

Structural aspects of the regulation of Parkinson's Disease-associated LRRK2 kinase by Rab GTPases



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Declaration

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Summary

The Rab family of small GTPases are the premier organisers of endocytic and secretory pathways in cells. They behave as molecular ‘on/off’ switches and in their active GTP-bound state recruit soluble effector proteins to coordinate sequential trafficking events. In recent years, Rab GTPases have been identified as key players in Leucine-rich repeat kinase-2 (LRRK2)-mediated signalling pathways. LRRK2 is a large multi-domain protein containing an enzymatic GTPase and kinase core surrounded by multiple accessory domains. Mutations in LRRK2 are found to be a significant risk factor for both heritable and sporadic development of Parkinson’s disease, and typically result in the over-activation of the kinase domain. LRRK2 phosphorylates a subset of Rabs including Rab8, Rab10 and Rab12 in the Switch 2 helix and modifies their interactions with effectors and regulatory proteins. This activity is regulated upstream by Rab29, which recruits LRRK2 to Golgi compartments *via* its N-terminus and activates the kinase domain. Furthermore, the related Rab32-subfamily GTPases, Rab32 and Rab38, also interact with the N-terminus and are important for the localisation and trafficking of LRRK2 in cells. The N-terminus of human LRRK2 begins with armadillo repeat motifs (ARM) followed by ankyrin repeats (ANK), however the molecular basis of LRRK2:Rab complex interactions within these domains is currently unknown. Here we show that all members of the Rab32-subfamily bind to an identical site within the ARM domain of LRRK2. Biophysical analyses reveal affinities in the low micromolar range and complex formation is GTP-dependent. The crystal structures of uncomplexed Rabs suggest residues within the switches that likely mediate interactions with LRRK2, and mutational analyses reveal a positively charged residue in Switch 1 is critical for binding of Rab32/38. However, the equivalent mutation in Rab29 is dispensable for the interaction. Furthermore, homology modelling and mutational studies of the LRRK2 ARM domain identify a negatively-charged surface that represents the Rab binding site. These structural and biochemical studies provide insight into the molecular interactions between LRRK2 and Rab GTPases, and raise interesting questions concerning the regulatory functions of Rab29 versus Rab32/38 in mammalian cells and their role in Parkinson’s disease.

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Abbreviations

Abbreviation	Definition
α -syn	α -synuclein
ALP	Autophagy-lysosomal pathway
AMP	Adenosine monophosphate
ANK	Ankyrin
ARM	Armadillo
β_2 AR	β_2 -adrenergic receptor
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CDR	Complementarity determining region
<i>C. elegans</i>	Caenorhabditis elegans
CL-MS	Chemical cross-linking with mass spectrometry
<i>C. tepidum</i>	Chlorobium tepidum
COP	Coat protein
COR	C-terminal of ROC
DA	Dopaminergic
DCT	Dopachrome tautomerase
DENN	Differentially expressed in normal and neoplastic cells
<i>D. discoideum</i>	Dictyostelium discoideum
<i>D. melanogaster</i>	Drosophila melanogaster
Drp1	Dynamin-related protein 1
DTT	Dithiothreitol
<i>E. coli</i>	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome antigen 1
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast protein liquid chromatography
GDF	GDI-Displacement Factors

GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GppNHp	Guanosine-5'-[(β,γ)-imido]triphosphate
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GSK-3 β	Glycogen synthase kinase-3 β
GWAS	Genome wide association study
IEX	Ion exchange chromatography
IMAC	Immobilised metal affinity chromatography
iPSC	Induced Pluripotent stem cell
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IS	Immune synapse
ITC	Isothermal titration calorimetry
HCV	Hepatitis C virus
HPLC	High performance liquid chromatography
Hsp90	Heat shock protein 90
HVD	Hypervariable tail domain
kDa	Kilo Daltons
LB media	Luria-Bertani media
LCV	Legionella containing vacuole
LECA	Last eukaryotic common ancestor
LIC	Ligation Independent Cloning
LRO	Lysosome-related organelle
LRR	Leucine-rich repeats
LRRK2	Leucine-rich repeat kinase 2
M6PR	Mannose-6-phosphate receptor
Mant	<i>N</i> -methylantraniloyl
MAP	Mitogen-activated protein
<i>M. barkeri</i>	<i>Methanosarcina barkeri</i>
MVB	Multivesicular bodies
MW	Molecular weight

NFAT	Nuclear factor of activated T cells
OD	Optical density
P-loop	Phosphate binding loop
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PI3K	Phosphoinositide 3-kinase
PC-1	Polycystin-1
PKC	Protein Kinase C
PMSF	Phenylmethanesulfonyl fluoride
PTEN	Phosphatase and tensin homolog
PINK1	PTEN-induced kinase 1
QUICK	Quantitative immunoprecipitation combined with knockdown
Rab11FIP2	Rab11 family-interacting protein 2
RabSF	Rab subfamily
RBD	Rab-binding domain
REP	Rab Escort Protein
RabGGTase	Rab Geranylgeranyl Transferase
RIPK	Receptor-interacting protein kinase
ROC	Ras of complex
ROS	Reactive oxygen species
SCV	Salmonella-containing vacuole
SAXS	Small angle X-ray scattering
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size exclusion chromatography
SLS	Static light scattering
SNARE	soluble N-ethymaleimide-sensitive-factor attachment protein receptors
SNP	Single nucleotide polymorphisms

SNpc	Substantia nigra pars compacta
<i>S.pombe</i>	Schizosaccharomyces pombe
STxB	Shiga toxin subunit B
TBC1	Tre-2/Bub2/Cdc16
TEV	Tobacco Etch virus
TGase	Transglutaminase
TGN	Trans-Golgi network
Tyr	Tyrosinase
Tyrp1	Tyrosinase-related protein 1
UPS	Ubiquitin-proteasome system

Chapter 1: Introduction

1.1 The superfamily of Small GTPases

Cell signalling cascades and the transport and exchange of cargo through the cellular endocytic and secretory pathways are essential processes. To maintain homeostasis these systems require an elaborate network of coordination between functionally and spatially distinct organelles. Thus, communication between compartments as well as the interactions connecting a cell to its environment are imperative to cellular function and survival. Correct tissue and organ development and function relies critically on the activity of these pathways, and disruption can lead to a variety of pathological conditions. The Ras superfamily of small GTPases comprises a large group of monomeric guanine nucleotide binding proteins that act as the key regulators of virtually all these fundamental processes. Several common structural and biochemical properties are shared among members of the superfamily. Small GTPases are conserved in their primary structure and generally share between 30-55% homology with one another [1]. All are typically between 20 – 40 kDa in molecular mass, and contain guanine nucleotide binding domains with consensus amino acid sequences. They also possess effector binding domains, and act as binary molecular switches defined by their nucleotide-bound state [2, 3]. Nonetheless, considerable versatility is observed in their specialised functions to regulate such diverse cellular processes. As a result, the superfamily has been further divided into five main families: Ras, Rab, Rho, Arf and Ran proteins [4]. Phylogenetic studies have determined the separation of these families was an early evolutionary event that predates the expansion of eukaryotes, and underscores the functional diversity of the superfamily [5].

1.1.1 *Ras GTPases*

The founding members of the small GTPases encode the Ras family, originally identified as human cellular homologs of the viral *ras* oncogene, which can be mutationally activated and found in some human carcinomas [1, 6-8]. Activation of the Ras oncogene can also stimulate the rapid proliferation and transformation of cultured human cell lines [9, 10]. Ras proteins are activated by receptors bound to extracellular growth factors on the cell surface and transduce downstream signals to regulate gene expression. The mitogen-activated protein

(MAP) kinase cascade has been extensively studied and results in the transcription of genes controlling cell growth and proliferation. Ras proteins are also well known to act through the phosphoinositide 3-kinase(PI3K)/Akt signalling pathway and are implicated in cell cycle progression, cell differentiation, migration and cytoskeletal dynamics [11].

1.1.2 Rho GTPases

The first members of the Rho family were identified in 1985 [12], and in 1992 insights into their cellular functions were revealed when constitutively activated Rho and Rac were shown to induce the assembly of actin stress fibres and lamellipodia [13, 14]. Regulation of the actin cytoskeleton remains the role for which Rho GTPases are principally known, however they are now known to also influence cell polarity, membrane transport pathways, microtubule dynamics, various enzymatic activities, and gene expression [15].

1.1.3 Arf GTPases

The Arf family are the most divergent proteins of the small GTPases, and are recognised as important regulators of vesicle budding from donor compartments via the assembly of coat protein complexes [5]. Arf1 is the best studied member and mediates the membrane recruitment of COP (coat protein) 1 for budding along the biosynthetic and secretory pathways. In this way, Arf1 drives the formation of clathrin-coated vesicles at the *trans*-Golgi network [16]. Arf proteins also activate lipid-modifying enzymes and play a role in phospholipid metabolism [17, 18]. Arf6 is functionally distinct from Arf1 and exhibits a unique subcellular localisation at the cell periphery. Arf6 is involved in both endocytosis/endocytic recycling and cytoskeletal reorganisation at the cell membrane [16].

1.1.4 Ran GTPases

There is only one Ran gene found in most eukaryotic organisms, apart from in plants, however it is the most abundant small GTPase in the cell. Ran is most closely related to Rab GTPases yet it is classified as its own family due to its unique function in nucleocytoplasmic transport [5]. Unlike other small GTPases, active Ran is not membrane associated and does not possess

a C-terminal lipid modification motif [19]. Ran-GTP interacts with both nuclear importin and exportin complexes to shuttle RNA and protein cargo back and forth [20]. Interestingly, Ran has also been implicated in a transport-independent role in mitotic spindle formation, DNA replication, and nuclear envelope assembly [21].

1.1.5 Rab GTPases

The Rab GTPases were originally identified through yeast genetics, and the first mammalian homologues were subsequently purified from rat brain (Ras-like protein in brain) [22, 23]. Now, 11 Ypt proteins in yeast and ~70 human Rab proteins have been identified, making them the largest member of the Ras superfamily. Rab GTPases are considered the master regulators of intracellular trafficking, and participate in vesicle formation, transport along cytoskeletal tracks, docking, and along with the soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs) drive fusion with target membranes [17, 24].

Different subsets of Rab GTPases associate with distinct organelles and transport vesicles, thus playing a significant role in defining organelle identity and function. The ability of Rab proteins to control phosphoinositide levels of their associated membranes has been documented [25]. A change in the identity of membrane-bound Rabs present can be sufficient to convert a transport compartment to a new functionally distinct organelle over time. For example, the exchange of Rab5 for Rab7 occurs during the progression from early to late endosomes [26, 27]. It has been shown that different Rabs found on the same organelle do not mix, but are instead localised to individual microdomains [28]. Rab proteins therefore must coordinate with one another and numerous effector proteins to choreograph rapid, bidirectional trafficking pathways (Figure 1.1)[29].

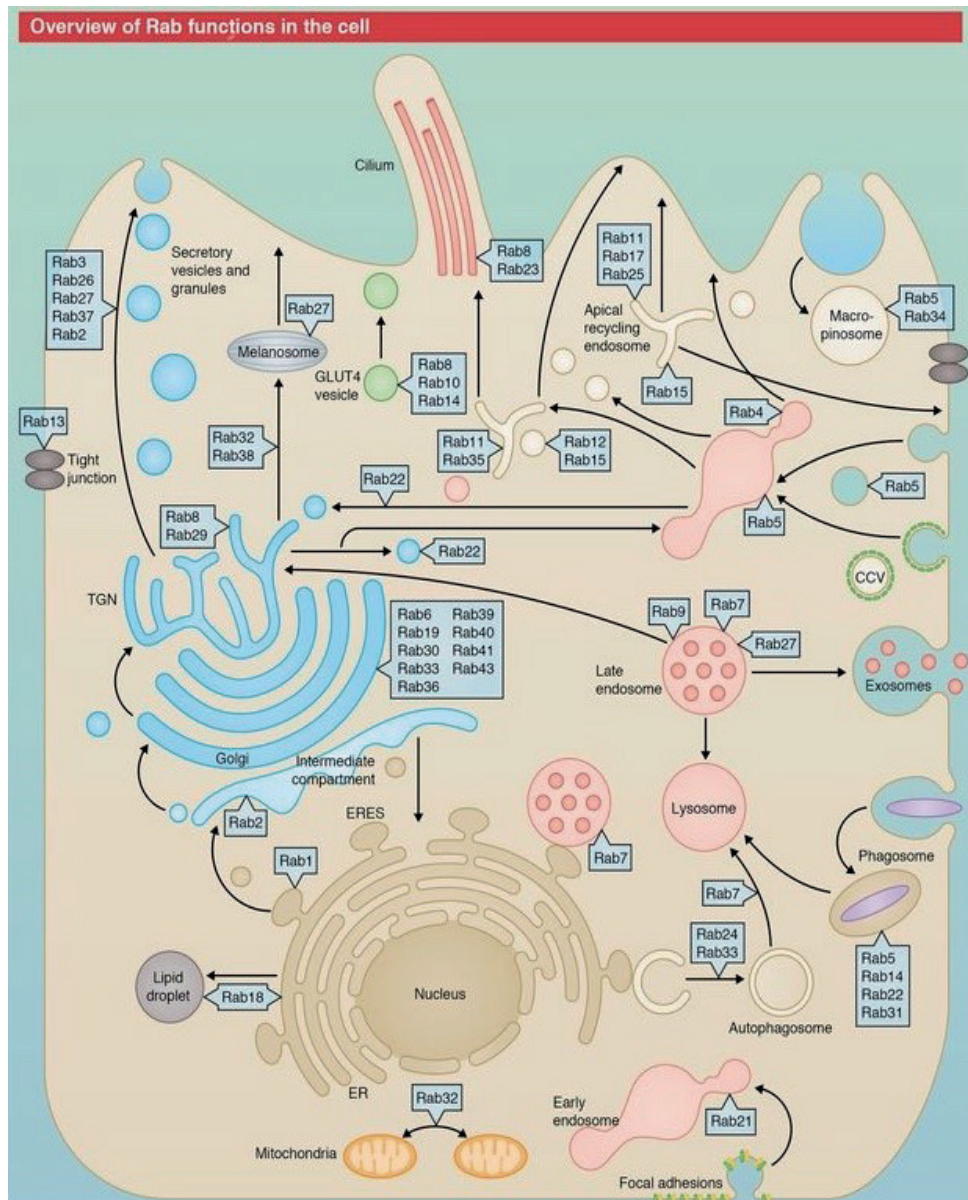


Figure 1.1. Overview of Rab functions in the cell.

Illustration of the components of the endocytic, secretory, and lysosomal/vacuolar trafficking pathways, and the Rab proteins associated with each compartment. Arrows indicate the direction of transport. Taken from Zhen and Stenmark (2015) [30].

1.2 Evolution of Rab GTPases

Evolutionary analyses have previously divided Rab GTPases into eight functional groups (Figure 1.2). Interestingly, phylogenetic trees revealed a phylogeny of function rather than of species, with Rab proteins of similar function and/or cellular localisation in different organisms co-segregating [31]. It is therefore probable that these Rabs share a common ancestry, and have maintained some of the properties from an initial set of 'ancestral' Rab proteins. Indeed, these eight functional groups may be representative of a minimal set of essential Rabs. In support of this theory only seven Rab proteins have been identified in the fission yeast *S. pombe*, the lowest of any organism investigated to date [31]. Over evolutionary history multiple gene loss and gene duplication/diversification events have occurred, and may be correlated with changes in cellular organisation. For example, Rabs linked to cilia and flagella – RabL4 and Rab23 – have been lost in most plants and fungi, and could be associated with these organisms gaining a cell wall [32]. In contrast, there has been a notable expansion in humans, who possess ~70 Rab GTPases. These paralogous genes have likely acquired subtly different roles, but remain spatially or functionally related. Such a large evolutionary expansion places emphasis on the importance of stringent control over trafficking events. Analyses predict the last eukaryotic common ancestor (LECA) already had a large repertoire of Rabs, consisting of at least 20 members [32].

Remarkably, other components of membrane trafficking, such as vesicle coat proteins or SNAREs, do not show such dynamic evolutionary patterns. This observation suggests that Rabs have directed the evolution and specificity of membrane traffic, and generated the diversity of organelle function with increasing complexity of the organism. The most evolutionarily conserved Rabs have ubiquitous expression profiles and regulate the most fundamental trafficking pathways, whereas less conserved Rabs confer a more specialised function and are frequently tissue/organ specific [33-35].

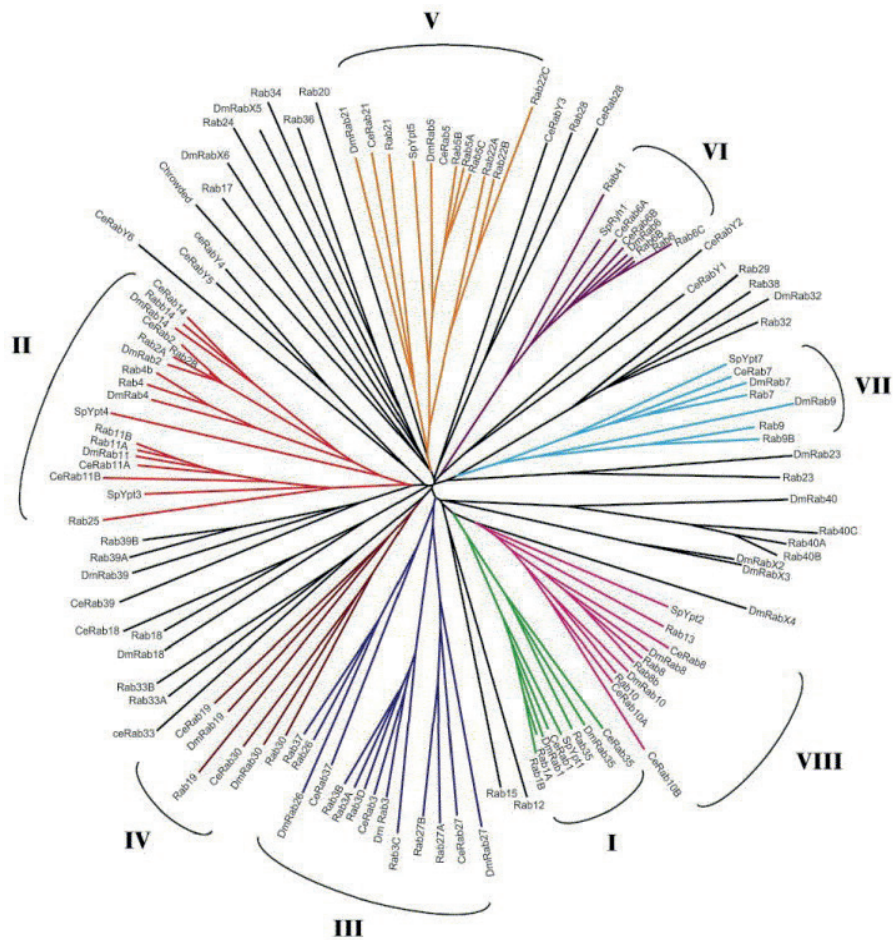


Figure 1.2. Dendrogram of the Rab family functional groups

Neighbour-joining tree diagram of human, nematode (Ce), fly (Dm) and fission yeast (Sp) Rab families. The Roman numerals (I– VIII) and coloured branches represent each evolutionary Rab functional group. Taken from Pereira-Leal and Seabra (2001) [31].

1.3 Structural properties of Rab GTPases

All members of the Ras superfamily share a similar G-domain fold, consisting of a mixed six-stranded β -sheet framed by 5 α -helices. This fold was first observed in the crystal structure of Ras in 1990 and later validated for Rab GTPases in 1999 after structure determination of Rab3a in complex with its effector Rabphilin-3a [36, 37]. Common sequence motifs had previously been identified and are referred to as G1-G3 for involvement in guanine binding and PM1-PM3 for phosphate/ Mg^{2+} binding [3]. A Mg^{2+} cofactor is essential for high affinity nucleotide binding and hydrolysis. The most important contributions are made by interactions of the β -, γ -phosphates and coordination of the Mg^{2+} ion with the sequence $\text{GX}_4\text{GK(S/T)}$ defining the P-loop ($\beta 1/\alpha 1$ loop) (PM1), and the interactions of the nucleotide base with the (N/T)(K/Q)XD motif (G2) (Figure 1.3) [38, 39].

The local arrangement of two regions, termed Switch 1 and Switch 2, defines the activity state of small GTPases and provides the main platform for effector protein binding. The switches undergo a conformational change that modifies protein activity depending on the nucleotide bound [40-42]. Whilst the overall conformation of the protein does not change significantly, inactive GDP-bound Rabs show structural variations and increased flexibility within these switch regions. In each case the active GTP-bound form is significantly more ordered (Figure 1.4C/D) [43]. The main chain NH groups of invariant residues Thr54 and Gly80 (Rab3a numbering) form two hydrogen bonds that bind the γ -phosphate of GTP, and gives way to a 'loaded-spring' mechanism. This intramolecular interaction holds the two switch regions in an ordered state, and upon GTP hydrolysis allows for structural relaxation into the GDP conformation (Figure 1.4B) [39]. The glutamine of the DX_2GQ motif (PM3) functions as a catalytic residue for the intrinsic GTP hydrolytic reaction [44]. Altogether, this universal switch mechanism is the basis for the biological activity of all small GTPases. The importance of conserved residues within these motifs can be exploited for experimental purposes to produce Rab protein variants with altered GTPase activity. The most commonly introduced 'activating' mutation is the replacement of glutamine in the DX_2GQ motif with leucine (QL mutant) preventing GTP hydrolysis and supposedly locking the Rab protein in the GTP-bound state. To generate an inactive 'GDP-locked' Rab mutant the P-loop Ser/Thr residue is substituted for an asparagine (SN or TN mutant) which disrupts the Mg^{2+} binding site and inhibits activity [44, 45].

Peripheral to the highly conserved G-domain, surfaces exhibiting high sequence variability between Rab proteins are formed by the $\alpha 3/\beta 5$ loop and the N/C-termini, and are termed 'complementarity determining regions' (CDRs) (Figure 1.4A) [37]. These regions are thought to play a role in determining the specificity of Rab-effector interactions. Additionally, the Rab C-terminus is often referred to as the hypervariable tail domain (HVD), and is where Rab sequences are most divergent from each other. This region is intrinsically disordered and varies in length between Rabs, creating a unique recognition surface for Rab targeting to specific membrane compartments [46, 47].

1.3.1 Rab family and subfamily motifs

Besides the conserved G and PM amino acid regions common to small GTPases, five Rab-specific motifs were also classified through sequence alignments. The Rab family motifs begin with RabF1 (IGVDF), a prototypical sequence of amino acids in the Switch 1 region. RabF2 (KLQIW), RabF3 (RFrsiT), RabF4 (YYRGA), and RabF5 (LVYDIT) all cluster around Switch 2 (Figure 1.4) [48]. Moreover, phylogenetic analysis of the mammalian Rab family have since revealed clusters of 'related' Rab GTPases that show unusually high homology and define subfamilies. Typically, these Rab proteins are functionally related or may be isoforms of one another, and their unique subset of effector proteins can often overlap. These regions have been termed Rab subfamily-specific motifs and are referred to as RabSF1 – RabSF4 [31, 48, 49]. In total, 44 Rab subfamilies have been classified in humans [50].

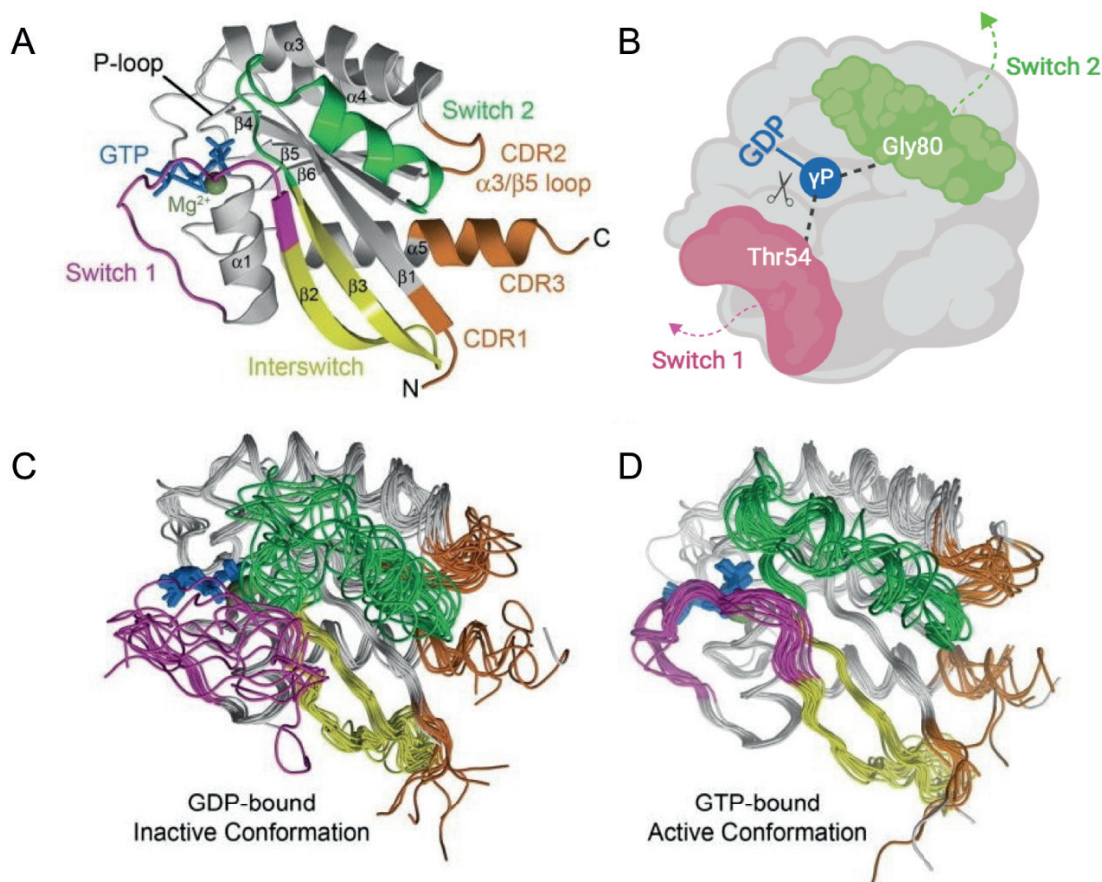


Figure 1.4. Structure of Rab GTPases

A) Ribbon model of Rab3a in complex with GTP outlining the general features of Rab GTPases (PDB code: 3RAB). Switch 1 is shown in magenta, Switch 2 in green, and the interswitch region in yellow. Complementarity-determining regions (CDRs) are coloured in orange. GTP is represented as blue sticks and the Mg^{2+} ion as a green sphere. B) A schematic diagram of the G-domain 'loaded spring' mechanism. The main chain NH groups of invariant residues Thr54 and Gly80 (Rab3a numbering) form two H-bonds with the GTP γ -phosphate. Release of the γ -phosphate after GTP hydrolysis allows the switch regions to relax into flexible conformations. C) Superposition of GDP-bound Rab GTPase structures demonstrating the increase in flexibility of the switch regions in the inactive state. Switch 2 in particular exhibits large structural variations. D) Superposition of active GTP-bound Rab GTPases whereby Switch 1 and Switch 2 adopt similar, well-ordered conformations. Adapted from Lee *et al.* (2009), Vetter and Wittinghofer (2001) [39, 44].

1.4 Cellular functions of Rab GTPases

1.4.1 Vesicle formation

The initial step in intracellular trafficking involves formation of the vesicle at the donor membrane. Early models theorised that bulk flow of material primarily occurred between various organelles. It is now understood that cargo selection into specific transport vesicles is a fundamental mechanism and is mediated by multiple factors including transmembrane cargo receptors, cytosolic coat complexes, Arf GTPases, Rab GTPases, membrane lipid composition, and membrane curvature. An example of Rab-mediated cargo sorting involves trafficking of the rhodopsin photoreceptor to primary cilia via a C-terminal VxPx targeting motif. The small GTPase Arf4 binds this motif and recruits Rab11, the Rab11/Arf effector FIP3, and the ArfGAP ASAP1 at the *trans*-Golgi to form a ciliary targeting complex [51]. A similar GTPase-dependent ciliary trafficking mechanism is observed for the receptor polycystin-1 (PC1) which binds a multimeric protein complex including Arf4, Rab6 and Rab11 via the same motif [52]. In a different case, recycling of mannose-6-phosphate receptors (M6PRs) from late endosomes to the *trans*-Golgi network is mediated by Rab9 and its effector TIP47, which functions as a sorting adaptor. Following recruitment to the membrane by Rab9, TIP47 recognizes the cytosolic tail of M6PRs resulting in cargo-specific coat assembly of late endosomal recycling buds [53].

1.4.2 Transport along the cytoskeleton

Translocation of vesicles requires directionality and efficacy of movement towards the intended destination. Targeted movement of vesicles is achieved through interaction with the cytoskeleton and its associated motor proteins, with actin filaments mediating local transport between compartments and microtubules facilitating long-range movement. It is currently unclear how different motor proteins with their associated cargos select the appropriate actin filament or microtubule within the cytoskeletal meshwork to facilitate transport to the intended target. Currently, transport is thought to be achieved via an intermittent search strategy involving ballistic movement and arrest states [54, 55]. Cargo may have multiple types of molecular motors associated, and switching between motor proteins and actin/microtubule tracks can occur at cytoskeletal intersections [56, 57]. Models suggest the

most efficient transport of cargo is achieved with microtubules radiating out from the centrosome and a thin actin cortex underneath the plasma membrane, as is found in the spatial organisation of a typical cell [54].

It is well established that the myosin class V family of actin motors associate with cargo vesicles in a Rab-dependent manner [58]. A well-studied example is the recruitment of myosin Va to melanosomes by Rab27a via its effector melanophilin, which then shuttles Rab27a-positive vesicles towards the cell periphery [59, 60]. In the case of myosin Vb, it is linked to Rab11-positive vesicles via the adaptor protein Rab11 family-interacting protein 2 (Rab11FIP2) in the endocytic recycling pathway [61]. More recently it has been shown that the myosin Va pool is shared between multiple different Rab proteins within the endocytic recycling and Golgi secretory systems, with the motor protein possessing three distinct Rab-binding domains [62].

Since microtubules are organised at the centrosome, high specificity in the attachment of particular motor proteins to vesicles is required. Kinesins are plus-end-directed motors and transport cargo towards the cell periphery, whereas minus-end-directed motors such as dyneins power vesicle traffic towards the peri-centriolar region of the cell. The kinesin motor Rabkinesin 6, which is essential for cytokinesis in mitotic cells, is a direct effector of Golgi-localised Rab6 [63-65]. Conversely, in Golgi-to-ER trafficking a different Rab6 effector, bicaudal D1, mediates attachment of Golgi vesicles to the dynein-dynactin motor complex [66]. How the same Rab GTPase can discriminate between different effectors depending on the physiological context remains poorly understood.

1.4.3 Docking and fusion to the target membrane

To initiate docking, long distance contacts are first made between the transport vesicle and the acceptor membrane through the recruitment of elongated tethering complexes by small GTPases. The early endosomal Rab5 protein has become an excellent model for the study of how Rabs coordinate with components of the vesicle docking and fusion machinery. The formation of giant early endosomes resulting from a GTPase-deficient Rab5 mutant proved to be an initial clue suggesting the involvement of Rab5 in vesicle fusion [67]. Effector screens subsequently identified a wide range of molecules, including SNAREs, that cooperate to form specialised complexes, however how these interactions serve to drive docking and fusion

events remains largely unknown. A direct interaction has been characterised between the Rab5 effectors early endosome antigen 1 (EEA1) and rabenosyn 5 with the endosomal SNAREs syntaxin 6 and syntaxin 13, and syntaxin 7 respectively [68-70]. Investigations into fusion of yeast vacuoles *in vitro* have also proven to be extremely insightful, particularly since they can be easily manipulated by both genetic and biochemical means. The assay demonstrated that the Rab7 homologue Ypt7 is required on both partner membranes, suggesting the need for bivalent Rab effector complexes [71]. This Ypt7-dependent tethering mechanism precedes the pairing of *trans*-SNAREs from opposite membranes and thus may be crucial in facilitating SNARE complex formation [72].

1.5 Regulation of Rab GTPases

Small GTPases exhibit high affinity for both GDP and GTP, but alone these proteins demonstrate only a slow interconversion between guanine-nucleotide states resulting in low intrinsic rates of GDP dissociation and GTP hydrolysis. While this excludes Rab self-activation, accessory regulatory proteins are therefore required to assist in the spatiotemporal control of Rab GTPase function.

1.5.1 Prenylation and Rab Escort Protein

When Rab proteins are first synthesized it is essential they first bind to the Rab Escort Protein (REP) which then presents the nascent Rab GTPase to Rab Geranylgeranyl Transferase (RabGGTase) for prenylation [73, 74]. REP exists in 2 isoforms in humans and consists of two separate domains. Domain I recognizes the globular G-domain and extended C-terminus of the Rab protein. RabGGTase then binds to REP domain II and recognizes conserved Rab C-terminal cysteine motifs – CC or CxC [75]. Double prenylation is observed in most Rab proteins and following the irreversible addition of the lipid tail the Rab:REP complex dissociates from RabGGTase with subsequent delivery of the Rab to its target membrane [76, 77]. REP can bind with similar affinities to both unprenylated and prenylated Rab proteins [78]. Overall, the addition of this C-terminal lipid moiety facilitates the spatial control of Rab GTPases within the cell.

1.5.2 Rab Guanine Nucleotide Exchange Factors

Guanine nucleotide exchange factors (GEFs) regulate Rab GTPases at distinct membrane surfaces, accelerating the rate of nucleotide dissociation by disrupting GTPase-nucleotide interactions [79, 80]. Importantly, GEFs catalyse nucleotide exchange in both directions but favour replacement with GTP due to its higher concentration in the cell, thus promoting the conversion of Rabs to their active state [81, 82]. In general, the displacement of Switch 1 is broadly employed by RabGEFs as a mechanism for nucleotide egress, dislodging the phenylalanine/tyrosine of a conserved hydrophobic triad spanning the switch regions that caps one face of the guanine ring. Additionally, the Mg^{2+} /phosphate site is disrupted, either directly by the GEF or as a result of conformational rearrangements [83].

Four types of structurally diverse RabGEFs have been identified thus far. The Vps9-domain containing proteins were the first characterised and are specific for the Rab5 subfamily [84, 85]. Rabex-5 is the best characterised Vps9 protein [86, 87], however there are at least 9 members in humans which contain a wide variety of different protein motifs [88]. All family members utilise a V-shaped tandem of α -helices to insert an invariant aspartate residue in close proximity to the conserved P-loop lysine. The interaction of these residues likely expels GDP by a charge repulsion effect [89, 90].

The DENN (differentially expressed in normal and neoplastic cells) domain family consists of 18 members in humans and possesses broader specificity, acting enzymatically on several different Rab proteins [91]. DENN domain proteins form a 2-domain closely packed heart-shaped tertiary structure with one longin domain commonly found in GEFs and a C-terminal lobe [92, 93]. DENN domain GEFs elicit their effect by pulling the Switch 1 into an open conformation and approaching a hydrophobic residue in the vicinity of the Rab phosphate binding site to induce a repulsive hydrophobic effect which compromises high affinity nucleotide binding [89].

In contrast, the Sec2 and TRAPP complex RabGEFs display narrow specificity. In yeast, Sec2p is recruited by the GTP-bound Rab Ypt32, and activates the downstream Sec4 GTPase in the exocytic pathway [94, 95]. In mammals, the Sec2p orthologue Rabin8 activates Rab8. Sec2p forms a parallel coiled-coil with two catalytic sites located on opposite sides of the dimer. While Sec2p does not insert residues directly into the nucleotide binding pocket, it induces large structural rearrangements in the switch regions and P-loop of Sec4 that are incompatible with nucleotide binding [96-98].

The GEF activity of the multi-subunit TRAPP complexes was originally identified in yeast toward Ypt1p, which mediates Golgi trafficking, followed by its homologue Rab1 in mammals [99, 100]. Yeast cells contain three forms of the TRAPP complexes, each possessing a stable core and varying in additional sets of subunits [101]. Two functionally distinct TRAPP complexes exist in mammals, while broadly similar to yeast TRAPPs they differ in their subunit composition and are believed to play different roles in the early secretory pathway of these organisms [102, 103]. The crystal structure of yeast TRAPP complex suggests two acidic residues approach the P-loop and help nucleotide dissociation via a charge repulsion mechanism [104].

1.5.3 Rab GTPase Activating proteins

GTPase-activating proteins (GAPs) increase the intrinsic rate of hydrolysis of GTP to GDP, allowing Rabs a more rapid return to their inactive conformation [80]. Unlike the diversity of Rab GEFs, Rab GAPs consist of one major family known as the TBC1 – (Tre-2/Bub2/Cdc16) domain GAPs. First described using yeast genetics in the early 1990's, there are now over 40 members identified in humans [105-107]. This conserved TBC domain is made up of an all helical fold comprising an N-terminal subdomain possessing most of the conserved motifs, and a variable C-terminal subdomain [108, 109]. For many Rab proteins no corresponding Rab GAP has been identified, however several GAPs have been shown to behave promiscuously toward multiple Rabs [82, 106, 110, 111]. While most GAPs utilise an Arginine finger together with the Gln residue provided by the DxxGQ PM3-motif of the substrate small GTPase for inactivation, TBC-domain GAPs were shown to possess an altered catalytic mechanism known as the dual-finger mechanism. TBC domain GAPs provide an Arginine finger analogous to other GAPs, however also provide a catalytic glutamine residue in trans that substitutes the need for the glutamine in the Rab PM3-motif [112]. During the hydrolysis reaction, the arginine provided by the GAP neutralises developing negative charges in the transition state, while the glutamine (regardless of the source) aligns a water molecule for an in-line nucleophilic attack of the γ -phosphate of GTP [89, 113]. There is only one GAP identified in humans to date that does not contain this conserved TBC domain – the Rab3GAP is a complex of 2 different proteins and acts specifically on members of the Rab3 family [114]. While no

structural data currently exists for Rab3GAP, it is believed to use a similar Arginine finger catalytic mechanism to other GAPs [115].

1.5.4 GDP Dissociation Inhibitors and GDI-displacement Factors

Extraction of Rab proteins from the membrane is essential to maintain the integrity of the Rab cycle after one round of vesicle trafficking. GDP dissociation inhibitors exclusively recognise prenylated, GDP-bound Rab proteins with high affinity, displacing them from the membrane and maintaining them in an inactive cytosolic form [78, 116, 117]. The structure of RabGDIs is highly conserved from yeast to mammals, and three isoforms of RabGDI have been identified in humans to date. GDIs are comprised of two separate domains, termed domain I and II, with domain I recognizing the globular Rab G-domain and domain II binding to and shielding the lipid tail from surrounding solvent [118-120].

It had been suggested that displacement of Rab GTPases from GDI is catalysed by proteins called GDI-Displacement Factors (GDFs). However, until now only two proteins with proven GDF activity, Pra1 and its yeast homolog Yip3, have been characterised [121, 122]. The *Legionella* protein SidM/DrrA was also proposed to have GDF activity, however structure solution of the DrrA/Rab1 complex in a nucleotide-free state indicated DrrA behaves as a RabGEF [123-125]. In general, the precise mechanisms of specific membrane targeting have yet to be elucidated, but probably involves a combination of GEFs, GDFs, effectors and other factors [126].

1.6 Effector proteins

Since different Rabs are localised to specific subcellular membrane compartments, soluble effector molecules are required to bind to implement downstream effects. As such, effectors are generally large modular proteins that also encompass other functional domains. Effector proteins interact with Rab proteins exclusively in their active GTP-bound form, with the ordered switch elements forming the primary binding interface. While the G-domain has been sufficiently conserved to allow binding to common regulatory partners such as REP and GDI, amino acid variations allow each Rab to discriminate between their own unique subset of

structurally unrelated effector proteins. As a result, the number of Rab effector proteins greatly exceeds the number of Rab GTPases. Among those characterised thus far include molecular motors, tethering proteins, sorting adapters, identity regulators, kinases and phosphatases [127]. Active Rabs recruit these cytosolic effectors via interaction of their Rab-binding domain (RBD). While small families of homologous RBDs have been identified, overall there is little to no sequence similarity observed between the RBDs from the various effectors [128].

1.6.1 Structural basis for effector specificity

The ability of Rab GTPases to recognise a diverse range of effector molecules is still not well understood, however certain determinants of specificity have been identified. Effectors interact predominantly with the surface formed by the nucleotide-dependent Switch 1-Interswitch-Switch 2 elements of Rab proteins. It has been noted that the active structures of some Rabs undergo very little conformational change upon effector binding. In this circumstance, non-conserved residues within the switch epitope are critical for achieving specific recognition [33]. Nonetheless, when the surface residues of Rab4 were mutated to match the residues involved in Rab5 RBD binding, the Rab4 mutant was still not capable of recognising Rab5 effectors. Mutation of additional core residues were required for the conversion [129]. While removed from the binding interface, non-conserved residues in the hydrophobic core of the Rab protein can influence the structural plasticity of the switches [130].

Other Rabs can undergo large conformational changes at the binding interface upon interaction with different effectors. In this case remodelling of the switch regions provides clear structural variation for the accommodation of various RBDs. A good example is seen in the rearrangement of Rab11 bound to FIP proteins showing significant changes in Switch 2, and the interaction of Rab11 with myosin-V in which both switches are extensively altered [131, 132]. The ability for Rabs to drastically modify the surface of the binding interface may allow promiscuity for effector binding.

Furthermore, an invariant hydrophobic triad within the Rab switch region is known to play an

important role in the specificity of most Rab:effector complexes. Interactions with effectors usually involve at least one of a conserved phenylalanine residue in Switch 1, a tryptophan in the interswitch region and a tyrosine/phenylalanine near the end of Switch 2. The side chain rotamers of this hydrophobic triad are influenced by the highly variable CDRs, which provides structural plasticity and therefore creates distinct binding surfaces [44, 130]. Since the CDRs overlap with both the switch regions and the RabSF motifs they may ultimately provide specificity determination for unique effector interactions [37, 44]. OCRL1 is an effector that can interact with multiple Rab proteins from different functional groups. It is thought to achieve this promiscuous behaviour by forming tight contacts with conserved amino acids and main chain atoms within the binding interface [133, 134]. OCRL1 also does not fully engage the hydrophobic triad and overall, its interactions with the most invariant components of the Switch-Interswitch allow for recognition by several unrelated Rabs.

1.6.2 Effector binding modes

Given their structural diversity, bioinformatic approaches to identify or predict novel Rab partners are limited, and effector proteins have mostly been identified through experimental methods such as yeast two-hybrid assays and GST tag pulldown assays [135, 136]. However, the growing number of Rab:effector structures available have revealed several shared modes of binding. These are classified as all α -helical, mixed α -helical, β - β zipping and bivalent binding [137].

All α -helical binding describes multiple conformations, ranging from coiled-coils to α -helical bundles. In most structures, two helices are found packed closely alongside the Rab Switch-Interswitch junction. Mixed α -helical binding shows a variation of this strategy, where a single α -helical segment interacts with the Rab Switch-Interswitch junction, while a second site of interaction is located outside this region. In this case, the interface formed by the single α -helix alone is insufficient for effector recruitment, with both interfaces being required for proper specificity and affinity. An additional binding mode finds the β 2 strand of the Rab interswitch involved in the formation of β - β zipping interactions. Here the strand from the effector protein is part of a larger β sheet and results in the formation of a contiguous β sheet that extends over both molecules. Lastly, bivalent binding involves the assembly of a heterotetramer, with two Rab proteins bound to a central homodimeric effector. The

restricted orientation to the membrane imposed by this type of assembly, and increased effector residence time are believed to be biological consequences of Rab:effector interactions of this nature [138]. Examples of Rab:effector interactions illustrating the various modes of binding are shown in Figure 1.5.

1.6.3 Functional networks of Rab:effector interactions

Certain effectors may be shared between different Rab proteins to functionally link their activities in networked cascades [29, 33]. Rab conversions can then occur whereby the activity of one Rab protein results in the sequential recruitment of the next GTPase coupled with simultaneous upstream inactivation via a specific GAP, and downstream activation via its cognate GEF to confer directionality [27, 139, 140]. In fact, proteins may encode both a RBD and a GEF/GAP domain thereby possessing dual functionality as an effector for one Rab GTPase while acting as a regulator for another [141]. In general, effector binding is assumed to be sequential and mutually exclusive. However, Vetter *et al.* (2015) showed that Rab11 is capable of binding FIP3 and Rabin8 simultaneously, forming a dual effector complex. A direct interaction between the effectors is also observed, likely stabilising the tertiary complex. This new evidence serves to further highlight how Rabs can function as important nodes in trafficking networks [142, 143].

