neuronal culture (*7*). Additionally, there is a decrease in the number of mature dendritic spines in $LRRK2^{-/-}$ neurons, which is accompanied by altered synaptic transmission (*8*). We therefore examined if KD of CLCs through lentivirus-driven expression of shRNAmiRs (*36*), would result in a similar actin-dependent phenotype. KD of nCLCa/b in cultured neurons was confirmed by Western blot (Fig. 2.3G) and we observe a loss of mature dendritic spines (Fig. 2.3H). 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 (*33*). 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*. (*3*), who observe that LRRK2 overexpression activates Rac1 resulting in decreased neurite outgrowth. However, Matta *et al.* (*9*) 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 (*3*).

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 (*37*). 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. 2.4Ai, 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. 2.4Aii-v). KD efficiency was verified by immunofluorescence on eye discs and Western blot of protein levels on adult head with a previously validated dlrrk antibody (*38*) (Fig. S2.6). Additionally, we used a previously established dlrrk UAS-RNAi line from Imai and colleagues (*38*) to show similar KD efficiency (Fig. S2.6B/C). No phenotype is seen with the four *UAS-RNAi* lines alone, even when driven off of the strong *GMR-GAL4* promoter (Fig. 2.4Avii-x). Moreover, the mild rough eye phenotype with Rac1 overexpression is

not enhanced with KD of an inward rectifying potassium channel (*UAS-irk3-RNAi*) as a control (compare Fig. 2.4Avi with 2.4Ai), but is largely rescued with KD of Slipper (Fig. S2.7), a *Drosophila* homologue of the mammalian mixed lineage kinase family shown to rescue the rough eye phenotype resulting from Rac1 overexpression (*39*). 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. 2.4Bi/ii with 2.4Aii/iii).

We next examined if 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 from Rac1 overexpression (Fig 2.4Ci-iii). A kinase-dead dlrrk mutant (dlrrk-3KD) (*38*) yielded similar rescue (Fig. 2.4Civ). No phenotype is seen with Clc or dlrrk, WT or 3KD alone (Fig. 2.4Cv-viii). These observations demonstrate that dlrrk and Clc regulate Rac1 activity *in vivo*.

Enhanced intrinsic kinase activity of LRRK2 pathogenic mutants (*40*) is correlated to increased neurotoxicity (*41*), 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 (*16-18*) 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 3 times with buffer A (20mM HEPES, pH 7.4, 0.83mM benzamidine, 0.23mM PMSF, 0.5μg/ml aprotinin/leupeptin) containing 5mM $CaCl_2$ 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 gluthathione-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, 0.5μg/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 63x oil objective and 20x 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 10x objective (0.25=N.A).

Statistical analysis

All statistical analyses were performed by one-way, parametric analysis of variance (ANOVA) or two-tailed Student t-tests, using GraphPad Prism 5 software. Error bars represent the mean ± sem. 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.

Antibodies and DNA constructs

Polyclonal antibody recognizing CLCa/CLCb, and affinity-purified rabbit polyclonal antibody recognizing CHC were generated as described (*42, 43*). 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 (*44*), GFP (Invitrogen), dlrrk (*38*). 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 (*45*) denoted here as myc-LRRK2 was from Addgene (#25361). GST-CLCb (1-211), GST-nCLCb (1-228), GST-nCLCb (1-165) were previously

described (*20*). 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 (*46*). 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 (*21*). 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 (*47*) 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*. (*48*). 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 (*30*) 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 (*49*).

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 (*38*). Flies were raised on standard fly food containing yeast, corn syrup and cornmeal at 25^{o}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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AUTHOR CONTRIBUTIONS

Conceived the experiments: AMAS, EAF, PSM. Designed the experiments: AMAS, EAF, PSM, MC, WR, PAB. Performed the experiments: AMAS, MC, WR, SL. Wrote the paper: AMAS, EAF, PSM.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest

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FIGURE LEGENDS FOR CHAPTER 2

Figure 2.1: 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. 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 boundaries as indicated. 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. For A-D/F, aliquots of the starting material (SM) equal to 10% of that added to the beads, was analyzed in parallel.

Figure 2.2: 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 3xHA-LRRK2 (2) using a primer pair with the antisense in the 3xHA insert and the sense primer in endogenous LRRK2. (3) Control unedited COS-7 cells using the same primer combination as in (2). (4) 1Kb marker. *(B)* Schematic diagram of the oligonucleotide used to

direct insertion of the 3xHA 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)* COS-7 clone E1 cells were fixed and processed for immunofluorescence using HA and CLC antibodies. Scale bar = 10 μm. *(E)* COS-7 clone E1 cells were fixed an processed for immunofluorescence using HA and EEA1 antibodies. Scale bar = 10 μm for bottom 6 panels and 25 μm for top 3 panels.

Figure 2.3: KD of LRRK2 and CLCs leads to Rac1 activation. *(A)* Lysates from COS-7 cells transfected with siRNA as indicated were processed for Western blot with antibodies recognizing the indicated proteins. *(B)* Band intensities of blots as in *(A)* are presented as % control siRNA. Two-tailed Student t-test, Mann-Whiteney *post hoc* test, ***$p < 0.001$, N = 3. *(C)* 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. An aliquot of the starting material (SM) equal to 5% of that added to GST-PAK-CRIB was analyzed in parallel. *(D)* Band intensities of blots as in *(A)* were presented as % control siRNA. Bars represent mean ± sem. One-way ANOVA, Bonferroni's *post hoc* test, ***$p < 0.001$, N=3. *(E)* COS-7 cells transfected with siRNA as indicated were fixed and processed for immunofluorescence using Phalloidin-647 and antibody recognizing CLCs. Scale bar = 10 μm. *(F)* Perimeter and area of cells as in *(A)* were measured 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 = 10 μm for the low mag and 2.5 μm for the high mag.

Figure 2.4: 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 (v22139* or *v22140)/longGMR-GAL4, UAS-Rac1W*), results in smaller eyes where most ommatidia are fused. *(iv/v)* Rac1 overexpression with KD of Clc (*UAS-Clc-RNAi (v22318* or *v106632)/+;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(v101174)/+; longGMR-GAL4,UAS-Rac1W/+*), has no effect. *(vii/viii)* KD of dlrrk with *GMR-GAL4/+; UAS-dlrrk-RNAi(v22139* or *22140)/+*, respectively as control have no effect on eye morphology. *(ix/x)* KD of Clc with *GMR-GAL4/UAS-Clc-RNAi (v22318* or *v106632)*,

respectively as control have 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, have no effect on eye morphology.

Figure S2.1: 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.

Figure S2.2: 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.

Figure S2.3: 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.

Figure S2.4: 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.

Figure S2.5: 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.

Figure S2.6: 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.

Figure S2.7: 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.

Table S2.1: 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
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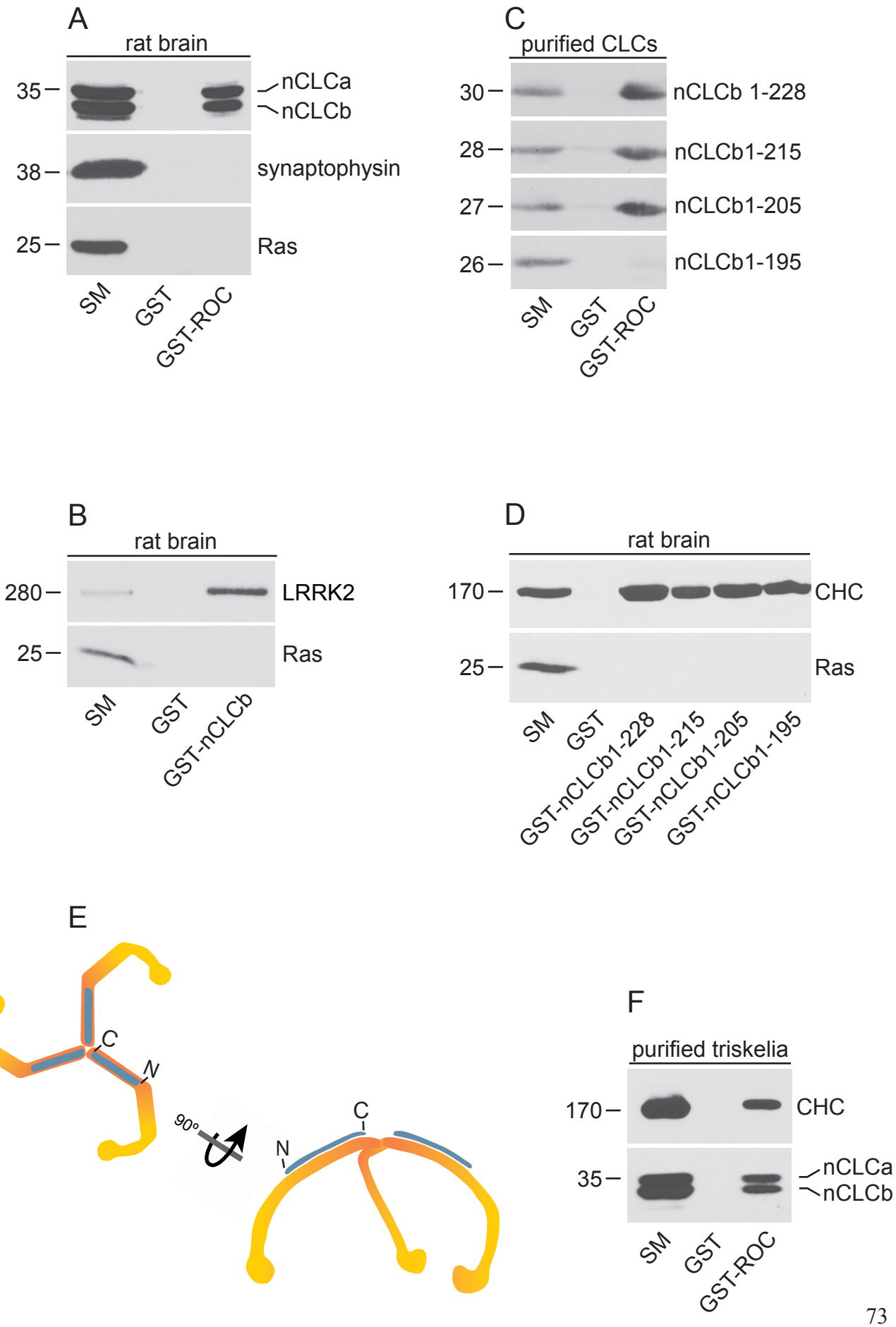
AGGACGAGGAAACTCTGAAGAAGTTGATA

Fig. 2.1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28
nCLCb1-215
27
nCLCb1-205
26
nCLCb1-195
SM
GST
GST-ROC
B
rat brain
280
LRRK2
25
Ras
SM
GST
GST-nCLCb
D
rat brain
170
CHC
25
Ras
SM
GST
GST-nCLCb1-228
GST-nCLCb1-215
GST-nCLCb1-205
GST-nCLCb1-195
E
C
N
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CHC
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nCLCa
nCLCb
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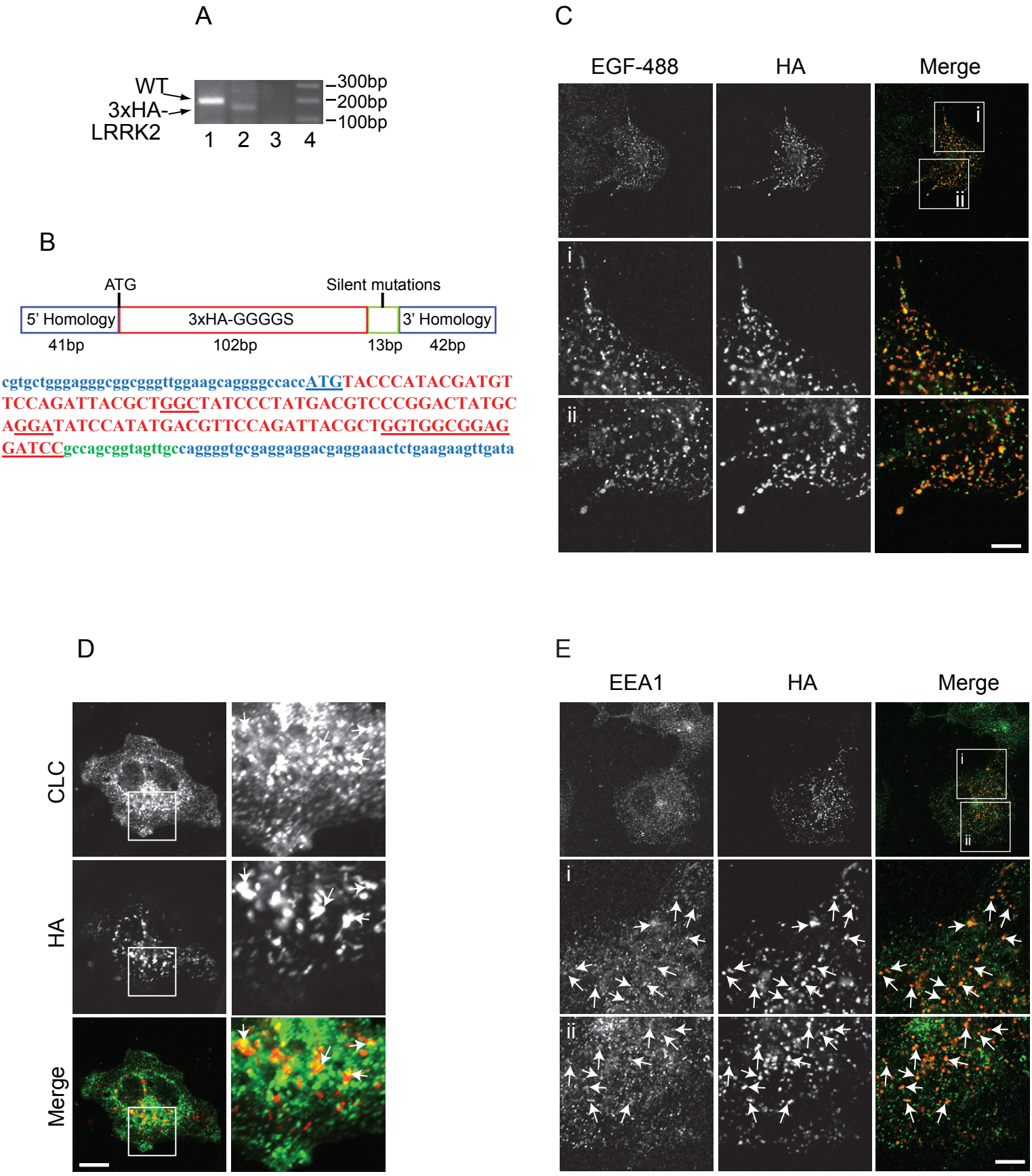
GST
GST-ROC