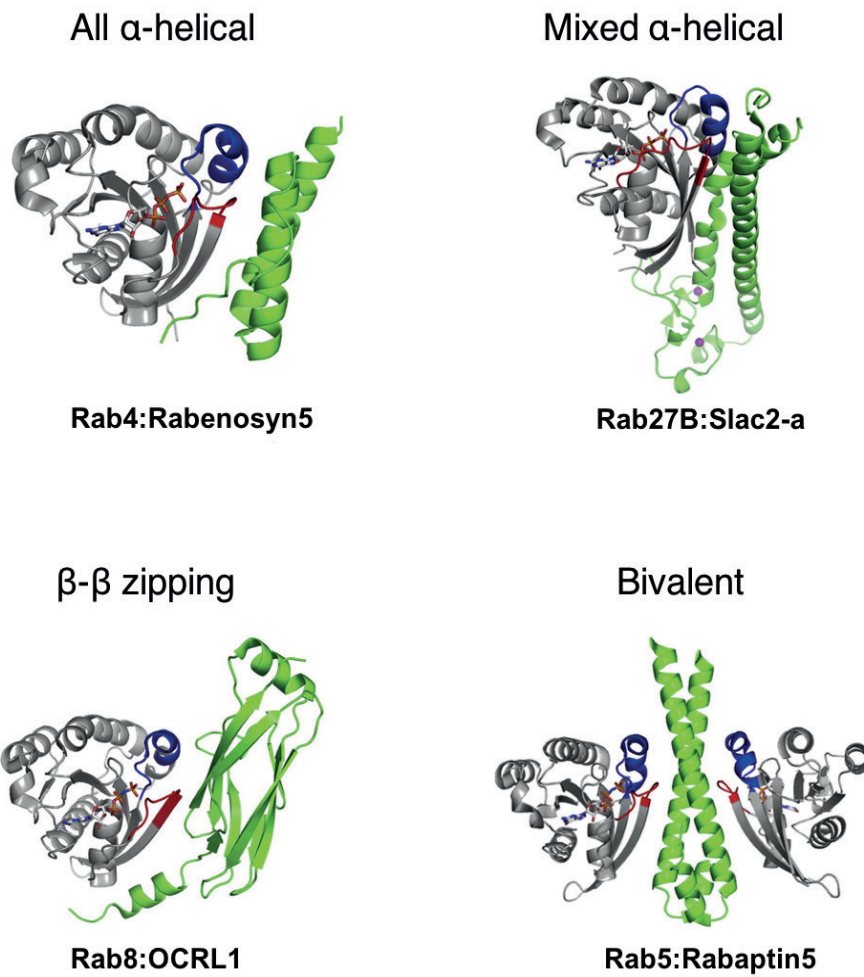


Figure 1.5. Examples of Rab:Effector binding modes

Rab proteins are shown in grey with Switch 1 and Switch 2 coloured red and blue, respectively.

Effectors are shown in green. Adapted from Khan & Menetrey (2013) [137].

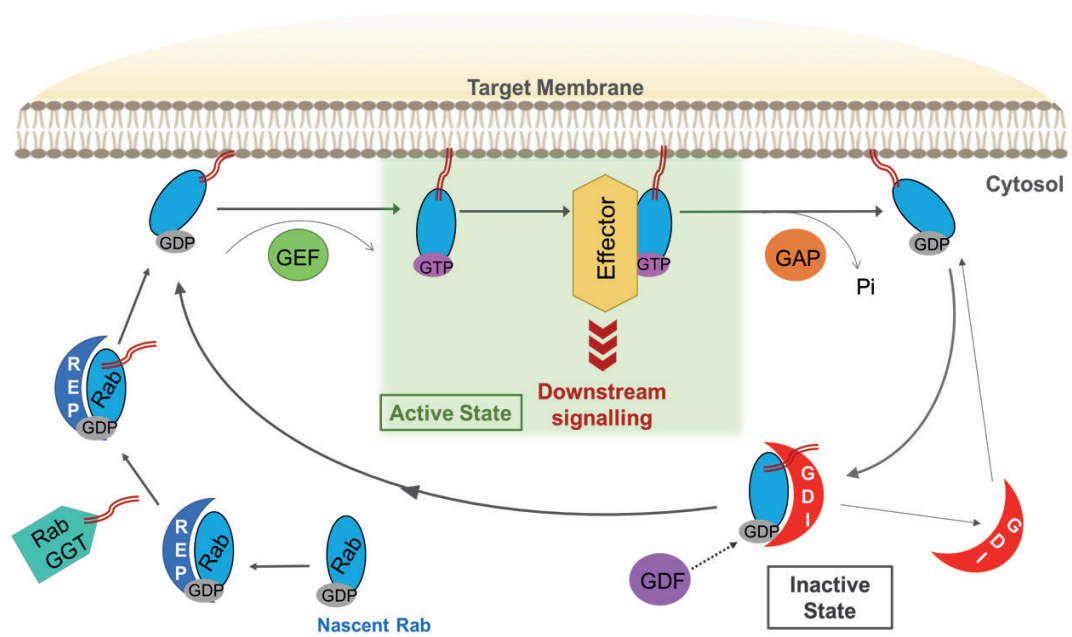


Figure 1.6. The Rab functional cycle

A nascent GDP-bound Rab associates with Rab Escort Protein (REP) and is presented to Rab geranylgeranyltransferase (RGGT) which prenylates one or two Rab C-terminal cysteine residues. REP escorts the prenylated Rab to its target membrane where it anchors via insertion of its lipid tail. Once membrane-associated, a guanine nucleotide exchange factor (GEF) catalyses the exchange of GDP for GTP and the activated Rab can interact with various effector proteins. Following the binding of its associated GTPase activating protein (GAP) accelerating the hydrolysis of GTP to GDP, a guanine nucleotide dissociation inhibitor (GDI) removes the inactive Rab from the membrane until the next cycle. Reinsertion of the Rab may be mediated by a GDI dissociation factor (GDF) following appropriate signalling.

1.7 Post-translational modifications of Rab proteins

Traditionally, controlling the activity cycle of Rab GTPases has been understood in the context of GEF, GAP, GDI and effector interactions, as previously outlined. In addition to the well characterised protein prenylation mechanism, our knowledge of the role of other post-translational modifications in the regulation of Rab targeting and Rab protein interactions has greatly expanded in recent times.

1.7.1 Phosphorylation

Phosphoregulation of Rab GTPases was first demonstrated in 1991, with Rab1Ap and Rab4p shown to be substrates of the kinase p34^{cdc2} during mitosis in both *in vitro* and *in vivo* experiments. Phosphorylated Rab1Ap was found to be predominantly membrane localised, while phospho-Rab4p was cytosolic [144, 145]. Following this, it was found that Protein Kinase C (PKC) mediated phosphorylation of Rab6C and induced its translocation to the cytosol [146].

More recently, it has come to light that Rab proteins are important substrates of certain kinases associated with Parkinson's disease. Activation of the mitochondrial PTEN-induced kinase 1 (PINK1) indirectly results in the phosphorylation of Rab8a, Rab8b, and Rab13 at a highly conserved serine residue (Ser¹¹¹) [147]. It was also shown that the ability of the GEF Rabin8 to activate Rab8A was significantly impaired by phosphorylation at this site [147]. The use of phosphorylation as a means of regulating Rab-mediated signalling was further enforced by the finding that Leucine-rich repeat kinase 2 (LRRK2) directly phosphorylates a subset of Rab GTPases (Rab3A/B/C/D, Rab5A/B/C, Rab8A/B, Rab10, Rab12, Rab29, Rab35 and Rab43) on an evolutionary conserved Thr/Ser residue in Switch 2, verified by both *in vitro* and *in vivo* experiments [148, 149]. Since the Switch 2 region forms a critical part of the Rab binding interface, it would be expected that phosphorylation at this site can alter binding of both effectors and regulatory proteins. Prominent examples will be elaborated upon in Section 1.16.1.

Currently, few of the counteracting phosphatases that target Rab proteins have been identified. Rab7 is reported to be dephosphorylated at two conserved residues, S72 and Y183, by the well-studied tumour suppressor PTEN. Dephosphorylation at these sites is required for

Rab7 targeting to endosomes, with phosphoregulation of Rab7 controlling its localisation and thus governing EGFR trafficking through late endosomes [150].

1.7.2 AMPylation and phosphocholination

The intracellular bacterial pathogen *Legionella pneumophila* utilises unconventional modifications of Rab GTPases to manipulate host cell signalling processes during infection. The bacterial effector protein DrrA was originally identified as a GEF for Rab1, redirecting it to the Legionella containing vacuole (LCV) [151, 152]. It was later observed that DrrA possesses a novel function in addition to its GEF activity, and can AMPylate the Switch 2 Tyr77 residue of Rab1B. The covalent attachment of adenosine monophosphate (AMP) locks Rab1 in an active GTP bound state by disrupting its interaction with GAPs [153]. In addition, the bacterial effector SidD is a de-AMPyase for Rab1 and its de-AMPylation activity is required for the release of Rab1 from LCVs [154-156]. In this way, *L. pneumophila* hijacks the activation and deactivation of Rab1.

Following the characterisation of Rab1 AMPylation, another novel modification of Rab proteins during *L. pneumophila* infection also emerged. The bacterial protein AnkX was shown to function as a phosphocholine transferase, resulting in the phosphocholination of Rab1 on Ser79 [157, 158]. It was determined that Rab35 was also a target for DrrA and AnkX, with phosphocholinated Rab35 showing a marked decrease in its association with the innate GEF Connecdenn [157]. The effector Lem3 was identified as a dephosphorylcholinase, although the biological significance of Rab phosphocholination still needs to be fully elucidated [159, 160]. Both AMPylation and phosphocholination were shown to also abolish GDI-complex formation with Rab1b and Rab35 [161].

1.7.3 Ubiquitination and serotonylation

Additional PTMs have been identified that modify Rab protein activity in both normal and infectious processes. Rab11 associated with the β_2 -adrenergic receptor (β_2 AR) can be ubiquitinated by the HACE1 ubiquitin ligase. Ubiquitination mediates the activation of Rab11 as demonstrated by increased recycling of β_2 AR, although the mechanism of activation

remains unclear [162]. Ubiquitination of several Rab GTPases associated with the endoplasmic reticulum by the bacterial SidE effector protein, secreted by *L. pneumophila*, has also been reported [163].

In the case of Rab4, another interesting modification involves the covalent attachment of serotonin by the enzyme transglutaminase (TGase). Serotonylation results in the constitutive activation of Rab4, triggering α -granule exocytosis from platelets [164]. Furthermore, TGase has also been shown to serotonylate Rab3a and Rab27a, thereby promoting insulin secretion within pancreatic β -cells [165]. Serotonylated GTPases then become inactivated by proteasomal degradation [165, 166].

Overall, the identification of PTMs as important functional orchestrators of Rab activity illuminates our understanding of the complexity of Rab protein regulatory mechanisms.

1.8 The Rab32 subfamily

The Rab32 subfamily of Rab GTPases consists of the members Rab32, Rab38 and Rab29 and are most closely related to the Rab23, Rab7 and Rab9 GTPases of supergroup VII. Within the clade Rab29 arises as a sister group of Rab32/38, and all possess a unique, ultra-conserved stretch of four amino acids (FALK) at the end of Switch 1 [167]. Although the significance of these conserved residues is currently unknown, they may confer important functional properties of the subfamily.

Rab32 was first identified in platelets [168], but has been shown to be highly expressed in the liver, kidney and heart, and to a lesser degree in the pancreas, lung, bone marrow, testis and colon [169]. The expression profile of Rab38 is much more specific and is mainly restricted to melanocytes, platelets, and lung alveolar type II cells [170-173]. Rab32 and Rab38 share 75% sequence identity. Both proteins perform similar functions organising the trans-Golgi network (TGN) and play critical roles in the biogenesis and regulation of lysosome-related organelles (LROs). LROs share common features with both lysosomes and endosomes but possess a unique morphology and cell type-specific compositions to carry out specialised functions. LROs include, but are not limited to, melanosomes, lytic granules, MHC class II compartments, platelet-dense granules, and lamellar bodies in alveolar endothelial cells, and thus function in pigmentation, immunity and pulmonary homeostasis [174, 175].

Rab29, also known as Rab7L1, is closely related to Rab32/38 sharing 57%/52% sequence identity respectively. It is ubiquitously expressed which is suggestive of a fundamental physiological function [176]. Like Rab32/38, it too functions in maintaining the integrity of the TGN, regulates phagocytosis, and controls retrograde trafficking in the endocytic pathway [176, 177].

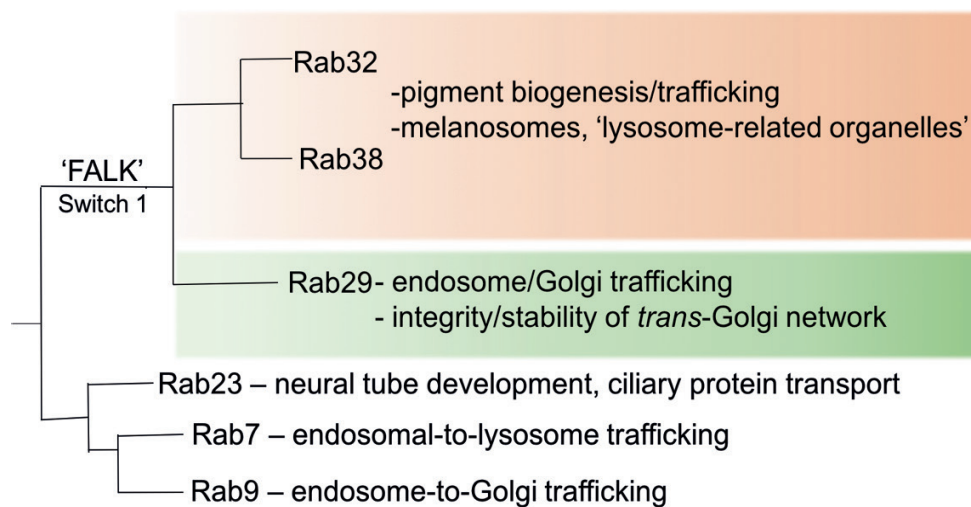


Figure 1.7. Simplified evolutionary tree of human Rab GTPases.

The Rab32 subfamily possess a conserved Switch 1 'FALK' motif not found in their closest relatives. Rab29 results as a sister group of Rab32/38. Adapted from Coppola *et al.* 2016 [167].

1.8.1 Role in melanogenesis

Rab32/38 have been best characterised for their role in endosomal trafficking and melanogenesis and coordinate the trafficking pathways of melanogenic enzymes such as tyrosinase (Tyr), tyrosinase-related protein 1 (Tyrrp1) and dopachrome tautomerase (Dct) [178, 179]. VARP (VPS9-ankyrin-repeat protein), the first Rab32/38 effector identified, mediates this process [180, 181]. The structure of the VARP Ankyrin Repeat-Containing Domain1:Rab32 complex was solved by X-ray crystallography, and represents the first Rab32 structure to be published (PDB code: 4CYM) (Figure 1.8) [182]. Dimerisation of the VARP:Rab32 heterodimer in the crystal was shown to also exist in solution and many GTPase:effector complexes are known to form dimers in this way [33, 44, 137]. Such bivalent interactions are hypothesized to impose further control over the selectivity and duration of effector binding [183].

It has been demonstrated that myosin Vc also acts as an effector for Rab32/38 and their interaction has been proposed to control trafficking of melanosomal cargoes like Tyrrp1 and VAMP7 to the melanosome via the actin cytoskeleton [184]. BLOC-3, a Rab32/38 GEF, and RUTBC1 which possesses GAP activity for Rab32/38 are thought to be recruited to the melanosome via Rab9 and thus regulate Rab32/38 cycling during this process [185-187]. Multiple disease states may arise from deregulation or disruption of Rab32/38 signalling pathways. Rab38 is found to be highly expressed in melanoma and presentation of a Rab38 peptide antigen at the cell surface can result in a specific antibody response in melanoma patients [188, 189]. Mutations in BLOC-3 are found in Hermansky-Pudlak syndrome in humans, a disorder characterised by impaired blood clotting and partial loss of pigmentation [187]. Rab38-deficient rats also serve as a model for the disorder [190], implicating dysfunction of Rab38 trafficking in the development of the disease. Furthermore, the underprenylation and reduced membrane targeting of Rab38 is causally linked to blindness in choroideremia patients, arising from ocular hypopigmentation and degeneration of the melanosome enriched retinal pigment epithelium of the eyes [191].

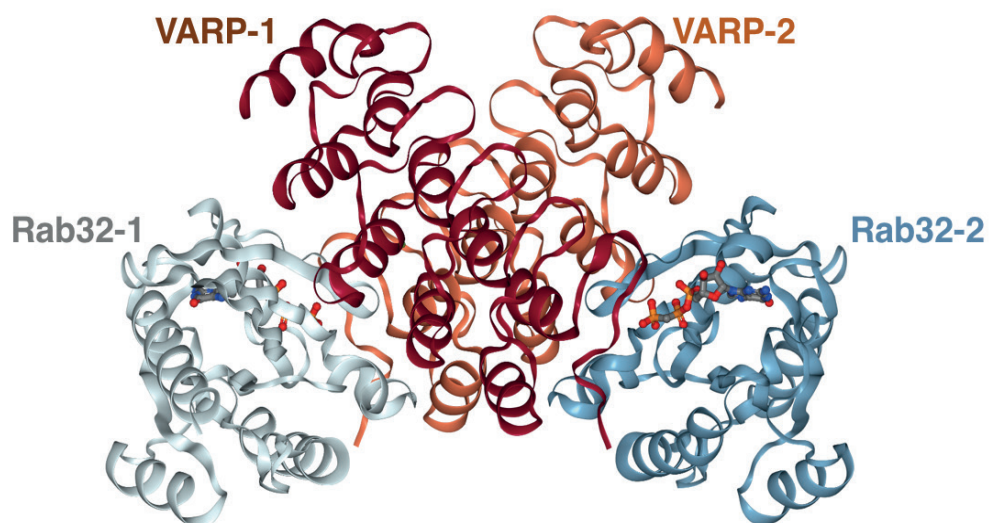


Figure 1.8. Structure of VARP ANKRD1:Rab32(GppCp) complex

Ribbon representation of the VARP:Rab32 complex. Each VARP subunit makes extensive contacts with both Rab32 molecules in the crystal structure giving rise to a dimer of heterodimers. Analytical gel filtration and size exclusion chromatography with multiangle light scattering provides evidence from the existence of this heterotetramer in solution (PDB code: 4CYM) [182].

1.8.2 Role in mitochondrial dynamics

Rab32 was first identified as an A-kinase anchoring protein and plays an important role in mitochondrial dynamics [169]. The mitochondrial-associated membrane (MAM) is an ER signaling hub that facilitates ER-mitochondria crosstalk. The dual localisation of Rab32 to ER and mitochondria suggested its importance in modulating MAM properties, ER calcium handling, apoptosis and PKA signalling [192-194]. Rab32 family proteins also interact with Drp1 (dynamin-related protein 1) to mediate mitochondrial fission events. Indeed, the control of mitochondrial dynamics is believed to be the most ancient role of the subfamily [195].

A connection between ER-mitochondria crosstalk and inflammation has been established [196, 197], and it has been reported that Rab32 is significantly upregulated during LPS induced neuroinflammation in a mouse model [198]. More recent findings demonstrate that ER stress induces Rab32 in the multiple sclerosis brain, subsequently altering mitochondrial dynamics and negatively impacting neurite outgrowth and neuronal function [199]. The *RAB38* locus has also shown a suggestive association in multiple sclerosis [200].

Autophagosome formation is reported to originate at the MAM, and Rab32 is required for this process [201, 202]. It is known that autophagy also affects lipid storage, and the role of Rab32 in lipid storage in *Drosophila* through autophagic processes was demonstrated by Wang *et al.* (2012)[203].

1.8.3 Role in infection

Small GTPases are well known to be involved in the phagocytic digestion of intracellular bacteria, and thus are a target for invading pathogens in order to promote the infection [204]. The Rab32 subfamily have been implicated in foodborne *Salmonella enterica* infection. The serovar *S. Typhimurium* can infect a wide range of hosts, and the *GtgE* gene product, a cysteine protease encoded by the bacterium, exhibits proteolytic activity towards the Rab32 subfamily. The ability to cleave and abolish the activity of these Rab GTPases has been linked to broad host selection in comparison to *S. Typhi* which only infects humans [177, 205]. The *Salmonella*-containing vacuole (SCV) is a modified phagosome where the bacteria survive and replicate following invasion [206]. Since the SCV constitutes a LRO, inactivation of Rab32 subfamily members may prevent the subsequent delivery of antimicrobial factors and thus contributes to the infectious mechanism. The Rab32 subfamily are proteolytically cleaved in

the Switch 1 region between residues G59 and V60 (Rab32 numbering), with GtgE exclusively targeting the GDP-bound form. The structure of catalytically inactive GtgE in complex with Rab32-GDP has been solved (PDB code: 5OEC/D)[207]. An additional *S. Typhimurium* effector SopD2 possesses GAP activity against Rab32 members, making Rab-GDP substrate readily available to GtgE and together ensuring GTPase activity is completely abolished [177, 208]. Curiously, it was noticed that GtgE was absent from the human adapted *S. Typhi* serovar, which causes typhoid fever, a life-threatening systemic disease. Intracellular replication of the bacterium is much slower than in cells infected with *S. Typhimurium*, perhaps advantageous for avoiding immune detection and leading to persistent infection. In this circumstance Rab29 was shown to be recruited to *S. Typhi*-containing vacuoles and may be hijacked for typhoid toxin transport to the cell periphery for extracellular release [177].

Rab32/38 and its effector proteins PHB/PHB-2 has been shown to be crucial in limiting the intracellular bacterial growth of *Listeria monocytogenes* in dendritic cells [209]. Furthermore, Rab32 was identified as a susceptible gene in a large-scale genome wide association study (GWAS) on leprosy, and is also known to localise to the *M. tuberculosis*-containing phagosome, however the precise roles of Rab32/38 in *Mycobacterium* infections have yet to be elucidated [210, 211]. More recently, Rab29 was also shown to also be targeted by the PknG kinase in mycobacterial infection. Although Rab29 was not a substrate for PknG kinase activity *in vitro*, a direct interaction was observed. Further analysis revealed PknG functions to block the conversion of Rab29-GDP to active Rab29-GTP, thus preventing its translocation to bacilli-containing phagosomes. Inhibition of Rab29 activity impairs the subsequent recruitment of downstream Rabs and effectors such as EEA1, Rab7 and LAMP2, which are crucial for the induction of phagosome-lysosome fusion during infection [212].

Rab proteins have also been implicated in viral infection, with Rab32 reported to be involved in the infection of Hepatitis C virus (HCV), a strong risk factor for the development of chronic liver diseases including liver cirrhosis and hepatocellular carcinoma [213]. It has been demonstrated that Rab32 activity may be modulated by HCV to facilitate virion assembly and promote infection [214].

1.8.4 Role in immune synapse assembly and ciliogenesis

The activity of the *S. Typhimurium* GtgE protease outlined in Section 1.8.3 was exploited for research purposes to elucidate the role of Rab29 in both immune and ciliated cells. Since neither Rab32 or Rab38 are expressed significantly in T-cells Rab29 was specifically depleted using transfected GtgE, resulting in a severe impairment in TCR recycling to the immune synapse (IS) [215]. Together with Rab8 and the intraflagellar transport protein IFT20, Rab29 was found to regulate trafficking of Rab11-positive endosomes carrying TCR cargo to the polarised centrosome via interaction with dynein motor proteins, ensuring their correct positioning for IS delivery [215, 216]. Rab29 participates in a similar pathway in ciliated cells where it functions to promote cilium growth. In this circumstance, Rab29 regulates the transport of endosome-associated ciliary cargo to the base of the cilium. Cilia length was significantly shortened and trafficking of the receptor *Smoothed*, which localises to the ciliary membrane in response to Hedgehog signalling, was inhibited in Rab29-depleted cells [215, 217].

1.8.5 Role in retromer trafficking

The retromer is a protein coat complex that mediates endosomal sorting and trafficking, recycling specific transmembrane proteins and receptors to the plasma membrane or the TGN. It is a heteropentameric complex composed of a conserved VPS26-VPS29-VPS35 trimer and a sorting nexin dimer [218]. The dimer is made up of either SNX1 or SNX2 in complex with SNX5, SNX6, or SNX32. Proteomic analyses have now identified at least 150 protein cargos trafficked through a retromer-dependent mechanism, however the cation-independent mannose-6-phosphate receptor (CI-M6PR) and Shiga toxin subunit B (STxB) are two of the best studied examples [219-221]. Rab29 also mediates the trafficking of M6PR [176], and a study by MacLeod *et al.* (2013) suggests that Rab29 interacts with Leucine-rich repeat kinase 2 (LRRK2) to regulate retromer function [222]. An additional link was documented by Waschbusch *et al.* (2019), providing evidence that Rab32/38 specifically interacts with the SNX6 transient subunit, and can affect both CI-M6PR and STxB trafficking in an SNX6-dependent manner [223]. It may be noteworthy that VPS35 and VPS29 are also direct binding partners of VARP, an effector of Rab32/38 outlined in Section 1.8.1 [88].

1.9 Rab GTPases in Disease

Given the importance of intracellular trafficking, perturbations in Rab function or their interaction partners often physiologically manifests in a range of disease states that can be infectious, endocrinological, neurological or even tumorigenic in nature. A number of common multifactorial human diseases are associated with Rab dysfunction; the progression of several cancers by loss of cell polarity or metastatic transformation [224], abnormal trafficking of the GLUT4 transporter in patients with type 2 diabetes [225], altered function of Rabs in post-Golgi trafficking and Huntington's disease [226], and evidence of Rab disruption in Parkinson's disease by α -synuclein [227] are prominent examples. Furthermore, rare monogenic diseases can arise from mutations in Rab proteins or their effectors, such as Griscelli syndrome, choroideremia, non-specific mental retardation, Charcot-Marie-Tooth disease, and Warburg Micro syndrome. Exhaustive reviews of Rab GTPases implicated in both monogenic and complex diseases are found elsewhere in the literature [35, 228, 229].

1.9.1 The Rab32 subfamily in Neurodegenerative Disease

Given their uniquely polarised structure and function, it is likely that neurons may be particularly sensitive to disruptions in vesicle trafficking. Abnormal endocytic trafficking is a feature of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Down's Syndrome and Parkinson's disease [230, 231]. For example, a hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (C9orf72) gene is the most common genetic cause of ALS and frontotemporal dementia (FTD) [232]. A novel interaction was recently characterised between the C9orf72 protein and Rab29, which together mediate *trans*-Golgi vesicle trafficking [233]. What's more, Rab38 has also been implicated in FTD following a genome-wide association study. Two non-coding single nucleotide polymorphisms (SNPs) identified at a locus encompassing both Rab38 and cathepsin C may result in decreased expression of Rab38 and is speculated to be the mechanism by which FTD association at this locus is mediated [234]. Perhaps most notably, there has been accumulating evidence in recent years implicating the Rab32 subfamily in the pathogenesis of Parkinson's disease, primarily mediated through their interaction with Leucine-rich repeat kinase-2 (LRRK2). The role of LRRK2 and its interaction with Rab GTPases will be further explored in the following sections.

1.10 Parkinson's Disease

Parkinson's disease is a neurological disorder first medically characterised in the 19th century by James Parkinson, and later expanded by Jean-Martin Charcot [235]. Pathologically, it is identified by the hallmark progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), and the presence of abnormal protein aggregates of α -synuclein (α -syn), defined as Lewy bodies, in surviving cells [236]. This resultant deficiency in dopamine neurotransmission within the basal ganglia leads to the motor symptoms which classically defined the disease, including bradykinesia, muscular rigidity, resting tremor, and postural and gait impairment. In recent years, however, the heterogeneous nature and symptomatology of Parkinson's disease has been recognised, and our understanding of the complexity of the disorder continues to evolve. Parkinson's disease arises from a complex association of both genetic and environmental factors affecting numerous cellular processes, and thus the risk factors are rather broad spectrum and difficult to universally define. With only 5-10% of PD cases exclusively familial in origin, the vast majority of cases are apparently sporadic, with age, gender, and ethnic background most likely to play a role in the development of the disease [237-239].

A number of causative genes resulting in monogenic forms of PD have been identified over the past two decades, the first of which were mutations in the *SNCA* gene encoding α -synuclein (α -Syn), a small protein widely expressed in the brain [240]. Since then, linkage analysis in rare kindreds with inherited parkinsonism have found 11 genes and at least 16 different loci associated with inherited forms of PD. Six of these genes have been proposed to mediate autosomal dominant forms of PD: *SNCA*, *LRRK2*, *VPS35*, *EIF4G1*, *DNAJC13*, and *CHCHD2*, most frequently as a result of missense mutations, but also through multiplications or splice-site alterations (Table 1.1) [239, 241].

Locus	Gene	Chromosome position	Inheritance	Protein	Function	Phenotype
PARK1/4	SNCA	4q22.1	Dominant, rarely sporadic	a-synuclein	Unclear	Broadly varying phenotype ranging from typical late-onset to severe atypical. Major component of Lewy bodies
PARK8	LRRK2	12q12	Dominant, sporadic	LRRK2	Ser/Thr kinase	Late onset, clinical phenotype similar to sporadic. Slow progression. Levodopa responsive
PARK2	parkin	6q26	Recessive, sporadic	Parkin	Ubiquitin ligase	Early onset, clinical phenotype similar to sporadic. Levodopa responsive
PARK6	PINK1	1p36.12	Recessive	PINK1	Mitochondrial kinase	Early onset, clinical phenotype similar to sporadic. Levodopa responsive
PARK7	DJ-1	1p36.23	Recessive	DJ-1	Chaperone	Early onset, slow disease progression
PARK9	ATP13A2	1p36.13	Recessive	ATP13A2	Transmembrane protein, putative ATPase activity	Juvenile onset, atypical parkinsonism. Levodopa responsive
PARK22	CHCHD2	7p11.2	Dominant	CHCHD2	Transcription factor	Late onset, clinical phenotype similar to sporadic. Levodopa responsive
PARK18	EIF4G1	3q27.1	Dominant	EIF4G1	Component of protein translation machinery	Late onset, clinical phenotype similar to sporadic. Levodopa responsive
PARK21	DNAJC13	3q22.1	Dominant	DNAJC13	Role in endosomal transport	Late onset, clinical phenotype similar to sporadic. Levodopa responsive
PARK16	SLC45A3 NUCKS1 RAB7L1 SLC41A1 PM20D1	1q32	Unclear	SLC45A3 NUCKS1 Rab7L1 (Rab29) SLC41A1 PM20D1	Various	Unclear
PARK17	VPS35	16q11.2	Dominant	Vacuolar protein sorting 35	Retromer component	Late onset, clinical phenotype similar to sporadic. Levodopa responsive

Table 1.1. Summary of Parkinson's disease-associated loci and genes. The subset outlined here comprise those best characterised to date. Adapted from Corti *et al.* (2011) [241].

1.11 An overview of Leucine-rich repeat kinase 2

LRRK2 was first identified in 2004 as the gene associated with the *PARK8* locus and is the most common genetic risk factor responsible for the development of PD, accounting for approximately 4% of familial Parkinson's disease and 1% of sporadic Parkinson's disease worldwide [239, 242-244]. LRRK2 is therefore an important candidate for a pleomorphic risk locus [245].

The *LRRK2* gene was found to be comprised of 51 exons and encodes a large 2527 amino acid (268 kDa) multi-domain protein. It is widely expressed in the brain, but also in the heart, liver, lung, skeletal muscle, kidney, pancreas, placenta, and immune cells [246]. LRRK2 belongs to the ROCO gene family, which consists of four members in mammals: LRRK1, LRRK2, DAPK1 (death-associated kinase 1) and MFHAS1 (malignant fibrous histiocytoma amplified sequence 1). Common to this group of proteins is a conserved ROC (Ras of complex proteins) GTPase domain, and COR (C-terminal of ROC) domain of unknown function. The ROC domain possesses high sequence similarity to Ras and other small GTPases [247, 248]. In LRRK2 a serine/threonine kinase domain, which is most closely related to the RIPK (receptor-interacting protein kinase) family of kinases, follows the ROC-COR supradomain, and together makes up the catalytic core of the protein [249-251]. This core is further surrounded by several protein-protein interaction domains (Figure 1.9).

Multiple lines of evidence indicate that LRRK2 is a functional dimer in cells, and dimerisation may be responsible for modulation of kinase activity via autophosphorylation [252]. Although monomeric LRRK2 is more abundant and principally cytosolic, enrichment at subcellular membranes is observed upon LRRK2 dimerisation and it is generally accepted this represents the active state of the protein [253-255]. Dimerisation is observed as a general feature among other ROCO kinases [256].

1.11.1 LRRK2 pathogenic and PD-associated mutations

Many pathogenic mutations in LRRK2 primary structure identified in familial PD result in clinical and pathological phenotypes that are indistinguishable from sporadic forms of the disease [257]. Over 80 missense mutations have been identified and are located over the entire LRRK2 protein (Figure 1.9) [258]. So far, seven of these mutations are considered

pathogenic (N1437H, R1441G/C/H, Y1699C, G2019S, I2020T) and are all clustered within the catalytic domains [241, 259]. The most common mutation is G2019S within the kinase domain. This substitution has high but incomplete penetrance, meaning not all carriers develop PD in their lifetime [260]. The G2019S mutation is particularly prevalent in certain populations. For example, this substitution accounts for 30% familial PD/13% sporadic PD in Ashkenazi Jews, and 37% familial PD/41% sporadic PD in North African Arab Berbers, but is rare in Asia [261, 262]. Mutations outside of the enzymatic domains have not been shown to segregate in a Mendelian fashion with the disease, and the pathogenicity of these rare nonsynonymous variants remains uncertain [263]. However, with the identification of common LRRK2 polymorphisms associated with an increased lifetime risk for developing late onset PD [264-267], it is clear that LRRK2 signalling may be central to the processes underlying both inherited and idiopathic PD. Much of the subsequent research has focussed on understanding the effects of these mutations on LRRK2 function in the context of PD.

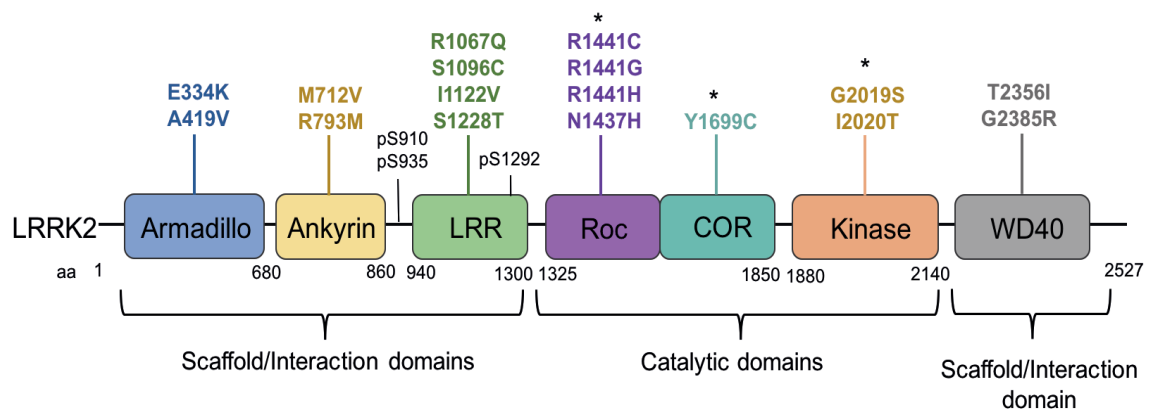


Figure 1.9. Schematic representation of LRRK2 primary structure

Domain organisation of LRRK2. Amino acid numbering represents approximate domain boundaries. Mutations associated with potential disease risk within each domain are indicated. Only those marked with asterisk * have confirmed pathogenicity. Residues Ser⁹¹⁰/Ser⁹³⁵ are constitutively phosphorylated and essential for binding of 14-3-3 proteins. Ser¹²⁹² is an important autophosphorylation site.

1.12 LRRK2 catalytic domains

The presence of a double catalytic core suggest these two enzymatic functions may regulate each other's activities. LRRK2 has been shown to undergo autophosphorylation *in vitro* and over 20 different autophosphorylation sites have been identified [252, 268, 269]. In particular, Ser1292 is an important marker of LRRK2 kinase activity *in vivo*, and phosphorylation at this site is enhanced by several PD mutations. Notably, an alanine substitution at this position can attenuate the cellular effects of pathogenic LRRK2 mutations *in vitro* [270].

The presence of multiple autophosphorylation sites within the Roc domain suggests that the kinase domain may intrinsically regulate the GTPase activity of LRRK2. While kinase activity does require a functional ROC domain, some reports have found it is not dependent on the nature of nucleotide bound [271, 272]. Pathogenic mutations found in the kinase domain (G2019S/I2020T) are which are mapped to the activation loop (Figure 1.9B) show an increase in the intrinsic kinase activity of LRRK2, yet the GTPase activity of these kinase mutants is unaffected [273, 274].

Studies of pathogenic Roc substitutions R1441C/G/H and Y1699C in the adjacent COR domain demonstrate increased affinity for GTP and compromised GTP hydrolysis, prolonging the 'active state' of the GTPase and leading to overactivation of the kinase domain [275-279]. Yet these mutants are dephosphorylated at Ser910/935/955/973 [280]. Strikingly, the introduction of concurrent mutations within both the GTPase and kinase domains can significant augment pathogenic effects. Insertion of the Roc R1441C mutation together with the G2019S kinase mutation results in a 7-fold multiplicative increase in LRRK2 activity [281]. The exact nature of the cross-talk between the kinase and the GTPase domains of LRRK2 still requires clarification. While there is some conflicting experimental evidence, it appears inter-domain regulation is pseudo-cyclical, with kinase-mediated autophosphorylation of the ROC domain modulating GTP binding which in turn further stimulates kinase activity. Overall, it is clear that intrinsic regulation between the catalytic domains is not unidirectional, but rather complex and interdependent.

1.12.1 Roc-COR supradomain

It has been suggested that the LRRK2 GTPase domain belongs to the GAD class of molecular switches, defined as G proteins activated by nucleotide dependent dimerisation [282, 283]. The first crystallographic structure of a human LRRK2 domain was that of the recombinant Roc domain in complex with GDP-Mg²⁺ determined by Deng *et al.* (2008) (PDB code: 2ZEJ) [284]. The structure reveals a unique dimeric GTPase fold consisting of a domain-swapped dimer with side chains contributed from both monomers forming the active site [284, 285]. However, this structure has been disputed as domain swapping is a common crystallographic artefact [286, 287]. Furthermore, Liao *et al.* (2014) showed that the human Roc domain is primarily monomeric in solution [279].

Due to difficulties in the expression and purification of human LRRK2 constructs, bacterial ROCO protein homologues have served as useful tools in the structural characterisation of conserved domains. In 2008 Gotthardt *et al.* solved the crystal structure of the tandem Roc-COR domains in a nucleotide-free state from the *Chlorobium tepidum* ROCO protein, in which a canonical G-protein domain is reported (PDB code: 3DPU) In this model, dimerisation is mediated by the C-terminal half of the COR domain, and additional experimental techniques have since supported this observation [288, 289]. A study using human LRRK2 showed that R1441C/G/H mutations in the Roc domain and the Y1699C COR mutation all weakened Roc-COR dimerisation, resulting in decreased GTPase activity [278].

The first nucleotide-bound Roc-COR structure (GDP-Mg²⁺) was later reported from the *Methanosarcina barkeri* Roco2 protein (PDB code: 4WNR). This study suggests that nucleotide binding induces a major structural change in the Roc-COR interface, however COR-mediated dimerisation is not dependent on the nature of the nucleotide bound (GDP or GppNHp) [290].

Subsequently, Deyaert *et al.* (2019) reported the crystal structure of the complete LRR-Roc-COR module from *C. tepidum* (PDB code: 6HLU) (Figure 1.10A) [291, 292]. Their model suggests dimerisation is mediated by more than just the C-terminal COR-COR interaction, with extensive contacts also observed between both Roc domains and the Roc and COR domains of adjacent subunits contributing to the total dimer interface [292]. In contradiction to the experimental data obtained from *M. barkeri* outlined above, Deyaert *et al.* showed dimerisation of the *C.tepidum* LRR-Roc-COR domains is dependent on the nucleotide bound. In their study, compact dimers are found in nucleotide-free and GDP-bound states, while an

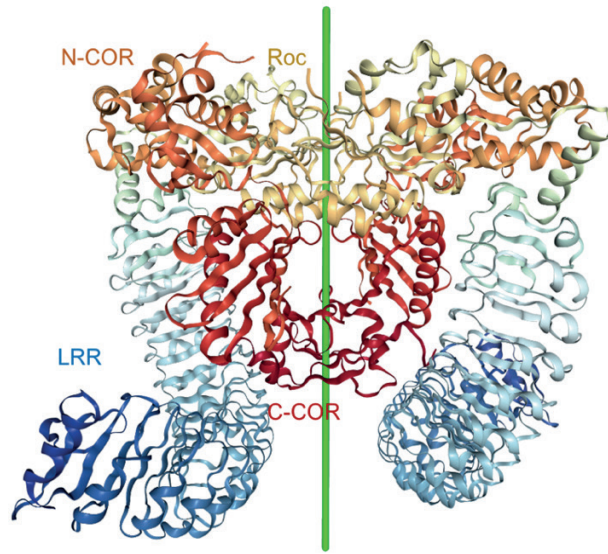
extended monomeric form is observed upon GTP binding. A mutation in this bacterial protein (L487A) analogous to PD-linked mutation (I1371V) in the LRRK2 Roc domain decreases GTPase activity by interfering with the dimer-monomer cycle [292-294]. It is clear much remains to be resolved in our understanding of oligomerisation and conformational cycling of Roco proteins.

1.12.2 Kinase domain

The Roco4 protein of the social amoeba *Dictyostelium discoideum* is currently the only structural model available for the LRRK2 kinase domain (Figure 1.10B). The Roco4 kinase shares 47% similarity to LRRK2, and is capable of phosphorylating LRRKtide, an artificial specific substrate of LRRK2. Roco4 kinase activity is also inhibited by various LRRK2 inhibitors and thus serves as a useful model for studying the LRRK2 kinase domain. Gilsbach *et al.* (2012) solved the structures of the wild-type Roco4 kinase and the PD mutants G1179S and L1180T corresponding to G2019S and I2020T in LRRK2 (PDB codes: 4F0F; 4F1M; 4F1O) [295]. Like almost all conventional kinases, the Roco4 kinase consists of a two-lobed structure, with cleft between the two lobes forming the conventional ATP nucleotide-binding pocket. This makes up the kinase catalytic site along with the activation loop and the α C-helix. They observed a requirement for dimerisation and autophosphorylation of the activation loop to induce the active conformation of the kinase, and revealed a potential mechanism for increased kinase activity of the G2019S mutation via an additional hydrogen bond stabilising the active configuration [295].

Despite intensive research into LRRK2 function since its discovery, it took more than a decade to identify bona-fide substrates of the kinase domain. As will be detailed further in Section 1.16.1 phosphoproteomics identified a subset of Rab GTPases that are phosphorylated by LRRK2 at a conserved Thr/Ser residue in Switch 2 [148]. The tumour suppressor p53, and the adapter protein p62/sequestosome-1, which functions in the autophagic degradation of cytoplasmic ubiquitin-containing inclusions, have also been identified as novel targets [296, 297].

A Roco protein LRR-Roc-COR domains in *C.tepidum*



B Roco4 kinase domain *D.discoideum*

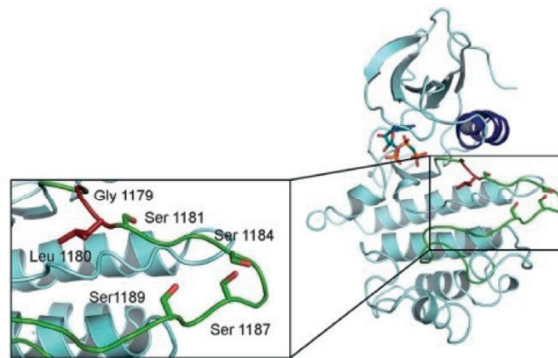


Figure 1.10. A) Ribbon model of the LRR-Roc-COR dimer from *C. tepidum*. LRR domains are displayed in blue, with dark gradient beginning at the N-terminus. The Roc domains are shown in yellow. The N-COR pseudo-domains are shown in orange and C-CORs are displayed in red. The green bar displays symmetry and distinguishes each monomer. (PDB code: 6HLU) B) Ribbon model of the active Roco4 kinase domain from *D. discoideum* bound to AppCH₂P represented by red sticks. The activation loop is shown in green and the regulatory α C-helix in blue. Residues homologous to LRRK2 PD mutations are shown in the enlarged view. (PDB codes: WT 4F0F; G1179S mutant 4F1M; L1180T mutant 4F1O). Adapted from Gilsbach *et al.* (2012)[295].

1.13 LRRK2 accessory domains

The armadillo, ankyrin and leucine-rich repeats are located N-terminally with respect to the Roc-COR domain, and the WD-40 domain at the C-terminus [298]. These repeat motifs are likely to be involved in the localisation and the regulation of LRRK2. Mills *et al.* (2012) carried out a detailed characterisation of these predicted accessory domains by homology modelling, however the domain boundaries remain controversial. Most importantly, some of these structures still need to be confirmed by experimental evidence.

1.13.1 Armadillo domain

There are 13 armadillo-type repeats, each of approximately 42 residues, comprising the first domain of LRRK2. One short α -helix, followed by two longer helices makes up each repeat and together form an extended super helix in the first 600 residues (Figure 1.11A) [299]. Characterisation of the interactions of cellular proteins with the LRRK2 armadillo repeats remains scarce presently. The small GTPases Rab32/38 have been shown to bind specifically to this region by yeast two-hybrid analysis, and fluorescence microscopy illustrates co-localisation of Rab32 and LRRK2 at recycling endosomes and transport vesicles [300]. Further work is required to elucidate how LRRK2 function is regulated by Rab32. Interaction of the FAS-associated death domain (FADD) protein, involved in the extrinsic apoptotic death pathway, was mapped to the ARM domain within residues 500-575 [301]. Intriguingly, it has also been predicted that a sequence of amino acids between residues 210-310 within the armadillo repeats could be responsible for LRRK2 aggregation in cells, and this aggregation is, in fact, protective when challenged with the PD neurotoxin 6-OHDA [302]. It is usually expected that protein aggregation leads to pathophysiology, as in many neurodegenerative disorders the smaller fibrils/oligomers are toxic. N-terminal mediated aggregation may have functional relevance in this circumstance, as the closely related LRRK1 protein does not demonstrate the same behaviour [303]. Several mutations have been identified within the armadillo repeats, however it has not been confirmed whether these amino acid substitutions are causative of PD. The E334K variation was found in two North American siblings with PD, and is predicted to lie within a 16-residue insert between repeats 6 and 7 [304]. The A419V variation was identified in several studies in Asian patients and its location is predicted to be

in the H3 helix of repeat 8 [266]. The mutations A211V and K544E were reported in two patients of Greek origin with autosomal dominant PD [305].

1.13.2 Ankyrin domain

A set of seven tandem 33-amino acid repeats were predicted for the Ankyrin (ANK) domain and a gently curved structure is formed from each repeat of two antiparallel α -helices followed by a loop (Figure 1.11B). Direct interactors of the ankyrin repeats have not been documented in the literature thus far, although Rab29 is suspected to bind within this domain [306]. A clue for a potential function comes from examination of other well-studied ankyrin domain-containing proteins. The crystal structure of the cyclin-dependent kinase 4 inhibitor (INK4) with cyclin-dependent kinase (Cdk6) in a heterodimeric complex illustrates the ability of ankyrin repeats to bind and inhibit protein kinase domains (PDB codes: 1BI7/1BI8) [307]. Thus, negative regulation of the LRRK2 kinase domain by the ankyrin repeats is plausible. Indeed, it has been shown that deletion of N-terminal region encompassing the ARM, ANK, and LRR domains results in greater autophosphorylation than in full length wild-type LRRK2 [252, 285]. As described in Section 1.14 the ankyrin domain lies in close proximity to the kinase domain in the full-length structure, further supporting this hypothesis. Identified variants M721W and R793M both map to the ankyrin repeats [308, 309]. These residues are evolutionarily conserved in LRRK2 orthologues and suggests they may be structurally or functionally important.

1.13.3 Leucine-rich repeats

The ankyrin domain is followed by a curved solenoid-shaped structure made up of 14 leucine-rich repeats (LRRs), each of approximately 24 amino acids. In addition to the LRR-Roc-COR module discussed in Section 1.12.1 (Figure 1.10A), the structure of the individual *C. tepidum* LRR domain is also available (PDB code: 5IL7). Overall, a curved β -sheet forms the concave face of the structure, with the convex surface containing a variety of α -helices, turns and loops. This provides a versatile structural framework for protein-protein interactions [310, 311]. Rab5b has been identified as a potential interactor with the LRR domain, as identified

by yeast two-hybrid screening of a human brain cDNA library [312]. Rab5b is also a substrate of LRRK2 kinase, with phosphorylation modulating Rab5 activity and interactions, acting as a negative regulator for Rab5b signalling [313].

Importantly, two conserved serine residues, Ser⁹¹⁰ and Ser⁹³⁵, located between the predicted boundaries for the ankyrin domain and the LRRs, are important phosphorylation targets and mediate the interaction of LRRK2 with 14-3-3 proteins, direct regulators of protein function. The 14-3-3 proteins are a family of homologous proteins that are highly expressed in the brain and regulate multiple aspects of brain function. Several PD-associated mutations that reduce or abolish Ser⁹¹⁰/Ser⁹³⁵ phosphorylation results in the disruption of 14-3-3 binding and the accumulation LRRK2 in cytoplasmic pools resembling inclusion bodies [314-317]. A large number of nonsynonymous variants have been identified within the LRRs that are potentially disease producing, with most found in the N-terminal half of the domain. These include R1067Q, S1096C, Q1111H, I1122V, I1192V, and S1228T [243, 304, 309, 318].

1.13.4 WD40 repeats

Finally, each WD40 repeat comprises a common length of 40 residues and seven such repeats have been identified in the C-terminus of LRRK2. Each WD40 repeat forms four β -strands. These β sheet structures adopt a closed formation and are arranged in a radial fashion resulting in a propeller-like shape [299]. Recently, the crystal structure of the human WD40 repeats at 2.6 Å resolution was reported, and also revealed a dimeric domain assembly which was confirmed to exist in solution (PDB code: 6DLO)(Figure 1.11C) [319]. Curiously, as seen in Figure 1.12, the WD40 domains are not found adjacent to each other in the low resolution full-length LRRK2 dimer. This observation leads to speculation as to whether WD40 dimerisation could act as an inhibitory mechanism. Another discrepancy with previous reports regarding Roc-COR mediated dimerisation (Section 1.12.1) arose with experimental data showing WD40 truncated-LRRK2 could not dimerise [320] Thus it is likely LRRK2 dimerisation depends on contributions from more than one domain.

Piccoli *et al.* (2014) identified 84 putative LRRK2 WD40 interactors by immunoprecipitation, Western blotting and mass spectrometry and demonstrated that this domain is capable of

binding to synaptic vesicles, suggesting its involvement in vesicle trafficking and the cytoskeleton [321, 322]. A T2356I variation was reported in two patients of British and Dutch backgrounds and may potentially disrupt the structural integrity of the WD40 domain [285, 323, 324]. The disease-related G2385R substitution is the most prominent WD40 mutation characterised [259, 325-327]. The introduction of this variation may perturb protein-protein interactions with cellular binding partners or with other LRRK2 functional domains. Indeed, in the structure the G2385 residue is located at the dimerisation interface and substitution to arginine compromised WD40 dimerisation in solution [319]. It has been shown that G2385R results in a reduction in LRRK2 kinase activity, and this variation can abolish the G2019S activating effect on the kinase domain [328]. These observations suggest that both a loss or gain of function can disrupt LRRK2 cellular homeostasis and contribute to the pathogenic mechanism.

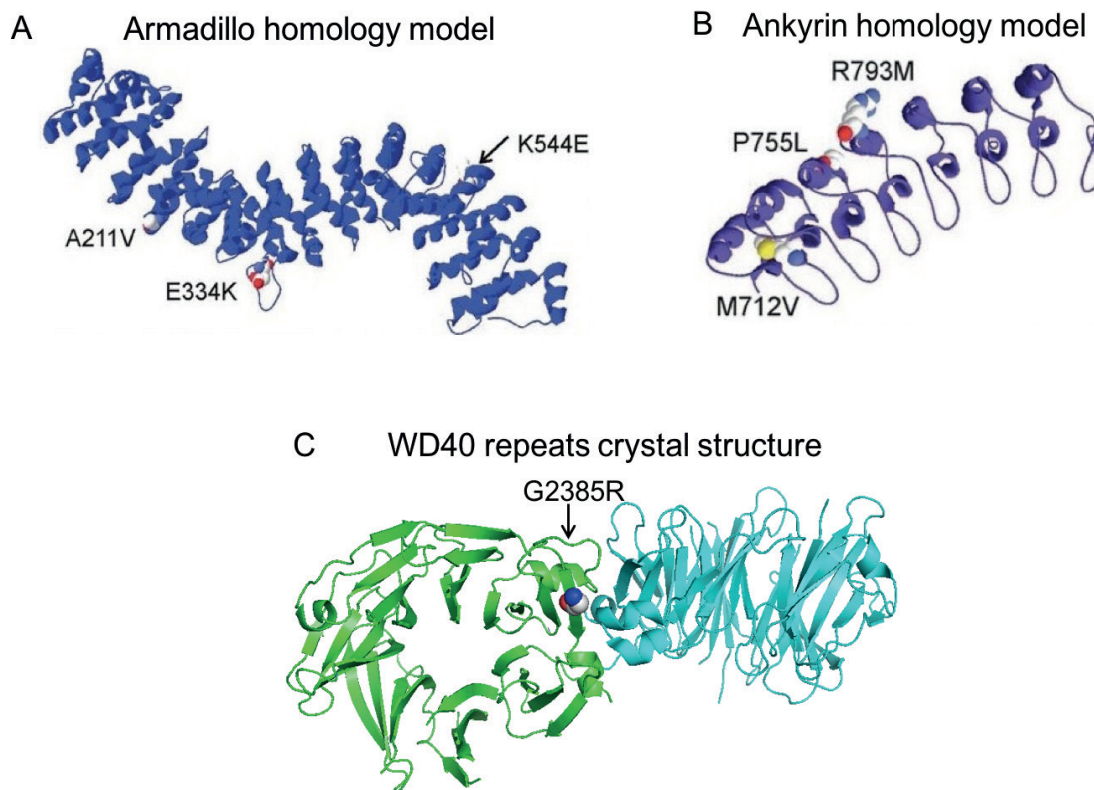


Figure 1.11. Homology and structural models of LRRK2 accessory domains

A and B) The armadillo and ankyrin N-terminal domains were modelled using known structures of similar repeat lengths; p120 catenin (PDB code: 3L6X) and importin- α (PDB code: 1EE4); ankyrinR (PDB code: 1N11). Sites of PD mutations are shown. Taken from Mills *et al.* (2012) [299]. **C**) Ribbon model of the human WD40 dimer illustrating a classical propeller-like fold. The PD-associated G2385R is highlighted and is found at the dimerisation interface (PDB code: 6DLO).

1.14 LRRK2 full length structure

Recently, an integrative structural biology approach yielded the first 3D model of full length LRRK2 at low resolution. A combination of computational modelling, negative-staining electron microscopy, small angle X-ray scattering (SAXS), X-ray crystallography, and chemical cross-linking with mass spectrometry (CL-MS) were employed to determine the complex structure, and revealed a compact dimeric intertwined architecture (Figure 1.12) [329-331]. This model sheds some light on general domain organisation and assembly. It suggests that the kinase and WD40 domains of one monomer interact with the ankyrin and LRR domains of the other, and further supports the Roc-COR domains forming the primary dimerisation interface [330]. However, because the fitting of the armadillo domain relied primarily on positioning it within the remaining available space in the EM density map, and the low resolution of 22 Å, further structural characterisation is required to correlate conformation, intramolecular and intermolecular interactions of LRRK2 with its activity and functions within the cell. A summary of all structural data pertaining to human LRRK2 and homologous Roco proteins is shown in Table 1.2.

Domain	Organism	Resolution	Techniques	PDB code
Roc	Human	2.0 Å	X-ray crystallography	2ZEJ
Roc-COR (nucleotide-free)	<i>C. tepidum</i>	2.9 Å	X-ray crystallography	3DPU
Roc-COR (GDP)	<i>M. barkeri</i>	2.9 Å	X-ray crystallography	4WNR
LRR-Roc-COR	<i>C. tepidum</i>	3.3 Å	X-ray crystallography	6HLU
LRR	<i>C. tepidum</i>	2.3 Å	X-ray crystallography	5IL7
Roco4 Kinase	<i>D. discoideum</i>	1.8 Å	X-ray crystallography	4F0F
WD40	Human	2.7 Å	X-ray crystallography	6DLO
LRRK2 full length	Various	~22 Å	Cryo-EM/CL-MS/SAXS/Computational modelling	N/A Guaitoli et al. 2016

Table 1.2. Current structural knowledge of human LRRK2 and LRRK2 homologues

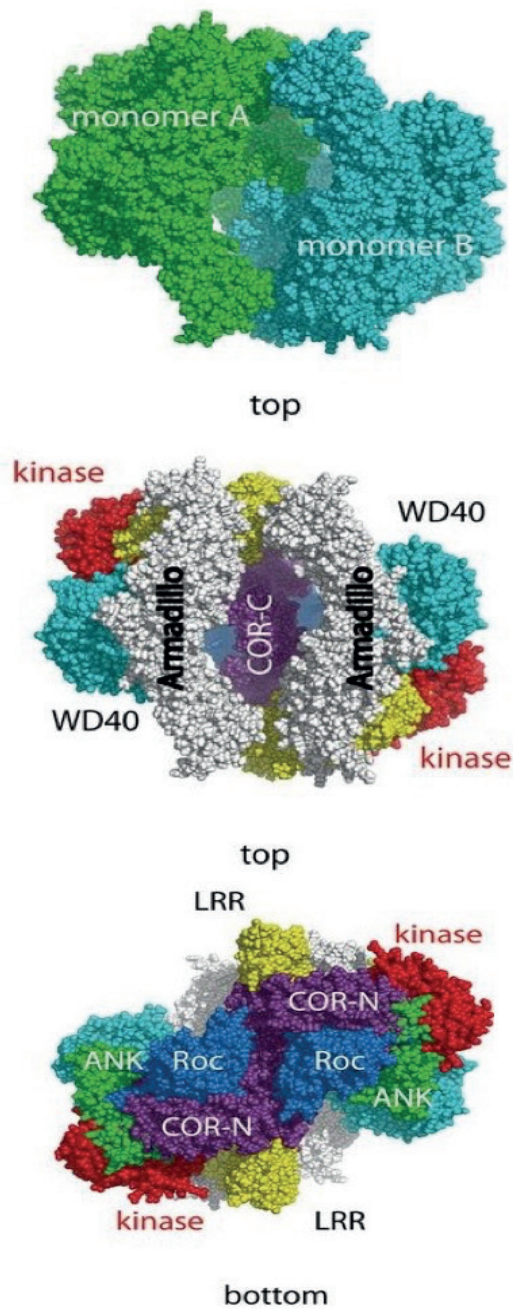


Figure 1.12. Integrative structural model of full length LRRK2

A) Dimeric space-filling model of LRRK2 with each monomer shown in green/cyan. B) Top and bottom view of LRRK2 model with domains indicated by colour. Armadillo – grey; Ankyrin (ANK) – green; LRR – yellow; Roc – blue; COR – purple; kinase – red; WD40 – cyan. Adapted from Guaitoli *et al.* (2016) [330].

1.15 LRRK2 Interactions and functions

Knowledge of the relationship of LRRK2 with other cellular proteins and its role in signalling pathways is crucial for understanding how pathogenic or PD-associated mutations manifest and result in disease. Porras *et al.* (2015) curated a LRRK2-specific interaction network by high-throughput screening which vividly demonstrates the enormous scope of LRRK2 interactions (Figure 1.13) [332]. It is thus unsurprising that LRRK2 has been implicated in numerous biochemical pathways. However, it is important to note that LRRK2 may exert different functions depending on the cell type in which it is expressed. Often, *in vitro* kinase assays can be difficult to replicate when specific signals may be required to exert an effect *in vivo*, and it is possible that these assays and results from lower complexity model organisms are not always transferable to mammalian systems. As a result conflicting lines of evidence frequently exist. Going forward it will of upmost importance to try to distinguish which of these observed effects are relevant under true physiological and pathophysiological conditions.

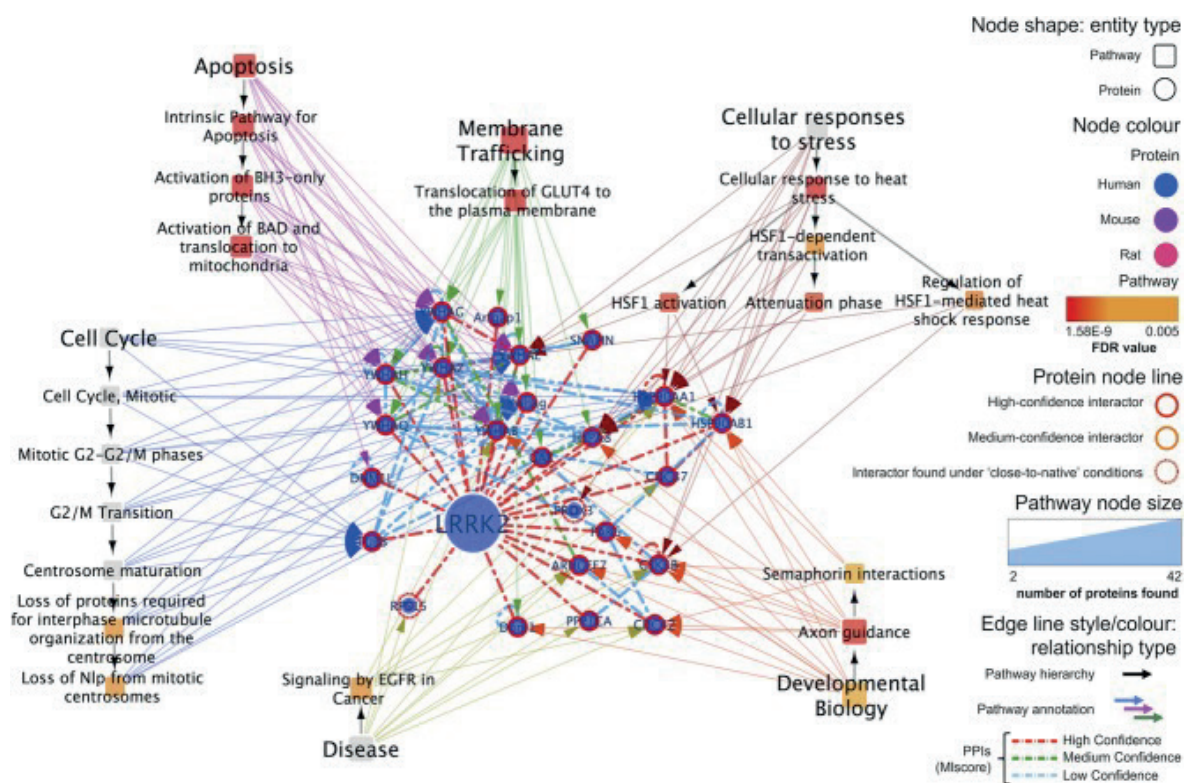


Figure 1.13. The LRRK2 Interactome

Visualisation of LRRK2 high-confidence interacting partners and pathways using the Reactome analysis tool. Taken from Porras *et al.* (2015) [332].

1.15.1 Signalling cascades

LRRK2 has been implicated in wide variety of cell signalling cascades, with two of the most prominent examples being the MAPK pathways and Wnt signalling. LRRK2 has been associated with several MAPK (mitogen-activated protein kinase) pathways after it was shown to bind and phosphorylate MAP2K3, -4, -6, and -7 *in vitro* [333, 334]. These pathways are involved in cellular stress responses and transmit signals downstream to effectors such as p38 and JNK. These effectors control cell proliferation and differentiation, apoptosis and the production of cytokines. Activation of MAP2K1 and -2 by LRRK2^{G2019S} resulted in the hyperphosphorylation of effectors ERK1 and ERK2. Hyperactivity of this pathway may be responsible for an increase in basal autophagy [335]. A large number of genes involved in cell proliferation, apoptosis, inflammation, synaptogenesis and synaptic maintenance are controlled by the transcription factor β -catenin, activated through Wnt (Wingless) signalling cascades [336]. The Roc-COR domain of LRRK2 has been shown to interact with Dishevelled proteins 1, -2 and -3 and is thought to enhance transduction of the WNT signal [337, 338].

Furthermore, the involvement of LRRK2 in protein synthesis was initially hypothesized based on its genetic interaction with elements of the TOR pathways in *Drosophila* [339]. LRRK2 was shown to phosphorylate the eukaryotic initiation transcription factor binding protein 4E-BP1 *in vitro*, however these results have not been reproduced in mammalian cells [340]. LRRK2 has also been reported to physically interact with the translation elongation factor eEF1A, and exhibits phosphorylation of several ribosomal proteins identified by unbiased proteomic screening and *in vitro* kinase assays [341, 342]. Taken together, LRRK2 may play an important role in both gene expression and protein synthesis pathways.

1.15.2 Cytoskeletal dynamics

Morphological abnormalities in neurite outgrowth and branching were among the first observed LRRK2 phenotypes, with neurite shortening resulting from LRRK2 overexpression and loss of LRRK2 function leading to increased neuron length and branching [343]. Involvement of LRRK2 in cytoskeletal dynamics has been supported by evidence of an association of LRRK2 with both actin and tubulin. Mexiner *et al.* (2011) performed a QUICK

(quantitative immunoprecipitation combined with knockdown) screen identifying actin isoforms and actin-associated proteins as LRRK2 interaction partners [344]. LRRK2 regulates the dynamics of F-actin enriched filopodia of growing neurites, and can alter actin dynamics and F-actin structure in brain neurons and skin fibroblasts [345, 346].

LRRK2 influences microtubule stabilisation through its interaction with α/β -tubulin and the microtubule-associated protein tau [347, 348]. The accumulation of hyperphosphorylated tau in neurofibrillary tangles is a hallmark pathological feature of the Alzheimer's brain, but is also seen in the brains of PD patients [349, 350]. A deficiency in tau phosphorylation was observed in the brain lysates from LRRK2 null mice [351]. While tau does not appear to be a direct substrate of LRRK2 kinase, there is evidence for a direct interaction between both proteins with LRRK2 regulating intracellular tau levels [352]. It is hypothesized that LRRK2 may facilitate phosphorylation indirectly by the recruitment of tau and the cdk5 kinase [353]. A separate study showed LRRK2 promotes tau phosphorylation via glycogen synthase kinase-3 β (GSK-3 β) [354]. While there is notable evidence for a relationship between tauopathies and Parkinsonism the exact interplay between these proteins remains poorly defined.

The ability for LRRK2 to form ordered filamentous structures along microtubules has been previously observed, and most pathogenic PD mutants, apart from the kinase G2019S substitution, enhance this type of oligomerisation [355, 356]. The LRRK2 Roc-COR domain mutations (R1441C, Y1699C) were shown to disrupt axonal transport *in vitro* and *in vivo* upon formation of these filamentous structures, and preferentially bind to deacetylated microtubules [357]. The rate of transport along microtubules is influenced by their acetylation status, with increased acetylation promoting axonal transport. These pathogenic mutants may thus assert an effect by interfering with α -tubulin acetylation [358, 359]. In addition, the histone deacetylase HDAC6 co-immunoprecipitates with LRRK2, and its inhibition can reverse the filamentous mutant phenotype and restore microtubule acetylation [297, 357].

1.15.3 Mitochondria and Reactive Oxygen Species

The long, high-branched, and unmyelinated axonal arbours of SNpc DA neurons requires a higher energy demand for normal function, and these neurons display a higher rate of oxidative phosphorylation [360, 361]. Approximately 10% of LRRK2 is present in the

mitochondrial fraction of cells overexpressing the protein and LRRK2 is associated with the mitochondrial outer membrane in rodent brains, suggesting a putative role in mitochondrial homeostasis [273, 362, 363]. Mitochondrial dysfunction arising from mutant LRRK2 may contribute to pathogenesis of PD, with abnormal mitochondrial function and morphology observed in patients carrying the G2019S mutation [364]. Multiple studies in mouse models and in human cell lines have been conducted demonstrating a variety of mitochondrial defects upon inhibition or overexpression of wild-type/mutant LRRK2 including mitochondrial fragmentation, uncoupling and depolarisation, increased susceptibility to chemical stressors, disrupted mitochondrial movement, and mitophagy [365-368]. Along the same vein, elevated levels of reactive oxygen species (ROS) is noted as a pathological feature of PD, with mitochondrial dysfunction linked to an increase in ROS production when mutant LRRK2 is expressed in cells [369, 370]. Moreover, induced pluripotent stem cells carrying the G2019S mutation showed increased sensitivity to H₂O₂ exposure with the activation of caspase-3 and cell death [371]. An extensive review of mitochondrial dysfunction arising from LRRK2 variants in different human cell lines and animal models was recently published [372].

1.15.4 Immune system

Chronic inflammation is a prominent feature of many neurodegeneration disorders, including PD, and can precede the development of classic neuronal pathology [373]. LRRK2 mRNA levels and protein expression are elevated in immune cells, and a robust induction of LRRK2 is observed in mouse microglia upon LPS-induced inflammation [374, 375]. LRRK2 has been described as a negative regulator of nuclear factor of activated T cells (NFAT) transcription factors, and thus abnormal LRRK2 activity could push immune cells toward a pro-inflammatory phenotype [376, 377]. Along with activation, LRRK2 modulates microglial migration through cytoskeletal dynamics and phagocytosis in response to pathological stimuli [375, 378]. Microglial activation is found in post-mortem PD brain tissue, and the pathological implications of LRRK2 in neuroinflammation serves as an appreciation for the relevance of astrocytes and microglia in PD as well as neurons [379]. Indeed, the importance of the role of LRRK2 in inflammation is solidified by its identification as a susceptibility gene for leprosy and Crohn's disease in GWAS studies [380, 381].

1.15.5 Protein quality-control mechanisms

Protein inclusions in the brains of PD patients may result from the disruption of the two major intracellular protein breakdown pathways: the autophagy-lysosomal pathway (ALP) and the ubiquitin-proteasome system (UPS). The observation that overexpressed LRRK2^{G2019S} resulted in a striking increase in the number of autophagic vesicles provided the first clue for a role for LRRK2 in autophagy [382]. LRRK2 was shown to localise to autophagic vesicles and multivesicular bodies (MVBs) [355], and while data from both cellular and animal model studies support a role for LRRK2 in autophagic flux, the results are conflicting as to whether LRRK2 is a positive or a negative regulator of these pathways [362, 383]. In LRRK2 knockout mice a biphasic alteration in macroautophagy was observed in the kidneys, with enhanced autophagy in young mice and reduced autophagy at old ages [384]. Studies with human fibroblasts showed an increase in basal macroautophagy in G2019S carriers, while a reduction was seen in induced Pluripotent stem cell (iPSC)-derived human dopaminergic neurons carrying LRRK2^{G2019S} [335, 385].

Recently, a study demonstrated LRRK2-mediated phosphorylation of leucyl-tRNA synthetase impaired autophagy, an effect augmented by the G2019S mutant [386]. It has also been reported that LRRK2 regulates autophagy through a calcium-dependent pathway. A molecular mechanism was suggested whereby LRRK2 localises to the lysosome resulting in Ca²⁺ release and lysosomal alkalinisation [387]. A more recent study demonstrated that LRRK2 and substrate Rab GTPases are recruited to stressed lysosomes and maintain their homeostasis [388].

The selective autophagic receptor p62/SQSTM-1 is a direct substrate of LRRK2 kinase, however LRRK2 itself is also a degradation target and the overexpression of p62 leads to the robust degradation of ubiquitinated LRRK2 [297, 389]. LRRK2 is also reported to be degraded by chaperone-mediated autophagy, and a reduction in LRRK2 removal via this process was noted for the G2019S form [390].

A relationship between LRRK2 and the ubiquitin-proteasome system has been documented. LRRK2 binds to the E3 ubiquitin ligase CHIP and heat shock protein 90 (Hsp90), and this complex regulates LRRK2 levels in cells [391, 392]. Both the overexpression and the loss of function of LRRK2 disrupts substrate clearance in the UPS pathway, and leads to an accumulation of proteins such as ubiquitin and α -Syn [393, 394]. This connection with α -Syn

is significant, with a large proportion of SNCA-positive Lewy bodies also containing LRRK2 [395]. Both wild-type and LRRK2^{G2019S} co-immunoprecipitate with α -Syn in human cell lines, and phosphorylation of α -Syn by LRRK2 has been considered, however whether there is a direct interaction between both proteins is still a matter of debate [396].

1.15.6 Vesicle dynamics

There has been accumulating evidence for a role of LRRK2 in vesicle dynamics and retromer function. As outlined above, LRRK2 localises to membranous structures in the mammalian brain, including endosomes, lysosomes, transport vesicles and mitochondria, and has also been shown to be enriched at the Golgi apparatus [363, 397]. Altered neurotransmitter release was observed in LRRK2^{G2019S} transgenic mice, and the G2019S mutation impeded synaptic endocytosis in G2019S-overexpressing flies, followed by restoration through pharmacological inhibition of LRRK2 kinase activity [398, 399]. LRRK2 silencing in primary cortical neurons demonstrated increased vesicle kinetics with altered recycling dynamics, and changes in vesicle distribution and the amount of ready releasable vesicles in PC12 cells expressing LRRK2^{G2019S} [321, 400]. Taken together, this data illustrates a role for LRRK2 in regulating presynaptic vesicle pools.

Interactions have also been documented between LRRK2 and the dynamin GTPase superfamily, members of which mediate membrane scission during clathrin-mediated endocytosis [401]. Furthermore, LRRK2 has been implicated in exocytosis of synaptic vesicles by phosphorylation of Snapin, therefore regulating SNARE complex functionality which mediates vesicle fusion to target membranes [402]. A role for LRRK2 in exocytosis was also implied by studies of lung cells in LRRK2 knockout rats [403].

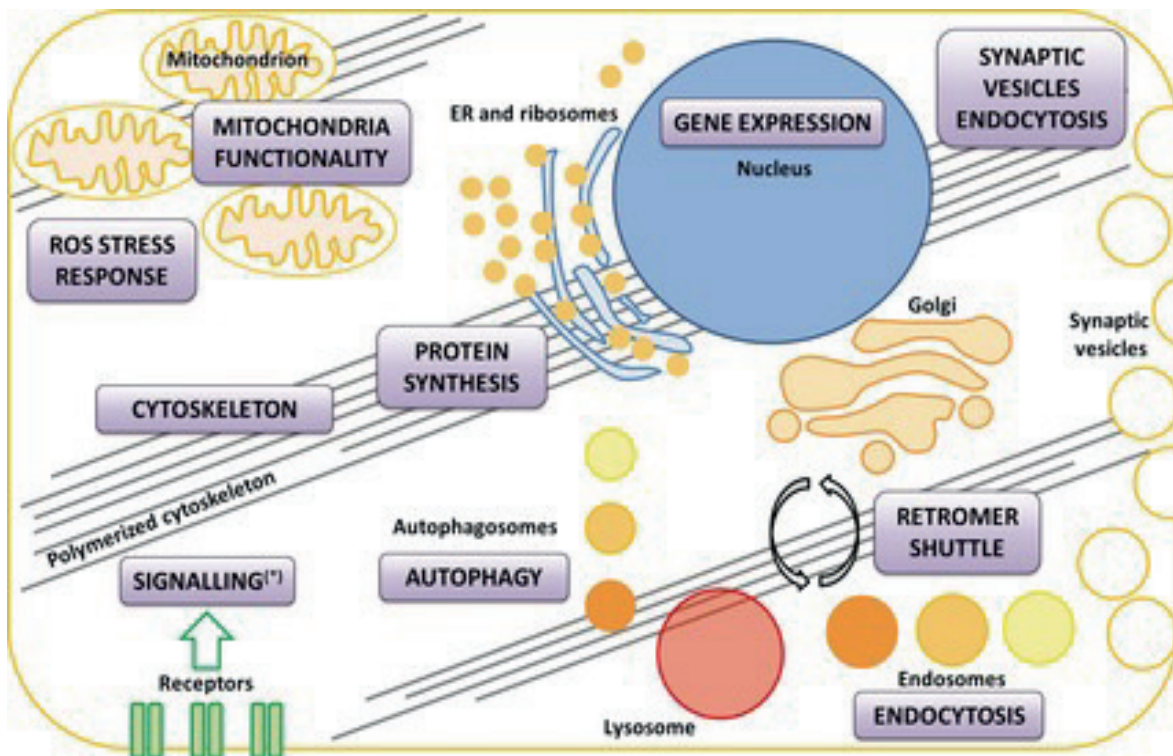


Figure 1.14. Overview of LRRK2 cellular functions

Illustration of the cellular processes associated with LRRK2 physiological and pathological function. Evidence suggests LRRK2 coordinates both interlinked and independent networks, however the precise role of LRRK2 may be dependent upon the cell type in which it is expressed. In many cases distinguishing which of the observed effects are a direct result of LRRK2 function from those that arise as an indirect consequence still needs to be established. Adapted from Wallings *et al.* (2015) [362].

1.16 LRRK2 and Rab GTPases

Of particular interest to our lab, the role of LRRK2 in vesicle dynamics outlined in Section 1.15.6 strongly links LRRK2 as an interaction partner for members of the Rab family of small GTPases. The association of LRRK2 with Rab5b has been shown to be of importance, whereby a significant reduction in synaptic vesicle endocytosis was observed upon siRNA knockdown of LRRK2 and a subsequent reversal of this phenotype was demonstrated upon co-expression of active Rab5b [312]. A homologue of LRRK2 (*Irrk*) in *Drosophila*, strongly interacts with Rab7 and together regulate the positioning of the lysosome. A mutant form of *Irrk* analogous to LRRK2^{G2019S} led to a disturbance in lysosome localisation [404]. Another study observed decreased Rab7 activity and thus a delay in late endocytic trafficking both in HeLa cells overexpressing LRRK2, and in fibroblasts from PD patients carrying a LRRK2^{G2019S} mutation compared to healthy controls [405]. Rab5 and Rab7 primarily associate with early and late endosomes, respectively. The resultant reduction in Rab7 activity may consequently affect the Rab5/Rab7 switch during the transition from early-to-late endosomal trafficking. Using the EGFR as a model degradative trafficking pathway, mutant LRRK2-transfected cells showed relatively normal co-localisation of fluorescent EGF with Rab5, however there was impaired entry of cargo into Rab7-positive compartments [405]. Taken together, this primary data provides a potential link between endolysosomal dysfunction and PD pathogenesis.

1.16.1 Rab GTPases as kinase substrates

A pivotal discovery was made when phosphoproteomic analyses identified a subset of Rab GTPases as bona-fide downstream physiological substrates of LRRK2 kinase. Phosphorylation by LRRK2 shows marked preference for threonine over serine residues [406]. Steger *et al.* (2016) demonstrated highly efficient phosphorylation of Rab1b, Rab8a, and Rab10 on a conserved threonine located in the Switch 2 domain, whereas Rab5b, Rab7a, Rab29, Rab12 and Rab39b with equivalent serine sites were phosphorylated to a lower degree [148]. In total 14 Rabs were shown to be phosphorylated by LRRK2 in an overexpression system, with at least 10 endogenously phosphorylated in cells. Rab5a was not an endogenous LRRK2 substrate, while experimental limitations did not allow for conclusive determination of

endogenous phosphorylation of Rab5b/c or Rab29 [149]. It was later shown that substrate Rabs must be membrane-associated and GTP-bound for LRRK2 phosphorylation [407].

As previously mentioned in Section 1.7.1, it is likely that the phosphorylation of Rabs within the switch region modulates their activity by controlling the interaction with numerous regulatory proteins and effectors. For example, phosphorylation of Rab8a interferes with its association with GDI, potentially prolonging its lifetime in the active state [148]. The same study showed the interaction of Rab8a with Rabin8 is also compromised by phosphorylation of Switch 2. A dominant interaction arising from Rab phosphorylation was recently identified, with the lysosomal effectors RILPL1 and RILPL2 preferentially binding to pRab10 and pRab8a respectively. Rab8a promotes cilia formation while Rab10 acts as a suppressor, and RILPL1/2 regulate their subcellular localisation [149, 408]. It was also shown that phosphorylation of Rab proteins by LRRK2 can affect intrinsic GTP/GDP exchange, with phosphorylation in some instances promoting the GTP-bound state [409].

In this way, fine-tuning of effector/regulator interactions can be precisely modulated in distinct cellular contexts. However, perturbations in Rab modifications may harbour significant consequences. For example, disruption of Rab7-dependent EGFR degradation could lead to aberrant growth signalling and implications in tumour development or progression. Furthermore, it has been reported that dysregulated phosphorylation in Rab1a, Rab3c and Rab35 induced neurotoxicity in primary cortical neurons, and expression of Rab35 phosphomutants caused significant degeneration of dopaminergic neurons *in vivo* [409]. Lastly, the aforementioned study on enhanced RILPL1 interaction with pRab10 noted that expression of LRRK2^{R1441C} resulted in decreased ciliation in mice cholinergic neurons. The authors speculate such a decrease can reduce the ability of cholinergic neurons in the striatum to sense Sonic hedgehog in a neuro-protective circuit that supports dopaminergic neurons [408].

Overall, it is clear that pathogenic LRRK2 mutations resulting in Rab hyperphosphorylation can disturb the Rab-cycling equilibrium, causing intracellular trafficking steps to become dysregulated.

1.16.2 LRRK2 and the Rab32 subfamily

Very little progress has been made thus far on the interaction of Rab proteins with LRRK2 accessory domains. In 2014, Waschbusch *et al.* reported a novel interaction of Rab32/38 with the N-terminal armadillo repeats of LRRK2. LRRK2 was found to co-localise with wild-type Rab32 at transport vesicles and pericentriolar recycling endosomes, supporting a role for Rab32/38 in LRRK2 intracellular localisation [300]. Furthermore, a missense mutation within the Rab32 interswitch (Ser71Arg) was very recently found to segregate with late-onset, autosomal-dominant PD in 3 unrelated families [410, 411].

There has been particular interest surrounding the role of Rab29 in the context of PD. Rab29 was identified as one of five genes found at the *PARK16* non-familial PD risk-associated locus. GWAS studies have documented a genetic interaction between Rab29 and LRRK2 and transcriptome analysis has suggested higher gene expression of Rab29 may affect disease risk [412, 413]. Evidence suggests a common pathogenic pathway for Rab29 and LRRK2, with individuals carrying common high-risk alleles at both loci showing a non-linear increase in PD susceptibility [222, 414]. Rab29 and LRRK2 double knockouts in mice exhibit an enlarged kidney morphology that is non-additive relative to single knockout phenotypes. In the same study the authors showed the Rab29 orthologue (GLO-1) acts upstream of LRRK2 (LRK-1) in *Caenorhabditis elegans* neurons and regulates axon elongation [415]. Following this, Purlyte *et al.* (2018) reported that Rab29 interacts with the LRRK2 N-terminus and confirmed Rab29 functions upstream as a master regulator of LRRK2, recruiting LRRK2 to the *trans*-Golgi network and significantly enhancing its kinase activity. Pathogenic LRRK2 mutants within the Roc-COR domain are more readily recruited and activated by Rab29 than the wild-type protein [306]. Significantly, despite high sequence identity shared among the subfamily a disparity has emerged between Rab32/28 and Rab29, with Rab29 suspected to possess a unique binding site within the LRRK2 ankyrin domain [306]. Curiously, as well as acting as a master regulator upstream of LRRK2, Rab29 may also be targeted downstream by LRRK2 kinase activity, whilst neither Rab32 nor Rab38 possess this conserved Switch 2 phosphorylation site.

Abnormal lysosomal phenotype and defective retromer complex function arising from a deficiency of the VPS35 component are observed in Rab29-LRRK2 pathway defects [222]. Deficiency of Rab29 recapitulates dopaminergic degeneration observed with LRRK2^{G2019S}

expression in a *Drosophila* model and, conversely, its overexpression rescues the LRRK2 mutant phenotypes. Expression of wild-type VPS35, which itself is associated with rare autosomal dominant forms of familial PD, can also rescue these defects [222, 416, 417]. A further link was established when – like Rab29 – the VPS35^{D620N} pathogenic mutation was also shown to enhance LRRK2-mediated phosphorylation of Rab8, Rab10 and Rab12 by an unknown mechanism [418]. What's more, as mentioned in Section 1.8.5 Rab32/38 are also linked to retromer function via their interaction with the SNX6 transient subunit [223]. Thus, while regulation of LRRK2 by the Rab32 subfamily is clearly multi-faceted and highly complex, evidence points to a common pathogenic mechanism involving retromer trafficking and lysosome functions. Furthermore, this evidence highlights the importance of accessory domains in regulating LRRK2 localisation and physiological functions. Further characterisation of these neglected interactions outside the catalytic core of LRRK2 may indeed provide important links to help to unravel the complexity of LRRK2 functionality in PD pathogenesis.

1.17 Scientific Objectives

In vertebrates LRRK2 has only one paralogue: LRRK1. Both proteins display a common conserved domain architecture with the exception that LRRK1 does not possess an armadillo domain or WD40 repeats. In fact, it has recently been uncovered that Rab7a is phosphorylated at the conserved Switch 2 site by LRRK1, promoting the interaction with its effector RILP [419]. Nevertheless, mutations introduced into LRRK1 are less toxic than the equivalent pathological LRRK2 mutations *in vitro*, and there is no known link between LRRK1 and Parkinson's disease [247, 303]. Thus, whilst all currently known LRRK2 pathogenic mutations cluster within the catalytic core, the importance of accessory domains should not be dismissed.

The Rab32 family are reported to bind to the N-terminus of LRRK2. The current data shows Rab32 and Rab38 play a role in controlling LRRK2 subcellular localisation, while Rab29 functions upstream as a master regulator of LRRK2 kinase activity [300, 306]. The Rab29 gene is also found within the *PARK16* PD risk-associated locus. Taken together, this evidence suggests the possibility that the N-terminus of LRRK2 and its associated interactions with the Rab32 subfamily may play an important role in disease pathogenesis. Further understanding of the nature of these interactions could provide valuable insight into the regulation of LRRK2.

Aim 1. Determination of the LRRK2 binding region and Rab nucleotide specificity

The Rab32/38 effector VARP is an ankyrin domain-containing protein, yet Waschbusch *et al.* (2014) demonstrate the binding of Rab32/38 to the armadillo repeats of LRRK2 and not the adjacent ankyrin domain. Through yeast two-hybrid analysis the authors identified Rab32/38 binding to a truncated ARM domain fragment (residues 1-552) [300]. It is hypothesized that Rab32/38 binding to LRRK2 is functionally redundant, and that Rab29 exhibits a unique interaction with the ankyrin repeats. This hypothesis regarding the Rab29 binding site arises from co-immunoprecipitation analyses, however a direct interaction specifically between Rab29 and the LRRK2 ANK domain has not been demonstrated to date [306]. Furthermore, the nucleotide-specificity of the Rab32 subfamily interactions with LRRK2 has not been fully addressed.

Here we will use LRRK2 truncation mutants to clarify the region of binding for each member of the Rab32 subfamily. We will also establish whether the interaction of the Rab32 subfamily with LRRK2 is GTP-dependent.

Aim 2. Structural analyses of the Rab32 subfamily and the LRRK2 ARM domain

The crystal structure of Rab32 has previously been solved in complex with VARP (GTP-bound form) and GtgE (GDP-bound form). However, the structures of Rab38 and Rab29 are currently unknown. Here we aim to apply X-ray crystallography techniques for structure solution of these Rab GTPases to complete the subfamily.

There is no structural data currently available at high resolution for the N-terminal regions of LRRK2. Human LRRK2 constructs have not been readily amenable to crystallisation, underscored by the extensive use of bacterial homologues in structural studies to date. In this work we use a homology model of the LRRK2 N-terminus. Together with Rab structural data we plan to identify candidate residues that are critical for mediating the LRRK2:Rab interaction.

Aim 3. Biophysical characterisation of LRRK2-Rab interactions

Lastly, binding experiments will be performed to determine the affinities of LRRK2-Rab interactions by different methods. Mutational analysis will also be carried out based on the candidate residues identified in Aim 2. Here we intend to validate and expand upon our structural data by using *in vitro* reductionist assays to further characterise the site(s) of interaction.

Chapter 2: Materials and Methods

2.1 Buffers and Recipes

2.1.1. Protein Extraction and Purification Recipes

- **Phosphate-buffered saline (PBS)** : 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4
- **Extraction Buffer**: 10 mM Tris, 300 mM NaCl, 10-40 mM imidazole, 10 mM β -mercaptoethanol (β -ME), pH 8
- **Elution Buffer**: 10 mM Tris, 300 mM NaCl, 200 mM imidazole, 10 mM β -ME, pH 8
- **Dialysis Buffer**: 10 mM Tris, 300 mM NaCl, 10 mM imidazole, 10 mM β -ME, pH 8
- **Low salt anion exchange buffer**: 10 mM Tris, 10 mM NaCl, 1 mM DTT, pH 8
- **High salt anion exchange buffer**: 10 mM Tris, 1 M NaCl, 1 mM DTT, pH 8
- **Gel Filtration Buffer**: 20 mM HEPES/Tris, 100-200 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$ pH range 7.0 - 8.0
- **HPLC Running Buffer**: 100 mM potassium phosphate, 8mM tetrabutylammonium (TBA), pH 6.5

2.1.2 SDS-PAGE Recipes

- **4X Loading Buffer**: 40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β -ME
- **20X MOPS Bis-Tris Running Buffer** : 2.5 M MOPS, 2.5 M Tris, 5% SDS, 50 mM EDTA, pH 7.7
- **10X Tris-Glycine Running Buffer**: 250 mM Tris, 2 M Glycine, 1% SDS
- **4X Separating Buffer**: 1.5 M Tris, pH 8.8
- **4X Stacking Buffer**: 1 M Tris, pH 6.8
- **Separating Gel formula 10.5%**: 3.5 mL Acrylamide (30% v/v), 3.5 mL ddH₂O, 2.5 mL separating buffer, 100 μ L 10% APS, 100 μ L 10% SDS, 10 μ L TEMED
- **Separating Gel formula 12%**: 4 mL Acrylamide (30% v/v), 3 mL ddH₂O, 2.5mL separating buffer, 100 μ L 10% APS, 100 μ L 10% SDS, 10 μ L TEMED

- **Separating Gel formula 15%:** 5 mL Acrylamide (30% v/v), 2 mL ddH₂O, 2.5 mL separating buffer, 100 µL 10% APS, 100 µL 10% SDS, 10 µL TEMED
- **Stacking Gel formula:** 670 µL Acrylamide (30% v/v), 3 mL ddH₂O, 1.25 mL stacking buffer, 50 µL 10% APS, 50 µL 10% SDS, 10 µL TEMED
- **Coomassie stain:** 10% Acetic acid, 40% Ethanol, 0.5% Brilliant blue powder
- **Destain:** 10% Acetic acid, 40% HPLC grade methanol

2.1.3 Agarose gel electrophoresis recipes

- **TAE buffer:** 40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.5
- **Agarose Gel (1% w/v):** 0.7 g agarose, 70 mL TAE buffer, 7 µL Nancy-520 dye

2.2 Methods

2.2.1 Polymerase chain reaction (PCR)

Routine PCR amplification was carried out according to the New England Biolabs protocol for Phusion® High-Fidelity DNA polymerase. The mix for a 50 µL reaction is outlined in Table 2.1 and the thermocycling conditions are outlined in Table 2.2. Primers were ordered from Eurofins Genomics (Europe) or Integrated DNA Technologies, Inc. (USA).