Fig. 2.2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300bp
200bp
100bp
1 2 3 4
B
ATG
Silent mutations
5' Homology
3xHA-GGGGS
3' Homology
41bp
102bp
13bp
42bp
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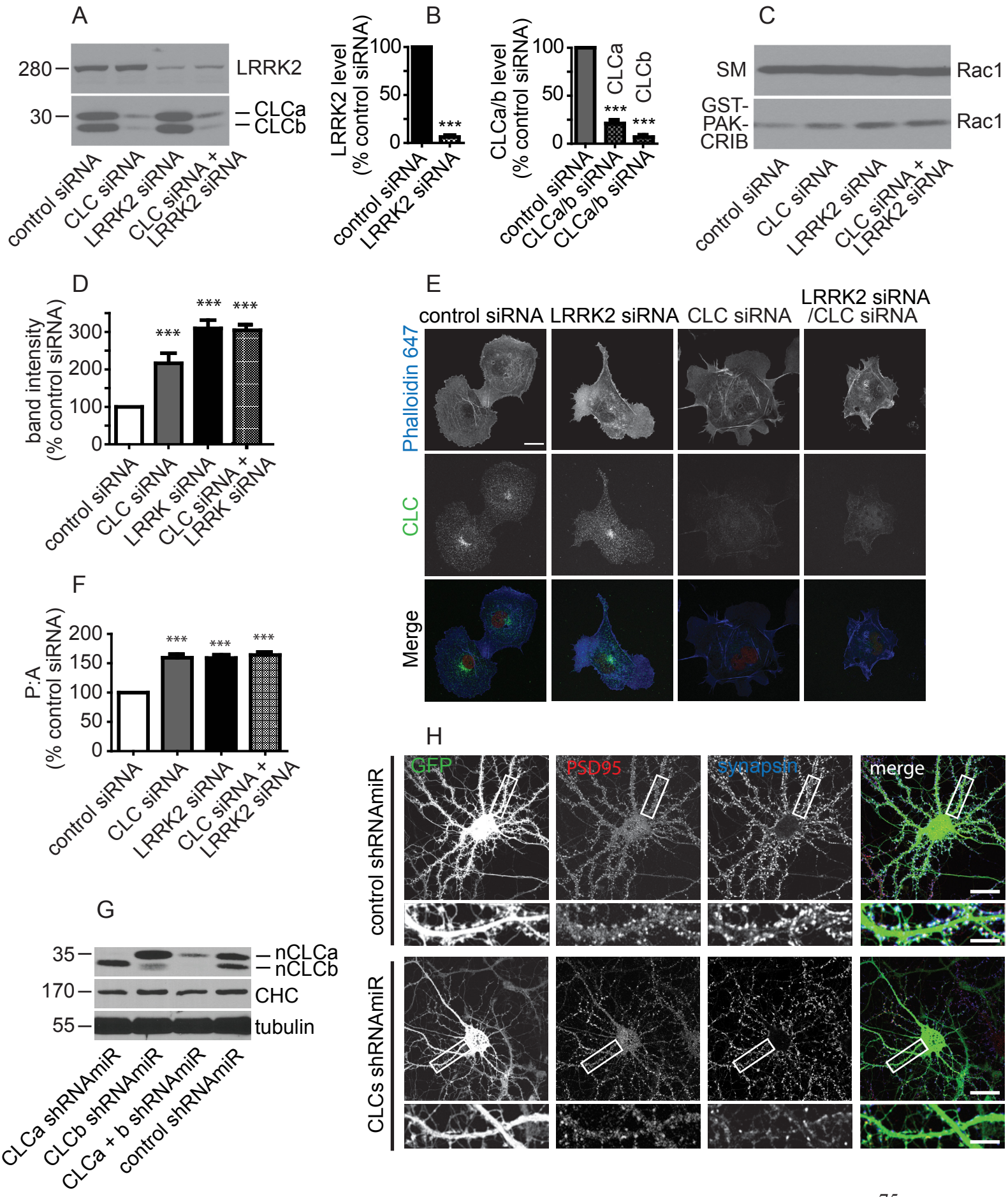
AGGATATCCATATGACGTTCCAGATTACGCTGGTGGCGGAG
GATCCgccagcggtagttgccaggggtgcgaggaggacgaggaaactctgaagaagttgata
C
EGF-488
HA
Merge
i
ii
D
CLC
HA
Merge
E
EEA1
HA
Merge
i
ii

Fig. 2.3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280
30
LRRK2
CLCa
CLCb
control siRNA
CLC siRNA
LRRK2 siRNA
CLC siRNA + LRRK2 siRNA
B
LRRK2 level (% control siRNA)
100
50
0
***
control siRNA
LRRK2 siRNA
CLCa/b level (% control siRNA)
CLCa
CLCb
control siRNA
CLCa/b siRNA
C
SM
Rac1
GST-PAK-CRIB
Rac1
control siRNA
CLC siRNA
LRRK2 siRNA
CLC siRNA + LRRK2 siRNA
D
band intensity (% control siRNA)
300
200
100
0
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control siRNA
CLC siRNA
LRRK siRNA
CLC siRNA + LRRK siRNA
E
control siRNA
LRRK2 siRNA
CLC siRNA
LRRK2 siRNA /CLC siRNA
Phalloidin 647
CLC
Merge
F
P:A (% control siRNA)
200
150
100
50
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CLC siRNA + LRRK2 siRNA
G
35
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55
nCLCa
nCLCb
CHC
tubulin
CLCa shRNAmiR
CLCb shRNAmiR
CLCa + b shRNAmiR
control shRNAmiR
H
GFP
PSD95
synapsin
merge
control shRNAmiR
CLCs shRNAmiR

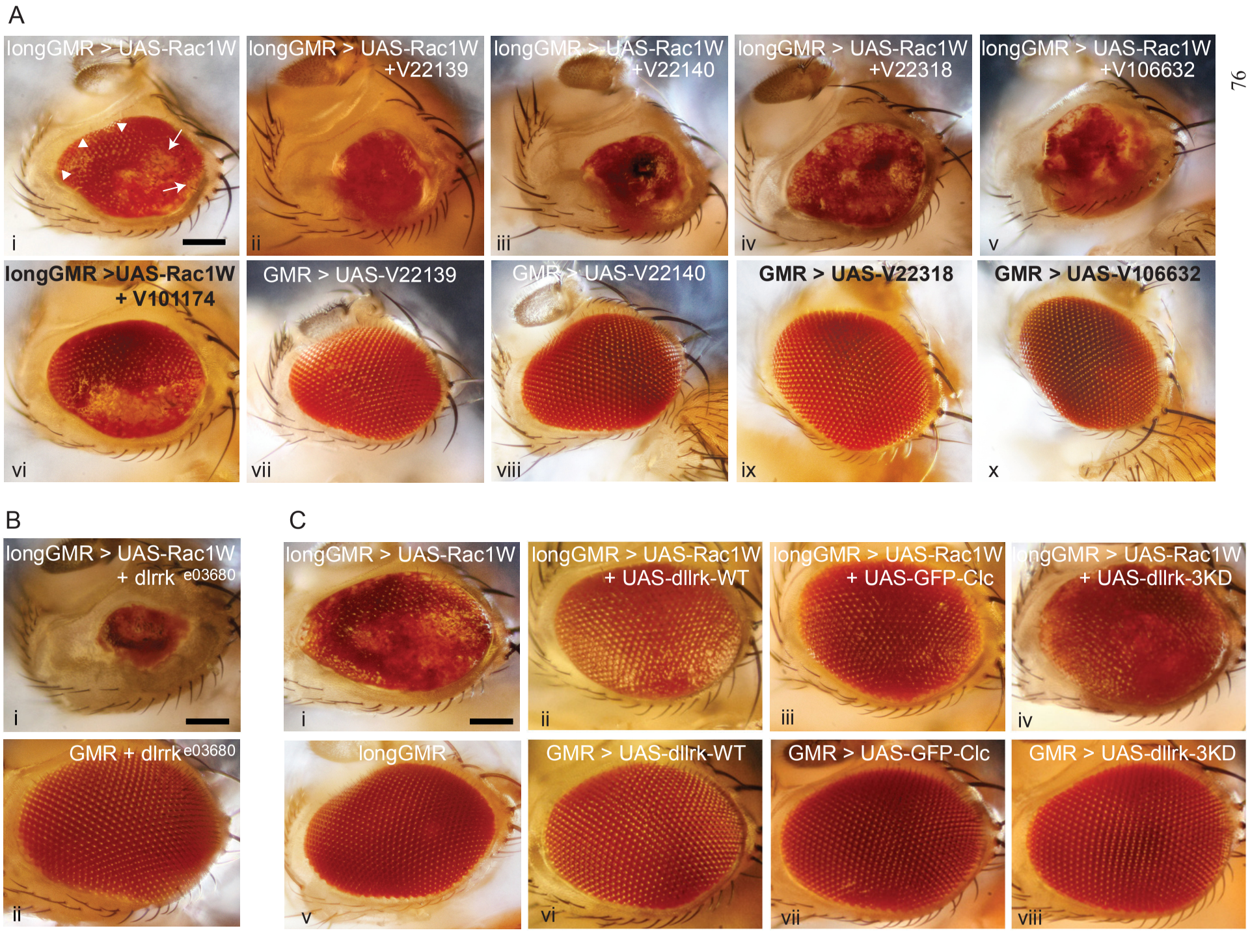

A
longGMR > UAS-Rac1W
i
longGMR > UAS-Rac1W +V22139
ii
longGMR > UAS-Rac1W +V22140
iii
longGMR > UAS-Rac1W +V22318
iv
longGMR > UAS-Rac1W +V106632
v
longGMR >UAS-Rac1W + V101174
vi
GMR > UAS-V22139
vii
GMR > UAS-V22140
viii
GMR > UAS-V22318
ix
GMR > UAS-V106632
x
B
longGMR > UAS-Rac1W + dlrrk e03680
i
GMR + dlrrk e03680
ii
C
longGMR > UAS-Rac1W
i
longGMR > UAS-Rac1W + UAS-dllrk-WT
ii
longGMR > UAS-Rac1W + UAS-GFP-Clc
iii
longGMR > UAS-Rac1W + UAS-dllrk-3KD
iv
longGMR
v
GMR > UAS-dllrk-WT
vi
GMR > UAS-GFP-Clc
vii
GMR > UAS-dllrk-3KD
viii

Fig. 2.4

Fig. S2.1

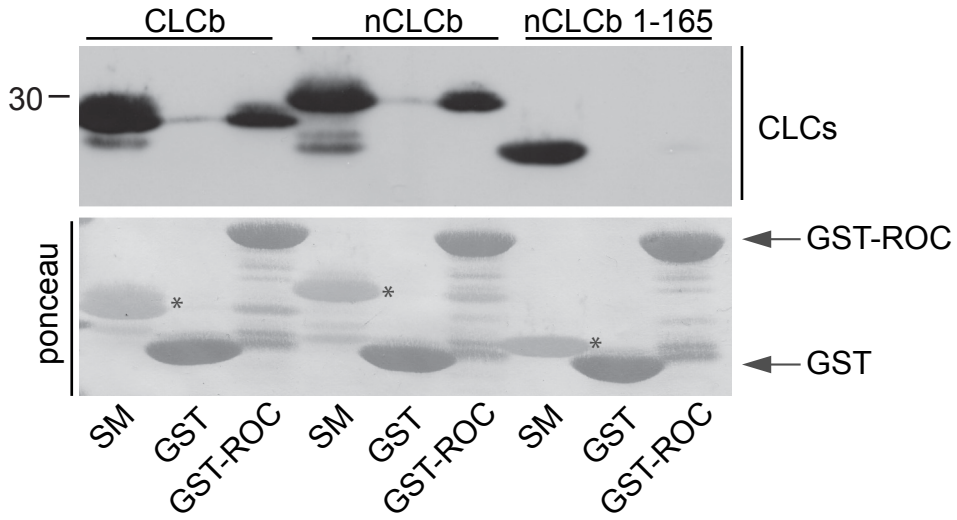
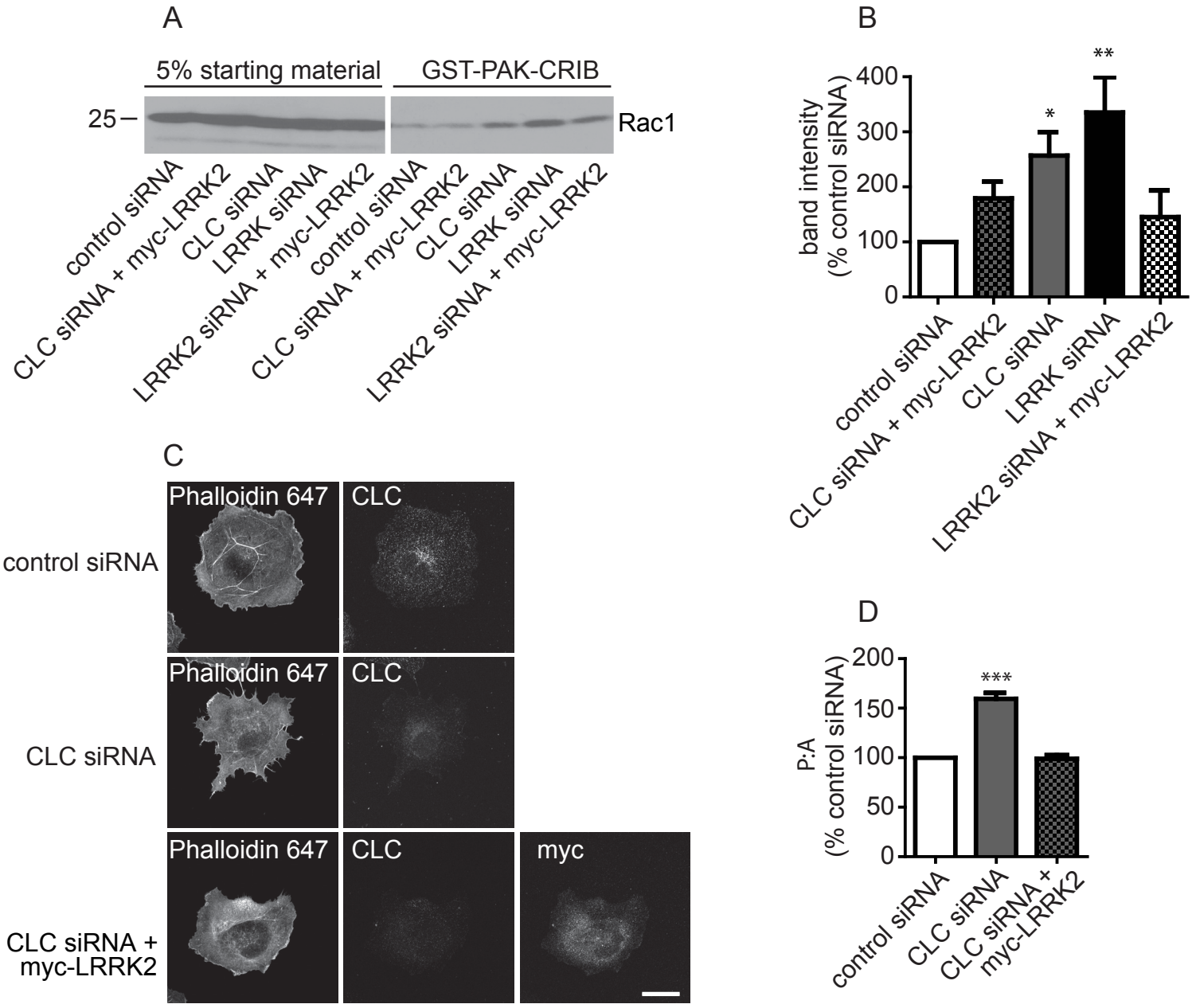