Chemical (in order of addition)	Volume (µL)
ddH ₂ O	X
5X Phusion® HF Buffer	10
10 mM dNTPs	1
10 µM forward primer	2.5
10 µM reverse primer	2.5
Template DNA	X
DMSO	1.5
Phusion® DNA Polymerase	0.5

Table 2.1. PCR reaction mix

Volumes of ddH₂O and template DNA were adjusted according to concentration of DNA. The recommended amount of DNA template for a 50 µL reaction is between 1 pg – 10 ng.

Step	Temperature (°C)	Duration	No. of cycles
Initial Denaturation	98	1 min	1
Denaturation	98	45 sec	30
Annealing	58	45 sec	
Extension	72	45 sec	
Final Extension	72	5 min	1

Table 2.2. PCR thermocycling conditions

Annealing temperature was adjusted according to the T_{melt} values of the primers used in each individual reaction.

2.2.2 Ligation independent cloning

Ligation independent cloning (LIC) into the pNIC-BSA4 vector was carried out by amplifying the desired insert using primers with the following 5' extensions.

Forward Primer 5' TAC TTC CAA TCC ATG-----

Reverse Primer 5' TAT CCA CCT TTA CTG TTA-----

ATG corresponds to the start codon of the insert, and TTA to the STOP codon. Amplification was carried out using conventional PCR thermocycling methods as described in 2.2.1. The PCR insert was then purified using the Isolate II PCR and Gel kit (Bioline) and eluted in 30 μ L sterile filtered water. Optimal DNA concentration for the LIC reaction is 15-20 ng/ μ L as recommended by New England Biosciences, and inserts were diluted accordingly.

Insert DNA was treated with T4 DNA polymerase in the presence of dCTP to generate cohesive ends using the reaction mix outlined in Table 2.3. Reactions were incubated for 30 min at 22 °C (295K) then for 20 min at 75 °C (348 K) in a thermocycler.

Chemical (in order of addition)	Volume Per Reaction (μ L)
ddH ₂ O	1.9
NE Buffer 2 (10X)	1
dCTP (25mM)	1
DTT (100mM)	0.5
BSA (10mg/mL)	0.1
Insert DNA	5
T4 DNA Polymerase	0.5

Table 2.3. Reaction mix for T4 treatment of PCR inserts

The *Bsa*I linearized pNIC28-BSA4 vector was already available for use in the lab. The stock vector had previously been treated with T4 DNA polymerase in the presence of dGTP as per the protocol in Table 2.3 to allow for complementary base pairing to the insert, and stored at -20 °C (253 K). A 'stuffer' fragment in any empty nonlinearized vector present includes the

SacB gene, allowing for negative selection on 5% sucrose. A 1 µL volume of the 'LIC-ready' vector DNA was added to 2 µL of T4 treated insert and incubated at room temperature for 10 min to anneal. The ligated construct was then transferred on to ice and transformed using DH5α cells as per protocol described in Section 2.2.6. Cells were plated onto kanamycin/sucrose plates and incubated at 37 °C (310 K) overnight.

2.2.3 Site-directed mutagenesis

Site specific amino acid mutations were introduced using either the Quikchange™ II (Aligent) or Q5® (NEB) site-directed mutagenesis protocols and temperature-cycling was performed as in Section 2.2.1. After cycling completion 1 µL *DpnI* restriction enzyme was added to the reaction and incubated at 37 °C (310 K) for 3 hours to digest template DNA, followed by 80°C (353 K) for 20 min to deactivate the enzyme. Transformation of the amplified product was carried out as described in Section 2.2.6 and individual clones sequenced (Section 2.2.7) to confirm introduction of the desired mutation.

2.2.4. DNA electrophoresis

The quality and the concentration of PCR products, template DNA and plasmid DNA were analysed by electrophoresis through 1% agarose gels. Gels were prepared by melting 70 mL of 1% w/v agarose in TAE buffer with gentle heating. When the melted gel was cool to touch 7 µL of Nancy-520 dye was added and mixed into the solution before the gel was poured and allowed to set in an ATTO AE-6100 gel box. The Generuler™ Express 1kb/10kb ladder was used depending on requirements. Samples were electrophoresed in TAE buffer at 100 V for 50 – 60 min and visualised on an ImageQuant LAS 4000 imager (GE Healthcare). DNA concentration was also confirmed by NanoDrop™ as described in Section 2.2.14.

2.2.5. Calcium²⁺ competent bacterial cells

To make *Escherichia coli* (*E. coli*) bacterial cells competent for plasmid uptake, an overnight culture without antibiotic was prepared from glycerol stocks as described in Section 2.2.8.

This culture was used to inoculate 5 mL fresh LB broth and incubated at 37 °C (310 K), 150 rpm until the optical density at 600 nm (OD_{600}) reached 0.6 – 0.8. The culture was then centrifuged at 3200 g for 10 min at 4 °C (277 K) and the supernatant removed. The cell pellet was gently resuspended in 1 mL of chilled 100 mM $CaCl_2$ solution, transferred to a 1.5 mL micro-centrifuge tube and incubated on ice for 30 min. The cell suspension was then centrifuged at 3200 g for 10 min at 4 °C (277 K), gently resuspended in 300 μ L of chilled 100 mM $CaCl_2$, 15% glycerol solution, and incubated for a minimum of 2 hr on ice. For transformation, 100 μ L cells were taken and the protocol carried out as in Section 2.2.6. The remaining volume was aliquoted and snap frozen in liquid nitrogen (-196°C, 77 K) for future use.

2.2.6. Transformation of *E. coli* bacterial strains

Plasmids were transformed into competent *E. coli* cells using a heat shock method. Following subcloning and mutagenesis the DH5 α strain was used to maximise transformation efficiency. The BL21(DE3) strain, engineered as a bacteriophage T7 promoter-based expression system, was chosen for recombinant protein production. Derivatives of BL21(DE3) cells also available in the lab are outlined in Table 2.4. Plasmid DNA (10-50 ng) was added to 50 μ L of appropriate host cells, the suspension mixed by gently tapping of the tube, and incubated on ice for 20 minutes to allow for DNA binding to the cell surface. The suspension was then heat shocked at 37 °C (310 K) for 3 min for plasmid uptake, followed by incubation on ice for an additional 2 min. A 1 mL volume of sterile LB broth (Miller) was added to cells and incubated at 37°C (310 K), 800 rpm for 45 – 60 min. Samples of poor transformation efficiency were subsequently centrifuged at 11,000 g for 5 min and 950 μ L supernatant removed. The cell pellets were resuspended in the remaining volume (~100 μ L). For high efficiency transformations 100 μ L was taken directly from the 1 mL culture without centrifugation. In both circumstances the 100 μ L volume was streaked onto LB agar plates with selective antibiotics according to the plasmid resistance gene. Agar plates were incubated overnight at 37 °C (310 K), followed by storage at 4 °C (277 K).

Antibiotics were used at the following final concentrations: Ampicillin (Amp^+): 100 μ g/mL; Kanamycin (Kan^+): 30 μ g/mL; Chloramphenicol (Cam^+): 34 μ g/mL.

Bacterial strain	Features
DH5 α	Contains <i>recA1</i> and <i>endA1</i> mutations, improves plasmid uptake, stability and yield of DNA [420]
BL21 (DE3)	Basic IPTG-inducible strain. Deficient in Lon and OmpT proteases[421]
BL21 (DE3) Star	Mutated in RNase E, RNase degradation is reduced[422, 423]
BL21-CodonPlus-RIL	Carries extra copies of the <i>argU</i> , <i>ileY</i> , and <i>leuW</i> tRNA genes. Cam ⁺ resistance [424]
BL21 (DE3) pLysS	Produces T7 lysozyme, reduces basal level expression of the gene of interest. Cam ⁺ resistance [425]

Table 2.4. Competent *E. coli* strains for plasmid DNA amplification and recombinant protein expression

2.2.7. Miniprep plasmid DNA purification

Single colonies obtained from transformation of DH5 α cells were inoculated into 5 mL LB broth (Miller) with appropriate antibiotic and incubated at 37 °C (310 K), 150 rpm overnight. The cultures were centrifuged at 3200 g for 10 min and the supernatant removed. The plasmid was purified from the cell pellet using the Bioline Isolate II Mini Plasmid Kit. To improve yield the manufacturer's instructions were optimized by increasing centrifuge spin length to 11,000 g for 10 min at the clarification step, and elution with 30 μ L of sterile filtered water replacing 50 μ L elution buffer at the final step. Purified plasmid DNA was sequenced at MWG Eurofins Germany or Functional Biosciences Wisconsin.

2.2.8. Glycerol stocks

Single colonies from agar plates were picked up with a sterile yellow tip and were used to inoculate 5 mL of sterile LB broth with appropriate antibiotic. The culture was grown at 37 °C (310 K), 150 rpm overnight. A 400 μ L volume of culture was then added to 200 μ L of 50%

glycerol for the creation of bacterial stocks to be stored at -80 °C (193 K).

2.2.9. Small scale expression test

Small scale expression tests were carried out for each recombinant protein construct prior to expression in large volumes to confirm the presence of soluble protein, and to establish optimal expression conditions. A 5 mL volume of sterile media with antibiotic was inoculated with 100 µL of 5 mL overnight starter cultures and grown at 37 °C (310 K), 150 rpm for ~ 1.5 hours. Media optimisation for protein expression could be carried out using LB broth, LB AutoInduction media (AIM), 2xYT broth, 2xYT + 1% glucose, and 2xYT AIM. When cultures had reached OD₆₀₀ between 0.6 - 0.8 gene expression was induced by addition of IPTG at 0.25 – 0.5 mM final concentration, excluding cultures grown using AIM. Induced cultures were typically incubated at 37 °C (310 K), 150 rpm for 3 hours, or at 18 °C (291 K), 150 rpm overnight.

The cultures were harvested by centrifugation at 3200 g at 4 °C (277 K) for 10 min. Cell pellets were stored at -20 °C (253 K) until required, or immediately resuspended in 1 mL extraction buffer and sonicated 2 x 20 pulses. The lysed cells were centrifuged at 11,000 g for 20 min at 4 °C (277 K) and both supernatant and pellet were retained. To each soluble fraction 30 µL of 50% HisPur™ Ni²⁺ resin was added, and samples rotated for 2 min at speed 20 on a spiral rotator to allow for His-tagged target protein binding. Once mixed the samples were centrifuged at 0.4 g for 1 min in a minifuge and the supernatant carefully removed so as not to disturb the Ni²⁺ agarose bead pellet. Rotation and centrifugation were repeated with 1 mL extraction buffer to wash. A 10 µL volume of 4X loading buffer was added to the Ni²⁺ agarose bead pellet and the samples retained for SDS-PAGE analysis as in Section 2.2.13. Insoluble cell pellets were resuspended in 1 mL ddH₂O and a 10 µL insoluble sample was diluted with 20 µL ddH₂O and 10 µL 4X loading buffer for ease of loading on an SDS-PAGE gel. All gel samples were stored at -20 °C (253 K) until required.

2.2.10. Large scale protein production

For expression of each recombinant protein, an overnight culture with selective antibiotic 1:50 volume of final large culture was prepared from glycerol stocks as previously described. Large volume flasks of 2xYT broth with selective antibiotic were warmed to 37 °C (310 K) prior to addition of overnight cultures. Large scale cultures were incubated at 37 °C (310 K), 150 rpm until an OD₆₀₀ of 0.4 was reached. Cultures were then moved to 18 °C (291 K). When cultures reached OD₆₀₀ 0.6 – 0.8, approximately 30 min later, gene expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.5 mM final concentration. Induced cultures continued to grow at 18 °C (291 K), 150 rpm overnight. Following the incubation period cultures were centrifuged at 1500 g for 15 min at 4 °C (277 K). To proceed immediately to protein purification cell pellets were resuspended in extraction buffer as described in Section 2.2.11. Alternatively, pellets were resuspended in 25 mL phosphate-buffered saline (PBS) and transferred to 50 mL falcon tubes. The PBS cell suspension was centrifuged at 3200 g for 10 min at 4 °C (277 K) and pellets were stored at -20 °C (253 K) until required. A summary of all constructs used for large scale protein production is given in Tables 2.5 and 2.6.

Construct	Vector	Description
Rab38 1-181 WT	pET15b	Globular G-domain of Rab38. C-terminal hypervariable region truncated
Rab38 1-181 Q69L	pET15b	Activating mutation. Prevents GTP hydrolysis
Rab38 1-181 R39Q	pET15b	Switch I mutant, loss of exposed positive charge
Rab38 1-181 R77/81Q	pET15b	Switch II mutant, loss of exposed positive charge
Rab38 1-181 I42A/E	pET15b	Switch I mutant, loss of hydrophobic residue often used as a binding interface
Rab32 1-198 WT	pNIC-Bsa4	Globular G-domain of Rab32. C-terminal hypervariable region truncated
Rab32 1-198 Q85L	pNIC-Bsa4	Activating mutation. Prevents GTP hydrolysis
Rab32 20-198 Q85L	pNIC-Bsa4	N-terminal truncation
Rab32 20-198 R55Q, Q85L	pNIC-Bsa4	Switch I mutant, loss of exposed positive charge
Rab29 1-177 WT	pNIC-Bsa4	Globular G-domain of Rab29. C-terminal hypervariable region removed
Rab29 1-177 Q67L	pNIC-Bsa4	'Activating' mutation. Prevents GTP hydrolysis
Rab29 1-177 '3cys' C84A, C120A, C127S	pET15b	Substitution of cysteines for improved crystal growth
Rab29 1-177 V156A	pNIC-Bsa4	Mutation in G5 motif to promote GTP binding
Rab29 1-177 E68S	pNIC-Bsa4	Switch II mutant to promote GTP binding
Rab29 1-177 K37Q	pNIC-Bsa4	Switch I mutant, loss of exposed positive charge
Rab11A 1-173 Q70L	pNIC-Bsa4	Control. Does not interact with LRRK2

Table 2.5. Rab32 subfamily constructs for large scale protein expression and purification

Constructs within the pNIC-BSA4 vector were sub-cloned in-house. Constructs within the pET15b vector were purchased from Genscript and encode a codon-optimised sequence for *E. coli* expression.

Construct	Vector	Description
LRRK2 1-552 WT	pNIC-Bsa4	Armadillo (ARM) repeats fragment identified in yeast two-hybrid screen
LRRK2 1-552 '2mut' D390N, D392N	pNIC-Bsa4	Loss of 2 negative charges towards C-terminus of ARM domain construct
LRRK2 1-552 '3mut' D390N, E391Q, D392N	pNIC-Bsa4	Loss of 3 negative charges towards C-terminus of ARM domain construct
LRRK2 1-552 '4mut' E386Q, D390N, E391Q, D392N	pNIC-Bsa4	Loss of 4 negative charges towards C-terminus of ARM domain construct
LRRK2 1-552 'Nmut' E192Q, E193Q	pNIC-Bsa4	Loss of 2 negative charges towards N-terminus of ARM domain construct
LRRK2 1-552 Δ CC 317-342	pNIC-Bsa4	Deletion of negatively charged loop
LRRK2 10-661	pNIC-Bsa4	Predicted full ARM domain
LRRK2 681-860	pLIC-MBP	Predicted Ankyrin (ANK) repeats
LRRK2 1-910	pET15b	Predicted full ARM and ANK domains

Table 2.6. LRRK2 N-terminus constructs for large scale protein expression and purification

Constructs within the pNIC-BSA4 and pLIC-MBP vectors were sub-cloned in-house. Constructs within the pET15b vector were purchased from Genscript and encode a codon-optimised sequence for *E. coli* expression.

2.2.11. Large scale protein purification

Cell pellets obtained from Section 2.2.10 were resuspended in 20 - 30 mL of chilled extraction buffer with 1 cOmplete™ protease inhibitor tablet and 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and homogenized using a glass homogenizer. The resuspended cells were then sonicated on ice for 3 x 2 min cycles, with a 2 min break between each cycle. Sonication settings were at a duty output of 35% and a power output of 4. The resulting lysate was centrifuged at 25,000 g for 30 min at 4 °C (277 K) and the supernatant retained for affinity purification.

All constructs used in this study were recombinantly expressed with an N-terminal cleavable hexa-histidine tag. Immobilised metal affinity chromatography (IMAC) was used as an initial purification step. The HisPur™ Ni²⁺-agarose or Co²⁺-agarose beads (5 mL of 50:50 suspended resin) were equilibrated in a gravity flow column with extraction buffer followed by loading of the cell supernatant. Once the supernatant passed through, the column was washed rigorously with extraction buffer supplemented with 40 mM imidazole to remove any proteins non-specifically bound to the beads. The progress of the wash could be monitored with Bradford drops as in Section 2.2.15. The His-tagged protein of interest was eluted from the column with elution buffer and collected. Cleavage of the His-tag was carried out by addition of 100 µL recombinant TEV protease (produced in house) or 10 units/mL thrombin (GE Healthcare), with overnight dialysis (Thermo Scientific Snakeskin Dialysis Membrane 14 kDa MWCO) to remove high imidazole concentration from protein elute. Following dialysis, 3 mL of suspended Ni²⁺-agarose resin was added to the protein solution and gently mixed using a spiral rotator for 20 – 30 mins at 4 °C (277 K). An additional 1 mL of fresh suspended resin was added to a gravity column and the protein/Ni²⁺ solution loaded for reverse IMAC purification. The flow-through containing cleaved protein was immediately collected and column washed with extraction buffer to collect any residual cleaved protein. Cleaved His-tags and any uncut protein remained bound to the Ni²⁺-agarose beads.

Following affinity chromatography steps, 10 – 20 mL of protein sample was collected. For binding studies, protein was then concentrated to 1 mL using an Amicon® Ultra centrifugal filter unit (Millipore™ 10 kDa MWCO) and loaded directly onto a Superdex™200 13/300 size exclusion chromatography (SEC) column. Protein intended for crystallisation trials was first

subject to ion exchange chromatography (IEX). The protein sample was dialysed for 3 hr in 1 L low salt buffer and loaded onto an anion-exchange MonoQ® column (GE Healthcare) mounted on the Akta purifier system. Low salt and high salt buffers were prepared and a 60% linear salt gradient was run over 30 mL to separate the components of the sample based on surface charge. The appropriate fractions were collected and loaded onto a Superdex™200 16/60 size exclusion column equilibrated with Gel Filtration buffer. The protein purity was checked at each step before proceeding by SDS-PAGE analysis as described in Section 2.2.13.

2.2.12. Rab nucleotide exchange reaction

A nucleotide exchange was performed by incubating the Rab proteins with 10 mM EDTA and a 10-fold molar excess nucleotide (non-hydrolysable GTP analogue GppNHp, or GDP as required) at room temperature for 10 min. To quench the exchange a final concentration of 15 mM MgCl₂ was added and the solution incubated on ice for a further 15 min. Excess nucleotide was removed by running samples through a PD10 column (GE Healthcare) or by gel filtration chromatography. For fluorescence studies *N*-methylantraniloyl (mant) derivatives of GppNHp and GDP were used for nucleotide exchange. On account of small volumes removal of excess mant nucleotide was achieved by overnight dialysis in gel filtration buffer using Slide-A-Lyzer™ dialysis cassettes (Thermo Fisher).

2.2.13. SDS-Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the presence and the purity of proteins. The desired percentage recipes for discontinuous Tris-Glycine SDS-PAGE as per the Laemmli system [426] are outlined in Section 2.1.2. NuPAGE 12% Bis-Tris precast gels (Thermo Fisher) were also used. Protein samples containing 4X loading buffer were heated at 99 °C (372 K) for 2 min prior to loading. Typically, 10-15 µL of sample and 10 µL of unstained protein molecular weight marker (Thermo Fisher) was loaded. Gels were run using a constant voltage of 150 V. Completed gels were washed briefly with water and stained with Coomassie Brilliant Blue. Gels were then treated with destain solution until protein bands became clearly visible. If greater sensitivity was required the SilverQuest™

silver stain kit (Invitrogen) was used for band visualisation as per the manufacturer's protocol.

2.2.14. Concentration measurements by NanoDrop™ spectrophotometry

The NanoDrop™ ND-100 spectrophotometer was used to estimate protein concentration at 280 nm wavelength. The spectrophotometer was first 'blanked' with an appropriate reference material. A 2 µL sample was then pipetted onto the lower measurement pedestal and the sampling arm closed to create surface tension for sample measurement. A spectral reading was initiated using the PC operating software. Readings were taken three times for each sample to account for discrepancies. The Beer-Lambert Law was used to relate the measured absorbance to the concentration of protein in the solution.

$$A = \epsilon \cdot c \cdot l$$

A = the absorbance represented in absorbance units (A)

ϵ = the molar extinction coefficient with units of litre/mol-cm and is a constant for a given molecule. The value of ϵ for the protein of interest can be obtained from the ProtParam website.

C = the analyte concentration in moles/litre

L = the path length in cm. The Nanodrop™ absorbance reading is represented as if measured with a conventional 1 cm path.

In the same way, the concentration of DNA samples was also estimated by measuring absorbance at 260 nm on the spectrophotometer.

2.2.15. Bradford protein assay

To detect the presence of protein in samples during the purification process, 3 µL of the sample was pipetted into a 30 µL drop of 1X Quick Start™ Bradford dye reagent. A colour change from red to blue indicated the presence of protein and the intensity of the blue colour could be used to visually estimate protein concentration.

For more accurate measurements of protein concentration, Bovine Serum Albumin (BSA) standard solutions were prepared at the following concentrations: 0, 150, 250, 300, 500, 600, 750 and 1000 µg/mL. Using micro-centrifuge tubes 20 µL of each standard was added to 480 µL of 1X Quick Start™ Bradford dye reagent and the solution mixed thoroughly. After a 5 min incubation period at room temperature the absorbance value of each standard solution at 595 nm wavelength was measured. A standard curve was generated through linear regression in Microsoft Excel by plotting the A_{595} of the standard solutions versus their concentrations. One 20 µL sample of the protein of interest was then subject to the same protocol, and the equation for the standard linear regression could be used to calculate the concentration of the sample.

2.2.16. Bicinchoninic acid assay (BCA assay)

The QuantiPro™ BCA assay kit (Sigma) was used as per the manufacturer's protocol. The working reagent was prepared by mixing 25 parts of Buffer A (Na_2CO_3 , $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6$, NaHCO_3 in 0.2 M NaOH pH 11.25) with 25 parts of Buffer B (4% (w/v) bicinchoninic acid pH 8) and 1 part of reagent C (4% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).

BSA standard solutions were prepared in the same concentrations as Section 2.2.15 above. Samples of the protein of interest were diluted over a wide range if necessary, to ensure the sample concentration value will fall within the limits of the standard curve. A 2 µL aliquot of each standard/sample was added to 16 µL of working reagent and the solutions incubated at 60 °C (333 K), 500 rpm for 30 - 60 min. A standard curve was generated using the NanoDrop™ BCA assay program measuring at 562 nm, and the protein sample concentration then determined using this software. In this circumstance water was used as the blank and the dye reagent + buffer without protein as the reference sample.

2.2.17. Analytical HPLC analyses of Rab GTPases

Rab proteins were purified as described in Section 2.2.11 and boiled for 10 min at 95 °C (368 K) to release the nucleotide, followed by centrifugation at 16,000 g for 30 min at 4°C to remove precipitated protein. The supernatant was mixed with HPLC running buffer at a 1:1

ratio and the samples loaded on an Acquity Ultra Performance LC® system (Waters Corporation) or a Varian 920 LC® machine (Agilent) equipped with a Zorbax 300SB-C18 column (Agilent). To verify the nucleotide state of the Rab from the elution profiles, pure solutions of 100 µM GDP, GTP and GppNHp (Sigma Aldrich) were run as standards.

2.2.18. Static light scattering

The FPLC system was connected in-line with the miniDAWN™ multi-angle light scattering system, followed by an Optilab® refractometer (Wyatt Technologies). A Superdex™200 10/300 size exclusion column equilibrated with gel filtration buffer was then loaded with 0.1–0.5 mg purified protein and allowed to run at a flow rate of 0.6 mL/min. Data processing and absolute molecular mass calculations were performed using the ASTRA software (Wyatt Technologies).

2.2.19. In vitro pulldown assays

To test for *in vitro* binding purified His-tagged Rab proteins (bait) were used to pull down untagged LRRK2 (prey). The proteins were mixed together in equimolar amounts with 40 µL Ni²⁺-agarose resin in final volume of 1 mL. The reaction mixture was agitated gently for 5 min, centrifuged at 0.1 g to pellet the resin, and the supernatant removed. The resin was washed three times with 1 mL extraction buffer before elution of bound protein with 50 µL elution buffer. Samples were then visualised by SDS-PAGE as per Section 2.2.13.

2.2.20. Isothermal titration calorimetry

Purified and nucleotide exchanged Rab38 and LRRK2 were co-dialysed against Gel Filtration buffer, and concentrated to 400 µM and 40 µM respectively. Calorimetric measurements were performed using the MicroCal™ ITC200 instrument. Titrations were carried out at 20 °C (293 K) with LRRK2 in the cell and Rab38 injected from the syringe. Data was analysed using Origin software 7.0, and curves were fit to a single site binding model.

2.2.21. Fluorescence assays

Fluorescence measurements were carried out at 20 °C (293K) in Gel Filtration buffer, using black 96-well assay plates (Corning). A final concentration of 0.5 - 1 µM mant-GppNHp/GDP bound Rab proteins were incubated with increasing concentrations of LRRK2 in 80 µL volumes. Fluorescence intensity measurements and fluorescence anisotropy data were obtained using the SpectraMax® M3 or M5 plate reader. The mant guanine nucleotide analogues were excited at 355 nm, and emission was detected at 448 nm. Background fluorescence of mant-associated Rab GTPases in the absence of LRRK2 was subtracted and non-linear regression curve fitting for affinity measurements was performed with MATLAB software, using the following quadratic equation to account for ligand depletion:

$$y = (K_d + RT + LT - ((K_d + RT + LT)^2 - 4 * RT * LT)^{1/2}) / 2$$

To analyse the effects of various mutants on binding, multiple intensity measurements were taken at 1 µM Rab(mant-GppNHp) incubated with 10 µM LRRK2 and the change in fluorescence was plotted as a bar graph using GraphPad Prism 8 software. Rab(mant-GDP) signal was also measured as a control.

2.2.22. Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) was carried using the ThermoFluor™ protocol. An 18 µL sample of purified LRRK2 protein (5-10 µM) was mixed with 2 µL of SYPRO Orange dye (10X stock) in a 96 well assay plate. A temperature gradient from 25 – 95 °C (298 – 368 K) was set on a RT-PCR machine, with temperature increasing in increments of 1 °C per min. As temperature increases, post-peak aggregation of protein-dye complexes may lead to fluorescence quenching. Truncation of the dataset to remove quenching provides a more accurate estimate of protein melting temperature. Dataset cut-off and non-linear fitting using the Boltzmann equation were carried out as per the protocol detailed in Huynh and Pertch (2015) [427].

2.2.23. Crystallisation condition screening

Protein samples considered to be sufficiently pure by SDS-PAGE analyses following IEX/SEC were subject to crystallisation trials. Proteins were concentrated to 5 - 15 mg/mL using Amicon® 10 kDa cut-off centrifugal filter units (Merck Millipore). Initial screening for successful crystallisation conditions was carried out with commercial 96 well sparse matrix screens using the sitting drop vapour diffusion method at 18 °C (291 K). The reservoir volume of 70 µL was pipetted into the Swissci MRC 296-well sitting drop plates (Molecular Dimensions) using a multi-channel pipette. A 150 nL volume of concentrated protein sample was then combined with 150 nL of reservoir solution for each condition using a Mosquito® robot (TTP Labtech) and sealed with clear tape. Drops were examined immediately after set up, then daily for the first week, and weekly thereafter. The crystallisation condition and morphology of any drops of interest were noted. Initial hits were then set up using manual hanging drop 24-well screens (XRL plates Molecular Dimensions) to replicate the original condition. To optimise the crystals, the precipitant concentration and the pH of the solution were altered around the initial condition. Rab38^{Q69L} crystals were grown in 0.2 M sodium potassium thiocyanate, 0.1 M sodium cacodylate pH 6.5, 15% PEG 4000. Rab29^{Q67L} crystals were grown in 1 M potassium sodium tartrate, 0.4 M NaCl, 5% glycerol, 0.1 M imidazole pH 8. Rab29 '3cys' crystals were grown in 2 M NaCl, 0.1 M HEPES pH 7. Crystals were harvested using mother liquor supplemented with 25% glycerol as cyroprotectant and flash frozen in liquid nitrogen at -196 °C (77 K).

2.2.24. Structure determination and refinement

Diffraction data were collected at the Synchrotron Soleil, Paris, France, and the Advanced Photon Source, Argonne, USA. Data were indexed using XDS and scaled with Aimless [428, 429]. The structures of both Rab38 and Rab29 were solved by molecular replacement using the structure of uncomplexed Rab32 previously solved in the lab (PDB code: 6FF8). Alternate cycles of model fitting and refinement were performed using the programs Coot and Phenix [430, 431].

Chapter 3:

Purification and characterisation of LRRK2 and the Rab32 subfamily of GTPases

3.1 Overview

Rab29 is a close relative of Rab32/Rab38 and together they encompass the Rab32 subfamily. All members are involved in the organisation of the trans-Golgi network and the regulation of lysosome-related organelles. Rab32/Rab38 are best known for their role in melanogenesis and their expression profiles are cell-type specific [168-170, 172, 179]. The function of Rab29 in cells is less well understood, however it is ubiquitously expressed and is known to play a role in retromer trafficking [176, 177]. Proteolytic targeting of the subfamily by intracellular pathogens promotes the infectious mechanism, highlighting their importance in antimicrobial defence [205]. In recent years, it has been reported that the Rab32 subfamily interact with the Parkinson's disease-associated kinase LRRK2. Previously, Rab32/38 were found to be important for targeting LRRK2 to transport vesicles and recycling endosomes, thus regulating the localisation of LRRK2 in the cell [300]. However, the functional consequence of this interaction within the cell remains to be elucidated. Significantly, Rab29 was shown to possess unique functionality towards LRRK2. Rab29 is required for the recruitment of LRRK2 to the trans-Golgi network and acts as a master upstream regulator of LRRK2 kinase activity [306]. Activation by Rab29 is further enhanced by PD-linked mutations found within the LRRK2 Roc-COR domain. The pathogenic mechanism giving rise to PD is not currently understood, however pathogenic mutations in LRRK2 most frequently result in the over-activation of the kinase domain [432]. Thus, the ability of Rab29 to regulate kinase activity may too have important consequences. Furthermore, the Rab29 gene is found within the *PARK16* locus, conferring an increased lifetime risk of developing the disease [412].

While all three members of the Rab32 subfamily bind to LRRK2, it is currently unclear what features distinguish Rab29 from its closest relatives to enable this important regulatory function. The Rab binding domain of the Rab32/38 effector VARP is found within its ankyrin domain [182]. LRRK2 also possesses an ankyrin (ANK) domain at its N-terminus, yet yeast two-hybrid assays, GST-pulldowns and co-immunoprecipitation experiments demonstrate

Rab32/38 bind within the adjacent LRRK2 armadillo (ARM) repeats in this case [300]. On the other hand, mutational studies suggest that Rab29 binds within the LRRK2 ankyrin domain at a site unique from Rab32/Rab38 [306]. However, a direct interaction has not yet been shown.

Considering their high sequence identity, it is interesting that members of the Rab32 subfamily may possess distinct binding sites on a shared interaction partner. Currently there is little biophysical or structural information available for these Rab GTPases to allow for speculation as to why this might be the case. Here the successful expression and purification of the LRRK2 N-terminus and all three members of the Rab32 subfamily is shown, and is critical for further investigations into the nature of these interactions *in vitro*. Conflicting results have frequently arisen in the study of LRRK2 function *in vivo*, as it is often difficult to discern direct effects from indirect consequences in cells. Experiments carried out *in vitro* simplify the system under study, and allow for a direct, controlled, and detailed analysis of protein-protein interactions, which is currently lacking for LRRK2 and the Rab32 subfamily in the literature. Using affinity pulldown analyses we will clarify the location of the LRRK2 binding site for all members of the subfamily. The preliminary pulldown data in this chapter on which much of the later work presented in this thesis is based was performed primarily by Dr. Dieter Waschbusch, a post-doctoral researcher in the Khan Lab (Trinity College Dublin).

3.2 Results

3.2.1 Purification of the Rab32 subfamily

The globular G-domains of Rab32, Rab38 and Rab29 were recombinantly expressed in BL21(DE3) cells at 18 °C (291 K) overnight and purified as described in Section 2.2.10. The His-tagged Rab can be visualised by SDS-PAGE following elution from the beads (Figure 3.1; lanes 1,4,7). Following removal of the flexible N-terminal His-tag, cut protein can be identified by a reduction in molecular weight of ~2 kDa. Excellent cleavage efficiency of TEV (Rab32 and Rab29) and thrombin (Rab38) is shown, with negligible amounts of uncut protein remaining (Figure 3.1; lanes 2,5,8). Removal of contaminants and a highly pure cleaved sample was achieved following a reverse Ni²⁺ IMAC step (Figure 3.1; lanes 3,6,9). Assuming the His-tag is accessible, affinity chromatography cannot distinguish between soluble folded protein and aggregates or misfolded protein. On account of this, the purified Rab proteins were then applied to a size exclusion chromatography (SEC) column as a final polishing step. All Rab32 subfamily proteins eluted in a single Gaussian peak at equivalent volumes from the Superdex™200 16/60 column, corresponding precisely to the expected molecular weight of Rab GTPase monomers. The final product for each Rab GTPase following SEC is seen in Figure 3.2 and shows high purity of the sample. These Rab proteins were then used for binding analyses or crystallisation trials as later described.

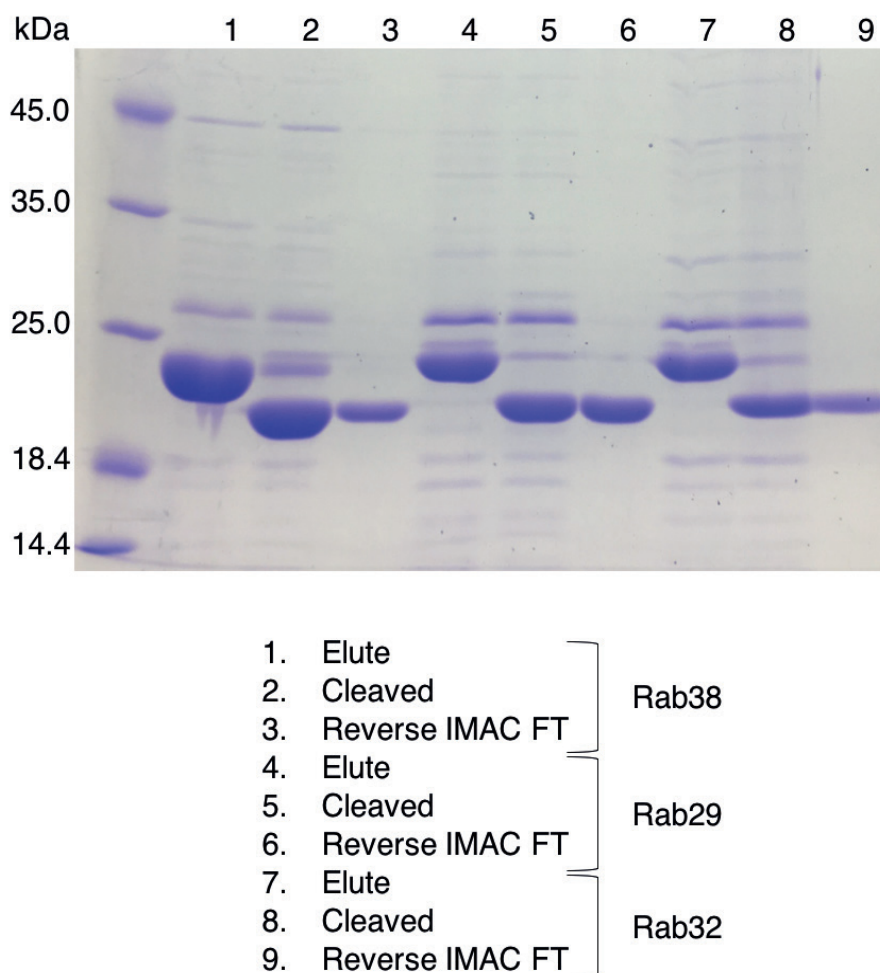


Figure 3.1. Initial affinity purification of the Rab32 subfamily

Affinity purifications of the Rab32 SF proteins is visualised by SDS-PAGE. His-tagged protein was captured by Ni^{2+} -agarose beads and following wash steps was eluted from the column with a buffer containing a high concentration of imidazole. The eluted proteins were cleaved overnight to remove the N-terminal histidine tag and passed through a second Ni^{2+} affinity column. This time all successfully cleaved protein flowed directly through the column, termed 'flow-through (FT)', and free His-tags and uncleaved protein remained bound to the beads, facilitating their removal. A high degree of purity was achieved for Rab GTPases following this initial purification step. (lanes 3, 6, and 9).

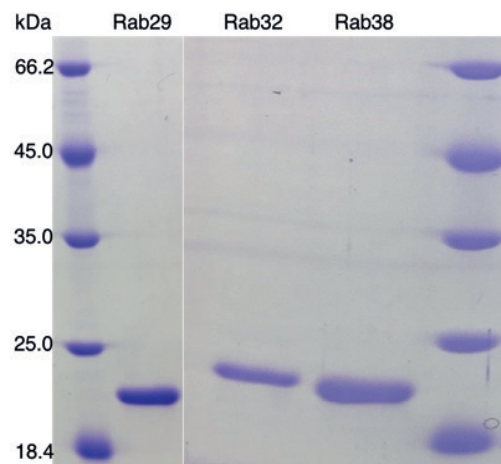
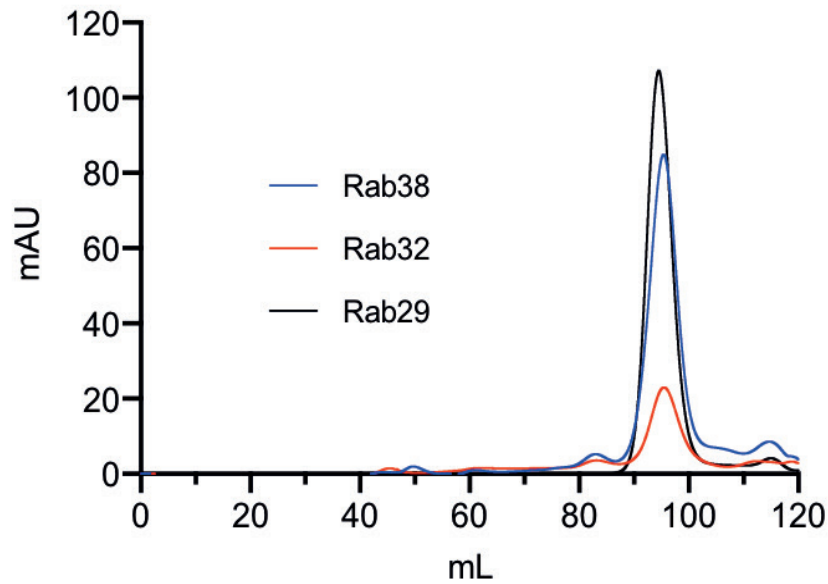


Figure 3.2. Size exclusion chromatography of the Rab32 subfamily

Cleaved Rab proteins obtained following initial affinity purification were concentrated and applied to a Superdex™200 size exclusion column. The resulting chromatographs are displayed as an overlay. All Rab proteins elute as folded monomers with a retention volume of ~95 mL, corresponding to the expected molecular weights of ~20 – 23 kDa. SEC fractions were pooled and purity could be visualised using SDS-PAGE. No visible contaminants are observed within the final samples.

3.2.2 HPLC analysis of the Rab32 subfamily

The SEC profiles of the Rab GTPases indicate a soluble monomeric protein in solution. Reverse phase high pressure liquid chromatography (HPLC) analysis was then conducted to verify the nucleotide-bound state of the Rab protein. For experimental purposes, it is important to maintain the Rab proteins in an active state. While intrinsic hydrolysis of GTP is typically slow, Rabs were exchanged prior to binding experiments or crystallisation trials with a non-hydrolysable GTP analogue (GppNHp) (Section 2.2.12) to ensure the active state was maintained. HPLC analysis was also performed to confirm the success of the exchange reaction. Control runs with pure nucleotide are shown in Figure 3.3, chromatographs show UV absorbance at 254 nm. GMP elutes at 1.7 mL, GDP at 2.8 mL, GppNHp at 3.7 mL, and GTP at 4.4 mL. Examination of Rab proteins determined that nucleotides bound to Rab29 and Rab38 elute from the hydrophobic C18 column at 4.3 mL and 4.4 mL respectively (Figure 3.4). These Rabs are found in their active GTP-bound form upon isolation and purification from *E. coli*. Rab29 could also be successfully exchanged with GppNHp, as seen with an elution volume at 3.5mL in Figure 3.5. Excess nucleotide from the exchange reaction was removed from the protein sample by SEC or dialysis prior to analysis by HPLC. In this circumstance, the Rab29 WT '3cys' construct was tested for use in crystallisation trials, later addressed in Chapter 4. Additional peaks suggest other species are present within the sample, indicating the exchange does not occur with 100% efficiency. Nevertheless, it is apparent that active GppNHp-bound Rab29 is the predominant species.

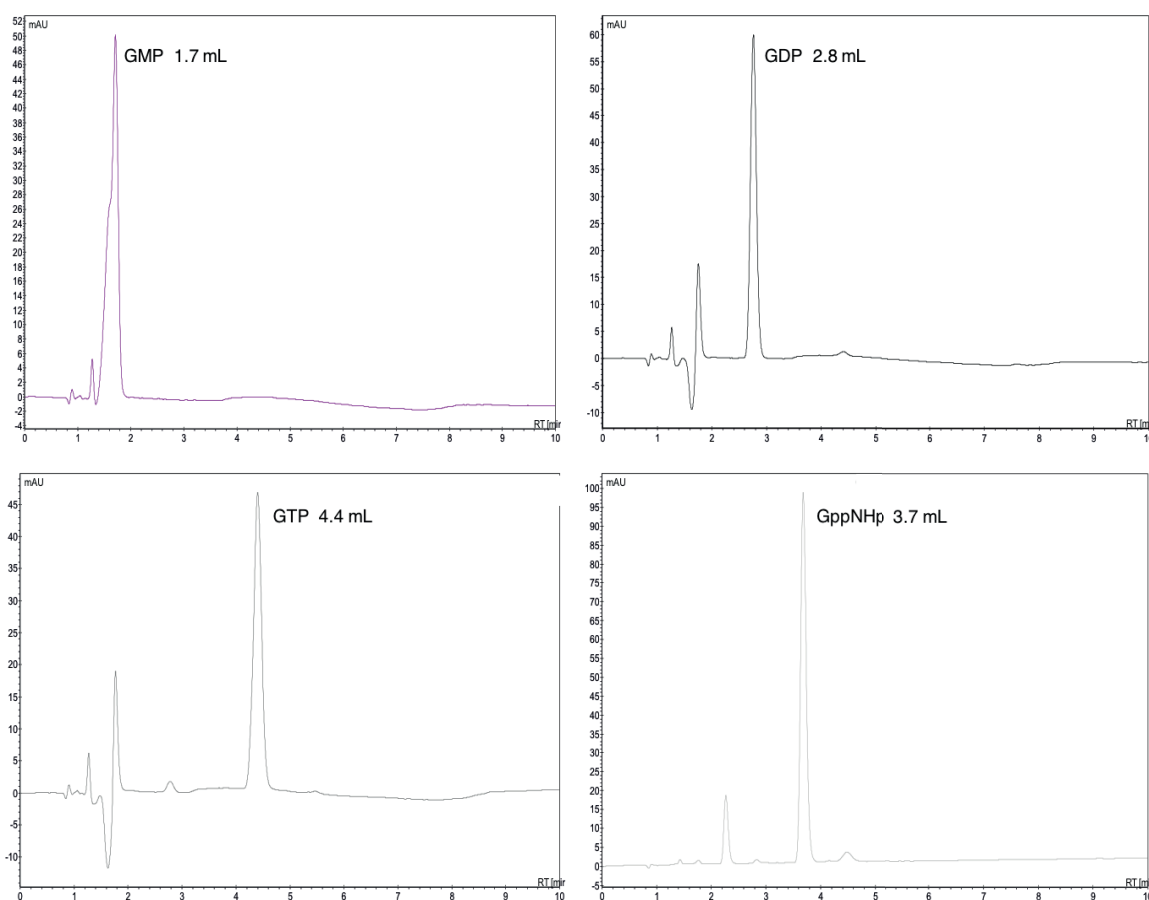


Figure 3.3. HPLC analysis of pure guanine nucleotides

Pure samples of guanine nucleotides were subjected to reverse phase HPLC as control samples to determine the elution profile for each species. The nucleotide and its characteristic retention volume on a C18 column are annotated. The polar nature of guanine nucleotides ablated the need for a gradient to be applied to the HPLC and each run was ceased after 10 mins.

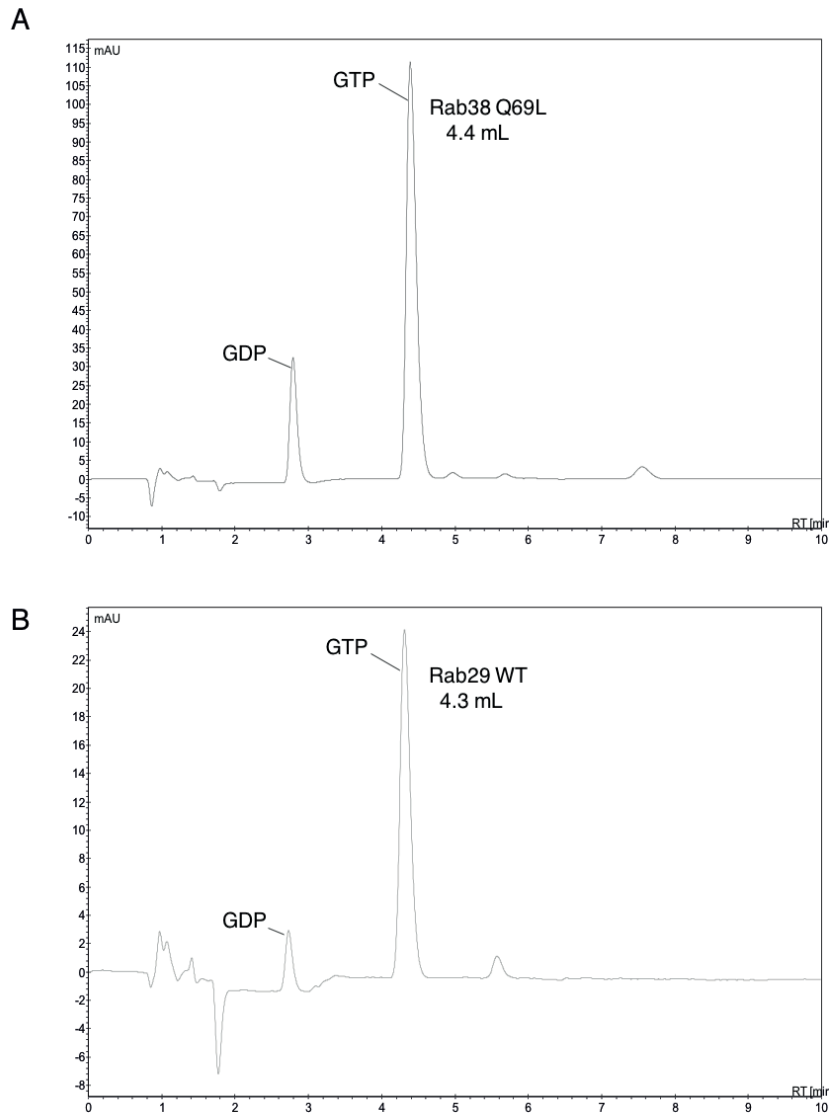


Figure 3.4. HPLC analysis of Rab-bound guanine nucleotides

Following purification samples of Rab38^{Q69L} and Rab29 WT were boiled at 95 °C to denature the protein and release the nucleotide bound. Free nucleotide was then subject to reverse phase HPLC and eluted peaks on a C18 column were compared with previously determined standards. Both Rab38^{Q69L} (A) and Rab29 WT (B) were found to be predominantly GTP-bound.

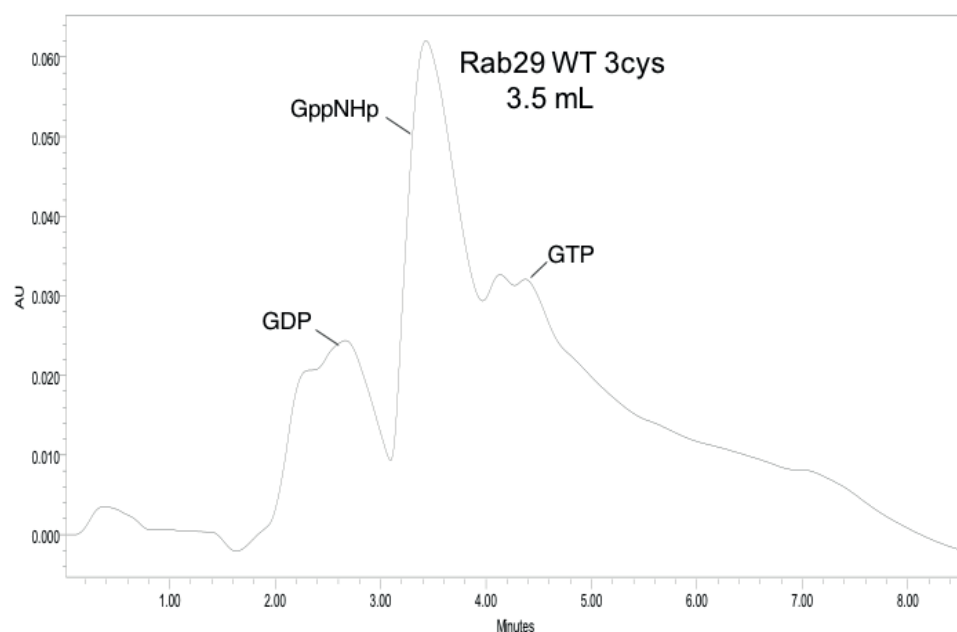


Figure 3.5. HPLC analysis of Rab29 following GppNHp exchange

To maintain Rab29 WT in its active state, purified protein was nucleotide-exchanged with a non-hydrolysable analogue of GTP (GppNHp). Reverse phase HPLC was used to determine the success of the exchange reaction. The Rab29 WT '3cys' construct was nucleotide-exchanged for use in crystallisation trials. A small aliquot of concentrated protein was boiled at 95 °C and free nucleotide was run on a C18 column. Eluted peaks are annotated with the corresponding nucleotide from previously determined standards. Multiple species are present indicating the exchange reaction did not occur with 100% efficiency. GppNHp is the predominant species within the sample.

3.2.3 Purification of the LRRK2 N-terminus

The LRRK2¹⁻⁵⁵² construct is a truncation of the armadillo repeats, originally identified as a Rab32 interaction partner in yeast two-hybrid experiments [300]. The protein was solubly expressed in BL21(DE3)RIL cells at 18 °C (291 K) overnight, and purified as described in Section 2.2.10. While impurities are also present within the sample eluted from IMAC, the band intensity of LRRK2 on SDS-PAGE demonstrates a good yield is achieved from *E. coli* expression (Figure 3.6). Following isolation of cut LRRK2 after a reverse Ni²⁺ IMAC step, charge variants were separated on a MonoQ[®] anion exchange column using a gradient up to 600 mM NaCl. The main LRRK2 peak began to elute at 17.5 mL, 27 mS/cm (Figure 3.6). The difficulty separating impurities from the desired LRRK2¹⁻⁵⁵² protein suggested these contaminants maybe be degradation products of LRRK2, despite the use of protease inhibitors during purification. The main peak tapered to a small shoulder as salt concentration increases. In an effort to obtain the most homogeneous sample, fractions from the main peak were collected excluding the right-hand shoulder. This peak was then subjected to size exclusion chromatography (SEC) to further improve the purity of the sample. Impurities and degradation products were successfully separated using this method and LRRK2 eluted at 64 mL from the Superdex™200 16/60 column (Figure 3.7). In the absence of appropriate protein samples to standardise the column in-house, a comparison was made with the data file provided by GE Healthcare showing the separation profile of test proteins under similar conditions. Using this reference chromatograph it was estimated that LRRK2¹⁻⁵⁵² elutes from the column at the volume expected of a dimer (125 kDa) in solution.

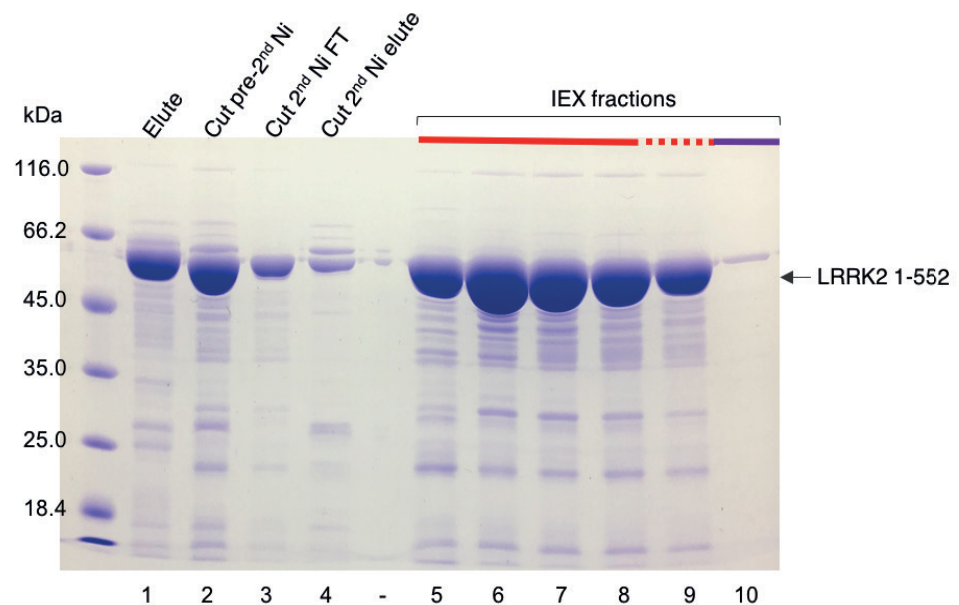
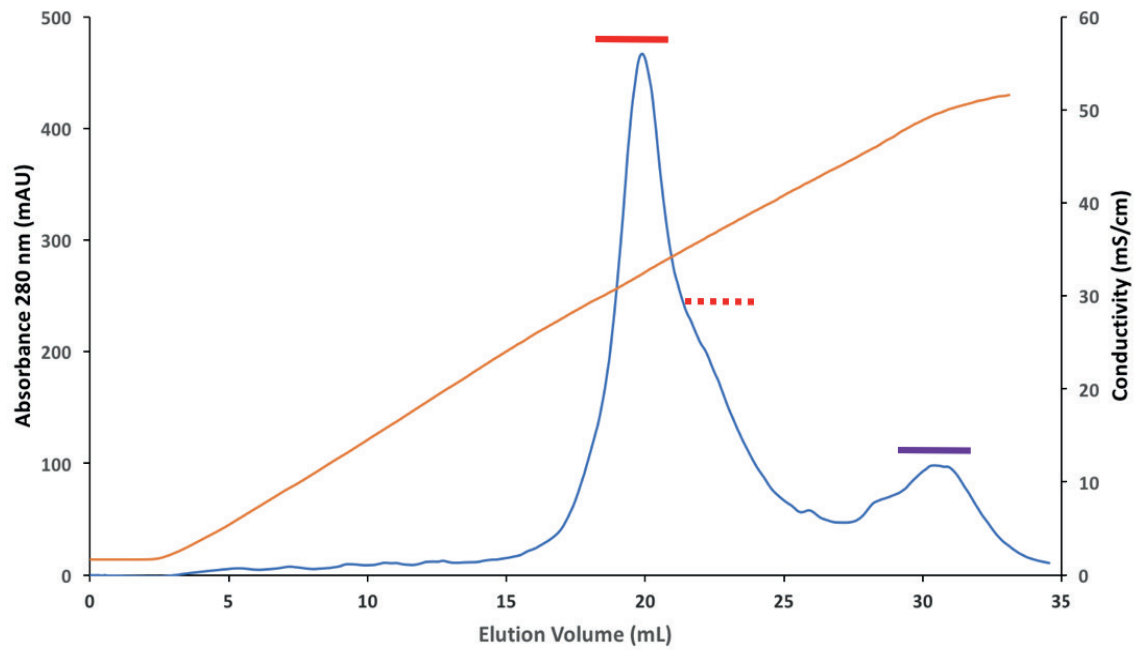


Figure 3.6. Affinity purification and ion exchange chromatography of LRRK2¹⁻⁵⁵²

Affinity purification and the subsequent ion exchange separation of LRRK2¹⁻⁵⁵² is visualised by SDS-PAGE. Following His-tag cleavage, protein obtained from the reverse Ni²⁺ affinity flow-through (FT) was loaded to a MonoQ[®] anion exchange column and a salt concentration gradient was applied. The resulting chromatograph is displayed. Fractions between 18.5 – 20.5 mLs corresponding to the main peak were collected and pooled for further SEC clean-up. Fractions within the peak shoulder (red dashed line) and the secondary peak (purple line) were also analysed by SDS-PAGE.

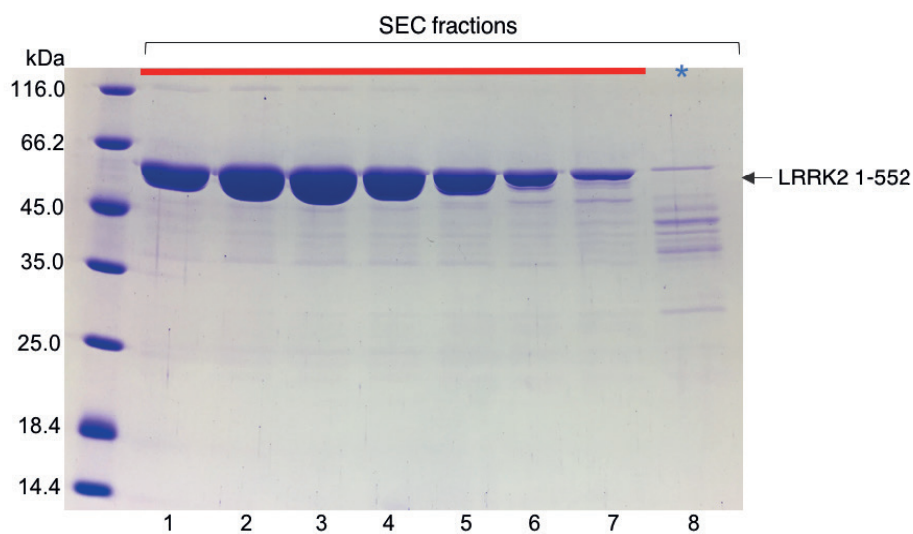
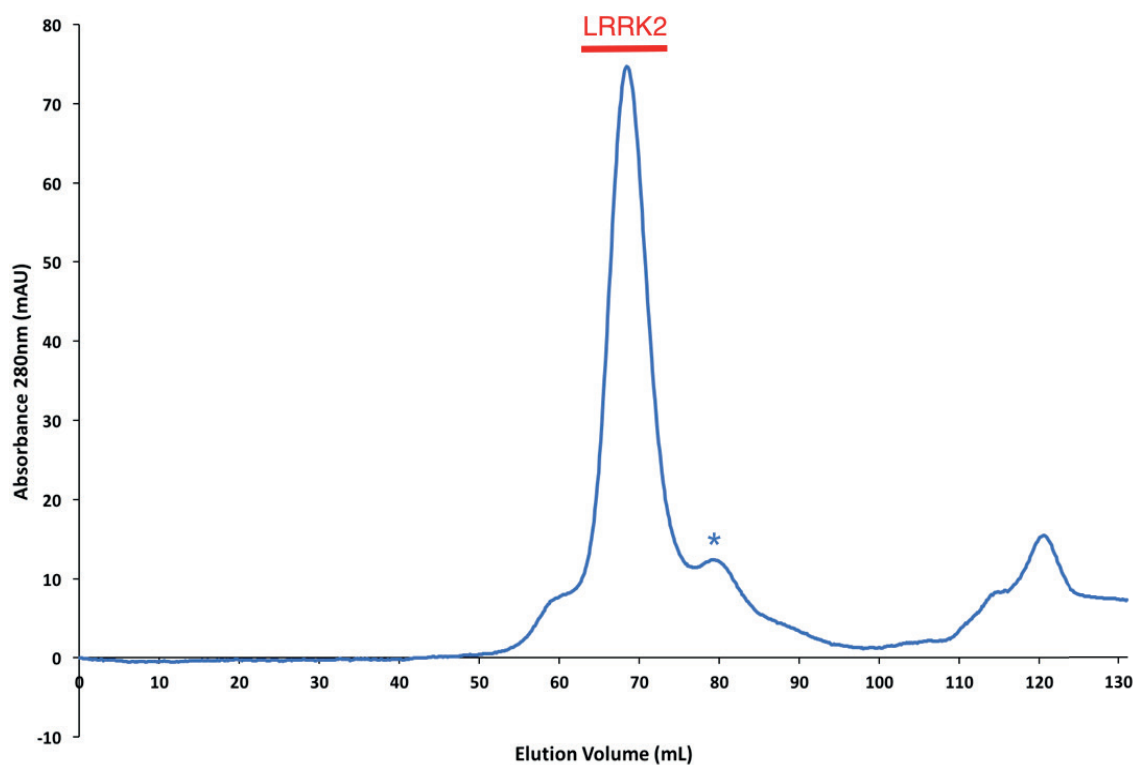


Figure 3.7. Size exclusion chromatography of LRRK2¹⁻⁵⁵²

Elution profile of LRRK2¹⁻⁵⁵² from a Superdex™200 column. SDS-PAGE analysis shows improved separation of LRRK2 from contaminants and degradation products. Fractions collected from 64 – 70 mLs were pooled for experimental use. The elution volume of the LRRK2 protein is indicative of dimer formation (125 kDa) in solution.

3.2.4 LRRK2:Rab38 complex formation

LRRK2 and the Rab32 subfamily were previously reported to interact based on both yeast two-hybrid and co-immunoprecipitation experiments. To demonstrate complex formation in solution, proteins were purified as previously described and excess Rab38 protein was combined with LRRK2¹⁻⁵⁵². The presence of a complex was determined by size exclusion chromatography and SDS-PAGE analysis. In Figure 3.8, the peak corresponding to the LRRK2:Rab38 complex elutes at 59 mL, and excess unbound Rab38 elutes at 90 mL. Guanine base has an absorbance maximum of 276 nm at pH 7. Thus, excess GppNHp from the Rab38 exchange reaction likely makes up the final large peak. There is no protein found within these final peak fractions, as tested by Bradford Assay (Section 2.2.14). A shift in elution profiles shown in Figure 3.9 also illustrates complex formation on the chromatograph as higher molecular weight species elute earlier from a size exclusion column. No change is observed in the Rab38 peak compared with a previous individual SEC run.

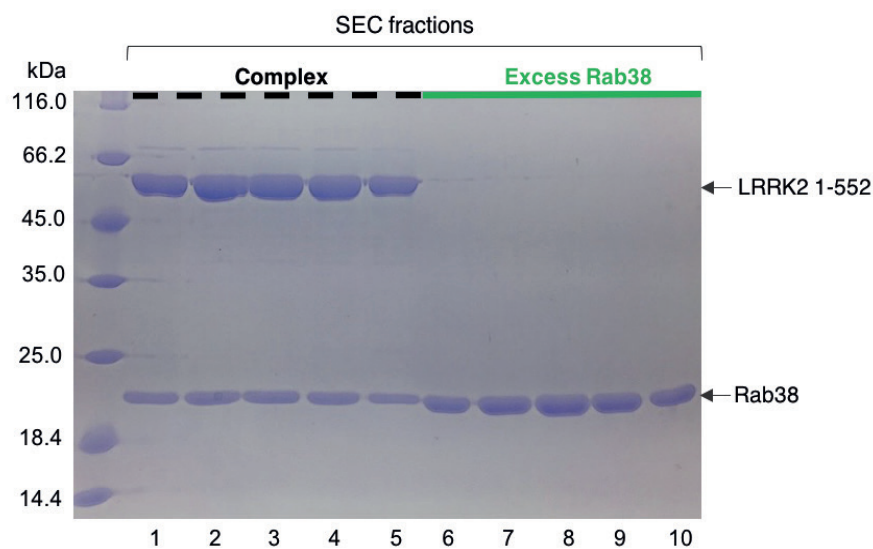
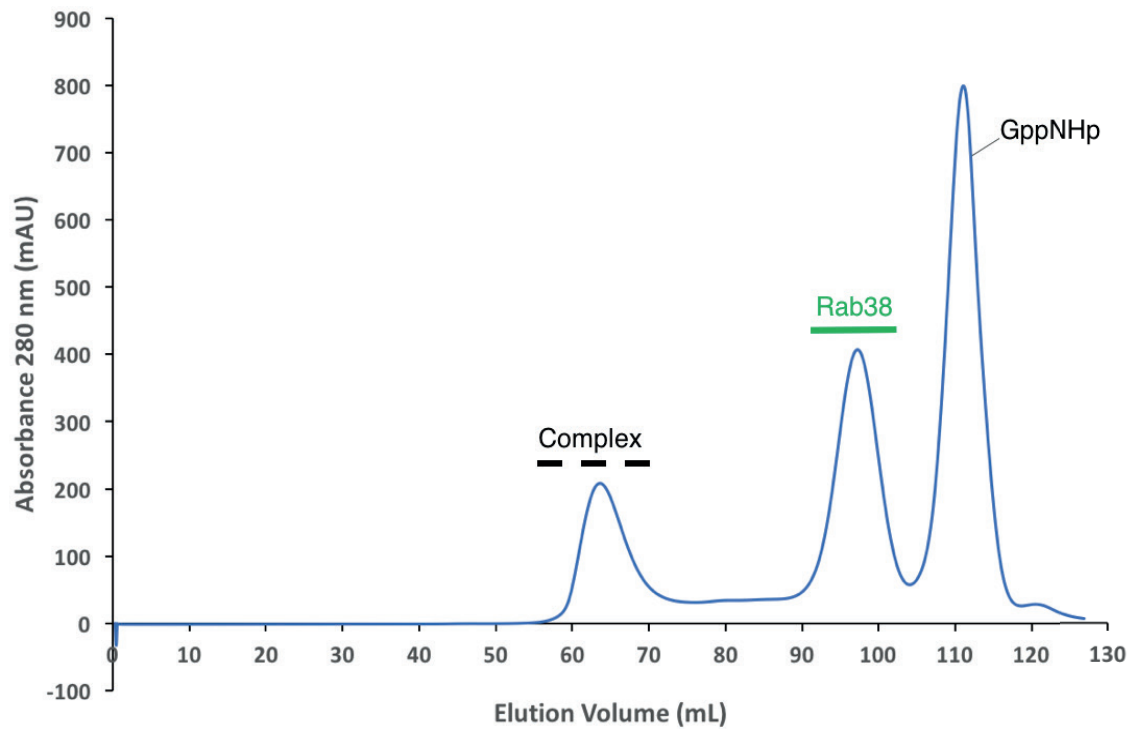


Figure 3.8. LRRK2:Rab38 complex formation

LRRK2¹⁻⁵⁵² and Rab38(GppNHp) interact and elute as a stable complex from a Superdex™200 size exclusion column (black dashed line). Excess Rab38 elutes at the expected volume. Excess GppNHp possesses maximal absorption at 276 nm and is represented by the final large peak at 110 mL. Fractions were analysed by Bradford reagent to confirm no protein is present in this peak.

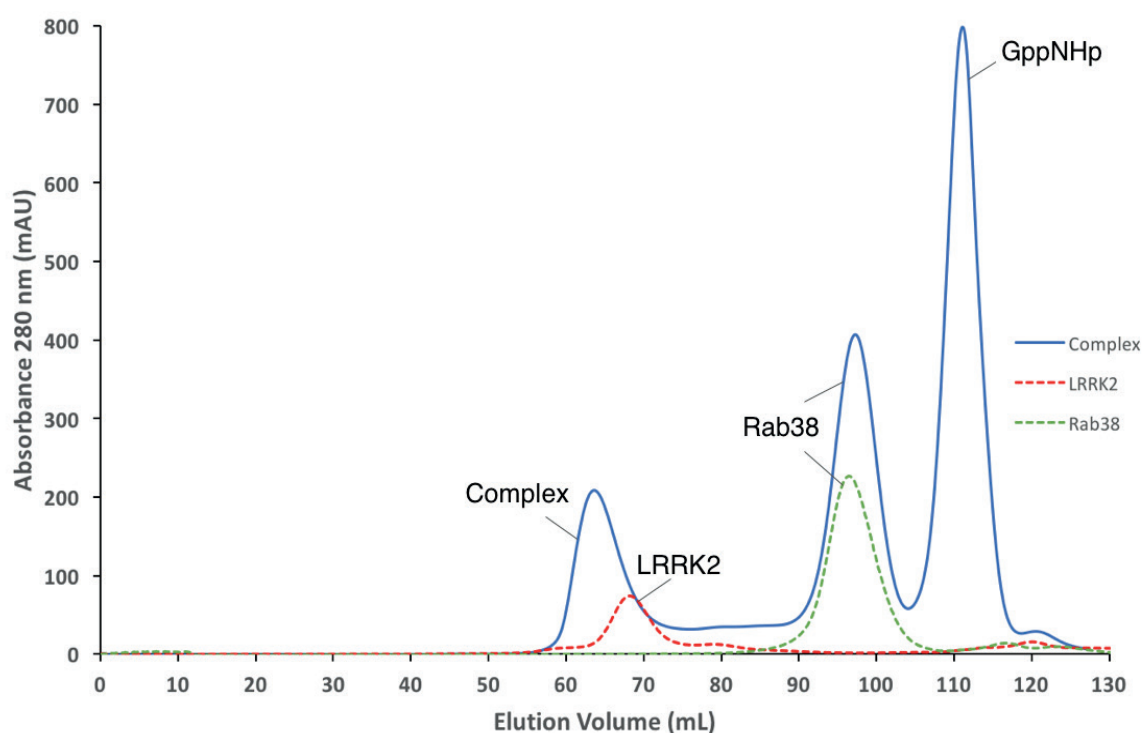


Figure 3.9. Size exclusion chromatography overlay

Chromatograph overlay demonstrating a shift in the elution profiles of LRRK2 in complex with Rab38 versus LRRK2 alone, suggestive of stable complex formation in solution. SEC run of the complex is shown as a solid blue line. LRRK2¹⁻⁵⁵² and Rab38 individual runs are shown as red and green dashed lines, respectively. Larger molecular weight species elute earlier from an SEC column. The Rab38 elution profile remains unchanged. Estimation of molecular weight based on elution volumes are as follows: LRRK2 1-552/Rab38 complex = 170 kDa; LRRK2 1-552 = 125 kDa; Rab38 = 20 kDa

3.2.5 Static Light Scattering of LRRK2

To confirm the observation of LRRK2 dimer formation reported in Section 3.2.3 by SEC, multi-angle light scattering (MALS) experiments of LRRK2¹⁻⁵⁵² alone and in complex with Rab38 were carried out under the same conditions. The miniDAWN™ system was coupled to the Superdex™200 10/300 column (SEC-MALS) as described in Section 2.2.18. The calculated molecular weight of a LRRK2¹⁻⁵⁵² monomer is 62.6 kDa. The average measured mass of the LRRK2 peak eluted from the column was 116 kDa (Figure 3.10A), consistent with that expected of a dimer, ~125 kDa. Upon subjecting the LRRK2:Rab38 complex to MALS measurements, a molecular mass of 162 kDa was obtained (Figure 3.10B). The molecular weight of the Rab38 G-domain is 20.6 kDa, thus the measured value aligns most closely with a 2:2 complex, whereby two Rab proteins are bound to a LRRK2 ARM domain dimer. The mass of excess unbound Rab38 was measured at 25 kDa.

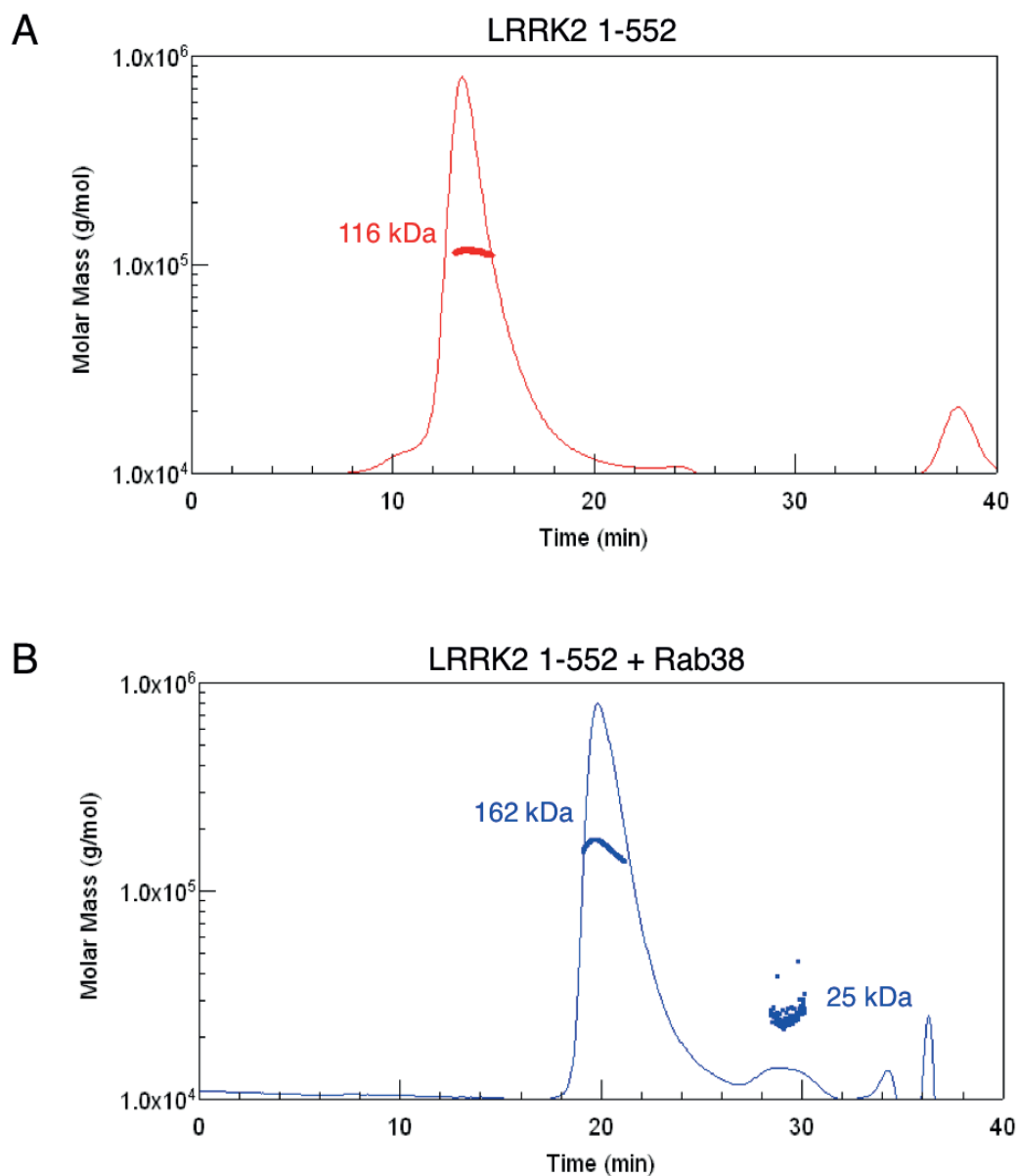


Figure 3.10. Static light scattering of LRRK2 ARM domain alone and in complex with Rab38

Multi-angle light scattering coupled to a size exclusion column was used for determination of absolute molecular mass of proteins in solution. A) LRRK2¹⁻⁵⁵² elutes as a single peak with a measured molecular mass of 116 kDa corresponding to a dimer. B) Measurement of LRRK2¹⁻⁵⁵² and Rab38 in solution determined a molecular mass of 162 kDa in the main peak corresponding closely to a 2:2 complex. A small secondary peak elutes later and represents excess unbound Rab38, with molecular mass calculated at 25 kDa. Manual injection of sample during the SEC-MALS experiment means that retention time of eluted peaks is not comparable between experiments in this circumstance.

3.2.6 Affinity pulldown assays

To address the disparity between the Rab32/38 binding site found within the first 552 residues of the LRRK2 armadillo repeats and the suspected interaction of Rab29 with the adjacent ankyrin domain, *in vitro* Ni²⁺ pulldown assays were performed using purified proteins. The experimental design of the pulldown assay using an affinity tag is given in Figure 3.11. Here, His-tagged Rab GTPases were used as 'bait' and untagged LRRK2 as 'prey' protein. LRRK2 constructs comprising of the armadillo and ankyrin domains (1-910), the predicted full-length armadillo repeats (1-661), and the truncated armadillo repeats (1-552) were used. A schematic of each N-terminal construct is provided in Figure 3.12. In line with previously published results and data given here in Section 3.2.4/5, both Rab32 and Rab38 bound to and 'pulled-down' all three LRRK2 constructs tested, suggesting the Rab32/38 interaction site lies within the first 552 residues (Figure 3.13A/B). To support the current published model of the Rab29 interaction with LRRK2, it was expected that Rab29 would 'pull-down' the 1-910 construct possessing both N-terminal domains, but would not bind to either armadillo domain construct. Unexpectedly, as with Rab32/38, binding was also detected to all constructs tested (Figure 3.13C). In contrast to our current understanding this lead us to the conclusion that all three members of the Rab32 subfamily likely bind within the first 552 residues of LRRK2. While Rab29 binding appears to be comparatively weak, it could be determined that the interaction with LRRK2 may be nucleotide-dependent. Visual inspection of band intensity indicates a stronger interaction with the active form of Rab29 (Figure 3.13C).

Additional pulldown experiments with the LRRK2 ankyrin domain alone (residues 681 – 860) were then performed to confirm this novel Rab29 data. Maltose Binding Protein (MBP) assists in maintaining the solubility of proteins during recombinant expression. Thus, in this circumstance a HisMBP-tagged ankyrin domain construct was used as bait and untagged Rab GTPases as prey. Following SDS-PAGE analysis, no binding was observed for any of the Rab32 subfamily members (Figure 3.14). Overall, this data clarifies the location of the Rab29 binding site, and support a new working model for the interaction of the Rab32 subfamily with the LRRK2 armadillo repeats.

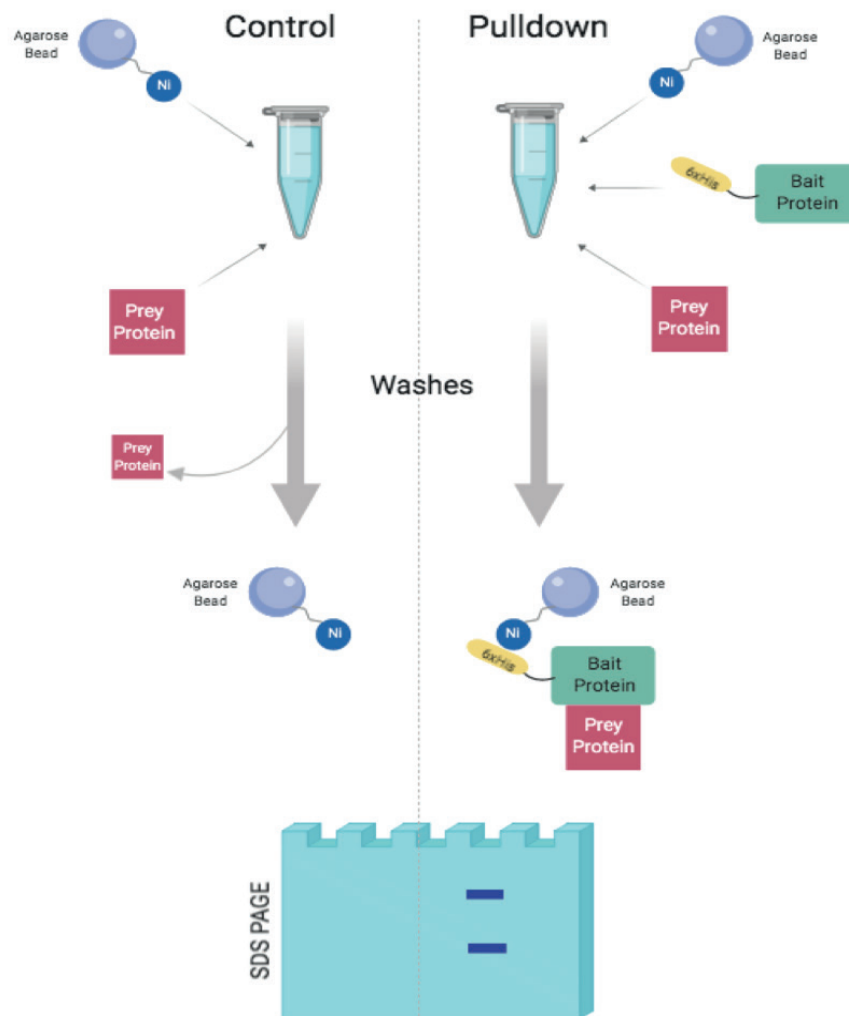


Figure 3.11. General schematic of an affinity pulldown assay

A His-tagged protein is bound by Ni^{2+} -agarose beads in a 1.5 mL minifuge tube. The immobilised protein acts as the 'bait' to capture a putative untagged interaction partner. Following stringent wash steps the remaining protein bound can be eluted from the beads with high imidazole and analysed by SDS-PAGE. A direct interaction can be confirmed by the visualisation of both bait and prey protein on the gel. As a control the untagged prey is incubated alone with Ni^{2+} agarose beads. Without the presence of a His-tag the prey should not be capable of binding the beads and is washed away. No protein is present following gel analysis.

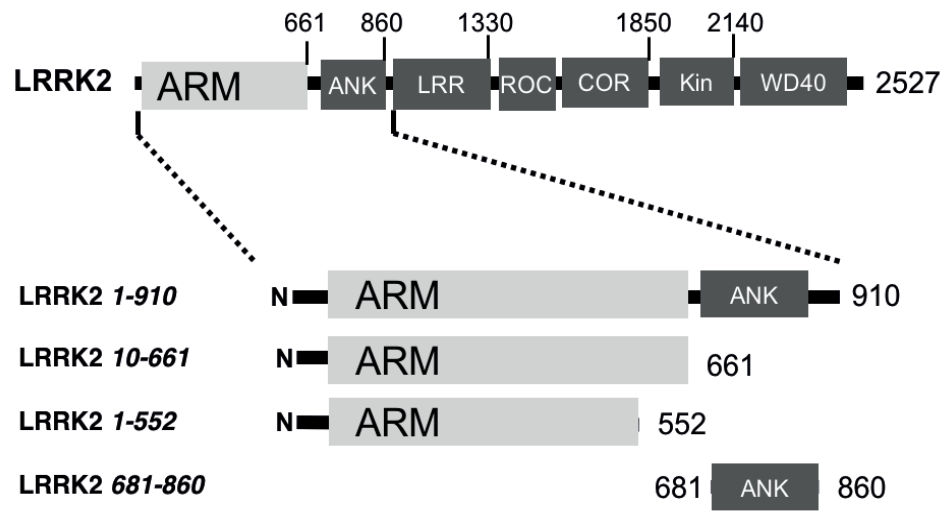


Figure 3.12. LRRK2 N-terminal constructs subject to pulldown analysis

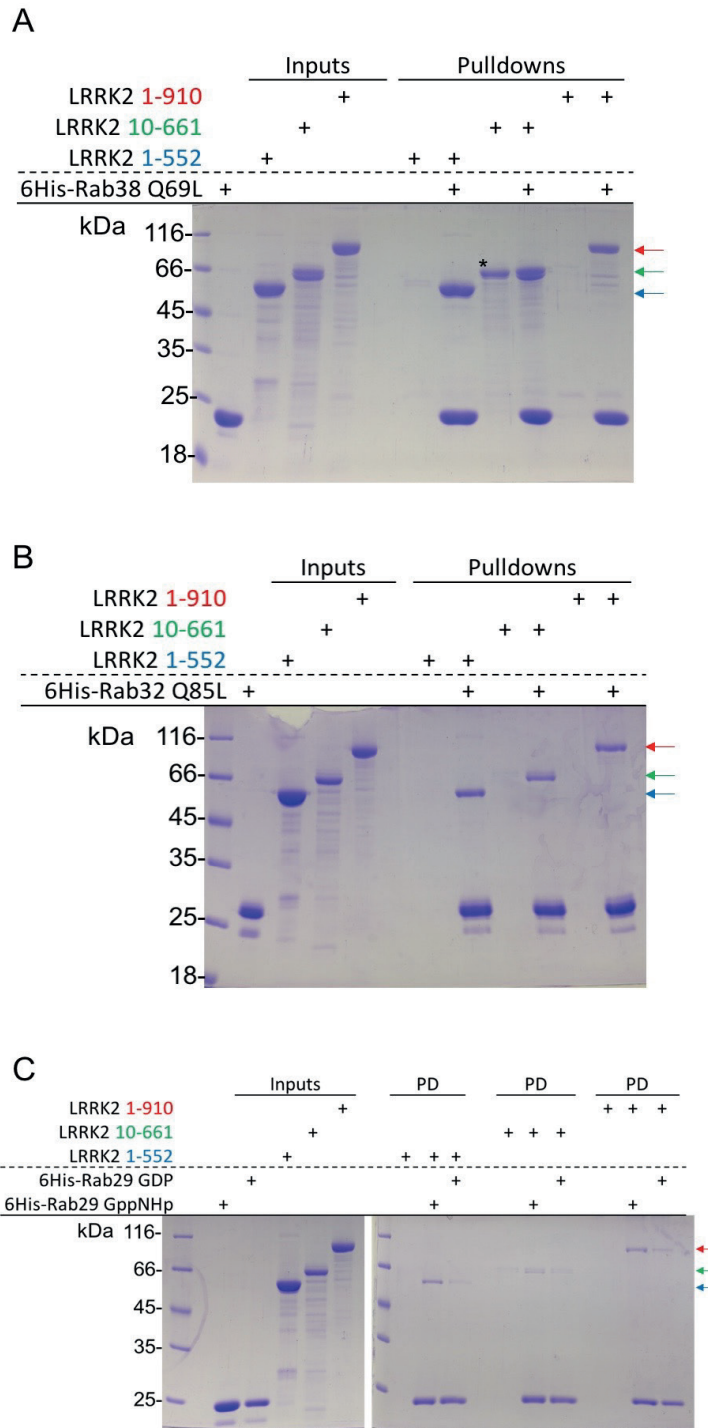


Figure 3.13. Interaction of the Rab32 subfamily with the LRRK2 N-terminus

Pulldown analysis using His-tagged Rab GTPases as bait and untagged LRRK2 N-terminal constructs as prey. Each member of the Rab32 subfamily binds to all constructs, indicating the binding site is located within the minimal 1-552 fragment. The asterisk in A) indicates high background binding for the LRRK2¹⁰⁻⁶⁶¹ construct. Comparison of active and inactive Rab29 in C) suggests the interaction with LRRK2 may be GTP-dependent. Experiments were performed in collaboration with Dr. Dieter Waschbusch.

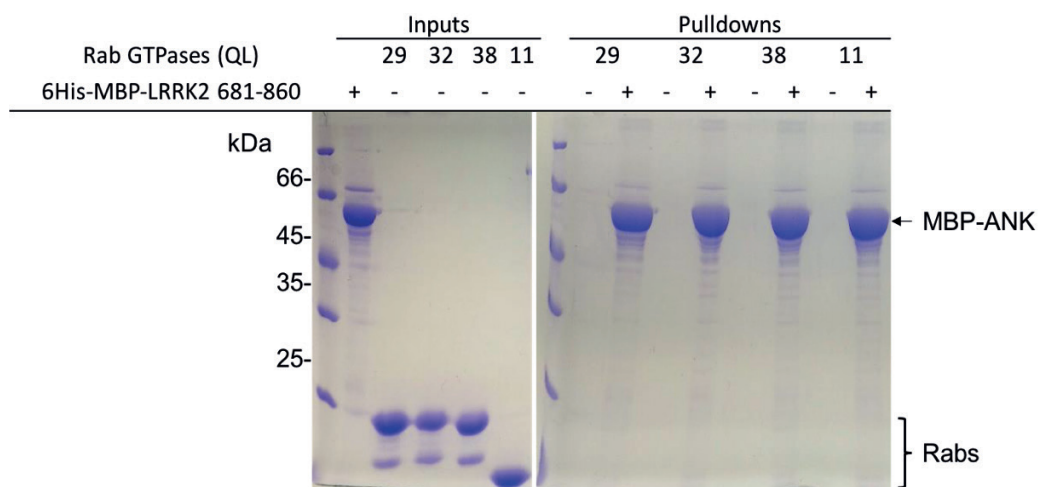


Figure 3.14. Interaction of the Rab32 subfamily with the LRRK2 ankyrin domain

Pulldown analysis using a HisMBP-tagged LRRK2 ankyrin construct as bait and untagged Rab GTPases as prey. No binding is observed with any of the Rab32 subfamily. Rab11 does not interact with LRRK2 and is included as an additional control. Experiment performed in collaboration with Dr. Dieter Waschbusch.

3.2.7 Rab GTPase competition assay

It was established in Section 3.2.6 that all three members of the Rab32 subfamily bind within the first 552 residues of LRRK2. Given their high sequence identity, it was then suspected all three Rabs may share the same binding site within this domain. To test the hypothesis, competition assays were performed using direct pulldowns with LRRK2¹⁻⁹¹⁰ as before. His-tagged Rab32/29 were used as bait and, in addition, untagged Rab38 was used to dope the samples. If the Rab proteins bind to different sites within LRRK2, a ternary complex would be expected, with His-Rab29/32, untagged LRRK2 and untagged Rab38 visible by SDS-PAGE. Conversely, a disruption of the interaction from the presence of Rab38 could determine whether the Rab32 subfamily members were competing for a common binding site. In this case any LRRK2 protein bound to untagged Rab38 would be washed away. No protein is 'pulled-down' in the control sample of LRRK2 and Rab38, which are both untagged and show no background binding to the Ni²⁺-agarose beads. Furthermore, no band corresponding to Rab38 is found in samples containing both Rab38 and His-tagged Rab29/32, demonstrating untagged Rab38 is removed during wash steps. SDS-PAGE analysis shows a clear reduction in the levels of LRRK2 'pulled-down' by Rab32 and Rab29 following incubation with Rab38 (Figure 3.15, indicated by asterisk). This confirms all three Rabs compete for binding to LRRK2 *in vitro*, and thus share the same or an overlapping site of interaction.