CLCb
nCLCb
nCLCb 1-165
30
CLCs
ponceau
GST-ROC
GST
SM
GST
GST-ROC
SM
GST
GST-ROC
SM
GST
GST-ROC

Fig. S2.2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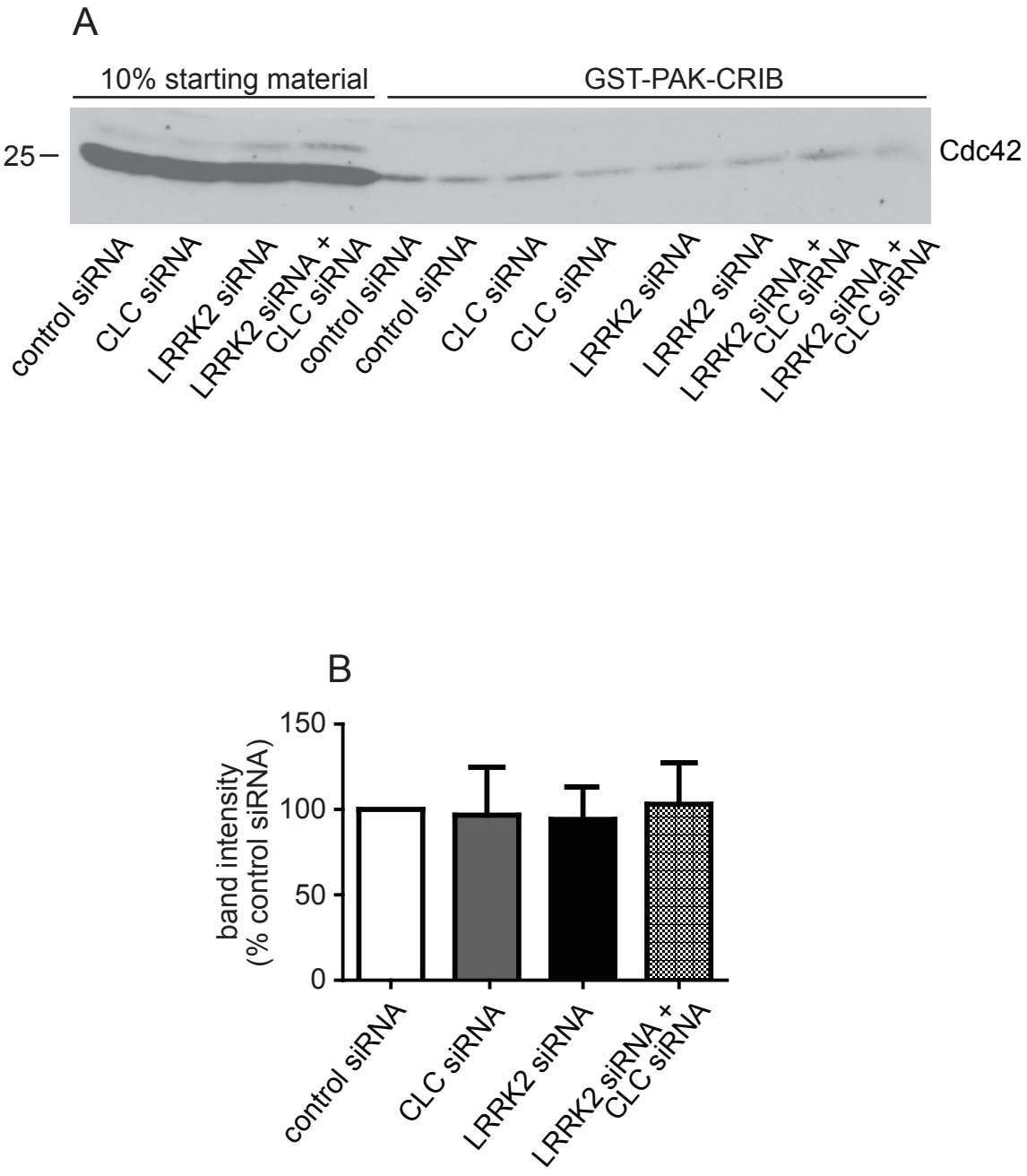
Rac1
control siRNA
CLC siRNA + myc-LRRK2
CLC siRNA
LRRK siRNA
LRRK2 siRNA + myc-LRRK2
control siRNA
CLC siRNA + myc-LRRK2
CLC siRNA
LRRK siRNA
LRRK2 siRNA + myc-LRRK2
B
band intensity
(% control siRNA)
400
300
200
100
0
*
**
control siRNA
CLC siRNA + myc-LRRK2
CLC siRNA
LRRK siRNA
LRRK2 siRNA + myc-LRRK2
C
Phalloidin 647
CLC
myc
control siRNA
CLC siRNA
CLC siRNA +
myc-LRRK2
D
P:A
(% control siRNA)
200
150
100
50
0
***
control siRNA
CLC siRNA
CLC siRNA +
myc-LRRK2

Fig. S2.3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GST-PAK-CRIB
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Cdc42
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CLC siRNA
LRRK2 siRNA
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control siRNA
control siRNA
CLC siRNA
CLC siRNA
LRRK2 siRNA
LRRK2 siRNA
LRRK2 siRNA + CLC siRNA
LRRK2 siRNA + CLC siRNA
B
band intensity (% control siRNA)
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100
50
0
control siRNA
CLC siRNA
LRRK2 siRNA
LRRK2 siRNA + CLC siRNA

Fig. S2.4

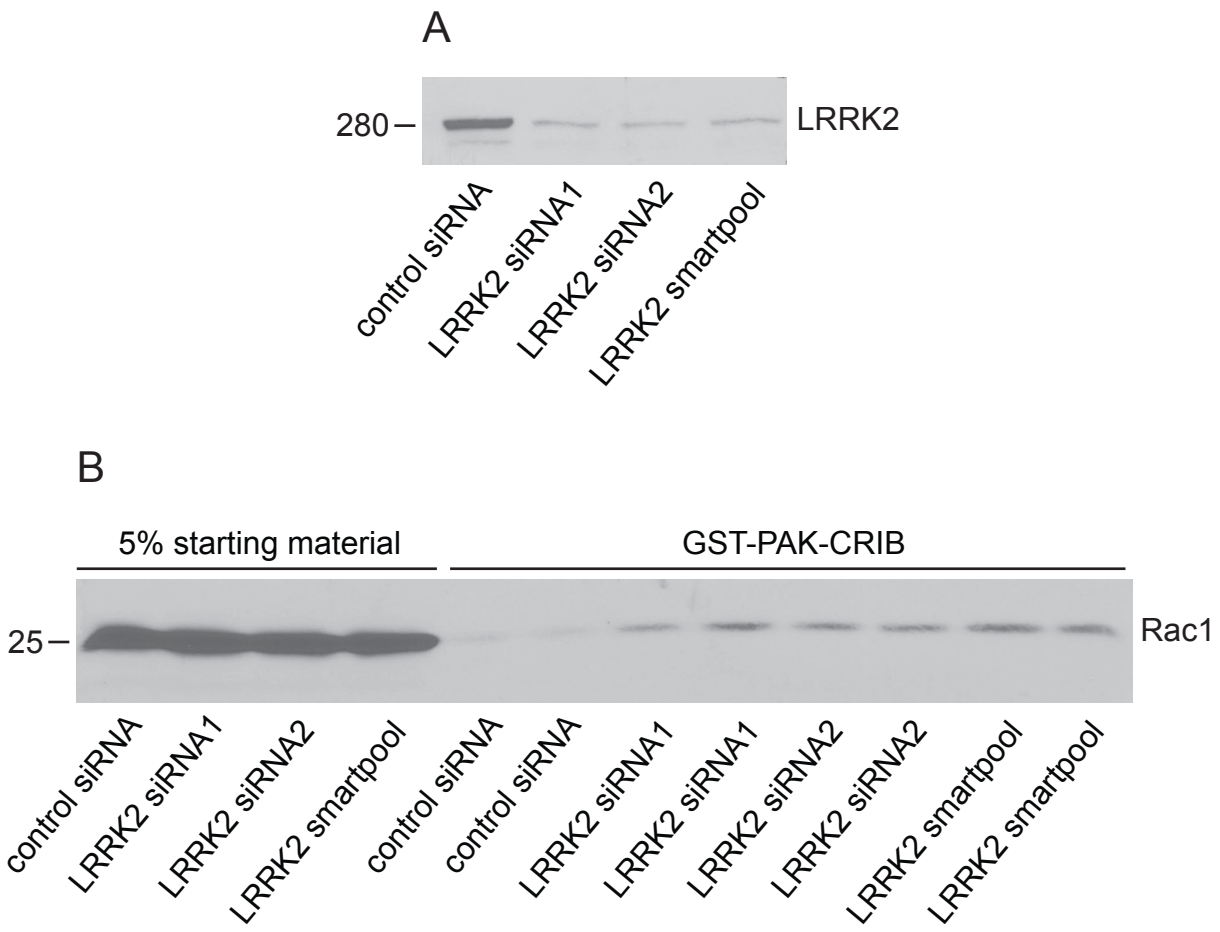
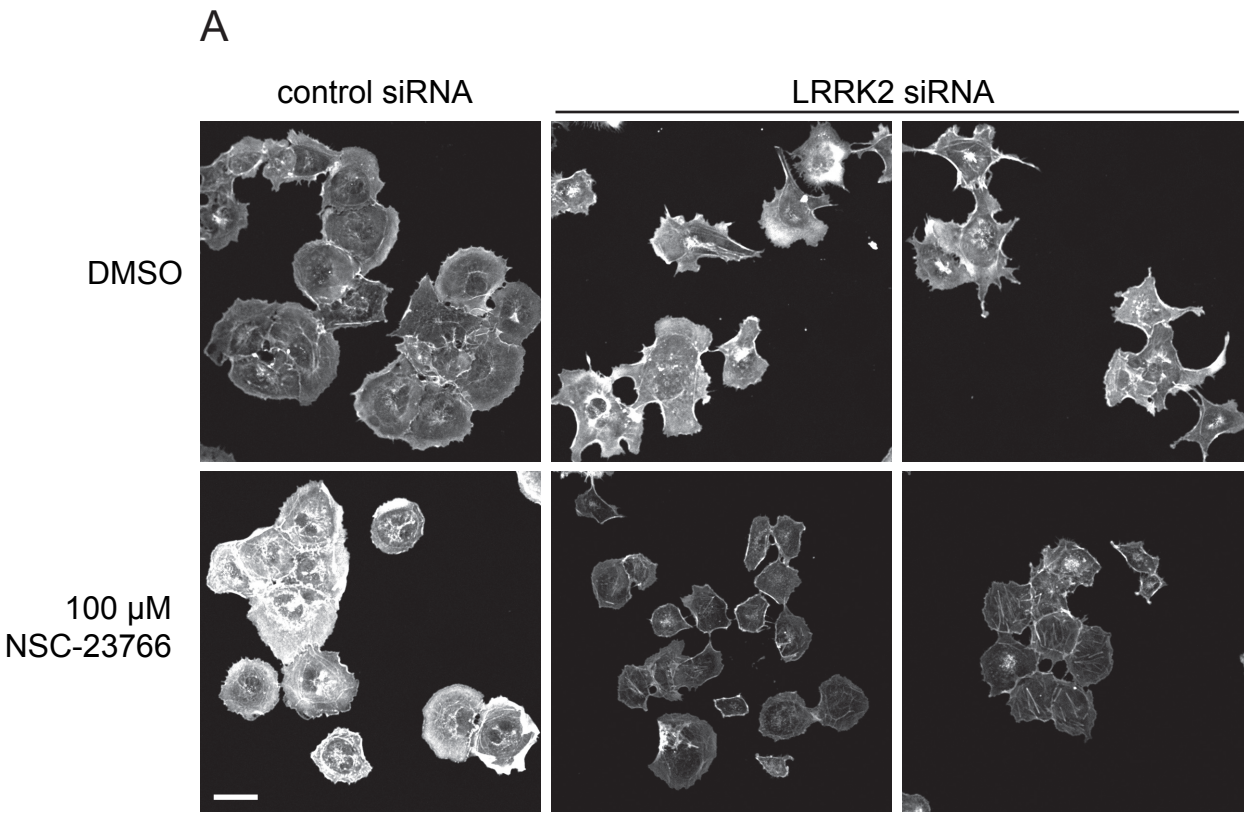

A
280
LRRK2
control siRNA
LRRK2 siRNA1
LRRK2 siRNA2
LRRK2 smartpool
B
5% starting material
GST-PAK-CRIB
25
Rac1
control siRNA
LRRK2 siRNA1
LRRK2 siRNA2
LRRK2 smartpool
control siRNA
control siRNA
LRRK2 siRNA1
LRRK2 siRNA1
LRRK2 siRNA2
LRRK2 siRNA2
LRRK2 smartpool
LRRK2 smartpool

Fig. S2.5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control siRNA
LRRK2 siRNA
DMSO
100 μM
NSC-23766

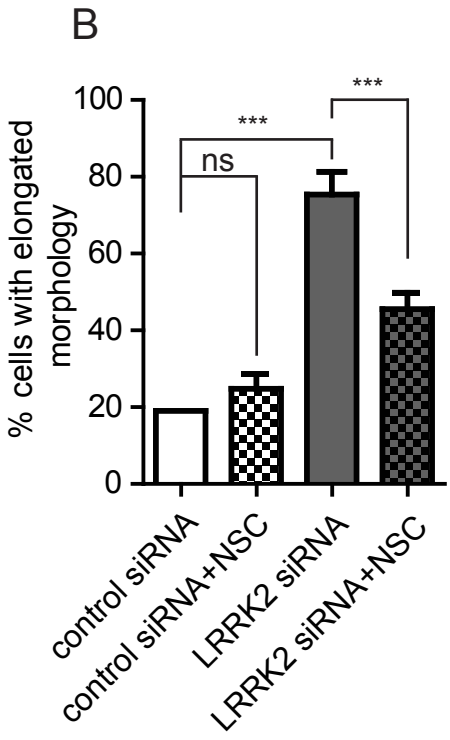
B
% cells with elongated morphology
100
80
60
40
20
0
***
***
ns
control siRNA
control siRNA+NSC
LRRK2 siRNA
LRRK2 siRNA+NSC

Fig. S2.6

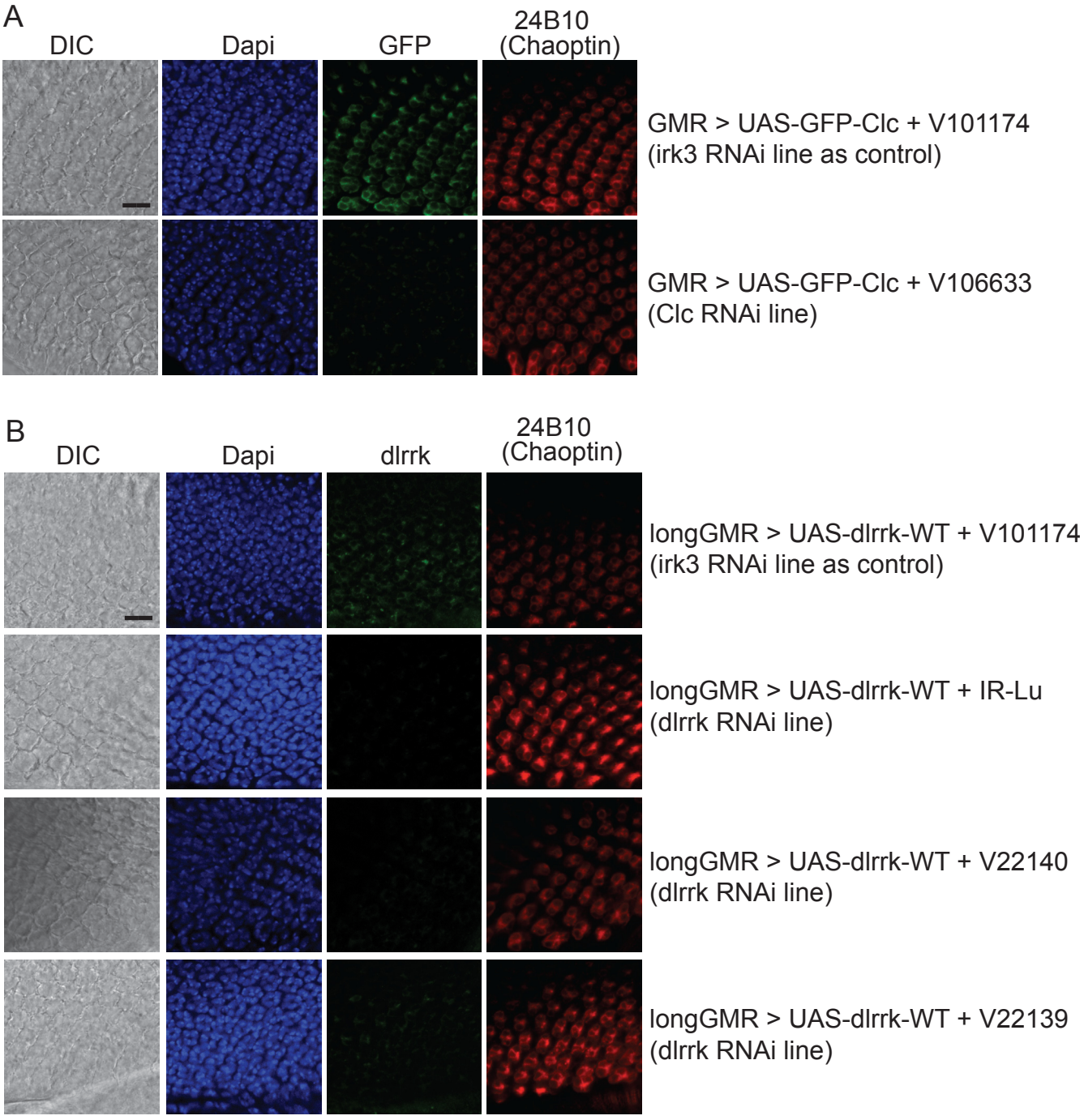
A
DIC
Dapi
GFP
24B10
(Chaoptin)
GMR > UAS-GFP-Clc + V101174
(irk3 RNAi line as control)
GMR > UAS-GFP-Clc + V106633
(Clc RNAi line)
B
DIC
Dapi
dlrrk
24B10
(Chaoptin)
longGMR > UAS-dlrrk-WT + V101174
(irk3 RNAi line as control)
longGMR > UAS-dlrrk-WT + IR-Lu
(dlrrk RNAi line)
longGMR > UAS-dlrrk-WT + V22140
(dlrrk RNAi line)
longGMR > UAS-dlrrk-WT + V22139
(dlrrk RNAi line)
C
long GMR > UAS-dlrrk-WT + V101174
long GMR > UAS-dlrrk-WT + IR-Lu
long GMR > UAS-dlrrk-WT + V22140
long GMR > UAS-dlrrk-WT + V22139
280
dlrrk
45
actin

Fig. S2.7


longGMR > UAS-Rac1W
i
GMR > UAS-V33516
ii
longGMR >UAS-Rac1W
+ V33516
iii

PREFACE TO CHAPTER 3

Parkinson disease (PD) is a devastating neurodegenerative disorder of unknown etiology (*1*). New technologies have led to the identification of a steadily growing number of PD-related genes over the last decade (*2-10*). Importantly, pinpointing the normal function of PD-gene proteins leads to new understanding of the cellular machineries and pathways that are altered in the disease process. *Leucine-rich repeat kinase 2* (LRRK2) mutations are the major cause of PD (*11*), yet much remains unknown of its physiological function. New insights are crucial for novel drug development, as current PD treatment options are merely symptomatic and do not prevent or retard neurodegeneration of the affected dopaminergic (DAergic) neurons (*12*).

Two transformative technologies have emerged in the last decade that allow for the ability to readily alter the genome of cells (*13*) and to convert adult cells into pluripotent stem cells, which can be further differentiated into numerous cell types including neurons (*14, 15*). These novel approaches have been used to study many disease mechanisms (*16-19*) and are even thought to provide a model for personalized medicine in the near future (*20-22*). As the normal function, and molecular mechanism(s) of LRRK2-PD remain largely ill-defined, these new tools are attractive for functional exploratory studies. The unpublished work presented in this chapter provides the basis for future studies to determine LRRK2 localization and physiological function in human DAergic neurons. We show an optimized protocol for differentiation of pluripotent stem cells into DAergic neurons and genome editing of LRRK2 for future localization studies.

CHAPTER 3

DOPAMINERGIC NEURONS IN A DISH: A PARKINSON DISEASE MODEL

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ABSTRACT

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common cause of dominant-inherited Parkinson disease (PD), yet our understanding of the physiological function(s) of LRRK2 remains incomplete. Dopaminergic (DAergic) neurons derived from human induced pluripotent stem cells (hiPSCs) provide a powerful system to study PD *in vitro*. So far, various DAergic differentiation protocols exist, with one major limitation that none obtain a highly differentiated final population of DAergic neurons. We now report successful refinement of current protocols that allows for robust and consistently reliable differentiation of hiPSCs into human DAergic neurons. We further inserted a Flag epitope tag under the endogenous promoter of LRRK2 in these hiPSCs for future localization and cell physiological studies of LRRK2 in DAergic neurons.

INTRODUCTION

Parkinson's disease (PD) is the second most common age-related progressive neurodegenerative disease of unknown etiology, characterized by motor and non-motor features affecting overall quality of life (*12, 23-26*). Currently, PD treatment is exclusively symptomatic, and does not halt or delay neurodegeneration of DAergic neurons – the neuropathological hallmark of PD (*12*). A lack of knowledge concerning the molecular events causing neurodegeneration in PD slows the development of neuroprotective therapies. Studying genes associated with PD has been the main focus of the field. Of particular interest are mutations in the *leucine-rich repeat kinase 2* (LRRK2) gene, as these comprise the most common genetic cause of both familial and sporadic PD (*11*). Importantly, LRRK2-associated PD is typically indistinguishable from sporadic PD cases (*27-29*), possibly suggesting common pathogenic pathways. Given the importance of LRRK2 for the study of PD, it is remarkable that its basic cell physiology functions remain poorly characterized. Understanding the normal function of LRRK2 may provide clues regarding early LRRK2 molecular and cellular alterations that eventually lead to the development of PD. In turn, this may lead to improved therapies aimed at halting or preventing disease progression.

To date, knowledge of PD pathophysiology has been mainly based on postmortem human brain studies due to the limited availability of living human brain tissue. This has restricted the understanding of disease onset and progression, as postmortem tissue merely corresponds to the end-stage of PD (*30*). Therefore, the LRRK2-PD field has relied heavily on *in vitro* assays using numerous established cell lines (eg. 293T (*31*), SH-SY5Y (*32*), COS-7 (*33*)) and primary rodent neuronal cultures to investigate molecular aspects of PD (*34-37*). The major limitation of these

approaches, however, is that the effect of LRRK2 mutations on DAergic neuron vulnerability cannot be specifically measured. LRRK2 knock-in/out rodent models have been developed with the aim of recapitulating disease in an *in vivo* animal model (*36, 38-47*). However, there is little consensus from these studies, as different groups have used diverse methods to obtain pathogenic LRRK2 rodent models (eg. human vs. mouse cDNAs, BAC clones, adenoviral vectors), reporting widely varying effects on the DAergic neuron population (*48, 49*). A promising new technology allows for the conversion of adult human fibroblasts into human induced pluripotent stem cells (hiPSCs), and subsequent differentiation into various cell types including neurons (*14, 15*). This novel approach provides unparalleled opportunities to study the physiological function of PD genes and their disease mechanisms in human neurons.

Midbrain DAergic (mDAergic) neurons originate from neural progenitors in the ventral midline of the embryonic neural tube, which corresponds to the floor plate region (*50, 51*). Three distinct steps, with corresponding transcription factors/molecular markers, drive the generation of mDAergic neurons from mDAergic neural progenitor cells: 1) regional and neuronal specification, 2) early, and 3) late differentiation (*52*). During step 1, sonic hedgehog (Shh), fibroblast growth factor 8 (Fgf-8), and Wnt1 are amongst the extrinsic factors that control regional and neuronal identity by regulating expression of a multitude of specific transcription factors, such as the floor plate marker Foxa2 and roof plate marker Lmx1a (*53*). In step 2, these progenitors undergo an early differentiation step resulting in immature mDAergic neurons (also known as neural precursor cells (NPCs)) that express βIII-tubulin (Tuj1) and Nurr1 amongst others (*53*). Subsequently, these immature mDAergic neurons differentiate into mature DAergic neurons that express tyrosine hydroxylase (TH), in addition to other markers also expressed in immature neurons (*53*). Thus, differentiation of hiPSCs into DAergic neurons relies on these

numerous factors that allow for the correct mDAergic patterning and final generation of DAergic neurons *in vitro*.

The ability to readily alter the genome of cells using CRISPR-Cas9 technology has recently emerged as a novel and powerful research tool (*13*). Using tag-insertion under the endogenous promoter of the gene of interest, genome editing allows for the localization of proteins which cannot be reliably detected using antibodies (*54*). Furthermore, patient hiPSCs bearing disease-associated mutations can be genome edited to wild-type sequences to create isogenic control cell lines (*55*). Importantly, CRISPR-Cas9 appears to have low off-target activity making this a reliable research tool to alter the genome of hiPSC (*56*).

Here, we set out to investigate the localization and cell physiological function of LRRK2 in human DAergic neurons, combining hiPSC and CRISPR-Cas9 genome editing techniques. We demonstrate that refinement of current protocols for differentiation of hiPSCs results in a highly differentiated final population of DAergic neurons with evidence of synapse formation, and obtain a CRISPR-Cas9 genome edited Flag-LRRK2 hiPSC line.