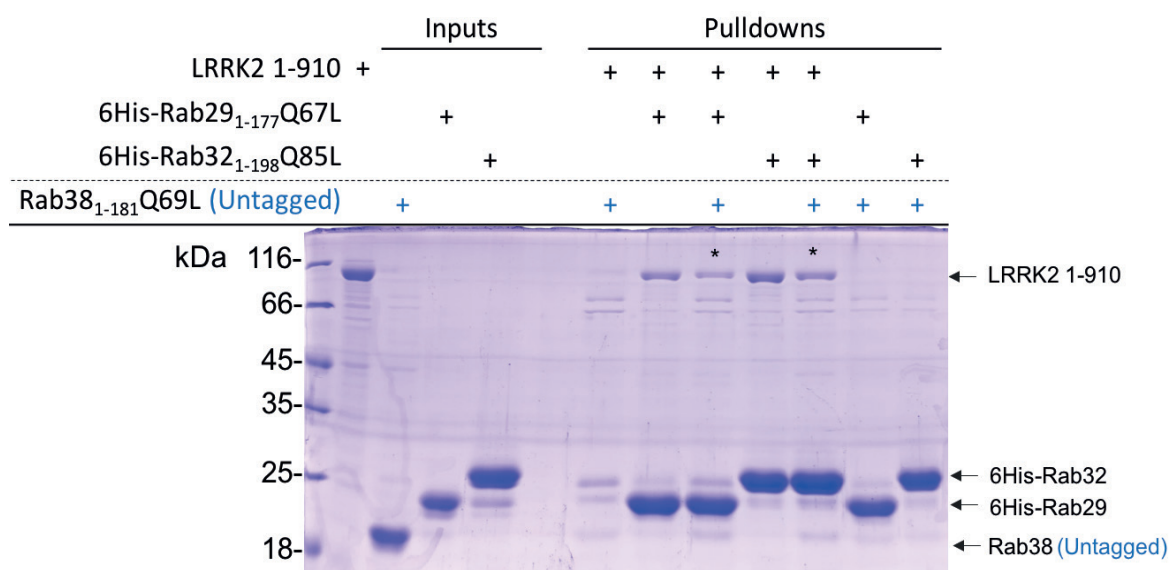


Figure 3.15. Rab competition assay

A competition assay was performed by doping pulldown samples with excess untagged Rab38 and is visualised by SDS-PAGE. The presence of Rab38 results in a decrease in the levels of LRRK2¹⁻⁹¹⁰ bound by His-tagged Rab29/32, marked by asterisks. This suggests the Rab32 subfamily share a common LRRK2 binding site. Experiment performed in collaboration with Dr. Dieter Waschbusch.

3.3 Discussion

3.3.1 Purification and HPLC analysis of Rab GTPases

To obtain high yields of pure protein for crystallographic and biophysical studies a successful purification strategy is essential. In this chapter, the successful isolation and purification of recombinant Rab32, Rab38 and Rab29 from bacteria using the T7 expression system is demonstrated. Expression using *E. coli* is easy to manipulate, safe, inexpensive and typically yields large amounts of the protein of interest. The purification of Rab GTPases proved to be straightforward with three consecutive purification steps required: immobilised Ni^{2+} -metal affinity chromatography, reverse IMAC, and size exclusion chromatography. Typically, the first step in purification is the most critical for achieving high purity, with size exclusion chromatography usually considered a final polishing step. Only minor impurities are present within the sample eluted from the affinity column. Depending on experimental requirements, it is not always necessary to remove the histidine-tag. However, reverse IMAC provides an additional clean-up step, with all Rab proteins achieving high purity at this stage (Figure 3.1). For Rab protein samples intended for crystallisation, the flexible His-tag was always removed. Each Rab construct is ~20 kDa, and the elution volume observed agrees with monomeric proteins of this size. This was determined by the protein standards chromatograph provided in the GE Healthcare data file. SDS-PAGE analysis of all Rab proteins reveals no detectable contaminants and high purity is achieved (Figure 3.2).

While SEC data shows correctly folded globular protein, it does not reveal the activity state of the Rab GTPases. In *E. coli*, intracellular GTP concentration is approximately 10-fold higher than GDP [433]. Thus, in an overexpression system lacking Rab regulators that control the activity cycle, it is expected that Rab GTPases are purified in their GTP-bound state. To confirm this assumption, Rab GTPases were denatured to release bound nucleotide. These samples were then subjected to reverse phase analytical HPLC, which is widely used for the separation of small molecules under 2000 Da. Reverse phase is the most common HPLC technique and uses a hydrophobic stationary phase and polar mobile phase. Here a C18 column was used which consists of a C_{18} carbon chain bonded to silica packing. Since guanine nucleotides are hydrophilic molecules an ion-pairing reagent, such as tetrabutylammonium (TBA), is included in the running buffer to increase hydrophobicity and retention time on the column. Although

the nucleotides are not significantly retained by the C18 column and elute rapidly, a unique retention profile for each nucleotide is observed (Figure 3.3). This allowed us to verify the activity state of the Rabs upon purification. Rab38 and Rab29 are predominantly GTP-bound, and thus are purified in their active state (Figure 3.4). A study had previously determined that 90% of Rab32 purified from *E. coli* was also in its GTP-bound form [207]. We were therefore confident Rab32 was in its active form upon purification and was not subject to HPLC analysis. Without the presence of GAPs, Rab GTPase activity is slow, and Rab32 was previously determined to be exceptionally slow with a hydrolysis constant of $k_{\text{hydr}} = 6.5 \times 10^{-6} \text{s}^{-1}$ [207]. However, to ensure purified Rab proteins remained active over the course of the experiment additional measures were taken. An activating mutation substituting the catalytic glutamine residue for leucine in Switch 2 was used, and is intended to 'lock' the Rab GTPase in its active state. Alternatively, a non-hydrolysable analogue of GTP, in which the oxygen atom bridging the β - and γ -phosphates is replaced with an amine (GppNHp), can be substituted into the Rab protein via an exchange reaction. The efficiency of the exchange reaction for Rab29 WT '3cys' was also tested by reverse-phase HPLC prior to crystallisation trials. The broad peaks indicate too high a sample load which impinges upon clear separation. Nevertheless, the various guanine nucleotide peaks can be identified and distinguished from one another (Figure 3.5). The Rab29 sample contains a mixed population, implying the nucleotide exchange reaction did not occur at 100% efficiency. However, GppNHp is the predominant species and with further consideration of the GTP also present it can be concluded that Rab29 is mostly found in its active conformation following nucleotide exchange.

3.3.2 Purification of the LRRK2 N-terminus

Achieving a high quality purification of the LRRK2 N-terminus proved more challenging than that of the Rab proteins previously described. LRRK2¹⁻⁵⁵² is a truncated construct of the armadillo repeats and was previously identified in a yeast-two hybrid screen as an interacting partner of Rab32/Rab38 [300]. It is the most soluble construct of the N-terminal region currently available and as a result most efforts in this work have focused on this LRRK2 protein fragment. Although not shown, the additional constructs used for pulldown analysis were subjected to an identical purification scheme.

A good yield of recombinant protein is obtained following affinity purification, with LRRK2 in far greater excess than contaminant bands also present (Figure 3.6). Recombinant protein overexpression can also induce the expression of native bacterial proteins in response to stress conditions. The binding of these proteins to affinity resins is typically due to native metal-binding functions or surface exposes clusters of histidine residues [434]. Following removal of the His-tag, cleaved LRRK2¹⁻⁵⁵² was subject to ion-exchange chromatography as an intermediate purification step. Since LRRK2¹⁻⁵⁵² has a theoretical isoelectric point (pI) of 5.4, the protein was loaded to a MonoQ[®] anion exchange column and a salt gradient applied up to 600 mM NaCl. The chromatograph shows the main LRRK2 peak followed by a small shoulder is not fully resolved (Figure 3.6). A relatively strong affinity to the resin is expected as LRRK2 should have significant net negative charge with experimental conditions at pH 8.0. It is unclear whether the lower molecular weight protein bands that elute with the intact protein are bacterial contaminants or LRRK2 degradation products. Considering the persistence of these bands following several stringent purification steps, LRRK2 degradation products seem most likely.

To achieve the most homogenous sample, fractions from the main IEX peak were pooled and subjected to size exclusion chromatography. A small shoulder at ~58mLs suggests the separation of higher molecular weight oligomers. The main peak shows even Gaussian distribution and there is successful separation of lower MW contaminants via this method (Figure 3.7). Curiously, the retention volume observed was indicative of a LRRK2 dimer in solution. SEC is not an absolute method for determination of molecular mass and is dependent upon the hydrodynamic radius of the molecule to be separated. Proteins with an elongated shape may elute at a position higher than the true molecular weight, and homology modelling predicts the LRRK2 armadillo repeats form an extended superhelical structure [299]. On account of this, static light scattering (SLS) was chosen to clarify this initial observation. SLS is an optical technique in which a polarized beam of light is used to illuminate a sample of interest and the scattering of this light by macromolecules in the solution provides information on their biophysical properties. Larger molecules scatter more light than smaller molecules, and the intensity of this scattered light is proportional to molar mass. Multi-angle light scattering (MALS) is a type of SLS in which scattered light is detected at multiple fixed angles. In Figure 3.10A, SEC-MALS of purified LRRK2¹⁻⁵⁵² revealed a molar mass of 116 kDa, which roughly corresponds to the expected molecular weight of a LRRK2 dimer (125 kDa). As

reviewed in Chapter 1, the COR domain is believed to be the main dimerization device for LRRK2 [288, 292]. However, dimerization is also known to occur in distal accessory domains. The human LRRK2 WD40 repeats were shown to dimerize in solution, despite being oriented in opposite directions in the dimeric full length model [319, 330]. Furthermore, deletion of the WD40 repeats prevents LRRK2 dimerization [320]. The ability of the LRRK2 N-terminus to form dimers has not been well documented in the literature thus far. The armadillo domains lie adjacent to one another in the full-length model, however do not appear to make significant contacts [330]. Given that the positioning of the armadillo repeats relied primarily on fitting it to the remaining space of the electron density map, and the low resolution of the structure (22 Å), this may not be an accurate representation of the domain architecture. Antoniou *et al.* (2018) recently described a dimeric LRRK2 ARM domain model *in silico*, using the human Importin-alpha1 dimer (PDB code: 3WPT) as template [301]. Experimental evidence to support N-terminal dimer formation was not given. In this work, we provide clear evidence of LRRK2 ARM domain dimerization *in vitro*. However, whether N-terminal dimerization is biologically or functionally relevant still needs to be established.

3.3.3 Interaction of the LRRK2 ARM domain with the Rab32 subfamily

Binding of Rab32/Rab38 to the LRRK2 armadillo repeats had previously been demonstrated by yeast-two hybrid, GST-pulldown and co-immunoprecipitation experiments [300]. Following successful purification of the Rab GTPases and the LRRK2 N-terminus, we sought to confirm the interaction in our system. Rab38 was chosen as a representative member of the subfamily to study the complex on account of high protein yield following purification, and previous preliminary evidence from the lab suggesting a strong interaction *in vitro*. A molar excess of Rab38 was added to LRRK2¹⁻⁵⁵² following IEX purification and the mixed solution was run through a Superdex™200 size exclusion column. SDS-PAGE analysis of the peak fractions shows the presence of both LRRK2 and Rab38, demonstrating the formation of a stable complex in solution (Figure 3.8). A shift in Peak 1 is visible upon overlay of chromatographs showing equivalent runs of LRRK2¹⁻⁵⁵² and Rab38 alone (Figure 3.9). Earlier elution from the column indicates the presence of a higher molecular weight complex. SEC-MALS could also be used in this circumstance to determine the absolute molecular mass and therefore infer the ratio of Rab protein bound to LRRK2 within the complex. Molecular mass

of the complex was measured at 162 kDa, closely corresponding to a complex comprised of 2 Rab molecules bound to a LRRK2 dimer (Figure 3.10B). The average molar mass values decrease towards the right side of the peak, suggesting heterogeneity in the sample. One possible explanation may be that LRRK2 and Rab38 are found in dynamic equilibrium with a fraction of unbound LRRK2 dimers in solution that are not well separated by the SEC column. This is likely considering Rab38 is not in large excess, indicated by the very small peak at 25 kDa molar mass.

Verification of protein binding in solution allowed us to proceed with Ni^{2+} affinity pulldown assays, here performed principally by Dr. Dieter Waschbusch, to confirm within which of the N-terminal domains each member of the Rab32 subfamily binds. Various LRRK2 N-terminal constructs were chosen, encompassing both predicted armadillo and ankyrin repeats, the full-length armadillo repeats as well as a truncated form, and the ankyrin domain alone. The interaction of Rab32/38 with the truncated LRRK2 ARM domain had been previously documented [300], and this was also observed for Rab38 by SEC in this study, as discussed above. Pulldown experiments with Rab32 and Rab38 (Figure 3.13A/B) further supported this hypothesis, with binding observed to all ARM domain-containing fragments. It is curious to note that Rab32/38 bind the ankyrin repeats of their shared effector VARP and yet there is no observable interaction with the LRRK2 ankyrin domain here. Given our understanding of Rab32/38 binding promiscuously to armadillo and ankyrin domains in different proteins, it is highly plausible Rab29 might possess a unique binding site within the LRRK2 ankyrin domain. However, this interaction was inferred from indirect evidence, whereby Rab29 was no longer capable of activating LRRK2 kinase activity upon introduction of mutations within the ankyrin domain [306]. To provide direct evidence for this suspected interaction, pulldown assays were performed (Figure 3.13C). Unexpectedly, like Rab32/38, Rab29 also bound to all armadillo domain constructs, indicating the Rab29 binding site is also contained within the first 552 residues of LRRK2. This was further corroborated by additional pulldown data demonstrating no observable interaction with the ankyrin domain construct (Figure 3.14). It appears that the ability of each of the Rab32 subfamily members to bind LRRK2 varied within the pulldown assays performed. Based upon band intensity, Rab38 appears to show the strongest affinity, followed by Rab32, and finally Rab29 demonstrating the weakest binding. However, this

approach is not quantitative and the relative affinities for this interaction have not yet been measured by quantitative methods. This will be investigated further in Chapter 5.

Having established the binding site for all members of the Rab32 subfamily is found within the truncated ARM domain, competition assays were then performed to determine whether these Rabs bound to the same or overlapping site in this region. In this case, untagged Rab38 was used to dope His-tagged Rab29/32 pulldown samples. The LRRK2¹⁻⁹¹⁰ construct was chosen to further check for any involvement of the ankyrin domain, since it is possible two Rab binding sites may exist. The visualisation of a tertiary complex – His-tagged Rab29/32, untagged LRRK2 and untagged Rab38 – by SDS-PAGE would suggest distinct Rab binding sites within the LRRK2 N-terminus. However, a decrease in LRRK2 binding to His-tagged Rab29/32 in the presence of Rab38 would indicate that the Rabs compete for binding to the same site. In Figure 3.15 a clear reduction in LRRK2 is seen upon introduction of Rab38 to pulldown samples of both His-tagged Rab29 and Rab32. Thus, all members of the Rab32 subfamily share a binding site in the LRRK2-N-terminus.

3.3.4 Conclusion

Overall, this is the first time a direct interaction with LRRK2 has been demonstrated for Rab29 *in vitro*, and, contrary to previous evidence, has been shown to occur within the armadillo repeats. A fascinating distinction has previously been reported between members of the Rab32 subfamily, whereby Rab29 can activate LRRK2 distally by an unknown mechanism, and functions as an upstream regulator of its kinase activity [306]. Rab32 and Rab38, on the other hand, do not possess this ability. This is further complicated by the findings in this chapter which show that all three members appear to bind to the same site within the LRRK2 armadillo repeats. Given that structure encodes function, determining the structures of these proteins is critical to our understanding of LRRK2 regulation via Rab GTPases. In the next chapter, we seek to identify structural determinants of the LRRK2:Rab interaction.

Chapter 4:

Crystallisation and structural analyses of LRRK2 and the Rab32 subfamily

4.1 Overview

X-ray crystallography is a powerful technique for determining the arrangement of atoms in a molecule, providing a high resolution model of its three-dimensional structure. The growth of biological crystals requires a homogenous, concentrated solution of the macromolecule under study, which is then brought to supersaturation through the addition of mild precipitants and the manipulation of various parameters such as pH, ionic strength, and temperature. Identification of appropriate conditions promotes self-assembly in which molecules are aligned in ordered, regularly repeating arrays in three dimensions to form crystals. Data can then be collected from the diffraction of X-rays from a single crystal. The X-ray scattering off the ordered, periodic arrays of molecules within the crystal results in a distinctive diffraction pattern. The electron density can then be reconstructed, and from this the mean positions of atoms in the crystal can be successfully determined. As of this year there are almost 143,500 structures available on the PDB, of which 90% have been determined by X-ray crystallography. Currently, it can be considered the most advanced technique for obtaining high resolution protein structures.

Rab GTPases all share a common G-domain fold, and superimposition of solved structures illustrates the high degree of similarity within the family. Nevertheless, Rabs display a high level of selectivity and each bind to their own unique subset of effectors. The molecular basis for recognition is poorly understood, however the flexibility of the switch regions and subtle side chain rearrangements are thought to be important for the adaptability of Rab GTPases. It is therefore essential to gather structural information to attempt to identify discrete differences that may confer specificity. There are two structures of the Rab32 G-domain currently available: Rab32(GTP) in complex with its effector VARP (PDB code: 4CYM [182]); and Rab32(GDP) bound to the Salmonella protease GtgE (PDB code: 5OEC [207]). Furthermore, the uncomplexed structure of Rab32 has also been previously solved within our lab (PDB code: 6FF8, *unpublished*). Here we set out to solve the structures of Rab38 and

Rab29, which are currently unknown, using X-ray crystallographic techniques. In doing so, we will have structural information on the complete Rab32 subfamily. This atomic level of detail will allow us to identify candidate residues that may be important for the LRRK2:Rab interaction.

Having established in the previous chapter that all members of the Rab32 subfamily bind to the LRRK2¹⁻⁵⁵² truncated armadillo fragment, the remainder of this work focusses on this construct. Structural work on LRRK2 to date has largely focused on the catalytic core domains, and there is little information available on the LRRK2 N-terminus. In the generation of the low resolution full length structure the armadillo domain was modelled in at the end, fitted using the remaining available space and thus may not be accurate. Crystallisation of human LRRK2 has proven challenging, and in this work we make use of a homology model produced in the lab using structural information from other armadillo domain-containing proteins. While we determined that all members of the Rab32 subfamily bind with the LRRK2 ARM domain, structural information determined here will allow us to further map the location of the binding site within this domain.

4.2 Results

4.2.1 Crystallisation of LRRK2 alone and in complex with Rab GTPases

LRRK2 and the Rab GTPases were purified as previously described. The purified proteins were then concentrated to 5 – 15 mg/mL and used to test conditions for crystallisation. SDS-PAGE analysis of protein purity for crystallisation trials is shown in Figure 4.1. Multiple 96-well sitting drop plates using a 1:1 ratio of protein to reservoir were set up using commercially available sparse matrix screens. For complex screening LRRK2 and Rab were combined in either a 1:1 or 2:1 ratio of Rab to LRRK2. Despite sustained efforts, no crystals of the LRRK2 ARM domain alone or in complex with Rab GTPases were obtained. To explain why LRRK2¹⁻⁵⁵² may be difficult to crystallise, differential scanning fluorimetry was carried out using the ThermoFluor™ protocol. LRRK2 was incubated with SYPRO Orange dye and a thermal denaturation assay was performed using a real-time PCR machine. The denaturation midpoint or melting temperature in which half the sample population is unfolded (T_m) was measured at 44 ± 0.2 °C, suggesting the LRRK2 ARM domain is relatively unstable (Figure 4.2).

An intrinsic disorder profile of LRRK2¹⁻⁵⁵² was also generated using multiple web-based prediction servers, and a region between residues 317-342 was flagged as disordered, likely to comprise a large loop between armadillo repeats 6 and 7 (Figure 4.3). A loop deletion construct removing residues 330 – 345 was engineered to reduce flexibility in this region and aid crystallisation. Despite removal of these residues no crystal hits were obtained. Pulldown analysis confirmed the LRRK2 deletion mutant did not affect binding with the Rab32 subfamily (data not shown).

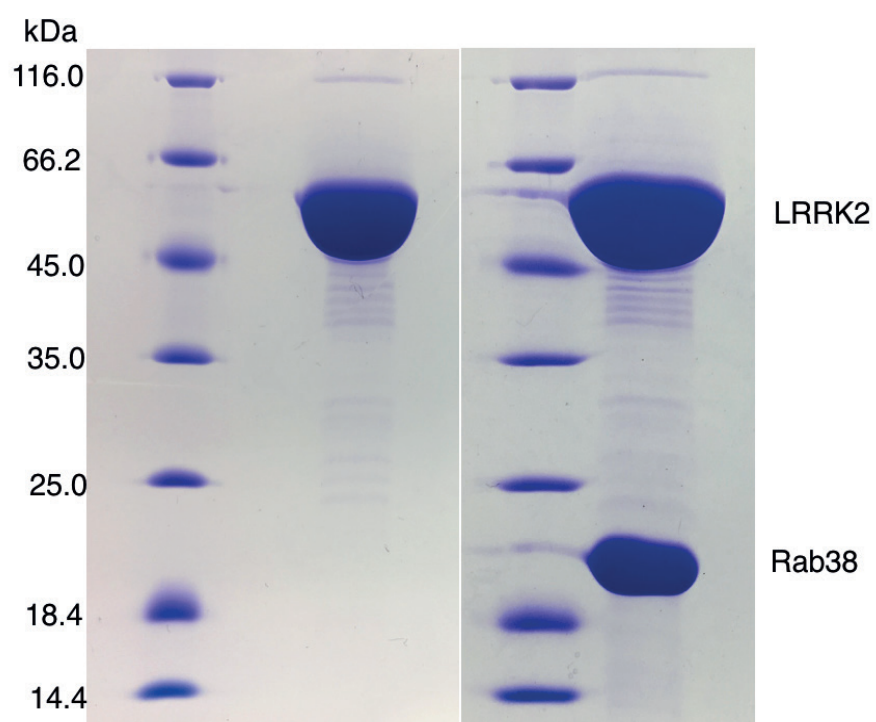


Figure 4.1. SDS-PAGE analysis of LRRK2¹⁻⁵⁵² alone and in complex with Rab illustrating purity for crystallisation

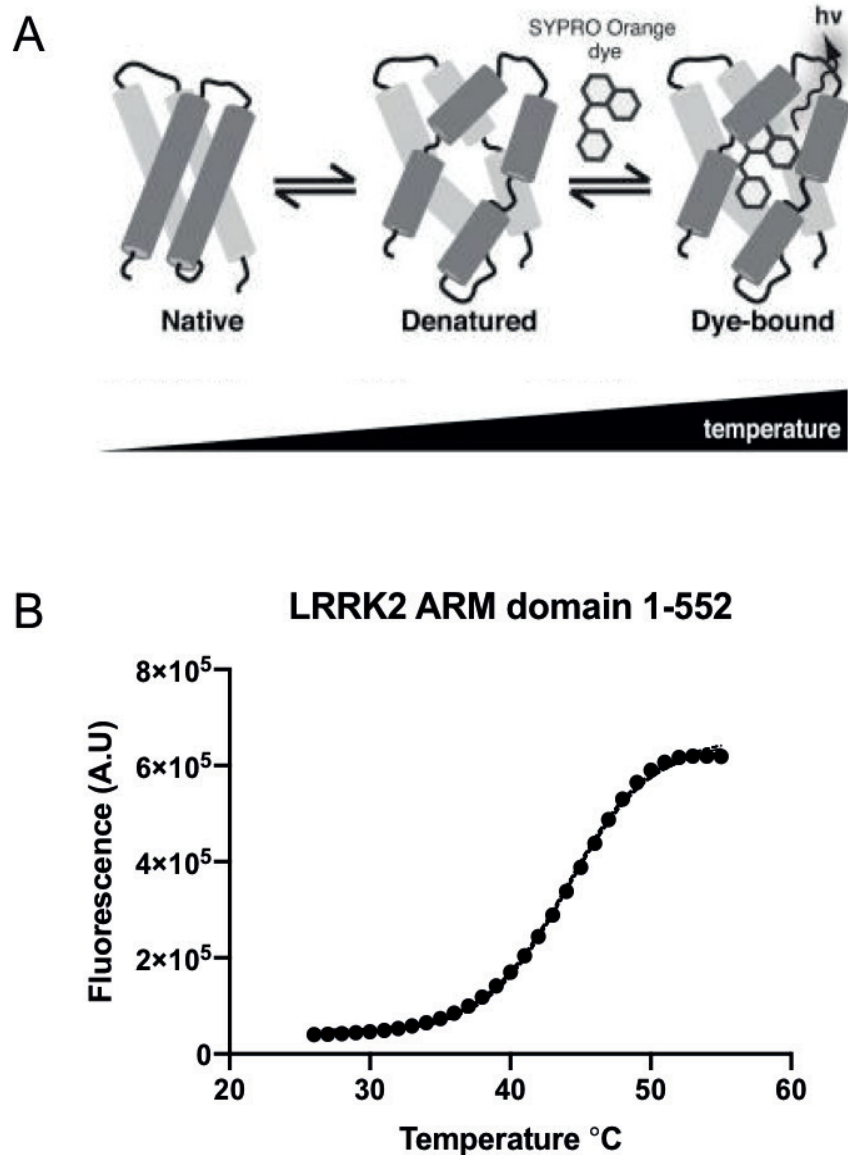


Figure 4.2. Thermal denaturation assay to assess LRRK2 stability

A) Diagram illustrating the process of thermal denaturation. A purified protein in its native state is slowly heated. Upon thermal denaturation of the protein hydrophobic regions become exposed, allowing the environmentally sensitive dye, SYPRO Orange, to bind. Binding to hydrophobic regions increases the fluorescence emission of the dye, serving as read out of protein unfolding. Image is adapted from Huynh and Partch 2015 [427]. B) Thermal denaturation curve for LRRK2¹⁻⁵⁵² armadillo domain fitted to a Boltzmann Sigmoidal curve. $T_m = 44 \pm 0.2^\circ\text{C}$.

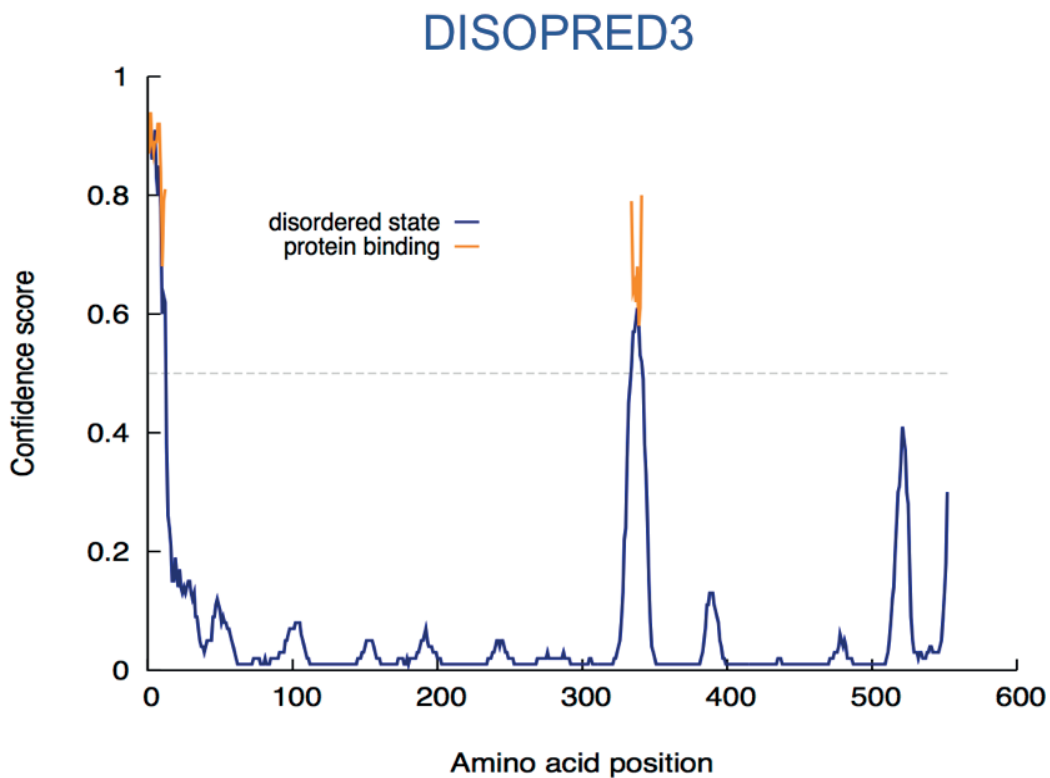
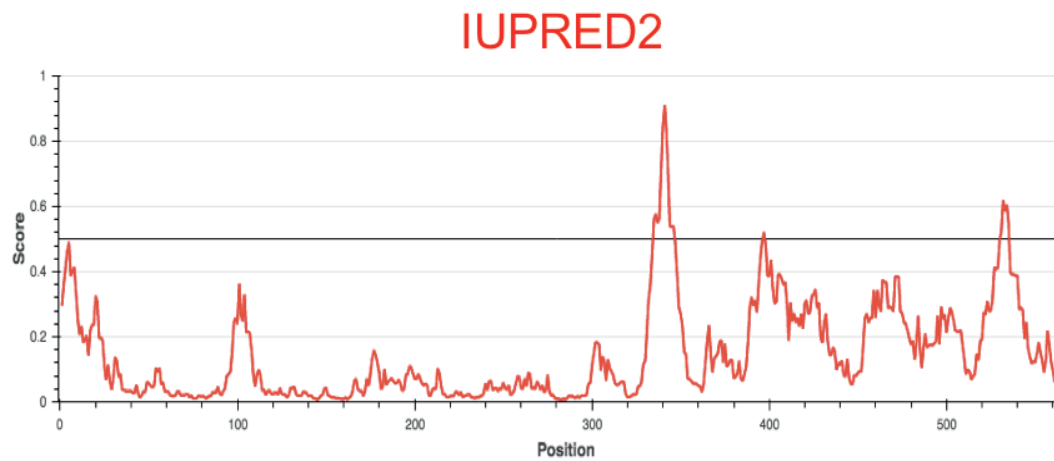


Figure 4.3. LRRK2 ARM domain intrinsic disorder profiles

Graphical outputs from IUPRED2 and DISOPRED3 web servers shows predicted disorder within the LRRK2 armadillo repeats. The loop region between residues 317 – 342 are commonly flagged among multiple prediction servers. DISOPRED3 also shows possible protein binding within this region [435] [436].

4.2.2 LRRK2 homology model

Due to the difficulties with crystallisation of the LRRK2 ARM domain outlined in Section 4.2.1, a model of the first 552 residues was generated *in silico* by Dr. Amir Khan, using the armadillo domain of mouse β -catenin (PDB code: 4EVP) as template (Figure 4.4A). The theoretical isoelectric point (pI) of LRRK2¹⁻⁵⁵² is 5.36, and upon examination of the predicted electrostatic surface potential, three distinct regions of negative charge were identified on the surface of the monomer that may be important for protein binding. These surfaces are highlighted in Figure 4.4B: Region 1 (yellow) = E192, E193; Region 2 (purple) = predicted loop 317-342; Region 3 (green) = D386, D390, E391, D392. As outlined above, the loop encompassing Region 2 had previously been deleted in an effort to improve crystallisation success. This deletion mutant and wildtype LRRK2 showed equivalent binding to the Rab32 GTPases, and thus this loop was disregarded as a potential site of interaction. The two remaining regions were considered candidate surfaces for the LRRK2:Rab interface.

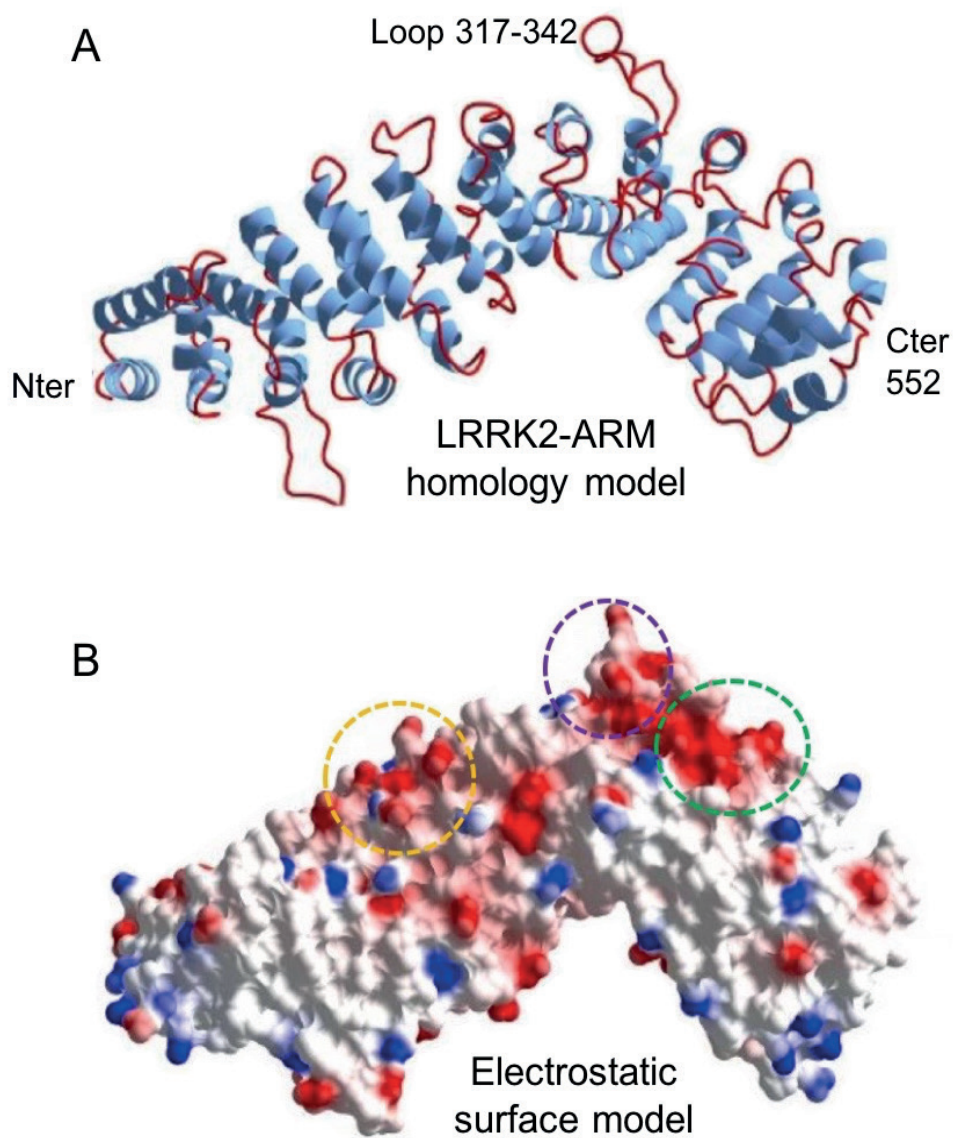


Figure 4.4. Homology model of the truncated LRRK2¹⁻⁵⁵² armadillo repeats

A) A LRRK2 homology model was generated by Dr. Amir Khan using the mouse β -catenin armadillo domain as template (PDB code: 4EVP). The ribbon model shows an extended helical structure. The disordered loop region between repeats 6 and 7 is highlighted. B) Surface potential map shows three distinct regions of negative charge that may be important for protein-protein interactions.

4.2.3 Crystallisation of Rab38

Purified Rab38^{Q69L}(GppNHp) concentrated to 7.3 mg/mL was used to test conditions for crystallisation. SDS-PAGE analysis of protein purity for crystallisation trials is shown in Figure 4.5. Several 96-well sitting drops with a 1:1 ratio of protein to reservoir were set up using commercially available sparse matrix screens. Crystals of Rab38 were observed after 24 hours in Morpheus® condition A9 (10% PEG 20,000, 20% PEG 550 MME, 0.03 M of each divalent cation – magnesium chloride, calcium chloride, sodium fluoride, sodium bromide, sodium iodide – and 0.1 M bicine/Trizma base pH 8.5) and exhibit a stacked plate-like morphology (Figure 4.6A). These crystals were harvested and tested in-house to confirm if they were proteinaceous in nature. The reflections seen in the diffraction image (Figure 4.6B) indicate a protein crystal and could be used for collection of a complete data set at a synchrotron source. Rescreening of Rab38 in additional 96 well commercial screens identified further hits in the Clear Strategy 1&2 screens. The best crystals were grown in 15% PEG 4000, 0.2 M potassium thiocyanate, 0.1 M sodium cacodylate pH 6.5 (Figure 4.6C). A diffraction data set to 1.79 Å resolution was obtained at the Synchrotron Soleil Proxima 2A beamline, France. The crystallographic data for Rab38 is outlined in Table 4.1.

4.2.4 Crystallisation of Rab29

Purified Rab29^{Q67L}(GppNHp) concentrated to 5.8 mg/mL was also used to test for crystallisation conditions as previously described for Rab38 (Section 4.2.3). After 24 hours crystals of Rab29 were observed in multiple conditions (Figure 4.7). All Rab29 crystals show various types of needle-like morphologies, and were too small to initially test in-house. The fan-like needles seen in Figure 4.7C were shot at a synchrotron source and diffracted to $\sim 4.4\text{\AA}$. To improve the quality of the data the most promising condition was optimised by varying precipitant condition, protein:reservoir drop ratio, and the addition of 2 - 5% glycerol as a nucleation inhibitor to encourage the growth of fewer larger crystals in manual screens. Following optimisation, the best crystals were grown in 1 M potassium sodium tartrate, 0.4 M NaCl, 5% glycerol, 0.1 M imidazole pH 8.0 (Figure 4.7D). These improved needles diffracted to $2.5\text{\AA}$ at the NE-CAT 24-ID-C beamline, Advanced Photon Source (APS), Argonne National Laboratory, USA, and the structure was solved by molecular replacement as before. Despite the protein being subjected to nucleotide exchange with non-hydrolysable GppNHp prior to crystallisation set-up, GDP was found to be present in the nucleotide binding pocket. It subsequently came to our attention that introduction of the 'activating' QL mutation in Rab29, which is intended to impair GTP hydrolysis, was reported to result in a significant increase in GTP dissociation compared to wild-type protein [413]. As a result, we immediately switched to using the wild-type Rab29 construct and within the sequence substituted 3 non-conserved cysteine residues (C84A, C120A, C127S) to further improve crystal morphology. This construct is referred to as Rab29 WT '3cys'. The purified protein was GppNHp-exchanged and confirmed by reverse phase HPLC as discussed in Chapter 3.2.2. Large, three-dimensional crystals were grown in 2 M NaCl, 0.1 M HEPES pH 7.0 over 48 hours (Figure 4.8). Fully grown crystals were then harvested rapidly after 24 – 72 hours to avoid nucleotide hydrolysis or dissociation. A $1.45\text{\AA}$ high resolution data set was obtained at the APS beamline. As will be outlined further in Section 4.2.6, the GDP-bound form of Rab29 also crystallised in this circumstance. The X-ray data statistics for Rab29^{3Cys}(GDP) are outlined in Table 4.1.

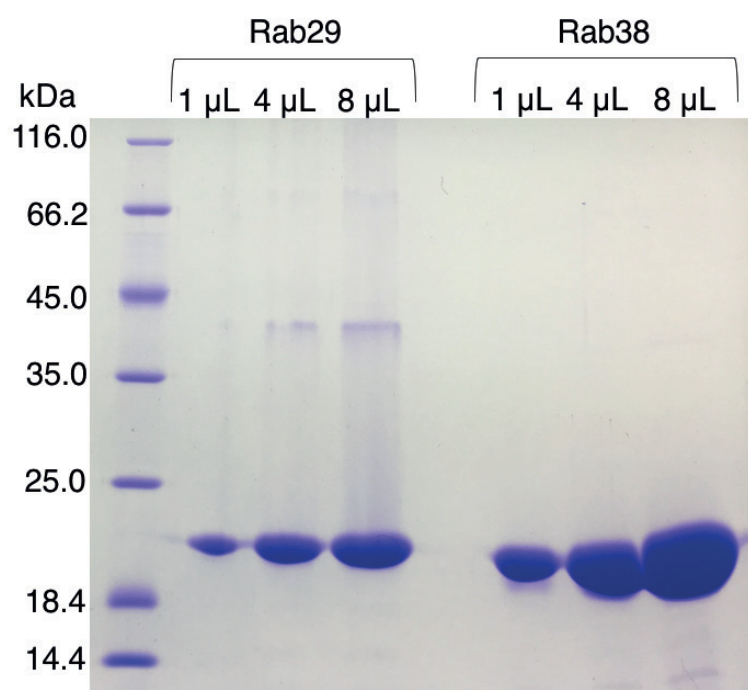


Figure 4.5. SDS-PAGE analysis of Rab29 and Rab38 crystallisation samples

SDS-PAGE analysis visualising the purity of Rab29^{Q67L} and Rab38^{Q69L} at 5.8 mg/mL and 7.3 mg/mL respectively that produced crystal hits.

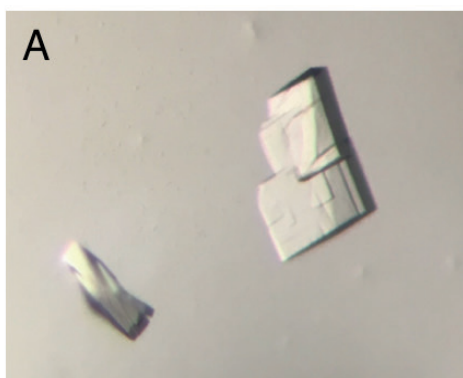
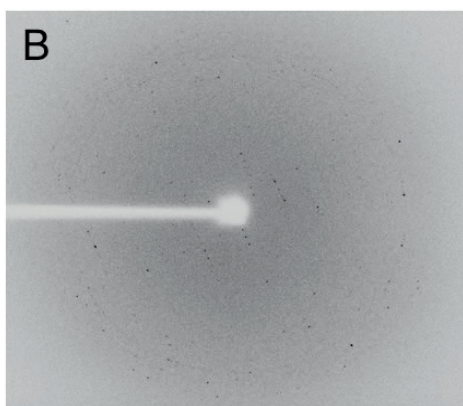
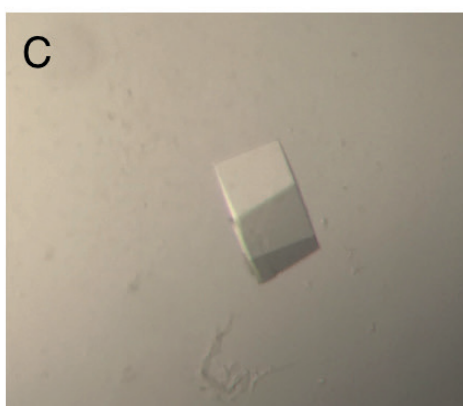


Figure 4.6. Rab38^{Q69L}(GppNHp) crystallisation hits

A) Three-dimensional crystals from initial hit show a layered plate-like morphology. Condition: 10% PEG 20,000, 20% PEG 550 MME, 0.03 M of each divalent cation – magnesium chloride, calcium chloride, sodium fluoride, sodium bromide, sodium iodide – and 0.1 M bicine/Trizma base pH 8.5.



B) Example of a diffraction image collected on the Rigaku R-Axis iV detector in-house from the crystal shown in A. Systematic reflections indicate it is a protein crystal.



C) An optimized Rab38 crystal produced high resolution diffraction data (1.79 Å) at a synchrotron source. Condition: 15% PEG 4000, 0.2 M potassium thiocyanate, 0.1 M sodium cacodylate pH 6.5.

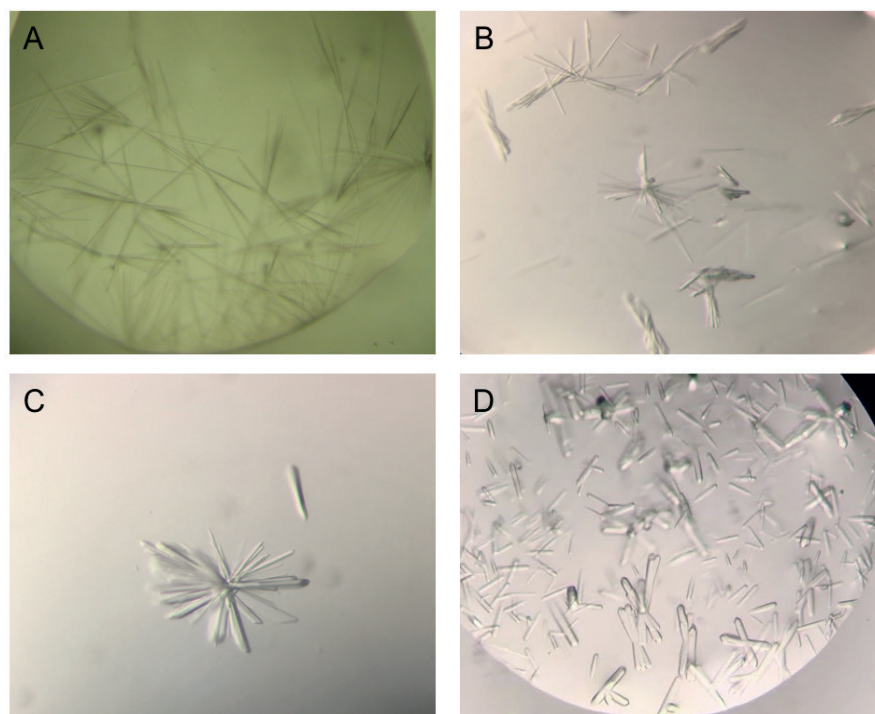


Figure 4.7. Rab29^{Q67L}(GppNHp) crystallisation hits

A) Thin hair-like needles. Condition: 10% w/v PEG 20 000, 20% v/v PEG MME 550, 0.02 M of each carboxylic acid (sodium formate, ammonium acetate, trisodium citrate, sodium potassium l-tartrate, sodium oxamate), 0.1 M bicine/Trizma base pH 8.5.