RESULTS

Differentiation of human induced pluripotent stem cells (hiPSCs) into DAergic neurons

To better understand the function of LRRK2 in DAergic neurons, we set out to differentiate hiPSC cells (NCRM1 line from STEMCELL Technologies) into human DAergic neurons. refined the established embroyid body (EB) protocol (*57*) with aspects from a monolayer protocol (*58*), for the generation of DAergic neurons from hiPSCs. The EB protocol consists of five steps; 1) culturing hiPSC, 2) generation of EB, 3) generation of nerual rosette, 4) differentiation into NPCs, and 5) final differentiation into DAergic neurons and their maturation. We adapted the EB protocol from Boyer *et al*. (*57*) by introducing two extra factors: Fgf-8 and CHIR-99021 (a small molecule known to strongly activate WNT signalling which induces Lmx1a expression in Foxa2 positive floor plate precursors) used in the monolayer protocol from Kriks *et al*. (*58*). These two factors increase the expression of Foxa2 and Lmx1a, which play important roles in mDAergic neuron development (*53, 59, 60*). Furthermore, we used SB431542 and Noggin – small molecules that stimulate rapid differentiation into neural lineages (*61*) – starting at the EB stage and continuing through the neural rosette stage, unlike Boyer *et al*. (*57*) who described its usage starting only at the rosette stage. Introducing these small molecules earlier jumpstarts the induction of mDAergic neurons from EB by 7 days, and potentially drives more cells to differentiate into DAergic neurons (Fig. 3.1). While maintaining hiPSCs we verified pluripotency with various stem cell markers (Fig. 3.2A). We generated DAergic neurons via differentiation of hiPSCs into NPCs, which characteristically express neuronal, NPC-specific, and pluripotency markers (Fig. 3.2B), and we subsequently converted the NPCs into

highly differentiated, TH-positive human DAergic neurons (Fig 3.2C). Thus, using our new protocol (that incorporates elements from Kirks *et al*. (*58*) into the Boyer *et al*. (*57*) protocol) we established consistent and reliable differentiation of hiPSCs into a highly differentiated final population of human DAergic neurons.

Characterization of DAergic neurons generated from hiPSCs

TH is the rate-limiting enzyme in the production of dopamine. It converts tyrosine to L-dopa, the precursor of dopamine (*62*). TH is expressed in noradrenergic neurons and at a higher level in DAergic neurons (*63*). The percentage of final DAergic neurons derived from hiPSCs appears to be highly variable, with as little as 3.6% to as much as 40% being reported (*64*). To assess the purity of DAergic neurons derived from hiPSCs using our protocol, we stained $6^{1/2}$ week old DAergic neurons for TH, βIII-tubulin to detect cell bodies, dendrites and axons, and MAP2 for cell bodies and dendrites selectively. The outcome of our new protocol shows highly differentiated, TH-positive human DAergic neurons (Fig. 3.3A). TH co-localizes to a high degree with the extensive network of MAP2, and partially with βIII-tubulin (Fig. 3.3A). DAergic neurons are known to have an elaborate axonal field which is reflected in the partial yet high degree of co-localization of TH with βIII-tubulin in our dense cultures (Fig. 3.2C and 3.3A lower panel). We further assessed whether these TH-positive human DAergic neurons also express DA transporter (DAT). DAT is required for DA reuptake from the synapse back into the cytosol, which terminates DA signalling by facilitating its synaptic clearance (*65*). In $5^{1/2}$ week old human DAergic neurons, DAT is expressed strongly in the cell body and in a punctate pattern in

dendrites, and co-localizes with TH (Fig. 3.3B). Thus, our hiPSC differentiation into human DAergic neurons yields a highly differentiated TH-, DAT-positive DAergic neuron population.

DAergic neurons have evidence of synapse formation and distribution of various organelles

To study LRRK2 localization in our human DAergic neurons we first characterized their synaptic networks. Evidence of rich synapse formation is readily observed in 7 week old DAergic neurons, using synapsin as a marker of presynaptic nerve terminals (Fig. 3.4A), suggesting that these neurons have matured into functional synaptic networks. We previously demonstrated that endogenous LRRK2 localizes to early endosomes, where it interacts with clathrin-light chains (CLCs) to regulate Rac1 activity in COS-7 cells and *Drosophila* (*33*). We thus determined CLCs distribution and the pattern of early endosomes in our human DAergic neurons (Fig. 3.4B,C). As expected, a distinct perinuclear pool of CLCs at the TGN, and more diffuse punctate pattern throughout the dendrites were observed (Fig. 3.4B). We previously showed that CLCs localize to the TGN (*66*), while its diffuse punctate pattern is reminiscent of clathrin coats at the plasma membrane and on early endosomes (*67-70*). Surprisingly, early endosomes appear to cluster in cell bodies and localize to dendrites but not axons (Fig. 3.4C). In *Drosophila*, dendritic arborization of branches has been attributed to early endosomal function (*71*), which may explain our observation of exclusive early endosomal staining in dendrites. LRRK2 function has further been associated with MPR trafficking from the TGN to endosomes. Overexpression of PD-mutant LRRK2-G2019S, which is a mutation that enhances the kinase activity, leads to altered MPR trafficking and impaired lysosomal function (*72*). Thus, to assess LRRK2 localization in DAergic neurons we determined TGN distribution (Fig. 3.4D). Vesicle-

associated membrane protein 4 (VAMP4) localizes to the TGN (*73*) and in our DAergic neurons VAMP4 displays a TGN pattern in cell bodies (Fig. 3.4D). Finally, LRRK2-G2019S *Drosophila* and knock-in mice show mitochondrial defects (*74, 75*) that may be of interest for LRRK2 localization. Therefore, we determined mitochondrial distribution in our hiPSC-derived DAergic neurons and observed abundant and spread-out mitochondrial networks in dendrites and cell bodies (Fig. 3.4E). Our effort to observe the synaptic network in our human DAergic neurons, as well as distinguish different organelles provides a solid base for further detailed LRRK2 localization studies.

Flag-LRRK2 CRISPR-Cas9 genome editing in hiPSCs

Endogenous LRRK2 recognition by immunofluorescence, using LRRK2 antibodies, has been shown to be unreliable (*76*). Hence, we recently used CRISPR/Cas9 technology to genome edit LRRK2, adding a triple HA-tag downstream of the endogenous promoter, in COS-7 cells, to determine LRRK2 endogenous localization (*33*). We have adopted the same strategy for hiPSCs, however, we have optimized our approach (Fig. 3.5A,B). First, we used a triple Flag-epitope tag instead of HA to reduce the costs of the expensive HA antibody, for insertion under the endogenous promoter of LRRK2 (Fig. 3.5A). Second, we used a puromycin selection cassette driving expression of a guide RNA and Cas9 for cleavage of the LRRK2 gene near the start codon (Fig. 3.5B). This allowed for puromycin selection to eliminate non-electroporated hiPSCs. Following electroporation of both the guide RNA-Cas9 and the triple Flag-epitope tag, and puromycin selection, the hiPSCs were dilution-cloned and screened by PCR (Fig. 3.5B). We screened 125 hiPSC colonies for genomic insertion of the triple Flag-epitope tag under the

endogenous promoter of LRRK2, and identified a genome edited hiPSC line positive for the triple Flag insertion (Fig. 3.5C). Thus, to assess the localization and cell physiological function of LRRK2, DAergic neurons will be generated from this hiPSC genome edited line in the future.

DISCUSSION

In this study we focused on refining an established DAergic EB protocol from Boyer *et al*. (*57*), in order to optimize the differentiation of hiPSCs into DAergic neurons for the purpose of studying LRRK2 localization and function. We have demonstrated that with our new EB protocol we can differentiate hiPSCs into a highly differentiated population of human DAergic neurons that contain an extensive synaptic network and well-defined organelles. We further generated a triple Flag-LRRK2 hiPSC genome edited line using CRISPR-Cas9 technology. Taken together, these results allow for future localization and cell physiological studies of LRRK2 in human DAergic neurons – a much needed requirement aimed at improving therapeutics for PD.

A better understanding of LRRK2's cellular functions may contribute to defining potential drug targets that improve DAergic neuron survival in PD. Since LRRK2-associated PD is typically indistinguishable from sporadic PD cases (*27-29*), and the majority of PD is sporadic (*11*), identification of LRRK2-related drug targets may have great consequences for the general treatment of PD patients. Our optimized EB protocol (Fig. 3.1 and 3.2) provides a method of obtaining a highly differentiated final DAergic neurons (Fig. 3.3. Moreover, our established CRISPR-Cas9 genome editing system provides us with a tool to generate triple Flag insertions into LRRK2 patient-derived hiPSCs in the near future, allowing us to study endogenous LRRK2 localization alongside our current wild-type triple Flag-LRRK2 line (Fig. 3.5).

Using DAergic neurons derived from patient hiPSCs, such as LRRK2-G2019S and R1441C/G, will further provide an *in vitro* tool for studying PD pathophysiology from start to finish. Such *in vitro* studies allow for a detailed mechanistic examination of the molecular

pathways involved in the initiation and progression of PD, in both familial and sporadic patient-derived DAergic neurons. Although these patient-derived hiPSCs will carry the genetic background of their donor, some caution is required, as reprogramming fibroblasts into iPSCs usually introduces mutations in the genomic DNA that lead to enhanced cell variability (*77, 78*). Furthermore, reprogrammed cells maintain characteristic DNA methylation signatures typical of their somatic tissue of origin, a phenomenon termed "epigenetic memory" (*79*), which also affects gene expression (*80*). Another concern using hiPSCs as a model for neurodegenerative diseases is that these cells essentially correspond to fetal neurons, not the matured neurons affected in patients (*81*). Provided that PD neurodegeneration progresses slowly over time, the idea of modeling PD in a dish has raised doubts (*81*). Nonetheless, the slow progression of PD most likely arises from early stage alterations in the affected pathways. Recent PD research shows pathological mechanisms in neurons derived from hiPSCs (*82-86*).

Determining endogenous triple Flag-LRRK2 localization in our DAergic neurons (Fig. 3.5) will be the first step to further define LRRK2 physiological function and identify potential disease pathways. We anticipate similar localization at early endosomes in DAergic neurons, as we previously observed in our genome edited COS-7 cells (*33*). Moreover, we predict that LRRK2 controls an early step in endo-lysosomal trafficking, as observed by us and others in established cell lines (*33, 87-89*). In general, alterations in endo-lysosomal trafficking lead to dysfunctional lysosomes and accumulation of undegraded macromolecules, toxic to the cell (*90*) which may, in part, contribute to the pathophysiology of LRRK2-associated PD. Future directions should focus on using LRRK2 patient-derived cells for *in vitro* drug screening to identify pharmacological agents capable of correcting disease phenotypes, as well as to gain

additional insight into the general molecular mechanisms that underlie PD-associated DAergic susceptibility.

MATERIALS AND METHODS

Antibodies

Polyclonal antibody recognizing CLCa/CLCb was generated as described (*91*). Rabbit monoclonal antibody was from Cell Signalling (EEA1). Rabbit polyclonal antibodies were from the indicated sources; synapsin (*92*), nanog, nestin, and TH (Abcam), and Vamp4 was a generous gift from Dr. Richard Scheller. Mouse monoclonal antibodies recognizing the following proteins were from the indicated commercial source; SSEA-4 and TRA-1-81 (Santa Cruz), MAP2 and Tuj1 (Millipore), PDH E2 (Abcam). Rat monoclonal antibody was from Millipore (DAT). Chicken polyclonal antibodies were from; TH (Millipore), and MAP2 (EnCor Biotechnology). Hoechst 33342, Alexa 488-conjugated donkey anti rabbit IgG, Alexa 488-conjugated goat anti rat IgG, Alexa 555-conjugated donkey anti mouse IgG, Alexa 555-conjugated donkey anti rabbit IgG, Alexa 647-conjugated goat anti mouse IgG, and Alexa 634-conjugated donkey anti chicken IgG were from Invitrogen Inc.

Immunofluorescence and microscopy

DAergic neurons derived from hiPSCs grown on poly-L-Ornithine and laminin-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.

Cells were imaged on a Zeiss LSM710 confocal microscope using a plan-apochromat 40x oil objective (Zeiss), and a Zeiss Axio Observer Z1 microscope using a plan-apochromat 20x objective (Zeiss).

Generation of DAergic neurons from hiPSCs

hiPSCs (NCRM1 line of STEMCELL Technologies) were grown in mTeSR1 media (Stemcell Technologies) for 6 to 7 days (Day 1-7) on Matrigel Matrix (Millipore) coated plates. Subsequently, hiPSCs were dissociated to single cells using gentle dissociation reagent (Stem Cell Research) for 7 minutes at 37°C. Two million cells were plated into a T25 flask without coating using media A (100% DMEM-F12 media (Gibco) with L-Glutamax (Life Technologies), N2 (Gibco), B27 (Gibco), BSA (Invitrogen), SB431542 (Tocris), Noggin (Gibco), Shh (GenScript), CHIR-99021 (Stem Cell Technologies), and Fgf-8 (GenScript)) with the addition of ROCK inhibitor Y27632 to support cell survival for (DAergic) EB formation. From day 8-14 changed media A every day to allow for DAergic EB formation and maintenance. Plated EBs on Poly-L-Ornithine (PLO) (Sigma)-laminin (Sigma) coated T75 flasks on day 15 for DAergic rosette formation using media A with addition of Y27632. From day 14-21 changed media A every day to allow for DAergic rosette formation and maintenance. Subsequently, DAergic rosette were dissociated to clusters and/or single cells on day 21 using gentle dissociation reagent for 7 minutes at room temperature and transferred to a new PLO-laminin coated T75 flask for DAergic NPC formation using media B (100% DMEM-F12 media with L-Glutamax, N2, B27, and BSA) with addition of Y27632. Throughout day 21-35 media B was changed every 2 days and cells were split every 6 days. Following these 2 weeks, DAergic NPCs were dissociated to

clusters and/or single cells on day 35 using gentle dissociation reagent for 5-8 minutes at 37°C and transferred to a new PLO-laminin coated T25 flask or coverslips for final differentiation into DAergic neurons over a minimum period of 6 weeks. From day 35-42 media C (100% DMEM-F12 media with L-Glutamax, N2, B27, BSA, Fgf-8, GDNF (Peprotech), BDNF (Peprotech), laminin, ascorbic acid (Sigma), and dCAMP (Sigma)) was used to change the media every 2 days. Followed by 3-5 days with media D (75% DMEM-F12 and 25% Neurobasal media (Life Technologies) with L-Glutamax, N2, and B27) and then 3-5 days with media E (50% DMEM-F12 and 50% Neurobasal media with L-Glutamax, N2, and B27). The next 3-5 days media F (25% DMEM-F12 and 75% Neurobasal media with L-Glutamax, N2, and B27) was used, and finally media G (100% Neurobasal media with L-Glutamax, N2, and B27) was used for the remaining 4-6 weeks to maintain the final DAergic neurons. Media D-G was changed every 2 days to maintain the differentiation of DAergic neurons.

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

hiPSCs were Neon electroporated with pSpCas9(BB)-2A-Puro plasmid (*93*) here denoted as plasmid encoding human optimized Cas9 with puromycin selection box from Addgene plasmids (#48139) and a guideRNA (gRNA) selective for LRRK2 flanking the start codon based on the protocol in Petit *et al*. (*54*). 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-electroporated with a large oligonucleotide encoding a triple Flag-epitope 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 Flag tag between residues 1 and 2 of LRRK2, driven by the endogenous LRRK2 promoter. Following Neon electroporation hiPSCs were plated and allowed to recover for 3 days, then puromycin treated for 5 days to select the transfected cells. Subsequently, small hiPSC colonies were transferred to a 96 well dish and allowed to grow. Finally, recombinants were identified based on PCR screening with primers that detected the endogenous LRRK2 (Supplemental Table 1) and all clones were frozen down. Our final clone 4 is probably a mix of both unedited (endogenous LRRK2) and CRISPR/Cas9 system edited (endogenous 3xFlag-LRRK2) cells, although a hemizygous addition of the 3xFlag-LRRK2 to all the cells cannot be ruled out at this point.

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FIGURE LEGENDS FOR CHAPTER 3

Figure 3.1: Differentiation of human induced pluripotent stem cells (hiPSCs) into DAergic neurons. An overview of the various steps, time, substrates, and media required to differentiate hiPSCs into DAergic neurons. *1)* hiPSCs are differentiated into *2)* DAergic embryoid bodies, followed by the formation of *3)* DAergic Rosette that differentiate into *4)* DAergic neural precursors (NPCs) from which final *5)* DAergic neurons arise. Images are representative of the various stages.

Figure 3.2: Culturing hiPSCs, NPC, and DAergic neurons. *(A)* Culturing hiPSCs. Two representative examples of immunofluorescence staining of the NCRM1 line of hiPSCs cells (STEMCELL Technologies): stained with Hoechst 3342 (a nuclei marker), and Nanog, SSEA-4 or TRA-1-81 (iPSCs markers). Scale bar, 20 μm. *(B)* Differentiation of hiPSCs into NPCs. NPCs were fixed and processed for immunofluorescence using Nestin (a marker of central nervous system progenitor cells), and TRA-1-8 (iPSCs marker) or Tuj1 (βIII-tubulin marker for cell bodies, dendrites and axons), and MAP2 (selective marker for cell bodies and dendrites) antibodies. Scale bar, 20 μm. *(C)* Generation of DAergic neurons from hiPSCs. 6 week old DAergic neurons generated from NPCs (as in *B*) were fixed and processed for immunofluorescence using tyrosine hydroxylase (TH, a DA neuron marker), Tuj1, and MAP2 antibodies. Scale bar, 20 μm.

Figure 3.3: Characterization of DAergic neurons generated from hiPSCs. *(A)* Majority of hiPSCs differentiates into DAergic neurons. $6^{1/2}$ week old DAergic neurons were fixed and

processed for immunofluorescence using tyrosine hydroxylase (TH), Tuj1, and MAP2 antibodies. Scale bar, 20 μm. The white boxes in the second row show a magnified view of the top row. *(B)* DAergic neurons generated from hiPSCs express dopamine transporters. $5^{1/2}$ week old DAergic neurons were fixed and processed for immunofluorescence using TH, and dopamine active transporter (DAT, a DA neuron marker) antibodies. Scale bar, 20 μm.

Figure 3.4: DAergic neurons have evidence of synapse formation and distribution of various organelles. *(A)* 7 week old DAergic neurons sere fixed and processed for immunofluorescence using synapsin (a marker of presynaptic nerve terminals), TH, and MAP2 antibodies. Scale bar, 20 μm. *(B)* $5^{1/2}$ week old DAergic neurons were fixed and processed for immunofluorescence using CLC (an endocytosis marker, present at plasma membrane and on endosomes) Tuj1, and MAP2 antibodies. Scale bar, 20 μm. *(C)* 7 week old DAergic neurons were fixed and processed for immunofluorescence using EEA1 (an early endosomal marker) Tuj1, and MAP2 antibodies. Scale bar, 20 μm. *(D)* $5^{1/2}$ week old DAergic neurons were fixed and processed for immunofluorescence using Vamp4 (a golgi marker) and Tuj1 antibodies. Scale bar, 20 μm. *(E)* 7 week old DAergic neurons were fixed and processed for immunofluorescence using PDH E2 (a mitochondrial marker) and MAP2 antibodies. Scale bar, 20 μm.

Figure 3.5: Flag-LRRK2 CRISPR-Cas9 genome editing in hiPSCs. *(A)* Schematic diagram of the oligonucleotide used to direct insertion of the 3xFlag 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). *(B)* Flow chart for hiPSC genome editing, starting with Neon electroporation of the puromycin selection plasmid with gRNA and the 3xFlag

olignucleotide, followed by iPSC recovery, puromycin selection, and PCR screening for positive clones with the 3xFlag insert. *(C)* PCR results of Flag-LRRK2 genome editing in hiPSCs. (1) 1kb marker, (2) LRRK2-WT from un-edited hiPSCs using primers that detect endogenous LRRK2, (3) Clone 4 is positive for 3xFlag-LRRK2 genome edited hiPSCs using the same primer combination as in (1).

Table S3.1: Oligonucleotides used to generate constructs for this study

Oligonuleotide name	Sequence
LRRK2-CRISPR_S1	gttccctgagcagcggacgttcat
LRRK2-CRISPR_AS2	gctcggagtacgtgaacac

Human LRRK2-3xFlag Ultramer oligonucleotide from Integrated DNA Technologies:

TATCAACTTCTTCAGAGTTTCCTCGTCCTCTTCGCACCCCTGGCAACTACCGCTGGCGGATCC
TCCGCCACCCTTATCGTCGTCATCCTTGTAATCTCCCTTGTCGTCATCGTCTTTGTAGTCGCCC
TTATCGTCGTCATCCTTGTAATCCATGGTGGCACCTGCTTCCAACCCGCCGCCCTCCCAGCAT
G

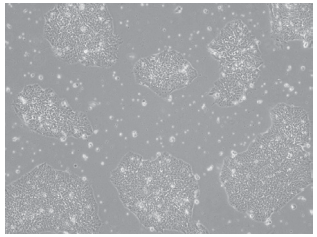
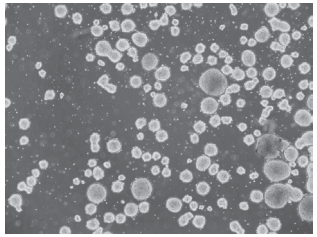
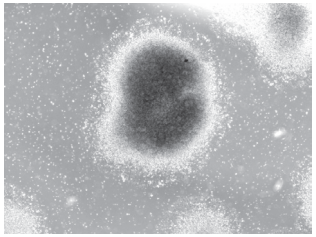
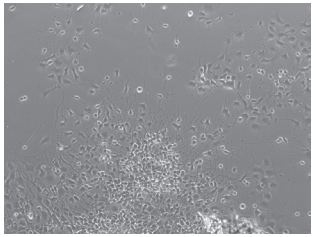
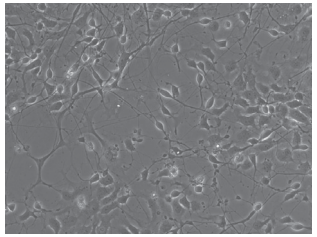
	Step 1	*Step 2*	*Step 3*	*Step 4*	*Step 5*
	hiPSC	DA-Embryoid bodies	DA-Rosette	DA-NPC	human DA neurons
					
Culture days:	6-7 days (1 week)	7 days (1 week)	7 days (1 week)	14 days (2 weeks)	minimum ~ 42 days (6-10 weeks)
Time line:	Day 1-7	Day 7-14	Day 14-21	Day 21-35	Day 35-77/105
Substrates:	Matrigel	---	PLO/Laminin	PLO/Laminin	PLO/Laminin
Media:	mTESR1	Media A	Media A	Media B	Media C-G

Fig. 3.1

Fig. 3.2

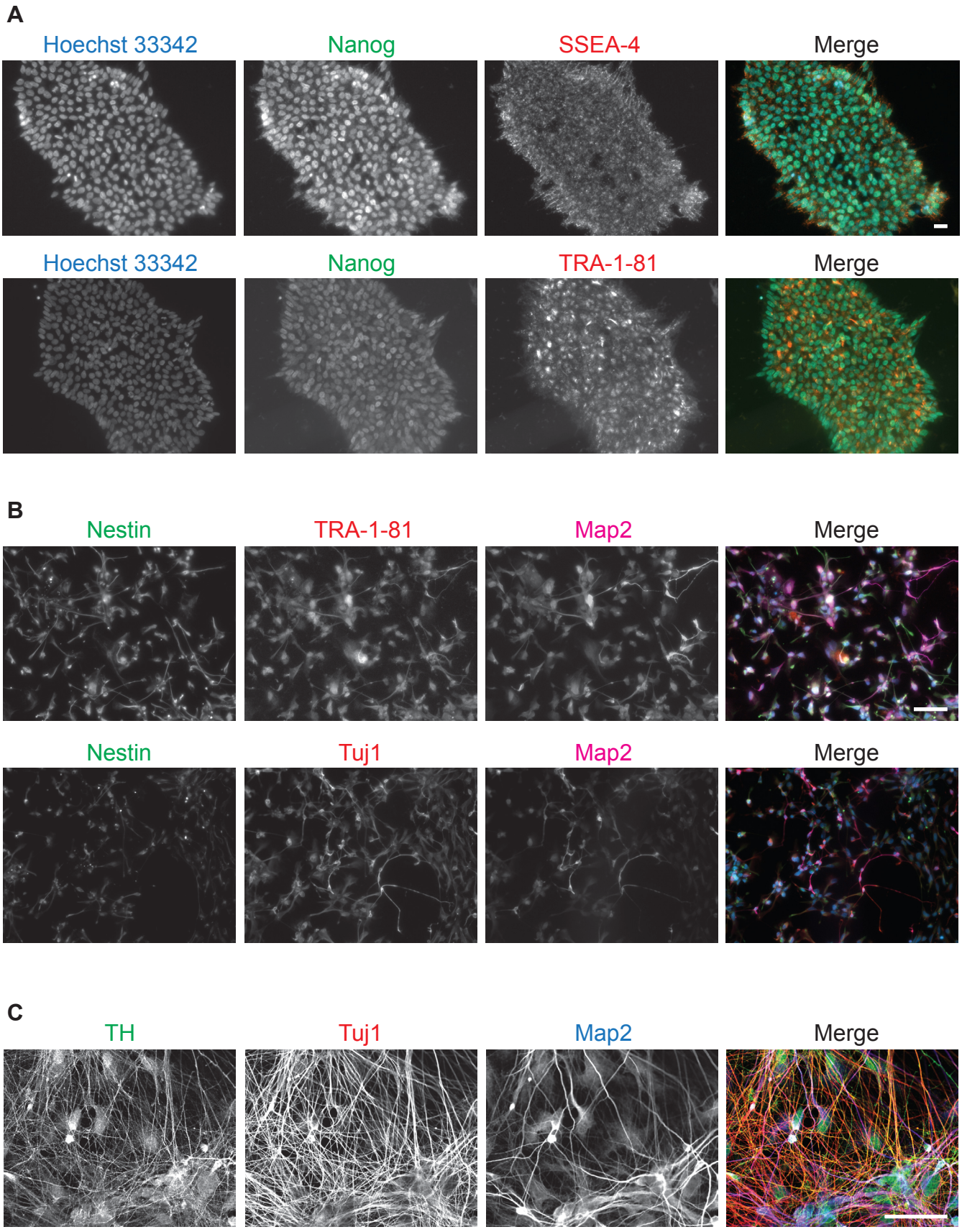

A
Hoechst 33342
Nanog
SSEA-4
Merge
Hoechst 33342
Nanog
TRA-1-81
Merge
B
Nestin
TRA-1-81
Map2
Merge
Nestin
Tuj1
Map2
Merge
C
TH
Tuj1
Map2
Merge