B) Thicker individual needles. Condition: 10% PEG 4000, 20% glycerol, 0.03 M of each NPS (sodium nitrate, disodium hydrogen phosphate, ammonium sulfate), 0.1 M MES/imidazole pH 6.5.

C) Needle/Plate-like fan. Data collected to 4.4 Å resolution. Condition: 10% PEG 4000, 20% Glycerol, 0.02 M of each alcohol (1, 6-hexanediol, 1-butanol, (RS)-1,2-propanediol, 2-propanol, 1, 4-butanediol, 1, 3-propanediol), 0.1M bicine/Trizma base pH 8.5.

D) Optimized rods produced from manual screening. A 2.5 Å data set was collected. Condition: 1M potassium sodium tartrate, 0.4 M NaCl, 5% glycerol, 0.1 M imidazole pH 8.0.

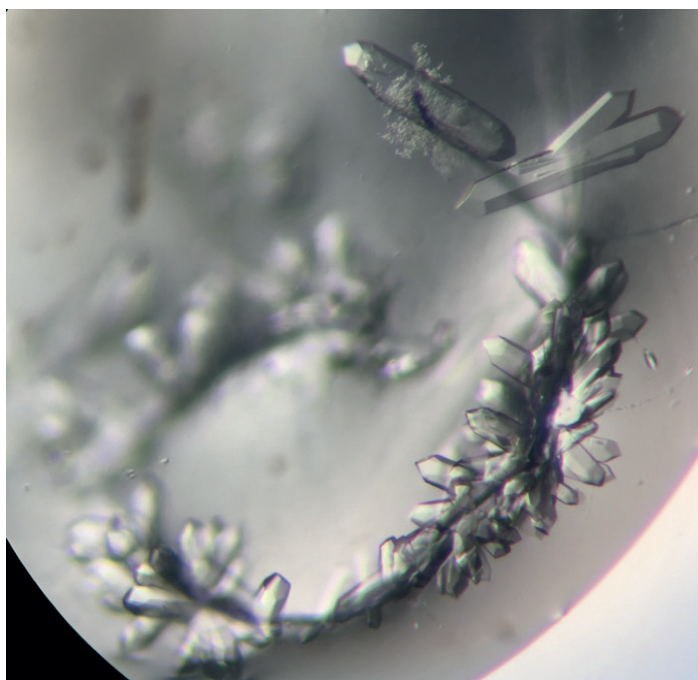


Figure 4.8. Rab29 WT '3Cys' crystals

Large, three-dimensional crystals from manual screening were grown over 48 hours and harvested over multiple time points. The optimized Rab29 crystals produced high resolution diffraction data (1.45 Å) at a synchrotron source. Condition: 2 M NaCl, 0.1 M HEPES pH 7.0.

Data Collection	Rab38 (GppNHP)	Rab29 (GDP)
Beamline	Proxima 2A	24-ID-C, APS
Detector	Eiger 9M	Pilatus 6M-F
Space group	P2 ₁	P3 ₁ 21
Unit cell length (Å)	70.04, 70.99, 74.49	71.96, 71.96, 86.35
Unit cell angles (°)	90, 102.21, 90	90, 90, 120
Wavelength (Å)	0.98	0.98
Low resolution limit (Å)	49.28 (1.83)	86.35 (1.47)
High resolution limit (Å)	1.79	1.45
Total no. reflections	455,161 (20,319)	450,712 (18782)
Unique reflections	66,651 (3,593)	46,405 (2,211)
Multiplicity	6.8 (5.7)	9.7 (8.5)
Completeness (%)	98.7 (83.2)	99.6 (93.5)
Wilson B-factor (Å ²)	20.1	21.8
R _{merge} (%)	7.5 (65.2)	5.2 (163.1)
R _{meas} (%)	8.2 (71.8)	5.5 (173.6)
Mn(I) correlation CC(1/2)	0.999 (0.867)	1.0 (0.68)
I/ σ all data	12.9 (2.4)	27.4 (1.4)
Refinement Statistics		
Model (chain/residues)		
A	1-131, 142-181	4-177
B	1-131, 143-180	
C	1-131, 142-180	
D	1-131, 142-181	
Ramachandran map (%)		
Favourable + allowed	100	98.26
Outliers	0	0
R _{work} /R _{free} (%)	19.02/21.23	16.51/18.43
Non-hydrogen atoms		
Protein	1396	5,587
Ligands	34	132
Mg ²⁺	1	4
Waters	194	700
Mean isotropic B-factor (Å ²)	25.0	29.38
R.M.S. deviations		
Bond lengths (Å)	0.005	0.02
Bond angles (°)	1.15	1.63
PDB code	6H DU	6HH2

Table 4.1. X-ray data and model refinement statistics

Values in parentheses correspond to the statistics for the highest resolution. Data collection and refinement was performed by Dr. Amir Khan. $R_{\text{merge}} = \sum_{hkl} \sum_j |I_{hklj} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_j I_{hklj}$; $R_{\text{meas}} = \sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$; Refinement residuals (R-factors), $R = \sum_{hkl} |F_{o,hkl} - F_{c,hkl}| / \sum_{hkl} F_{o,hkl}$.

4.2.5 Structural analysis of Rab32 and Rab38

The structure of Rab38 was solved by Dr. Amir Khan using molecular replacement with uncomplexed Rab32-GTP (PDB code: 6FF8, *unpublished*). As anticipated, structure solution of Rab38 revealed a classic $\alpha\beta$ G-domain fold, with the active conformation of the protein bound to GppNHp. The active structures of Rab32 and Rab38 are shown in Figure 4.9A. The high sequence identity (75% within the G-domain) is reflected in highly convergent switch conformations and near-identical topologies. The generation of electrostatic potential maps allowed for the visualisation of charged regions on the surface of the molecules (Figure 4.9B). Distinct regions of surface charge were observed around the suspected effector binding interface. For both Rabs, Switch 1 and latter portion of Switch 2 possess positive surface charge while the interswitch and beginning of Switch 2 contains negatively-charged residues. Given the previous identification of negatively-charged surfaces on the LRRK2 ARM domain (Section 4.2.2), it was decided to further investigate the positively-charged Rab switches. As aforementioned, the theoretical isoelectric point (pI) of LRRK2¹⁻⁵⁵² is calculated at 5.36, while the Rab32 subfamily G-domains range from pI = 8.0 – 8.4. It was therefore hypothesized that charge complementarity may be important for LRRK2:Rab binding. Closer inspection of the crystal structures of the active Rabs identified these surface-exposed candidate residues: Arg39 in Switch 1 and Arg77/Arg81 in Switch 2 (Rab38 numbering). For Arg39 and Arg77 the equivalent charges at these positions are conserved among the Rab32 subfamily, while Arg81 is also found among the wider family (Figure 4.9C). An overlay of the active structures in Figure 4.10 emphasizes the high level of structural identity in protein tertiary structure. The side chain orientations of the positively charged switch residues occupy similar positions in both structures (Figure 4.10B) The importance of these residues to the LRRK2 interaction will be further investigated in Chapter 5.

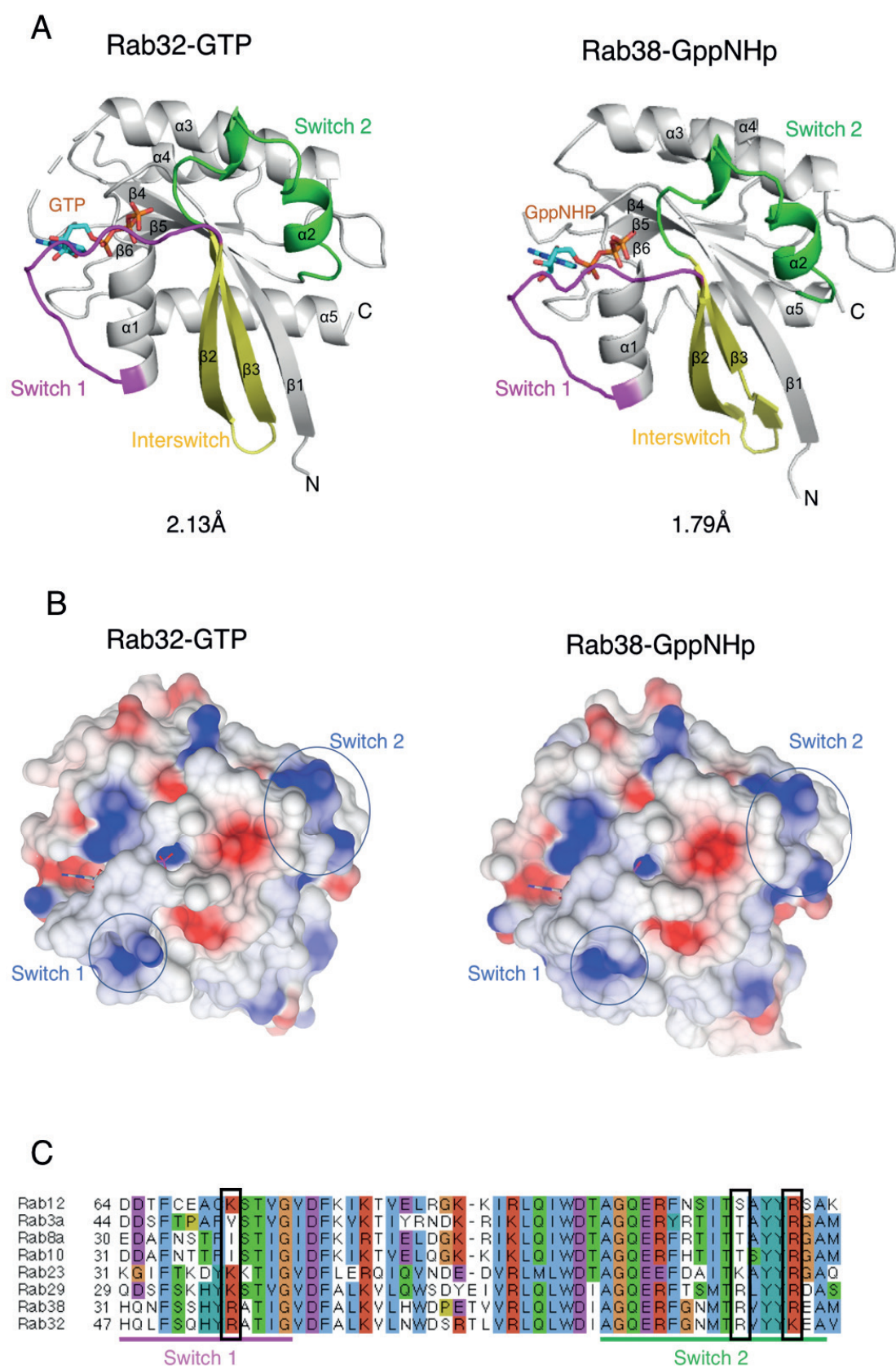


Figure 4.9. Active structures of the Rab32 and Rab38 G-domains

A) The active structure of Rab32^{Q85L}(GTP) was previously solved in the lab and used to solve the X-ray structure of Rab38^{Q69L}(GppNHp) by molecular replacement. The switch and interswitch regions are highlighted and typically make up the effector binding interface. Visual inspection of the proteins indicate highly convergent structural features. B) Electrostatic surface model shows regions of charge on the surface of Rab32 and Rab38. Two areas of positive charge within Switch 1 and Switch 2 were identified as candidate residues for the interaction with negatively charged surfaces on the LRRK2 ARM domain. C) Multiple sequence alignment with a subset of Rab GTPases show the single Switch 1 positive charge at position 39 and Switch 2 positive charge at position 77 are conserved within the Rab32 subfamily. The charge at position 81 is also found within the larger family (Rab38 numbering).

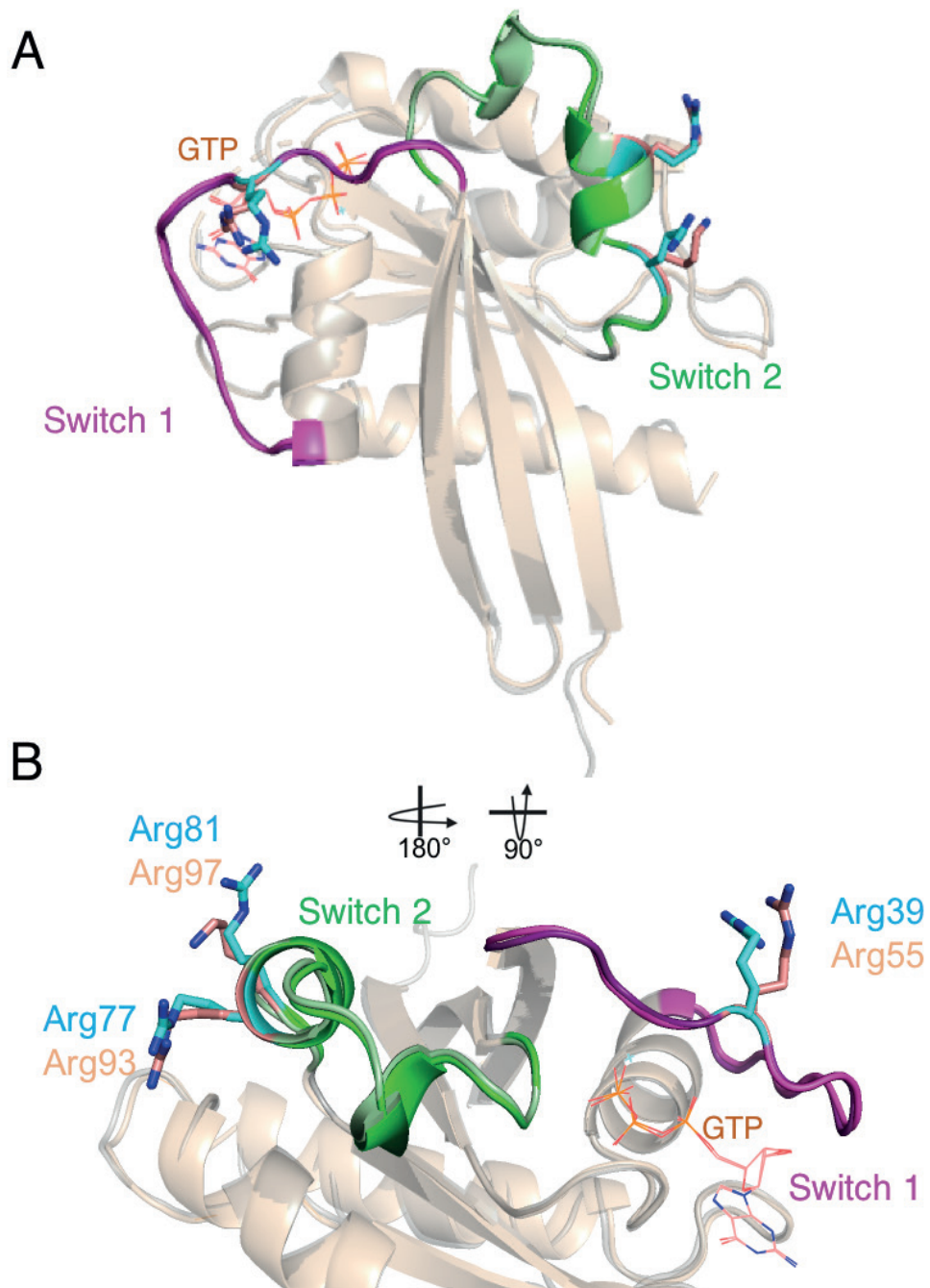


Figure 4.10. Positively-charged residues within the Rab32 subfamily switches

A) Overlay of Rab32(GTP) and Rab38(GppNHp) show highly convergent structural features, particularly in the switch regions which comprise the effector binding interface. Rab32 and Rab38 are shown in wheat and grey respectively. Switch 1 is coloured in pink and Switch 2 in green for both proteins. B) Visualisation of the surface exposed positive charges identified as candidate residues for the LRRK2:Rab interaction. Rab38 residues are shown in cyan, Rab32 residues are shown in salmon.

4.2.6 Structural analysis of Rab29(GDP)

Despite expression of the wildtype construct and exchange with GppNHp, the newly solved structure of Rab29^{3cys} revealed that the inactive form of the protein had crystallised. While this new GDP-bound structure also maintains the typical architecture of Rab GTPases, significant structural changes are observed within the switch regions. The partial unwinding of the $\alpha 1$ helix by one turn preceding Switch 1 likely creates additional flexibility, allowing Switch 1 to adopt an unusual open conformation which is stabilized by crystallographic contacts (Figure 4.11A). An overlay with the active and inactive structures of Rab32 (PDB codes: 6FF8, 5OEC) in Figure 4.11B illustrates the extent of this shift. A comparison of representative Rab(GDP) structures deposited in the Protein Data Bank shows flexibility within the switches, however in general all occupy a similar position within the molecule (Figure 4.11C). Currently, the high resolution model of Rab28(GDP-3'P) is the only other Rab structure known to also exhibit an equivalent Switch 1 shift in its inactive conformation (PDB code: 2HXS) (Figure 4.11D). Rab28 is only distantly related to Rab29, sharing 30% residue identity, and 49% similarities between both proteins. Furthermore, the Switch 1 of Rab GTPases is typically found as an extended loop without defined secondary structure elements, whilst the structure reported in this work is definitively helical in nature.

The Switch 2 of Rab29 shows slight conformational variability but is broadly similar to the inactive Switch 2 conformations observed in most other Rab GTPases. A glutamate residue in Switch 2 of inactive Rab29 is found protruding into the nucleotide binding pocket and occupies the position of the γ -phosphate of GTP (Figure 4.12A). Curiously, the Switch 2 of Rab32(GDP) exhibits an almost identical conformation and the equivalent glutamate residue (E86) occupies the same space in this structure (Figure 4.12B). In the active structures of Rab32/38 this glutamate residue is oriented in the opposite direction and is solvent exposed. The presence of an additional helical or pseudo-helical turn at the beginning of Switch 2 is unique to the GDP-bound Rab32/Rab29 structures and flips the glutamate residue inwards, pointing the side chain towards the nucleotide binding site. This Switch 2 conformation is incompatible with GTP binding, with a severe steric clash observed upon modelling the γ -phosphate into the available space (Figure 4.13A). Thus, in an effort to bias crystallisation towards the active form of Rab29, a single-site mutation was generated at this site. The Glu68 was substituted for a serine residue, which possesses a short side chain whilst maintaining a

similar polar character (Figure 4.13B). HPLC analysis revealed partial exchange of the Rab29^{E68S} protein with GppNHp. The remaining population was predominantly GTP-bound, and thus the protein was known to be active upon crystallisation set-up (Figure 4.13C). However, no crystal hits have yet been observed with this construct to date.

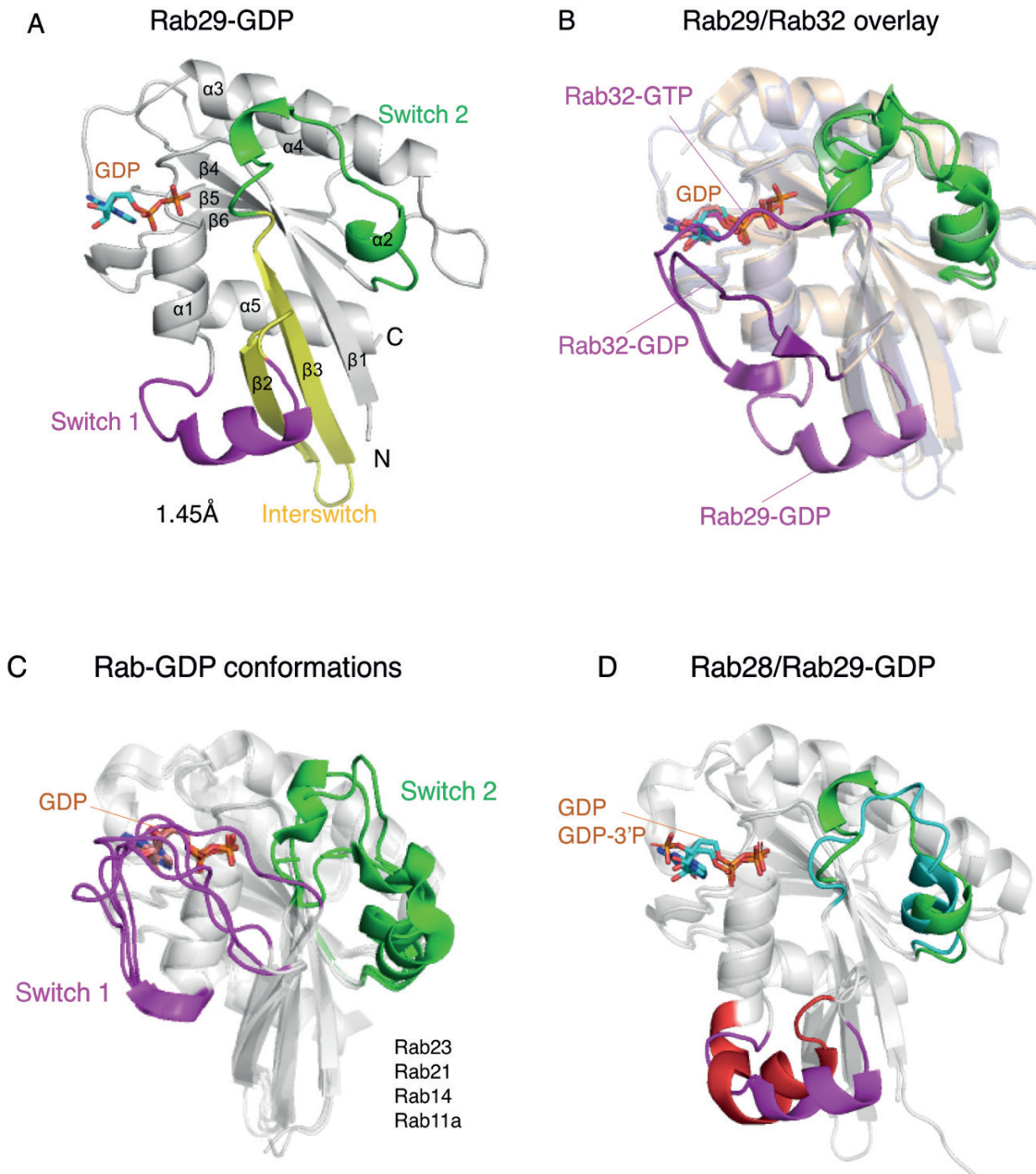


Figure 4.11. Structural comparison of Rab29(GDP)

A) X-ray structure of Rab29^{3cys}(GDP) at 1.45 Å resolution reveals a typical G-domain fold but extensive rearrangements of the Switch 1 region. The switches and interswitch are highlighted. B) Overlay of Rab29(GDP) with the structures of both active and inactive Rab32 showing a large conformational shift in Rab29 Switch 1. C) Overlay of a subset of inactive Rab(GDP) structures show structural variability in the switch regions, however most occupy a broadly similar position within the molecule. D) Structural comparison with Rab28(GDP-3'P) reveals a similar open Switch 1 conformation. For Rab29 Switch 1 and Switch 2 are coloured pink and green respectively. Rab28 Switch 1 and Switch 2 is shown in red and cyan.

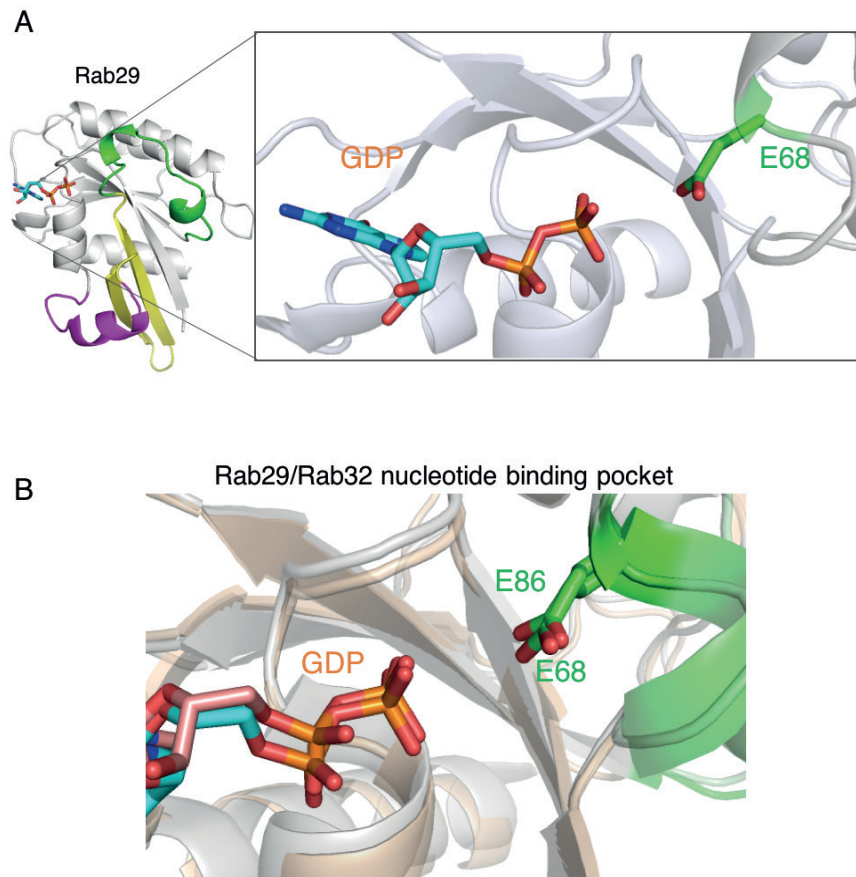


Figure 4.12. Nucleotide binding pocket of Rab29(GDP)

A) Closer inspection of the Rab29(GDP) reveals the side-chain of Glu68 extends into the nucleotide binding pocket, occupying the space of the γ -phosphate of GTP. B) Comparison with the Rab32(GDP) shows the equivalent Glu86 residue in the same orientation and may represent a unique structural feature of the Rab32 subfamily.

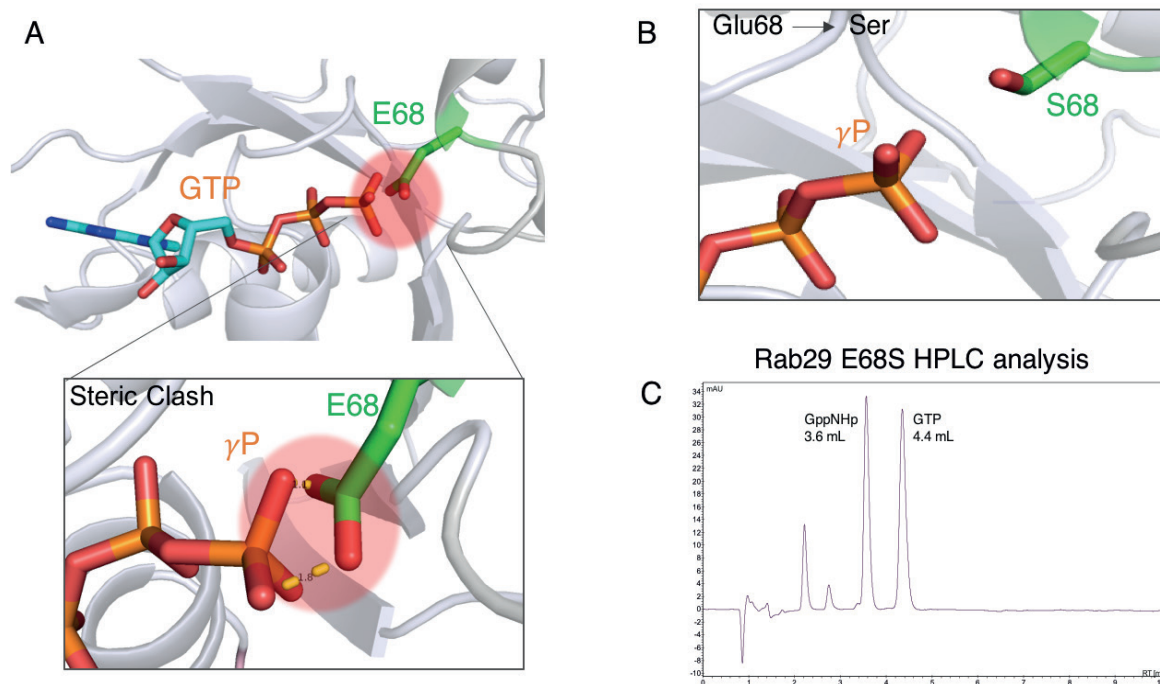


Figure 4.13. Single site mutation of Rab29 E68 to promote GTP binding

A) Insertion of GTP in the nucleotide binding site results in a steric clash between Glu68 and the GTP γ -phosphate. B) Substitution of Glu68 with a serine residue to accommodate GTP within the nucleotide binding pocket. C) Reverse phase HPLC shows a mixed population of GppNHP-bound and GTP-bound Rab29^{E68S}. The protein is mostly found in its active conformation.

4.3 Discussion

4.3.1 LRRK2 crystallisation trials and homology modelling

Crystallisation can be defined as the self-organisation of molecules or macromolecules into periodic arrays over three dimensions. For successful protein crystallisation, both compositional homogeneity and conformational stability are required. A homogenous sample of the protein of interest can be achieved through the optimisation of an expression and purification strategy. The purification scheme for LRRK2 was previously addressed in Chapter 3, and typically yielded high purity for crystallisation trials, as shown in Figure 4.1. Assuming compositional homogeneity is achieved, protein stability is also a fundamental prerequisite for crystallisation, and the protein must remain stable over the timeframe of a crystallisation experiment. Given the high degree of purity obtained for LRRK2, protein stability was examined as a potential roadblock to crystallisation success.

Differential scanning fluorimetry was carried out for the LRRK2¹⁻⁵⁵² construct using the ThermoFluor™ protocol with SYPRO Orange dye. The quantum yield of the dye increases dramatically upon binding to hydrophobic regions and so the denaturation of proteins can be monitored with increasing temperature [427]. The resultant thermal denaturation curve for LRRK2¹⁻⁵⁵² is given in Figure 4.2, with a melting temperature (T_m) = 44 °C. Thermal stability is a useful metric as it shows good correlation with the crystallisability of a protein. For example, lysozyme, which is easily crystallised, has a measured T_m of 71 °C [437]. Furthermore, a study of the bacterial pyridoxal 5'-phosphate (PLP) dependent transaminase BioA demonstrated notable improvement in crystallisation success and quality as T_m increased from 44.8 °C to 85.8 °C upon varying buffer conditions and additional PLP supplementation [438]. A large scale study analysing hundreds of different proteins found that 49% of those with a T_m > 45 °C yielded crystals. A rapid decrease in crystallisation success was noted for proteins with lower T_m measurements, with only 23% of proteins with a T_m < 43 °C crystallising [439]. The midpoint of LRRK2 denaturation can be considered relatively low, and suggests the protein may be unstable. However, the T_m measured falls directly between reported values for crystallisation outcome and so does not provide a clear gauge for the crystallisability of the 1-552 construct.

Given their inherent flexibility, disordered regions of a protein also hinder periodic self-assembly. Web-based prediction servers are useful tools for quickly analysing protein secondary structures and intrinsically disordered regions. Within the LRRK2 ARM domain, a predicted loop between residues 317-342 was commonly identified across several servers. The results from the commonly used IUPRED2 and DISPRE3 are shown in Figure 4.3. The predictability of unstructured regions is based on primary amino acid sequence, since these regions have been found to show compositional bias. Typically, disordered regions contain a reduced residue alphabet and since they have a large solvent-accessible area show a prevalence of polar and charged residues [440]. They are frequently depleted in 'order-promoting' hydrophobic or bulky amino acids [441]. It has been observed that short disordered stretches are mostly negatively charged. The ARM domain residues chosen for deletion 330 – EEKNENQENDDEGEED – 345 exhibit this property, with a composition made up of 63.8% Glu+Asp residues. Several additional N-terminal constructs encompassing the complete armadillo repeats (10-661) and the ARM+ANK domains (1-910) were also engineered to remove this flexible loop. The first 10 amino acids within the sequence had also been flagged as disordered and were removed. Despite efforts to optimize the LRRK2 ARM domain, further screening did not yield any crystal hits. There are several additional techniques that may be utilized to improve crystallisation success, including reductive alkylation, surface-entropy reduction and *in situ* proteolysis, however these methods are beyond the scope of this thesis work [442].

While X-ray crystallography has been enormously successful, it is clear the crystallisation process can be considered the bottleneck of the technique. To aid in this study, Dr. Amir Khan generated a homology model of the first 552 residues of LRRK2 to provide insight into possible Rab32 subfamily binding sites. The model built within our lab is in agreement with those previously published in the literature [299, 301]. Analysis of the surface electrostatics revealed three distinct regions of negative charge that may represent the site of interaction with the Rab32 subfamily (Figure 4.4). The negatively charged loop, termed 'Region 2' was identified by the DISOPRED3 server as a potential site for protein binding. Furthermore, the Parkinson's disease risk associated mutation E334K is found within this region. However, multiple servers had also identified this stretch of amino acids as likely be disordered. As aforementioned, the residues from 330 – 345 were therefore deleted to aid crystallisation

trials. Pulldown assays (not shown) confirmed Rab binding was not affected by these deletion mutants, and thus this region could be eliminated as a potential Rab32 subfamily binding site. Analyses of Region 1 and Region 3 will be further explored in Chapter 5.

4.3.2 Crystallisation and structure solution of Rab38(GppNHp)

Rab38^{Q69L} crystals of different morphologies were obtained from several unique conditions. The stacked plate-like crystals from the Morpheus® screen appeared overnight. Visual inspection suggested the crystals were proteinaceous in nature and were harvested to test in-house (Figure 4.6A). The pattern of reflections shown in Figure 4.6B confirmed the assumption that they were protein crystals. Additional screening identified further hits and diffraction data was collected at the Synchrotron Soleil Proxima-2A beamline. A 1.79 Å data set was obtained from the crystal shown in Figure 4.6C. Molecular replacement (MR) is a technique utilizing a known molecular model to solve the crystal structure of a related molecule [443]. The high resolution data set and the availability of the uncomplexed Rab32(GTP) structure previously solved in the lab (PDB code 6FF8, *unpublished*) allowed for straightforward structure solution using MR. The active Rab38 structure revealed an almost identical architecture to Rab32, (Figure 4.9A), and are also highly convergent in their switch regions. This observation was expected given their high degree of sequence identity and known overlapping functions within the cell.

Protein surface models can be useful for visualising the size and shape of molecules, and the knowledge of charge distributions across a surface can be used to determine how molecules might interact with one another. The surface electrostatics for Rab32 and Rab38 are shown in Figure 4.9B, and again are almost identical in nature. Surface charges within the switches and interswitch were of particular interest for our study, since these regions typically make up the effector binding interface. Positive charge was identified in both Switch 1 (Arg39) and the latter half of Switch 2 (Arg77 and Arg81), while surface-exposed negatively charge from Asp45 is found in the interswitch, and Glu70 at the beginning of Switch 2. Here amino acids are numbered according to the Rab38 sequence, however all residues identified are conserved among the Rab32 subfamily (Figure 4.9C).

4.3.3 Identification of candidate residues mediating the LRRK2:Rab interaction

Given that the theoretical pI of LRRK2 is 5.36 and the G-domains of the Rab32 GTPases is 8.0-8.4, it was speculated that charge complementarity may be important for binding. While both positive and negative charges were identified on the effector binding interface of the active Rabs, the negatively-charged surfaces previously determined for LRRK2 led us to primarily consider the positively charged switch regions as candidate residues for the interaction. Furthermore, Switch 1 and Switch 2 undergo the largest conformational transitions between active and inactive states, and play a large role in determining how Rabs control their association with binding partners. Structural overlay of active Rab32 and Rab38 reveals a single solvent exposed arginine residue in Switch 1 and a double positive charge in Switch 2. In each case, the side chain orientations are similar between Rab32 and Rab38 (Figure 4.10). It is also evident that the side chain angles are offset from one another between Switch 1 and Switch 2 in each case. Thus, it is possible LRRK2 may engage with one switch region more extensively than the other. The importance of these positively-charged switch residues will be further probed in Chapter 5.

4.3.4 Crystallisation and structure solution of Rab29(GDP)

It was possible to obtain crystals of Rab29^{Q67L} in multiple conditions across a diverse range of commercial screens. The best initial conditions were all observed in the Morpheus screen, which utilizes PEGs and polyols as the main precipitants and includes various low molecular weight ligands to enhance the solubility and stability of proteins for crystallisation. All initial Rab29 crystals exhibited needle-like morphologies which are not suitable for obtaining useful diffraction data (Figure 4.7A-C). Further optimization produced thicker wedges that diffracted to 2.5 Å, at the Advanced Photon Source, Argonne National Laboratory (Figure 4.7D). Upon structure solution it became apparent that despite use of the Rab29^{Q67L} activating mutant, the inactive GDP-bound form of the protein had crystallised. Following this, it then came to our attention that in the case of Rab29 the QL mutant resulted in a constitutively inactive form of the protein, as previously reported by Beilina *et al* [413]. We know from the structure solution of Rab38^{Q69L}, as well as numerous other Rab proteins in the literature, that this substitution is typically successful. However, in certain circumstances introduction of this mutation is ineffective or can disrupt protein function [444]. Caution should be used going

forward and the impact of ‘activating’ mutations should be thoroughly tested prior to their use in both *in vivo* and *in vitro* experiments.

Previously, HPLC analysis had shown that the wild-type Rab29 protein was predominantly GTP-bound upon purification (Section 3.2.2). As a result, we immediately switched to using the wildtype construct exchanged with GppNHp in an attempt to crystallise the active form. To improve crystal quality three non-conserved cysteines (C84A, C120A, C127S) were also substituted within the sequence to avoid potential disulfide-mediated aggregation or oxidation which can introduce heterogeneity into the sample. This strategy proved extremely successful upon re-screening, with large three-dimensional crystals growing in 24 – 48 hours (Figure 4.8). Crystals were harvested rapidly to avoid nucleotide hydrolysis or dissociation over time. Diffraction data to 1.45 Å resolution were collected from these optimized crystals at the APS, Argonne. This data is considered very high resolution, close to 1.2 Å where full atomic resolution is achieved [445]. Despite the use of the Switch 2 wildtype Rab29^{3cys} and exchange with GppNHp, it was found that the inactive GDP-bound form had again crystallised (Figure 4.11A). Previous HPLC analysis had shown a mixed population within the Rab29 WT ‘3cys’ sample, with a significant GDP peak present. Here the conformation of Switch 1 in the inactive form is stabilised by crystallographic contacts. It is therefore possible stabilisation of the flexible Switch 1 loop in this way is energetically most favourable for crystallisation. The structures of inactive GDP-bound Rab proteins show increased flexibility and variation in their switch regions, however these changes are typically subtle and occupy a broadly similar position within the molecule (Figure 4.11C). In the case of Rab29(GDP), the open Switch 1 conformation is unusual and arises from the partial unwinding of the preceding α 1 helix, likely providing additional flexibility. There is currently only one other Rab structure in the PDB showing such large nucleotide-dependent flip in Switch 1. Both active and inactive forms of Rab28 are available, and a displacement of ~25 Å is observed for Switch 1 upon binding GDP [446]. While the structure of Rab29(GTP) has not been solved, it can be speculated that this protein undergoes a similarly large Switch 1 transition. The overlay of Rab29 and Rab28(GDP-3’P) (PDB code: 2HXS) is given in Figure 4.11D for comparative purposes. In most Rab structures, Switch 1 is found as an extended loop, while for Rab29 and Rab28 this region is definitively helical in nature. Frequently, in Rab(GDP) structures deposited in the PDB Switch 1 is disordered and is therefore missing from the structural model. A common feature of both

these structures is the very high resolution at which they were solved; 1.45 Å and 1.1 Å for Rab29 and Rab28 respectively. Thus, it is possible these structures reveal a physiologically relevant conformation that may not be resolved at lower resolutions.

An additional feature of interest was noted in the Rab29(GDP) structure, involving the protrusion of the E68 side chain into the nucleotide binding cavity which would otherwise be occupied by the γ -phosphate of GTP (Figure 4.12A). The position of this side chain would result in a steric and electrostatic clash with the GTP γ -phosphate and so a structural rearrangement is required to facilitate the active state. This side-chain conformation has previously been observed in the structure of Rab32(GDP) in complex with the Salmonella protease GtgE (Figure 4.12B). GtgE specifically targets and cleaves the inactive form of the Rab32 subfamily within Switch 1, between residues G59 and V60 (Rab32 numbering) [177]. Curiously, these residues constitute part of the RabF1 motif which is highly conserved among all Rab GTPases. Wachtel *et al.* (2018) identified the conformation of Glu86 as a structural feature contributing to GtgE specificity towards Rab32(GDP) [207]. The finding that the equivalent Glu68 in Rab29(GDP) also occupies the same position suggests this side chain orientation may be a common feature of all Rab32 subfamily members and could play a role in targeting by GtgE. The crystal structure of Rab38(GDP) needs to be obtained to further support this hypothesis.

In an attempt to bias Rab29 towards its active conformation, a serine substitution was introduced for Glu68 in the wild-type background to better facilitate the presence of GTP within the nucleotide-binding pocket (Figure 4.13B). Following an exchange reaction for Rab29^{E68S}, HPLC analysis showed a mixed population of GppNHp and GTP nucleotides. Nevertheless, all protein within the sample was considered active upon crystallisation set-up. No crystals hits were upon prior to submission of this work, and further screening for appropriate conditions will be required.

4.3.5 Conclusion

In summary, crystallisation attempts of the LRRK2 N-terminus and analysis of the troubleshooting techniques applied are reviewed in this chapter. The generation of a homology

model for LRRK2¹⁻⁵⁵² allowed for the identification of negatively-charged surfaces that may represent the Rab binding site. Furthermore, two novel Rab GTPase structures solved by X-ray crystallography are presented. The structure of Rab38(GppHp) aligns almost identically to that of Rab32(GTP) and together allowed for the identification of common candidate residues, conserved within the subfamily, that may play a role in binding LRRK2. The structure of Rab29(GDP) revealed an unusual Switch 1 conformation, however Switch 2 corroborates with structural features previously identified in Rab32(GDP) that may confer specificity to the protease GtgE. In Chapter 5, biophysical studies and mutational analyses will be conducted to further characterise the interaction and scrutinise the relative contributions of noteworthy residues identified here.

Chapter 5:

Biophysical and mutational analyses of the LRRK2:Rab interaction

5.1 Overview

There are approximately 70 Rab GTPases encoded within the human genome, and while many exhibit specific expression profiles, it can be assumed a large number of different Rab proteins are present in any given cell type. Since all Rabs share a common architecture, the interactions between Rabs and their effectors must be highly specific. Effector proteins preferentially bind to the active GTP-bound Rab, typically recognising the conserved switch regions. These effector molecules are therefore capable of distinguishing explicitly between many structurally similar Rab interfaces. Several modes of binding have been classified for Rab:effector interactions, however it has been difficult to discern the precise molecular determinants of Rab:effector specificity to date [137]. Rab32/38 are known to interact with the ankyrin domain of their shared effector VARP [182]. The LRRK2 N-terminus contains both an armadillo (ARM) and an ankyrin (ANK) domain, yet the binding site for the Rab32 subfamily is not found within the ankyrin domain in this case. Here we aim to further characterise the interaction of the Rab32 subfamily with the armadillo repeats of LRRK2.

Binding affinity is the strength of the binding between two molecules that reversibly interact, and is typically measured and reported as the equilibrium dissociation constant (K_d). The smaller the K_d value the tighter the biomolecular interaction. Previous *in vitro* thermodynamic and kinetic investigations have shown Rab proteins bind to their cognate effectors with wide ranging affinities, from low nanomolar to low micromolar K_d values reported [134, 447-450]. While the binding of the Rab32 subfamily to the LRRK2 ARM domain has been demonstrated by qualitative methods in the literature and by pulldown analysis previously in this study, there have been no quantitative measurements of the interaction to date. Characterisation of binding affinities and thermodynamic parameters is critical for understanding molecular relationships and how this applies to biological function. Here we measure Rab binding affinities to the LRRK2 ARM domain using various biophysical techniques, and establish whether this interaction is nucleotide-dependent.

Following examination of the LRRK2¹⁻⁵⁵² homology model and the Rab crystal structures in Chapter 4, candidate residues suspected to be important for the interaction were identified. Based on the structural results, in this chapter we generate site-specific mutants to further probe the interaction and test whether these residues are important for recruitment. Using pulldowns and fluorescence assays we will map more precisely the location of the Rab binding domain within the LRRK2 armadillo repeats and how the Rab32 subfamily mediate this interaction.

In addition to its upstream regulatory function, Rab29 is also among a subset of Rab GTPases determined to be bona-fide substrates of LRRK2 kinase. These Rab GTPases, including Rab8A and Rab10, possess a conserved Thr/Ser phosphorylation site within Switch 2 not found in Rab32/38. Phosphorylation at this site has been shown to effect binding of both regulatory proteins and effectors to modify signalling pathways [148]. Rab29 is unusual, with adjacent threonine and serine phosphorylation sites found at this location. Both residues have been shown to be phosphorylated, however there is conflicting evidence pertaining to which is the dominant site [297, 451]. Curiously, a doubly-phosphorylated Rab29 peptide was also identified by mass-spectrometry analysis [149]. Purlyte *et al.* (2018) showed that Rab29 (T71/S72A) activated LRRK2 kinase to the same extent as wild-type, indicating phosphorylation is not a requirement for LRRK2 activation. However, the Rab29 (T71/S72E) phosphomimetic failed to activate LRRK2. Neither of the single phosphomimetic mutants induced this effect [306]. Here, we provide some preliminary data to examine whether Rab29 phosphorylation can alter binding to the LRRK2 armadillo repeats, potentially representing a cyclical self-regulatory mechanism.