Fig. 3.3

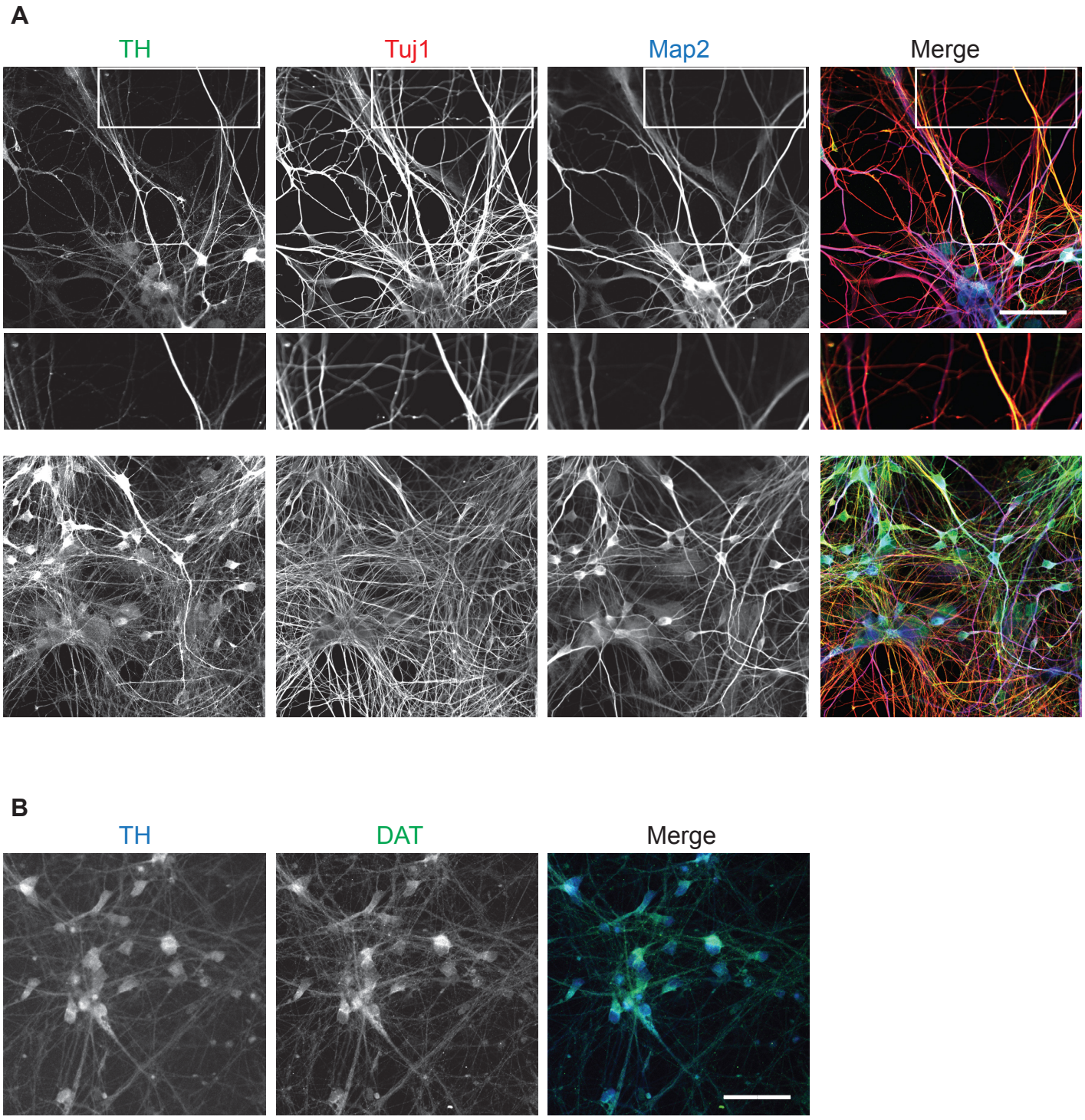


Fig. 3.4

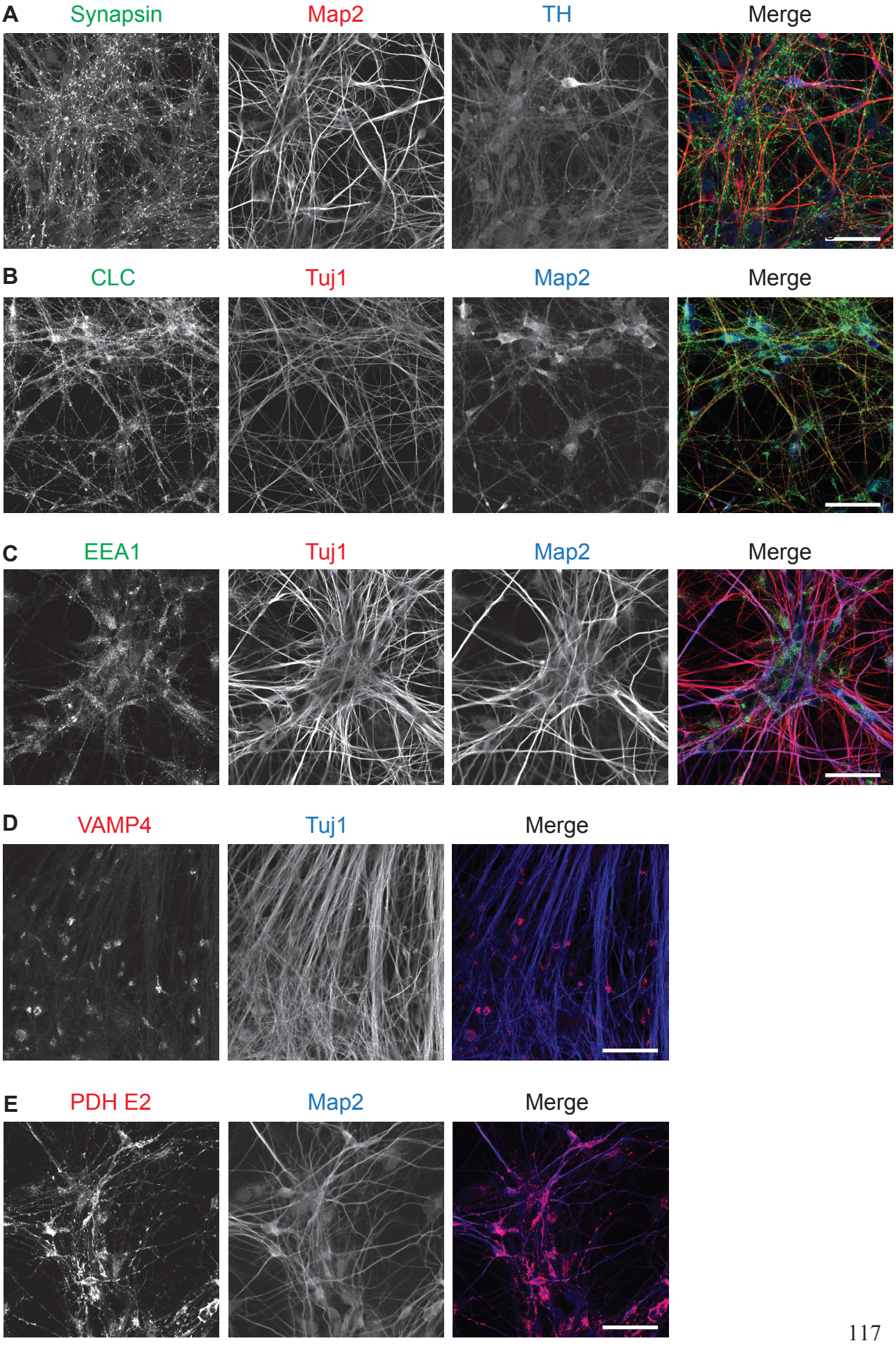
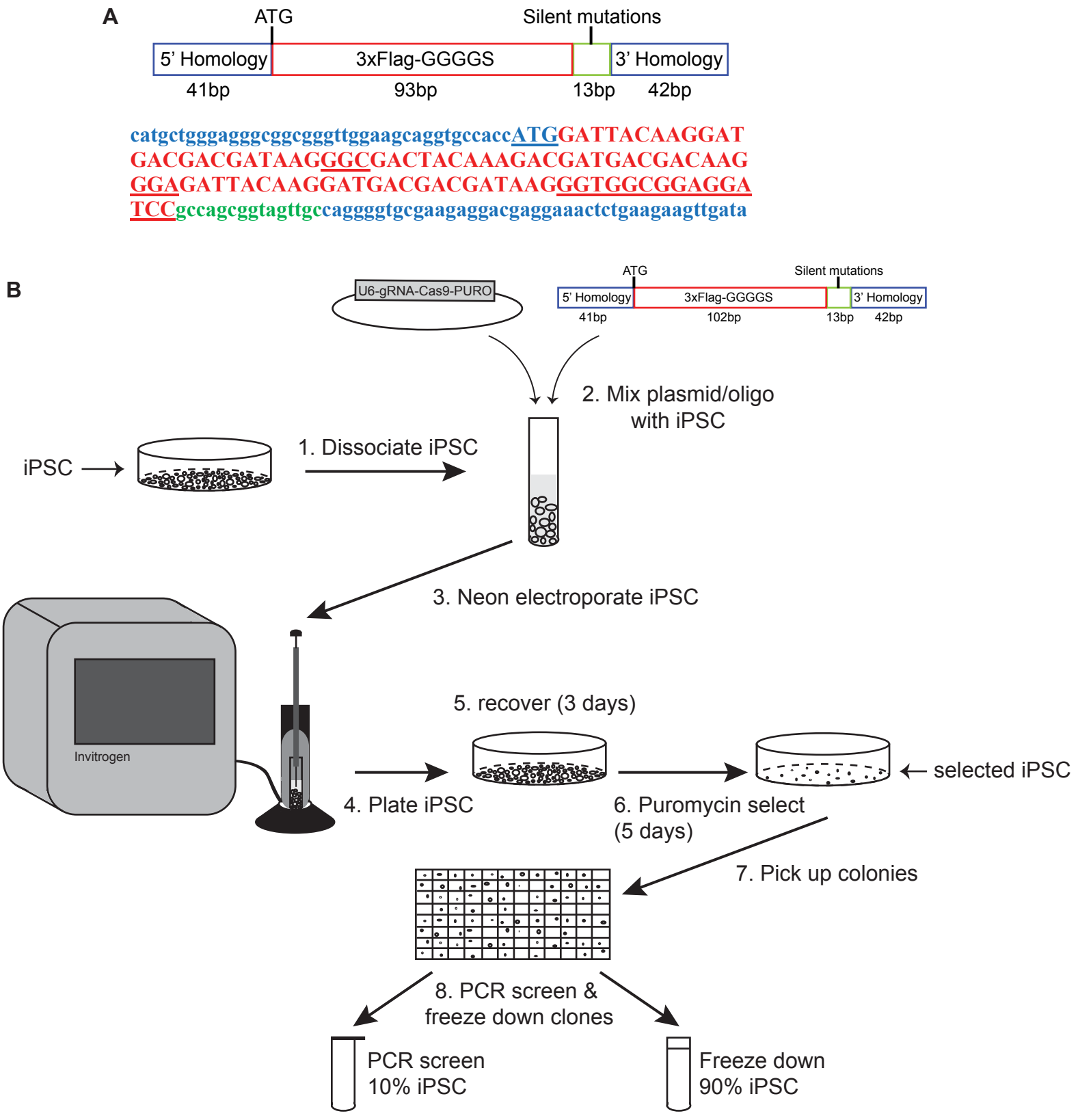

A
Synapsin
Map2
TH
Merge
B
CLC
Tuj1
Map2
Merge
C
EEA1
Tuj1
Map2
Merge
D
VAMP4
Tuj1
Merge
E
PDH E2
Map2
Merge

Fig. 3.5


A
ATG
Silent mutations
5' Homology
3xFlag-GGGGS
3' Homology
41bp
93bp
13bp
42bp
catgctgggagggcggcgggttggaagcaggtgccaccATGGATTACAAGGAT
GACGACGATAAGGGCGACTACAAAGACGATGACGACAAG
GGAGATTACAAGGATGACGACGATAAGGGTGGCGGAGGA
TCCgccagcggtagttgccaggggtgcgaagaggacgaggaaactctgaagaagttgata
B
U6-gRNA-Cas9-PURO
ATG
Silent mutations
5' Homology
3xFlag-GGGGS
3' Homology
41bp
102bp
13bp
42bp
2. Mix plasmid/oligo
with iPSC
iPSC
1. Dissociate iPSC
3. Neon electroporate iPSC
Invitrogen
5. recover (3 days)
selected iPSC
4. Plate iPSC
6. Puromycin select
(5 days)
7. Pick up colonies
8. PCR screen &
freeze down clones
PCR screen
10% iPSC
Freeze down
90% iPSC
C
1
2
3
400bp
300bp
200bp
3xFlag-LRRK2
WT

CHAPTER 4 GENERAL DISCUSSION AND CONCLUSION

4.1 Summary

The work presented in Chapters 2 and 3 reports on the cell physiological function of LRRK2 (*1*), and presents an adaptation of a new technique to generate human DAergic neurons in which endogenous LRRK2 is Flag tagged. Both studies are aimed at informing our fundamental understanding of how LRRK2 functions in the cell, to ultimately provide potential novel drug targets that could halt the progression of PD. In Chapter 2 we investigated the role of a new interacting partner of the ROC domain of LRRK2. The ROC domain is a functional GTPase (*2-4*) that regulates the intrinsic kinase activity of LRRK2 (*2, 5, 6*). In general, GTPases have interacting partners via which they regulate numerous cellular membrane trafficking processes (*7*). However, the interacting partner(s) of LRRK2's ROC domain remained largely unknown. We found that LRRK2 interacts directly with CLCs via its ROC domain and localizes to early endosomes together with CLCs. LRRK2 and CLCs function, both *in vitro* and *in vivo*, in a pathway that controls actin-dependent cell morphology via the regulation of Rac1 activity. These findings identify a novel pathway that connects the clathrin machinery to the cytoskeleton and PD, while also ascribing a role for LRRK2 in the endo-lysosomal system.

In Chapter 3 we determined the optimization of currently existing protocols for hiPSC-derived DAergic neurons, and applied CRISPR-Cas9 technology for genome editing of these hiPSCs, with the aim of studying LRRK2's cellular functions in DAergic neurons. Numerous cell types can be derived from hiPSCs (*8*), which holds great promise for multiple applications in dentistry (*9*), autoimmune diseases (*10*), dermatology (*11*), cardiology (*12*), as well as

neurodegenerative diseases such as PD (*13*). Although many labs use hiPSCs to generate DAergic neurons (*14-19*), the various protocols published to date have not been successful at generating a high percentage of final DAergic neurons (*20*). We therefore explored refinement options to enhance the final population of DAergic neurons derived from hiPSCs. We found that incorporating elements from Kriks *et al*. (*21*) in an established EB differentiation protocol from Boyer *et al*. (*22*) increased the final yield of DAergic neurons considerably. As our aim was to study the cellular function of LRRK2 in DAergic neurons, and LRRK2 antibodies have been shown to be unreliable (*23*), we further used CRISPR-Cas9 technology to insert a Flag-epitope tag under the endogenous promoter of LRRK2. Our findings provide a solid basis for future localization and cell physiological studies of LRRK2 in human DAergic neurons.

4.2 CLCs as novel binding partners of LRRK2

With most focus on the kinase domain, less is known regarding the other modules of LRRK2, including the ROC domain. Numerous interacting partners of LRRK2 have been revealed, yet only three were known to interact with LRRK2 via its ROC domain (tubulin (*24*), Snapin (*25*). We identified CLCs as novel binding partners that directly interact with LRRK2's ROC domain. We further determined the binding region in CLCs to a previously uncharacterized 10 amino acid stretch in the C-terminus, hereby identifying a new CLCs binding domain specific for LRRK2.

CLCs were initially thought to function exclusively as universal regulators of CCV formation in CME (*26*). However, no changes were observed in either CCV formation or the endocytosis of various cargo including transferrin receptor, EGFR and low-density lipoprotein

receptor upon knock down of CLCs in various cell lines frequently used to study CME (*27-29*). Instead, CLCs have been found important mediators of actin dynamics. CLCs bind directly to HiP1R (*30, 31*), and this interaction is required for HiP1R localization to CCPs and/or CCVs (*28*). HiP1R binds actin (*32*) and knock down of either HiP1R or CLCs results in over assembly of the actin cytoskeleton and formation of abnormal actin structures (*28, 33*). Thus, CLCs function as an important hub to recruit proteins to clathrin-coated structures, in addition to negatively regulating actin assembly. Furthermore, it was recently shown that in non-polarized cells actin polymerization is not required for CME (*29*). In contrast, in any cell where the plasma membrane is under tension (e.g. polarized epithelial cells, osmotic swelling, or mechanical stretching) CME depends on actin assembly (*29*). Importantly, knock-down of CLCs in these cells disrupts CME and leads to enhanced actin assembly and altered actin dynamics (*29*), suggesting that CLCs are required for actin engagement in actin-dependent forms of CME.

Actin rearrangements are essential for many cellular processes. For instance, the complexity of neuronal shape and structure requires extensive actin dynamics (*34*). We show for the first time that CLCs are also involved in spine formation. Spine morphology, size, and number are all highly dependent on actin dynamics regulated by small GTPases, such as Rac1 (*35, 36*). CLC knock down in cultured hippocampal neurons results in the loss of mature dendritic spines, thereby phenocopying the decreased number of mature dendritic spines observed in LRRK2$^{-/-}$ neurons (*37*). Moreover, similar to our findings, expression of a constitutively active Rac1 in cultured hippocampal neurons leads to a reduction in dendritic spines, with the remaining spines depicting irregular shapes (*38*). Therefore, the dendritic spine formation phenotype may potentially be ascribed to dysregulation of Rac1 activity by CLCs and LRRK2. As actin dynamics are important for synaptic connectivity and cell survival (*34, 39, 40*),

dysregulation may therefore promote neuronal susceptibility to cellular stress (*40*). Intriguingly, active Rac1 can sensitize cells to cell death via the induction of apoptosis (*41, 42*), and can enhance DAergic neuron vulnerability and death upon oxidative stress (*43*). Thus, dysregulation of Rac1 activity may, in part, contribute to the pathophysiology of PD by destabilizing actin dynamics, which in turn may reduce neuronal survival.

4.3 LRRK2 and CLCs as regulators of Rac1 activity

An important organizer of early endosome membranes is clathrin, which forms a bilayered clathrin coat on the early endosome through interactions with multiple endosomal membrane proteins (*44-48*). This clathrin coat is important for sorting specific cargo for degradation in the endo-lysosomal system (*45, 48*). CLCs are a component of bilayered clathrin coats on early endosomes where they function as a hub to recruit various proteins, including LRRK2 as we discovered (*1*). We further demonstrated, using *in vitro* and *in vivo* knock down experiments, that CLCs and LRRK2 interact biochemically and functionally to negatively regulate Rac1 activation. Inhibition of Rac1 activity by CLC/LRRK2 may be occurring at several regulatory points, such as: (a) inhibition of a Rac1 GEF, or (b) mislocalization and/or phosphorylation of Rac1.

Interestingly, Rac1 is endocytosed and activated on early endosomes, after which Rac1 recycles back to the plasma membrane to direct local actin polymerization and cytoskeletal changes (*49*). In the central nervous system, Rac1 controls numerous cellular events including spine formation, axon and dendritic outgrowth and branching, alongside directing motility (*50*). Multiple Rac GEFs have been reported to regulate Rac1 activity in a spatiotemporally dynamic

manner that is required in the nervous system (*51*). β-Pix, one of these Rac1 GEFs (*52*), participates in various signalling pathways that modulate neurite and dendrite outgrowth as well as motility (*53-55*). LRRK2 interacts with and phosphorylates β-Pix, which is also a known GEF for LRRK2 (*56*), causing uncharacterized effects on β-Pix function. Phosphorylation of β-Pix can alter its GEF activity towards target proteins (*57*). Therefore, it is conceivable that LRRK2 phosphorylation of β-Pix could reduce its GEF activity towards Rac1, and in turn locally inhibit or reduce Rac1 activity. Thus, CLCs recruit LRRK2 to early endosomes to regulate Rac1 activity, possibly via β-Pix phosphorylation, consequently affecting local actin and cytoskeletal rearrangements. Altered Rac1 activity could hereby contribute to the reported spine (*37*) and neurite outgrowth phenotypes in primary neurons from LRRK2$^{-/-}$ mice (*55, 58, 59*).

In general, the subcellular localization of small GTPases such as Rac1 is crucial to their proper cellular functioning. To be active, small GTPases have to associate with membranes (*60, 61*). Proper regulation of membrane association and dissociation is therefore essential to maintain small GTPase homeostasis. We demonstrate that knock down of LRRK2 and/or CLC in cells and the *Drosophila* eye leads to enhanced Rac1 activity. As Rac1 activity is dependent on membrane association, if CLC/LRRK2 are involved in regulating Rac1 membrane association/dissociation at the early endosome, loss of either CLC or LRRK2 may deregulate Rac1 activity. A potential mechanism is Rac1 phosphorylation by LRRK2. Recently, LRRK2 was shown to control and regulate membrane association via phosphorylation of Endophilin A (*62-64*). In agreement with such a mechanism, Rac1 phosphorylation has been shown to decrease its activity (*65*), and abrogate its function to regulate membrane ruffling and lamellipodia (*65-67*). Thus, it is plausible that LRRK2 could phosphorylate Rac1 to control and mediate its localization to early endosomal membranes and thereby regulate its activity. The effect of

CLC/LRRK2 either controlling Rac1 GEF activity, and/or mislocalization of Rac1 by LRRK2 phosphorylation are both mechanisms that could explain the enhanced Rac1 activity we observed following CLC/LRRK2 knock down.

4.4 Endocytic mayhem in neurodegenerative disease

In neurons, endosomes are challenged by trafficking over long axonal distances from the synapse back to the cell soma to deliver proteins, such as neurotrophins, that are crucial for neuronal development, survival, and synaptic plasticity (*68, 69*). Over the last decade, trafficking in the endo-lysosomal system has emerged as a common biological function affected in multiple neurodegenerative disorders such as PD, ALS and HSPs. For example, *ALS2*, encoding for alsin a GEF for Rab5, is mutated in a recessive juvenile form of ALS leading to a truncated form of the protein that no longer associates with early endosome antigen 1 (EEA1) on early endosomes (*70-72*). This process may hamper not only cargo sorting but also endosomal maturation, hence deregulating membrane flow through the endosomal system. Furthermore, mutations in genes causative of HSPs have been linked to failure of the ESCRT machinery, revealing a role for altered endo/lysosomal trafficking in these motor neuron pathologies (*73-79*). Thus, several endo-lysosomal proteins have been associated with neurodegenerative disorders when mutated, emphasizing the importance of functional membrane trafficking pathways.

Importantly, our discovery of CLCs as direct LRRK2 binding partners provides yet another link between the clathrin machinery and PD. Alterations in CME and endosomal trafficking are emerging as a central theme in PD, as mutations in multiple endocytic proteins are associated with PD. For example, mutations in synaptojanin, a lipid phosphatase required for

CCV uncoating in CME (*80, 81*) were recently found in Iranian and Italian consanguineous families with early onset PD (*82, 83*). Mutations in auxilin, a DNAJ domain-containing protein that recruits the ATPase Hsc70 to facilitate disassembly of the clathrin cage (*84, 85*), have been identified in families featuring autosomal recessive juvenile parkinsonism (*86, 87*). Evidence of a relationship between retromer retrograde trafficking from endosomes to the TGN and PD comes with the observation that an autosomal-dominant mutation in RME-8 is causative of late onset PD (*88*). RME-8 is a DNAJ domain-containing protein, shown to regulate retromer complex function in the formation of retromer-derived vesicles (*89-92*). RME-8 also interacts with Hsc70, which drives uncoating of CCVs, and RME-8 also regulates clathrin-coats on endosomes (*90*). Mutations in Vps35, a component of the same retromer pathway, also cause late onset PD (*93, 94*). Although to date the physiological effect of most of these PD-mutated endocytic proteins remains unclear, dysfunctional endocytic trafficking clearly contributes to PD pathology.

Trafficking in the endo-lysosomal system is critical for degradation and essential to cellular homeostasis. We demonstrated for the first time that endogenous LRRK2 localizes to early endosomes where it co-localizes with CLCs and EEA1. Previously, accumulation of EGFR in the late endosomal compartment and its delayed degradation was observed upon expression of LRRK2-G2019S (*95*). This delay was attributed to a decrease in Rab7 activity impeding its fusion with lysosomes (*95*). In agreement with a function for LRRK2 at the endosomes, *Drosophila* LRRK2 homologue interacts both with Rab5 on early endosomes, and Rab7 on late endosomes where it regulates Rab7-dependent perinuclear lysosomal positioning (*96*). Similar to EGFR degradation delays, expression of LRRK2-G2019S in *Drosophila* causes alterations in late endosomes and lysosomes (*96*). Maturation of Rab5-positive early endosomes to Rab7-positive late endosomes occurs via Rab5-dependent effector recruitment that activates Rab7 (*97*).

LRRK2 was recently shown to phosphorylate Rab5 which accelerated its GTPase activity (*98*). Thus, the enhanced kinase activity of LRRK2-G2019S would reduce the levels of active Rab5, and thereby result in EGFR degradation delays due to a halt in the transition of early to late endosomes. Consistent with this hypothesis, expression of LRRK2-G2019S in astrocytes diminishes the lysosomal capacity of the cells (*99*). Additionally, LRRK2 transport in the endo-lysosomal system appears to be modulated by Rab32, another small GTPase involved in membrane trafficking events at endosomes (*100*). Our data suggest a new regulatory role for LRRK2 at endosomes. We clearly demonstrate that LRRK2, together with CLCs, controls Rac1 activity and thereby dictates actin cytoskeletal alterations. It is thus possible that CLCs recruit LRRK2 to the early endosome to control an early step in degradative membrane trafficking as well as cellular actin dynamics. Alterations in such mechanisms may, in part, contribute to the pathophysiology of LRRK2-PD.

4.5 LRRK2 function in hiPSCs and PD drug development

LRRK2 mutations are the most common genetic cause of PD (*101*), but more importantly, in contrast to other PD genes, clinical symptoms of LRRK2-associated PD are typically identical to idiopathic PD (*102-104*). Thus, it is conceivable that the underlying molecular pathways contributing to disease are similar for LRRK2 and idiopathic PD. Studying LRRK2's fundamental biological function as well as its disease-associated mutants may therefore also provide critical insights into the pathways altered in idiopathic PD. hiPSCs are a powerful novel human *in vitro* model to study PD which allows for the use of patient derived DAergic neurons and their isogenic controls. Given the lack of understanding of LRRK2's basic cellular

functions, we aimed to investigate LRRK2's role in DAergic neurons derived from an established control hiPSC line, instead of using patient derived DAergic neurons. As DAergic differentiation efficiency is problematic (*20*), we successfully combined elements from two protocols (*21, 22*) to enhance the final population of DAergic neurons. Caution is still required as our final cultured neurons are not entirely DAergic and neither do they contain their striatal targets, hence they do not fully represent the human brain in a dish. Furthermore, we have used immunofluorescence to show neuronal connectivity, however we have not performed electrophysiological experiments to functionally examine neuronal connectivity. Nonetheless, the new technique of generating human DAergic neurons remains a powerful *in vitro* tool to study PD, and our optimized protocol yields highly differentiated DAergic neurons. Moreover, having obtained CRISPR-Cas9 edited triple Flag tagged endogenous LRRK2 hiPSCs, we will be able to study the localization and function of LRRK2 in DAergic neurons. So far, research has almost exclusively focused on G2019S-patient derived DAergic neurons and shown: i) an increase in α-synclein most likely due to lysosomal-autophagic flux dysfunction (*17, 105, 106*), and ii) reduced neurite length and arborization (*105, 106*) compared to control neurons. However, the underlying molecular mechanism(s) of these G2019S phenotypes, assumed to be caused by increased kinase activity of LRRK2, remain unclear. Importantly, none of these studies have shown endogenous LRRK2 localization or cellular function. Thus, future efforts should be focused on obtaining a better understanding of LRRK2's cellular functions, as such knowledge may also contribute to defining potential drug targets that improve DAergic neuron survival in PD.

Importantly, current PD treatment exclusively addresses symptomatology, and does not slow down, halt or reverse the progression of DAergic neurodegeneration. A wide variety of medical and surgical treatments are available for PD, all aiming to: i) increase dopamine levels,

and (ii) decrease muscarinic cholinergic levels in the brain (*107*). Pharmacological drugs used to treat motor symptoms are: Levodopa, monoamine oxidase B (MAO B) inhibitors, DA agonists, COMT inhibitors, anticholinergic agents, and Amantadine (*107-110*), but their effectiveness declines over time as the disease progresses. Surgical intervention, such as deep brain stimulation, can also be used in a subset of PD patients (*111*), and countless efforts are being invested in stem cell implantation possibilities, first using fetal mesencephalic tissue (*112-122*) and now using hiPSCs (*123, 124*) to replace the missing DAergic neurons. PD patients would, however, greatly benefit from unraveling the underlying molecular pathways altered in PD in more detail, as this would allow for targeted drug design that may improve therapeutics for PD and halt its progression. Thus far, evidence suggests that LRRK2 plays diverse functions in the endocytic system (*25, 62-64, 95, 96, 98, 99, 125-130*). Beyond localization and functional studies using our triple Flag tagged endogenous LRRK2 hiPSCs, future research may include a knock down target screen of known endocytic proteins to identify novel interacting partners that influence LRRK2 localization and function. Such a screen could provide much needed novel drug targets which may aid in the design of more specific drugs that target the affected PD pathway(s) and inhibit DAergic neuron degeneration. As LRRK2-associated PD patients present with identical characteristics as idiopathic patients (*102-104*), which account for 90-95% of all PD cases (*131*), functional LRRK2 drug screens could therefore have broad implications for the treatment of PD.

4.6 Conclusion

The work presented in this thesis addresses the cellular function of LRRK2, and presents optimizations to generate highly differentiated human DAergic neurons in which endogenous LRRK2 is triple Flag tagged. First, we identified CLCs as novel interacting partners of LRRK2. We discovered that LRRK2 functions in concert with CLCs on endosomes to limit Rac1 activation and control actin cytoskeleton dynamics. Secondly, we aimed to study LRRK2 cellular function in DAergic neurons. For this purpose, we adapted currently existing hiPSCs differentiation protocols to attain a highly differentiated and mature final population of DAergic neurons. We then used CRISPR-Cas9 technology to genome edit hiPSCs. These studies advance our fundamental understanding of LRRK2's cellular function while also providing a new tool to study LRRK2 in human DAergic neurons in the future.

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