5.2 Results

5.2.1 Isothermal titration calorimetry

LRRK2:Rab38 complex formation in solution had been demonstrated qualitatively by SEC in Section 3.2.4. Affinity pulldown experiments also demonstrate binding for all members of the subfamily (Section 3.2.6). For a quantitative approach, isothermal titration calorimetry was selected to examine the LRRK2:Rab38 interaction. A 40 μM solution of LRRK2¹⁻⁵⁵² was used in the calorimetric sample cell and Rab38 was concentrated to 400 μM for iterative injection from the syringe. Representative experiments for active and inactive Rab38 are shown in Figure 5.1. The energy of binding events associated with each injection is measured directly in $\mu\text{cal/sec}$. This heat change is represented as a peak which returns to baseline as the instrument returns the sample cell and a reference cell to equal temperature. The quantity of heat measured is in direct proportion to the amount of binding and is measured until the reaction reaches equilibrium. The negative peaks recorded for the LRRK2:Rab38 interaction indicate an exothermic reaction with heat released upon binding. The area of each peak is integrated and plotted against the molar ratio of ligand to protein, and the resultant sigmoidal binding curve is shown directly underneath the raw titration profile in each case (Figure 5.1). A clear dependency for the active form of Rab38 is observed, with no significant heat change detected for the interaction of Rab38(GDP) with LRRK2. For Rab38^{Q69L} the average of two titrations yielded a K_D value of 1.57 μM for binding to LRRK2, and an average K_D of 0.88 μM was measured for the interaction of LRRK2 with Rab38(GppNHp). All kinetic parameters obtained from peak integration and curve fitting to a single-site binding model are presented in Table 5.1.

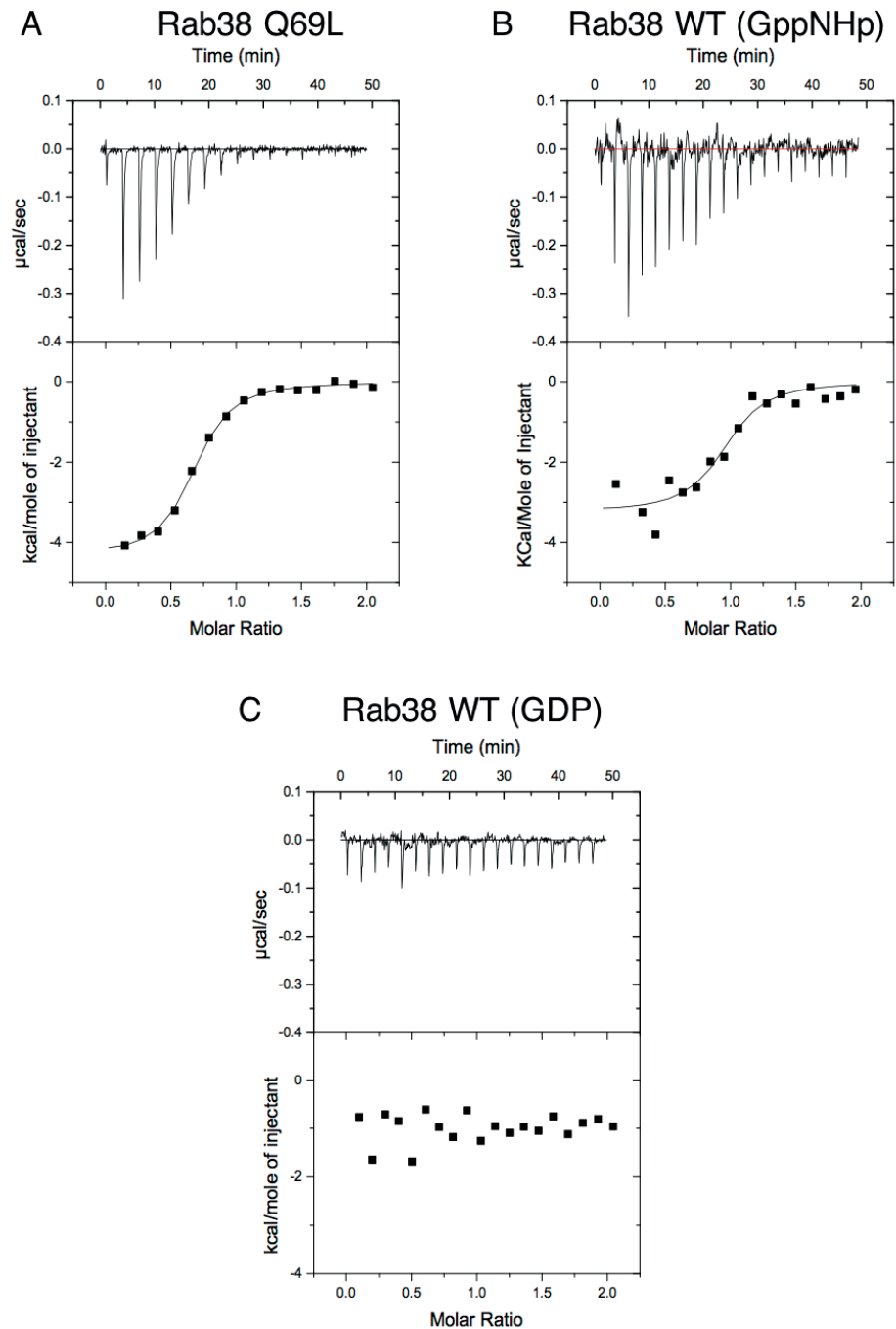


Figure 5.1. Quantitative measurement of the LRRK2:Rab38 interaction by isothermal titration calorimetry. Titration profiles and peak integration curves of A) active Rab38^{Q69L}; B) active Rab38 WT(GppNHp); C) inactive Rab38 WT(GDP) into LRRK2¹⁻⁵⁵². The interaction with LRRK2 critically depends on the activity state of Rab38. Experiments performed in collaboration with Dr. Amir Khan, Dr. Dieter Waschbusch, Dr. Aoife Kearney and Dr. Patrick Lall.

Rab38	K_d (μM)	ΔH (cal/mol)	ΔS (cal/Mol/deg)	Stoichiometry (N)
WT-GppNHp	1.07	-2934	17.3	0.86
WT-GppNHp	0.69	-3999	18	0.921
Q69L	1.54	-5806	6.8	0.725
Q69L	1.60	-4344	11.7	0.651

Table 5.1. Thermodynamic parameters for the LRRK2:Rab38 interaction obtained from isothermal titration calorimetry

5.2.2 Fluorescence anisotropy

Rab32 and Rab29 were not amenable to ITC due to protein precipitation at high concentrations, thus an alternative method to study the interaction with LRRK2 was required. Using a fluorescently labelled (N-Methylanthraniloyl, mant) GppNHp analogue bound to the Rab GTPases, a fluorescence anisotropy assay was developed using an equilibrium binding strategy. A 0.5 μ M sample of each mant-labelled Rab GTPase was incubated with increasing concentrations of LRRK2 and the anisotropy of the fluorescent molecule was measured following excitation at 355 nm. Baseline fluorescence readings of each Rab were taken to ensure all proteins were equally exchanged and subtracted from LRRK2 measurements to give the change in anisotropy (Δr) upon complex formation. For each Rab protein, an equilibrium binding curve was plotted and the dissociation constant was calculated using a quadratic formula in MATLAB to account for ligand depletion. This method provided binding affinities for each member of the Rab32 subfamily in good agreement with the previous ITC data, with binding affinities all estimated in the low micromolar range (Table 5.2). Inactive forms of the Rab32 subfamily bound to mant-GDP show no measurable binding and provide further evidence of nucleotide dependency for complex formation. Rab11(mant-GppNHp) does not interact with LRRK2 and was used as an additional control.

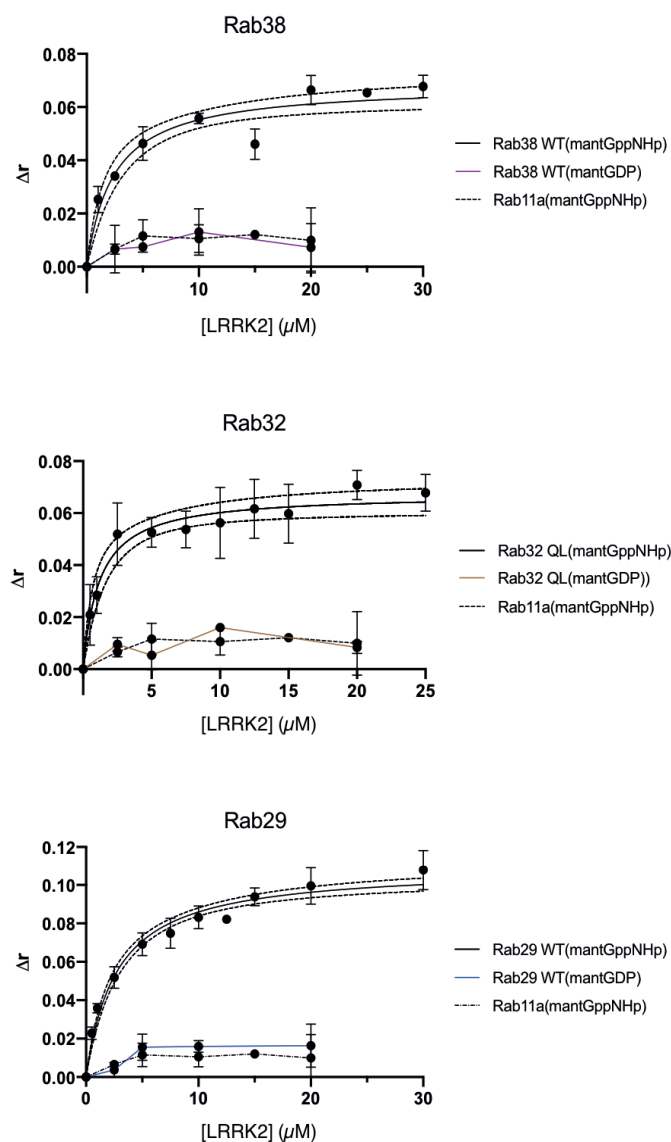


Figure 5.2. Quantitative measurements of the LRRK2:Rab32 subfamily interaction by fluorescence anisotropy. Samples of 0.5 μM Rab(mant-GppNHp) were incubated with increasing concentrations of LRRK2¹⁻⁵⁵² and anisotropy was measured using the SpectraMax M5 plate reader and the preconfigured 'Fluorescence Polarization' protocol on the SoftMax Pro software. The mant fluorophore was excited at 355 nm and emission was detected at 448 nm. Background fluorescence of Rab GTPases in the absence of LRRK2 was subtracted from readings to obtain the change in anisotropy (Δr) upon complex formation. Three independent samples ($n = 3$) were measured for each LRRK2 concentration within one experiment for each Rab protein. The final fluorescence value for each sample was obtained from the average of 3 readings. The error bars represent the standard deviation of the three samples at each concentration and the dotted lines indicate the 95% confidence bands. Binding affinities are in the low micromolar range with K_d measured between 1-3 μM . Inactive forms of the Rab32 subfamily bound to mant-GDP and Rab11(mant-GppNHp) which does not interact with LRRK2 were used as controls.

	K_d (μ M)	Goodness of fit (R^2)	95% Confidence Interval (CI)
Rab38 WT	2.29	0.938	0.61 – 3.96
Rab29 WT	2.64	0.9635	1.55 – 3.7
Rab32 Q85L	1.12	0.9324	0.61 – 1.74

Table 5.2. Binding affinities of active Rab GTPases bound to LRRK2¹⁻⁵⁵² ARM domain by fluorescence anisotropy. Values were calculated using MATLAB software.

5.2.3 Mutational analyses of the LRRK2 ARM domain

Homology modelling of LRRK2¹⁻⁵⁵² (Section 4.2.2) revealed three predicted clusters of negative charge on the surface of the molecule that may be important for mediating the interaction with the Rab32 subfamily. Residues 330-345 within a negatively charged loop constituting 'Region 2' had previously been deleted to aid in crystallisation trials and so were ruled out as a candidate binding site (Figure 5.3A). Multi-site mutants were generated to investigate the role of the negative charge in the remaining regions of interest. In each case, mild mutations were chosen to investigate the effect of the charge without disruption of protein structure. Pulldown analysis of the LRRK2 'Nmut' (E192Q, E193Q) construct showed no observable difference in binding to Rab38 and was ruled out as the site of interaction (Figure 5.3B). In contrast, introduction of the LRRK2 '2mut' (D390N, D392N) showed a decrease in binding to both Rab29 and Rab38 (Figure 5.3C). In this case, the band intensities were quantified, and the ratio of LRRK2 binding to Rab38 and Rab29 is presented as a bar chart to aid with visualisation (Figure 5.3D). To further probe this site two additional mutations then were introduced; LRRK2 '3mut' (D390N, E291Q, D392N) and '4mut' (E386Q, D390N, E291Q, D392N). While these proteins were susceptible to proteolysis during purification, as seen by the lower MW bands present in Figure 5.3E, sufficient amounts of intact LRRK2 were purified to confirm that the interaction with Rab38 was abolished.

The LRRK2 '2mut' protein was stable upon purification and exhibited a similar size exclusion elution profile to the wild-type construct (Figure 5.4B). Since the effects of the '2mut' protein upon Rab binding were relatively mild, direct fluorescence measurements were chosen to better quantify the impact of this mutant. Fluorescence titrations were first carried out with 1 μ M Rab and increasing concentrations of LRRK2 to determine the optimal LRRK2 concentration to use for testing. The signal at 10 μ M LRRK2 was chosen, as at this point the change in fluorescence intensity began to plateau (Figure 5.4C). Rab29(mant-GppNHp) was chosen to measure the impact of LRRK2 '2mut' upon binding since it showed the greatest change in signal upon complex formation. In Figure 5.4D, LRRK2 '2mut' induces a smaller change in fluorescence intensity over multiple readings compared with LRRK2 wild-type, indicating a reduced ability to bind Rab29. These fluorescence intensity measurements further validate the initial pulldown results and overall, these findings suggest that the negatively charged residues in the region 386 – 392 contribute to Rab recognition.

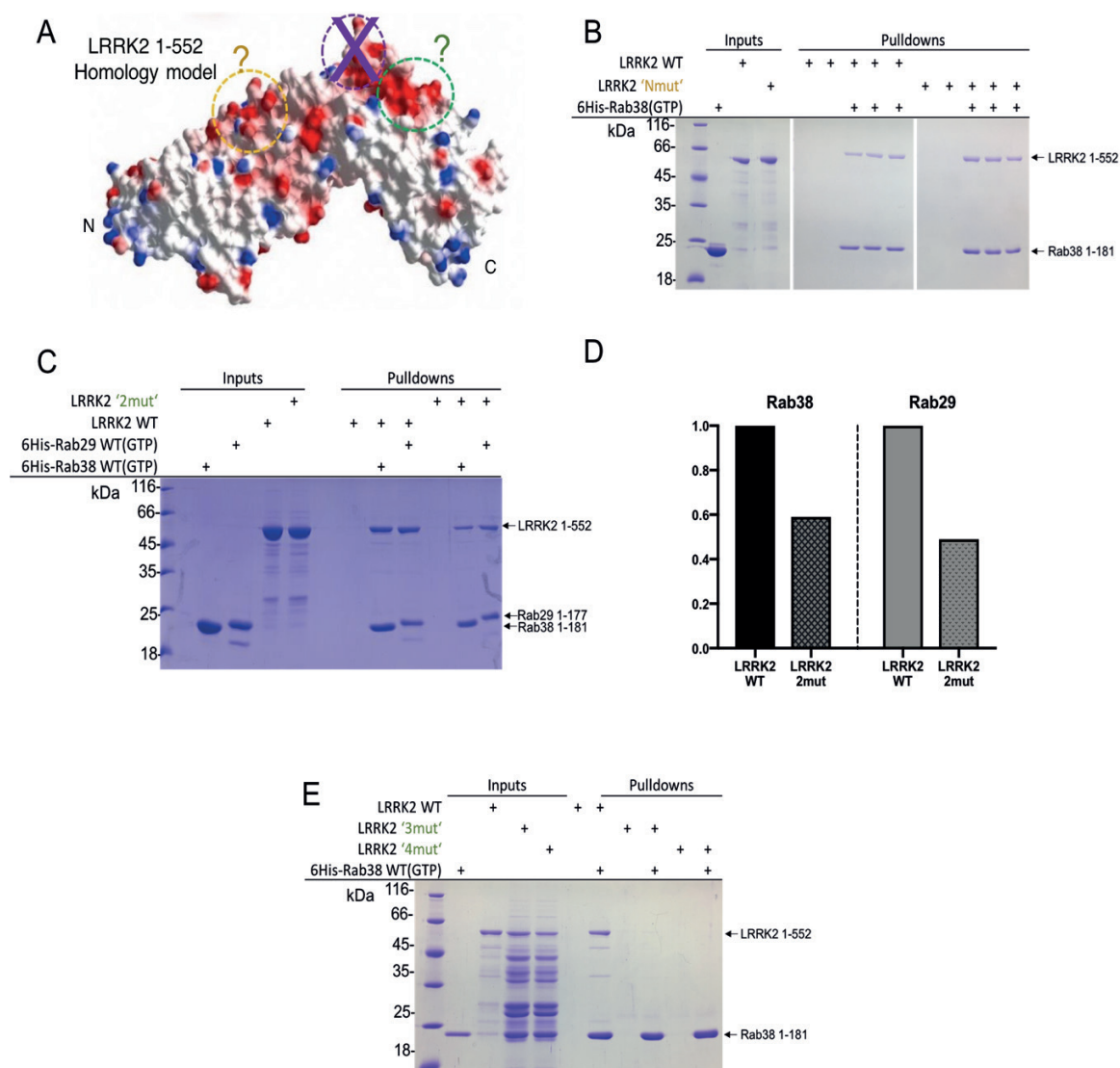


Figure 5.3. Mutational analyses of the LRRK2 armadillo domain

A) Homology modelling of LRRK2¹⁻⁵⁵² identified three candidate regions for Rab binding. Residues within Region 2 (purple) has previously been deleted and were excluded as the site of Rab interaction. B) Pulldown analysis of LRRK2 'Nmut' (E192Q, E193Q) in Region 1 (yellow) showed no effect upon Rab binding. C) Pulldown analysis of LRRK2 '2mut' (D390N, D392N) within Region 3 (green) showed a mild reduction in binding both Rab38 and Rab29. Pulldown performed in collaboration with Dr. Dieter Waschbusch. D) Densitometric quantification of LRRK2 '2mut' pulldown SDS-PAGE results. Pulldowns were quantified normalizing the Rab38/29 WT control to 1 in each case. E) Pulldown analysis of LRRK2 '3mut/4mut' proteins shows complete ablation of Rab38 binding. Significant protein degradation occurs upon introduction of these additional substitutions within Region 3, as seen from 'input' samples.

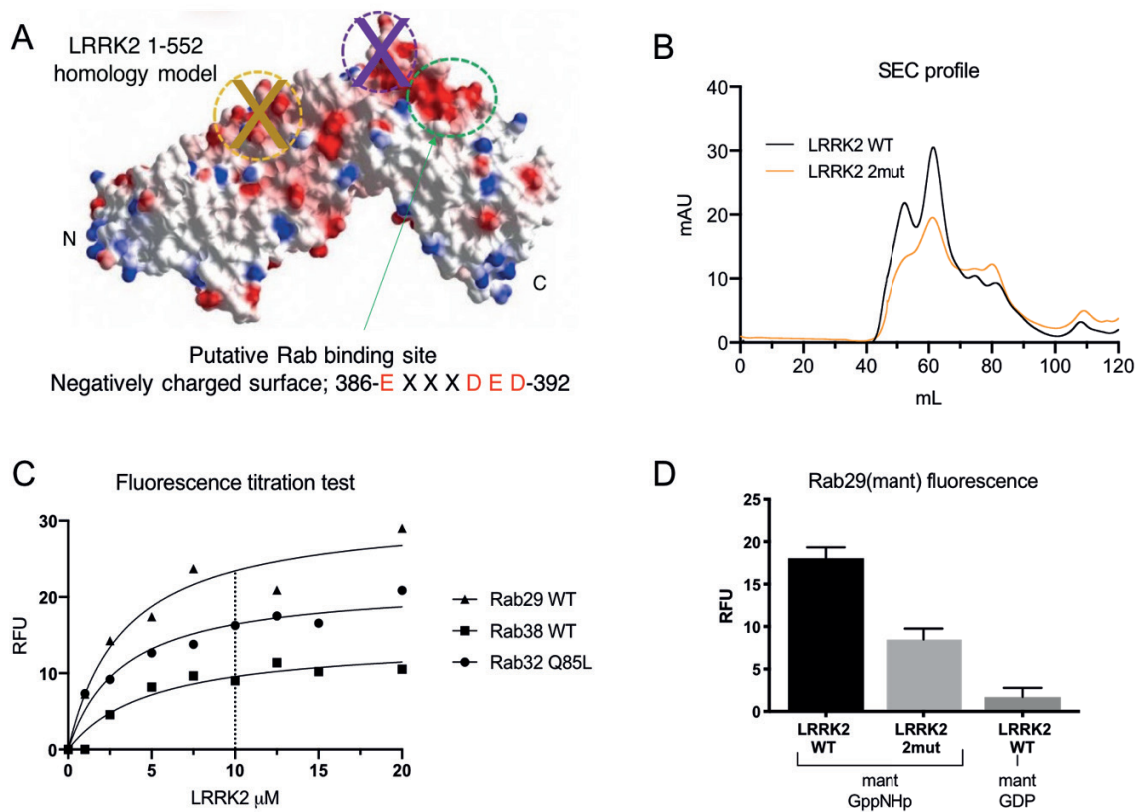


Figure 5.4. SEC and fluorescence analyses of mutant LRRK2¹⁻⁵⁵²

A) Residues within Region 3 (green) contribute to Rab binding. B) SEC chromatographs of LRRK2¹⁻⁵⁵² WT and '2mut' proteins show similar elution profiles. The peak at 60 mL was collected for fluorescence studies. C) Fluorescence titration experiments for each Rab GTPase (1 μ M) show an increase in fluorescence intensity upon LRRK2¹⁻⁵⁵² binding. Measurements were made using the SpectraMax M3 plate reader. Each point represents a single sample at each LRRK2 concentration. The value of each point was obtained from the average of 3 readings. Background fluorescence of Rab GTPases in the absence of LRRK2 was subtracted to obtain the change in fluorescence intensity. RFU = relative fluorescence units. D) The impact of the LRRK2 '2mut' mutation on Rab29 fluorescence intensity was tested. Sample wells containing 1 μ M mant-labelled Rab29 were incubated with 10 μ M LRRK2¹⁻⁵⁵² WT/2mut. Four independent replicates for each LRRK2 construct were measured within one experiment. For each replicate sample the value was obtained from the average of 3 separate readings. The mean $\pm$ SEM of the 4 samples for each construct is plotted as a bar graph. Experiment performed in duplicate (n=2), representative graph is shown. A decreased change in fluorescence intensity is seen upon introduction of LRRK2 '2mut' compared with wild-type, indicating a disruption in binding Rab29. The interaction is reduced by ~50%. The small signal change measured for inactive Rab29(mantGDP) indicates no interaction with LRRK2 WT and shows the increase in fluorescence intensity is a direct result of complex formation and does not arise from non-specific interactions.

5.2.4 Mutational analyses of the Rab32 subfamily

Structure solution of Rab38(GTP) reported in Chapter 4, and the availability of Rab32 structures in the literature, allowed the identification of positively charged, solvent exposed residues in the switch regions that could be important for binding to the negatively charged LRRK2 ARM domain site (Figure 5.5A). Beginning with Rab38, site-directed mutagenesis generated a single switch 1 mutant (R39Q), and a double switch 2 mutant (R77/81Q) to examine the importance of the positive charge at these sites. Pulldowns using GppNHp loaded His-tagged Rab38 mutants was carried out with cleaved LRRK2 and analysed by SDS-PAGE as before. Examination of the Coomassie-stained bands showed the introduction of the Rab38^{R39Q} Switch 1 mutation resulted in a visible reduction in LRRK2 binding, while the double switch 2 (R77/81Q) mutant had no observable effect (Figure 5.5C). Band intensities were also quantified and are shown in Figure 5.5D. Additional mutations of a conserved hydrophobic residue in Switch 1 (I42A, I42E), which is widely exploited as a binding interface by the Rab family, did not significantly reduce binding to LRRK2 (Figure 5.5B).

To test if this observation also holds true for the other members of the Rab32 subfamily, the equivalent Switch 1 mutations were introduced to generate Rab32^{R55Q} and Rab29^{K37Q} proteins. Comparison of SEC profiles show a shift of the Rab38^{R39Q} peak towards the left, eluting at an earlier volume than Rab38 WT (Figure 5.6A). Given that the change is relatively small, this observed shift is likely to be insignificant and may be due to freeze-thaw effects or differing experimental conditions. Furthermore, both Rab32 and Rab29 Switch 1 mutants behave identically to their wild-type counterparts, ensuring these substitutions do not result in aggregation or protein misfolding (Figure 5.6B&C).

As with the LRRK2 mutations described in Section 5.2.3 above, pulldown results for the Rab38^{R39Q} Switch 1 mutant were also validated by fluorescence intensity experiments. Measurement of the intensity change following incubation of 1 μ M Rab38(mant-GppNHp) with 10 μ M wild-type LRRK2¹⁻⁵⁵² is shown in Figure 5.7A. A decrease in the fluorescence change is observed for Rab38^{R39Q} compared to the wild-type protein, and is similar to the signal measured for inactive Rab38(mant-GDP). This suggests the interaction is severely disrupted by elimination of the Switch 1 positive charge. Rab11(mant-GppNHp), which does not interact with LRRK2, is used as an additional control. Fluorescence intensity

measurements of Rab32 elicited a similar effect. In this case, 1 μ M Rab32^{R55Q} also showed a considerable reduction in fluorescence signal upon incubation with 10 μ M LRRK2¹⁻⁵⁵² WT (Figure 5.7B). Importantly, the baseline fluorescence readings were similar for all mutant and wild-type proteins (not shown), confirming that fluorescence nucleotide was equally bound and the activity state of each Rab GTPase was the same in each case. Thus, it can be concluded that for Rab32/38 the positively-charged arginine in Switch 1 is critical for mediating the interaction with LRRK2.

A positively-charged lysine residue is found in the equivalent position in Rab29 Switch 1. Curiously, removal of this charge did not impact significantly upon the ability of Rab29 to interact with LRRK2¹⁻⁵⁵². Pulldown analysis showed no observable difference in LRRK2 binding to Rab29 WT and Rab29^{K37Q} proteins (Figure 5.8A&B). This data was further supported by fluorescence intensity measurements in which introduction of Rab29^{K37Q} showed no significant impact upon binding LRRK2 compared with wild-type Rab29 response (Figure 5.8C). Thus, in contrast to Rab32/38, the positively-charged lysine residue in Switch 1 of Rab29 appears dispensable for the interaction. Overall, these results suggest differences in the mode of Rab29 binding to the same LRRK2 ARM domain site.

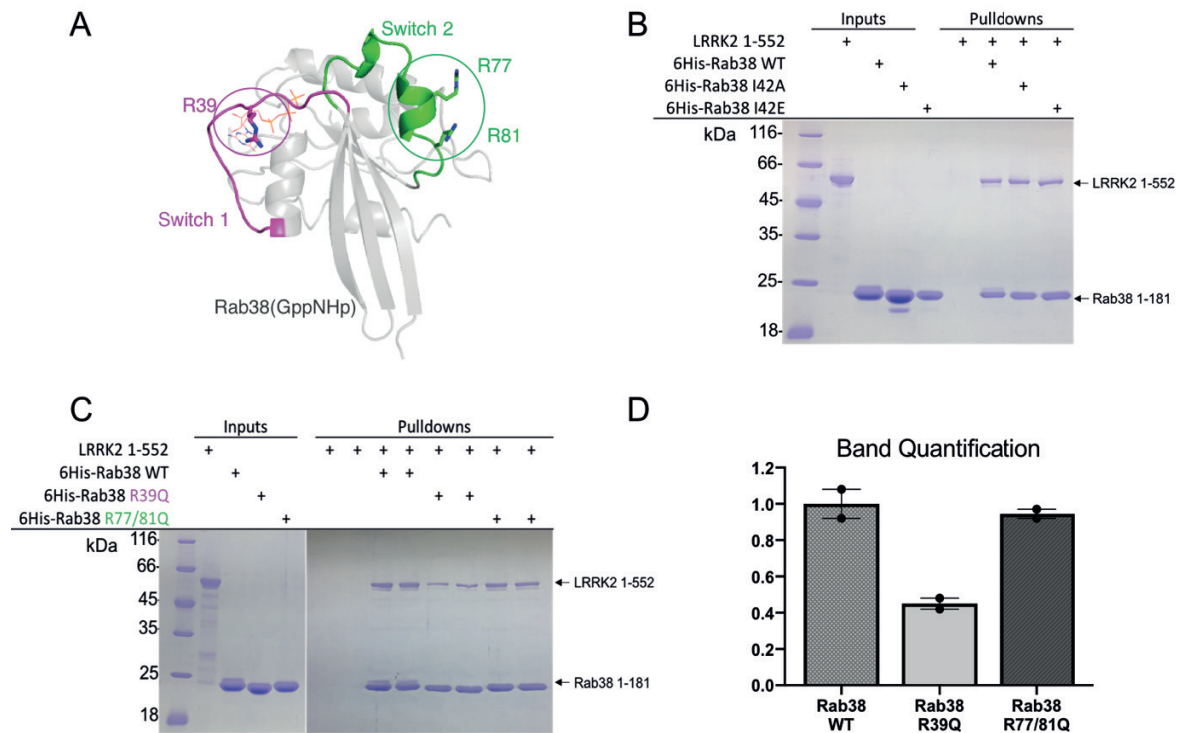


Figure 5.5. Mutational analyses of the Rab38 switches

A) Structural studies of the Rab32 subfamily revealed positively charged residues within Switch 1 and Switch 2 that may be important for binding LRRK2¹⁻⁵⁵². B) Substitution and pulldown analysis of a hydrophobic interswitch residue frequently exploited in Rab-effector interactions shows no effect upon LRRK2 binding. C) Pulldown analysis determines the removal of a positive charge in Rab38 Switch 1 (R39Q) causes a considerable reduction in LRRK2 binding. Mutation of a double positive charge in Switch 2 (R77/81Q) shows no effect. D) Quantification of Coomassie-stained pulldown bands represents Rab38 pulldowns as a bar graph. Pulldowns were quantified normalising the Rab38 WT control to 1. Individual values are shown and error bars represent the range in intensities measured.

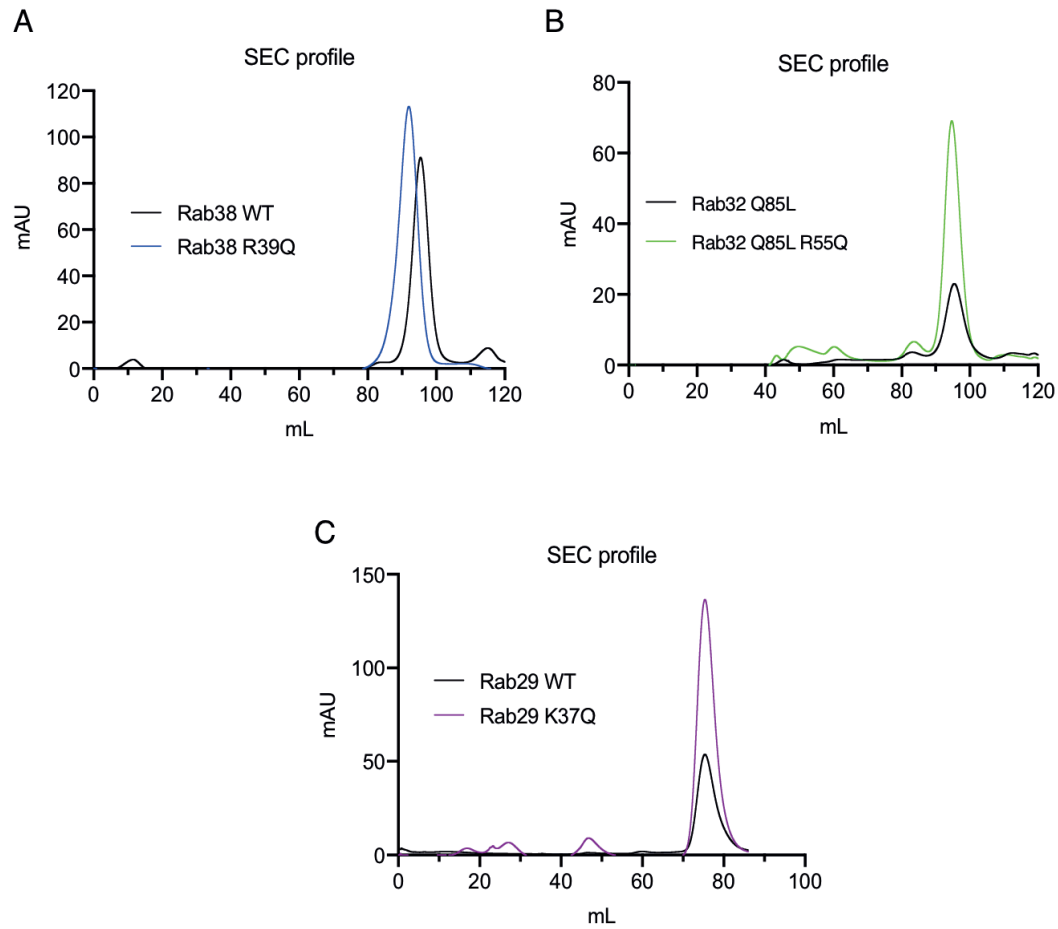


Figure 5.6. Size exclusion chromatography of Rab Switch 1 mutants

A) SEC chromatographs show a slight shift in the retention volume of Rab38^{R39Q} B&C) SEC chromatographs shown no change in the elution profiles of Rab32 and Rab29 following substitution of the Switch 1 positive charge.

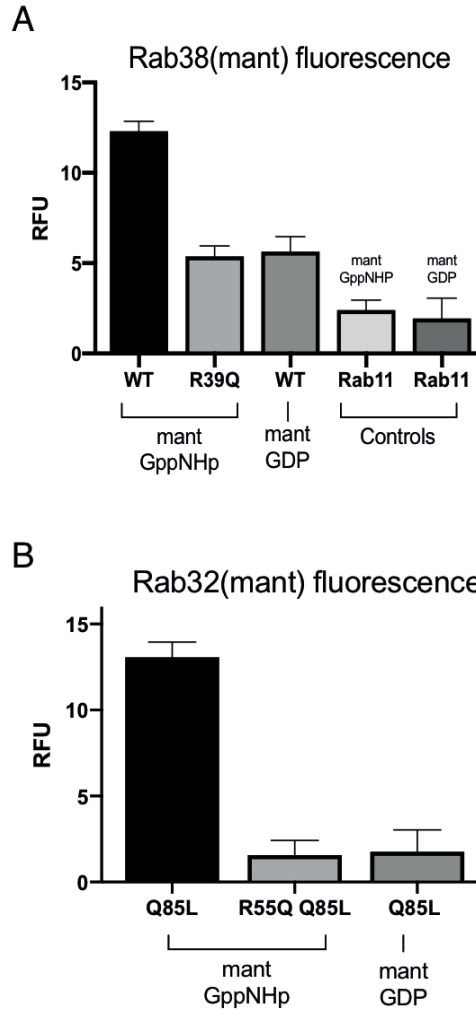


Figure 5.7. Fluorescence intensity measurements of Rab38 and Rab32 Switch 1 mutants.

Following incubation with 10 μ M LRRK2¹⁻⁵⁵² WT, the fluorescence intensity of 1 μ M mant-labelled Rabs were measured on a SpectraMax M3 plate reader. Background Rab fluorescence in the absence of LRRK2 was subtracted from readings and the change in fluorescence plotted as a bar graph. RFU = relative fluorescence units. A) Introduction of Rab38^{R39Q} Switch 1 mutant reduces LRRK2 binding to levels measured for inactive Rab38(mant-GDP). The fluorescence signal for LRRK2 binding to mant-labelled Rab11 is also measured as a control. Four independent replicates for each Rab38/11 construct were measured within one experiment. For each replicate sample the value was obtained from the average of 3 separate readings. The mean $\pm$ SEM of the 4 samples for each construct is plotted. Experiment performed in duplicate (n=2), representative graph is shown. B) Introduction of Rab32^{R55Q} Switch 1 mutant reduces LRRK2 binding to levels measured for inactive Rab32(mant-GDP). Here the Switch 1 mutation was generated on the 'activating' Rab32 Q85L background. Five independent replicates for each Rab32 construct were measured within one experiment. For each replicate sample the value was obtained from the average of 3 separate readings. The mean $\pm$ SEM of the 5 samples for each construct is plotted. Experiment performed in duplicate (n=2), representative graph is shown. RFU = relative fluorescence units.

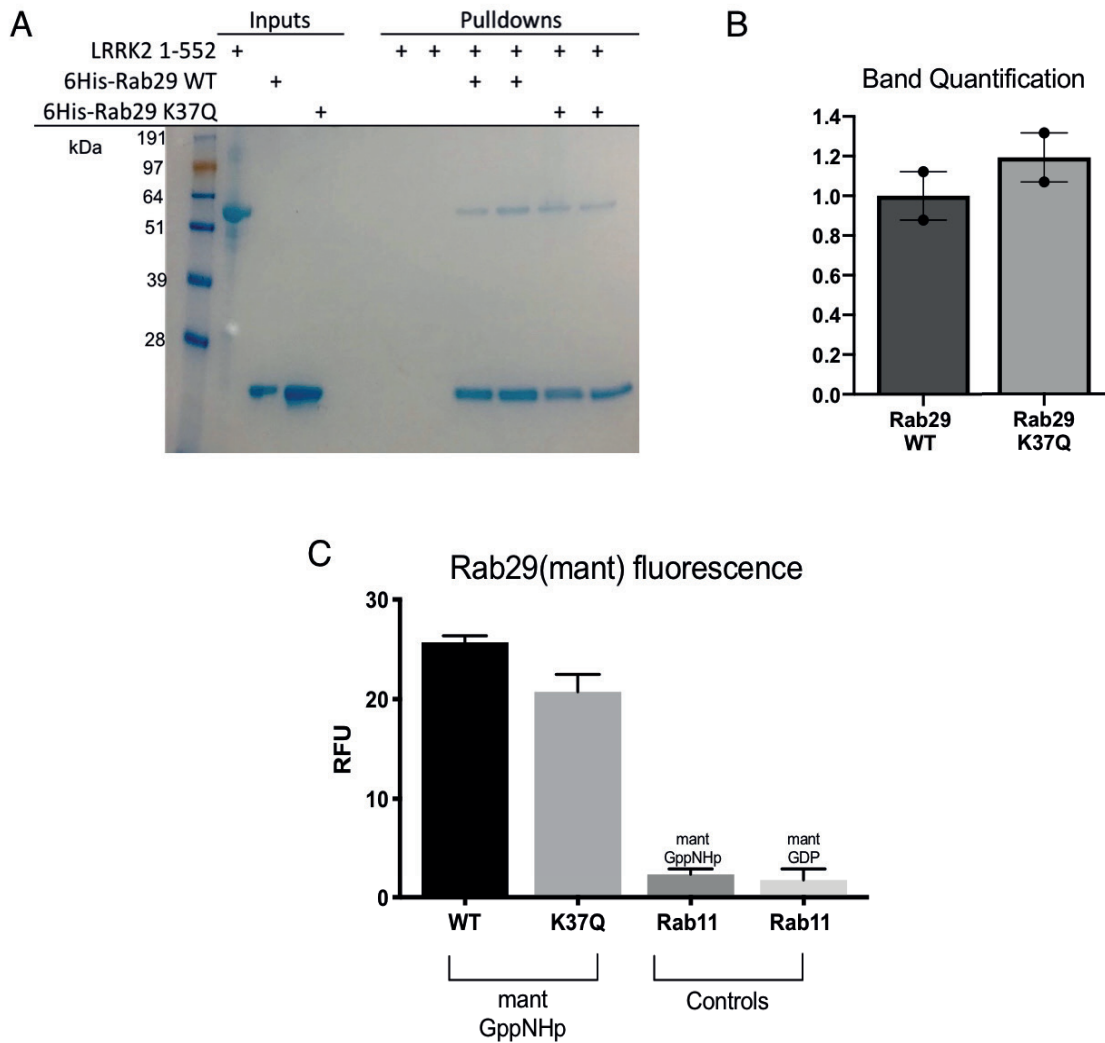


Figure 5.8. Mutational analyses of the Rab29 Switch 1 mutant

A) Affinity pulldown assay shows no observable difference in LRRK2¹⁻⁵⁵² binding to Rab29 WT and Rab29^{K37Q}. B) Band quantification of the pulldown gel confirms the ability of Rab29 to interact with LRRK2 is not impacted by loss of the Switch 1 positive charge. Pulldowns were quantified normalising Rab29 WT control to 1. Individual values are shown within the bar graph and error bars represent the range in intensities measured. C) The fluorescence intensity of mant-labelled Rab29 WT and Switch 1 mutant were measured following incubation with 10 μ M LRRK2¹⁻⁵⁵² WT. The fluorescence signal for LRRK2 binding to mant-labelled Rab11 was also measured as a control. Five independent replicates for each Rab29/11 construct were measured within one experiment. For each replicate sample the value was obtained from the average of 3 separate readings. The mean $\pm$ SEM of the 5 samples for each construct is plotted. Experiment performed in duplicate (n=2), representative graph is shown. No significant decrease in fluorescence intensity is observed for Rab29^{K37Q} binding to LRRK2. RFU = relative fluorescence units.

5.2.5 Rab29 as a LRRK2 substrate

Rab29 is also a downstream substrate of LRRK2 kinase activity, and possesses two adjacent phosphorylation sites within Switch 2 (Figure 5.9). Given that ablation of the positive Rab29 Switch 1 charge in Section 5.2.4 did not drastically impact upon LRRK2 binding, we set out to determine whether post-translational modification of Rab29 in Switch 2 could directly affect its ability to bind to the LRRK2 ARM domain. Site specific phosphomimetic mutants were generated for each position (T71E and S72E), as well as a double Switch 2 substitution (T71/S72E). Pulldown analysis showed no significant difference in LRRK2 binding to the single phosphomimetics compared to wild-type Rab29. The SDS-PAGE gel was silver-stained to improve visualisation (Figure 5.10A&B). In this case the polyacrylamide gel was silver-stained for improved sensitivity. On the other hand, introduction of the double phosphomimetic mutations resulted in a notable decrease in the ability of Rab29 to interact with LRRK2 (Figure 5.10C). Negative controls show no background binding of LRRK2 however there are bands visible at the same molecular weight as the Rab GTPases, despite having no Rabs protein present in these samples. This is likely due to dirty beads and these intensities were subtracted from the pulldown bands to achieve the correct ratio of LRRK2 binding to His-tagged Rab GTPases, plotted in Figure 5.10D. SEC elution profiles given in Figure 5.10E show the Rab29 T71/S72E double mutant behaves similarly to the monomeric wild-type protein and is folded correctly in solution.

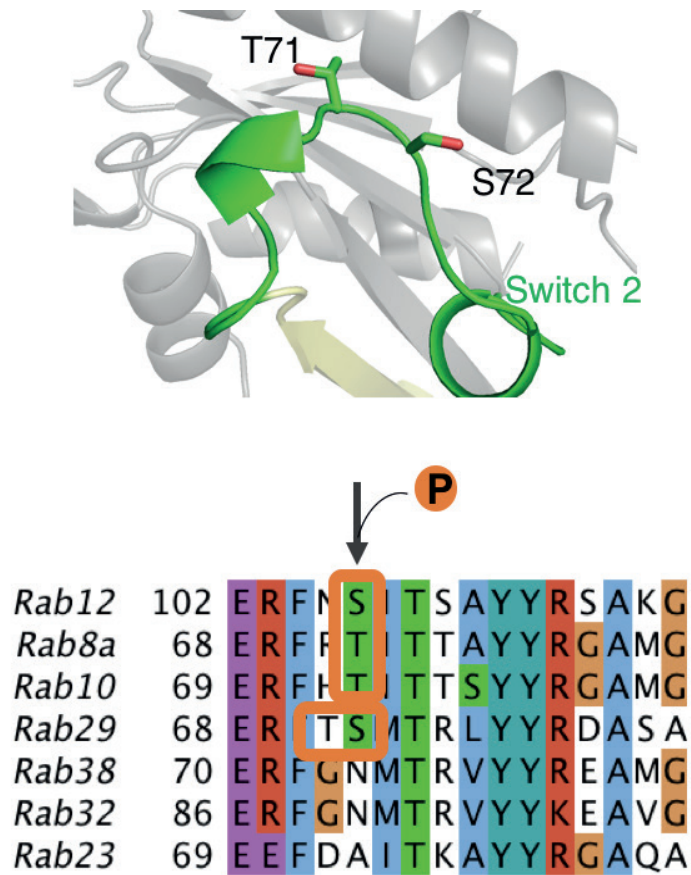


Figure 5.9. Rab29 phosphorylation sites

Adjacent phosphorylation sites in Rab29 Switch 2. Rab32/38 and the related Rab23 are not LRRK2 substrates and do not possess the conserved Ser/Thr phosphorylation site.

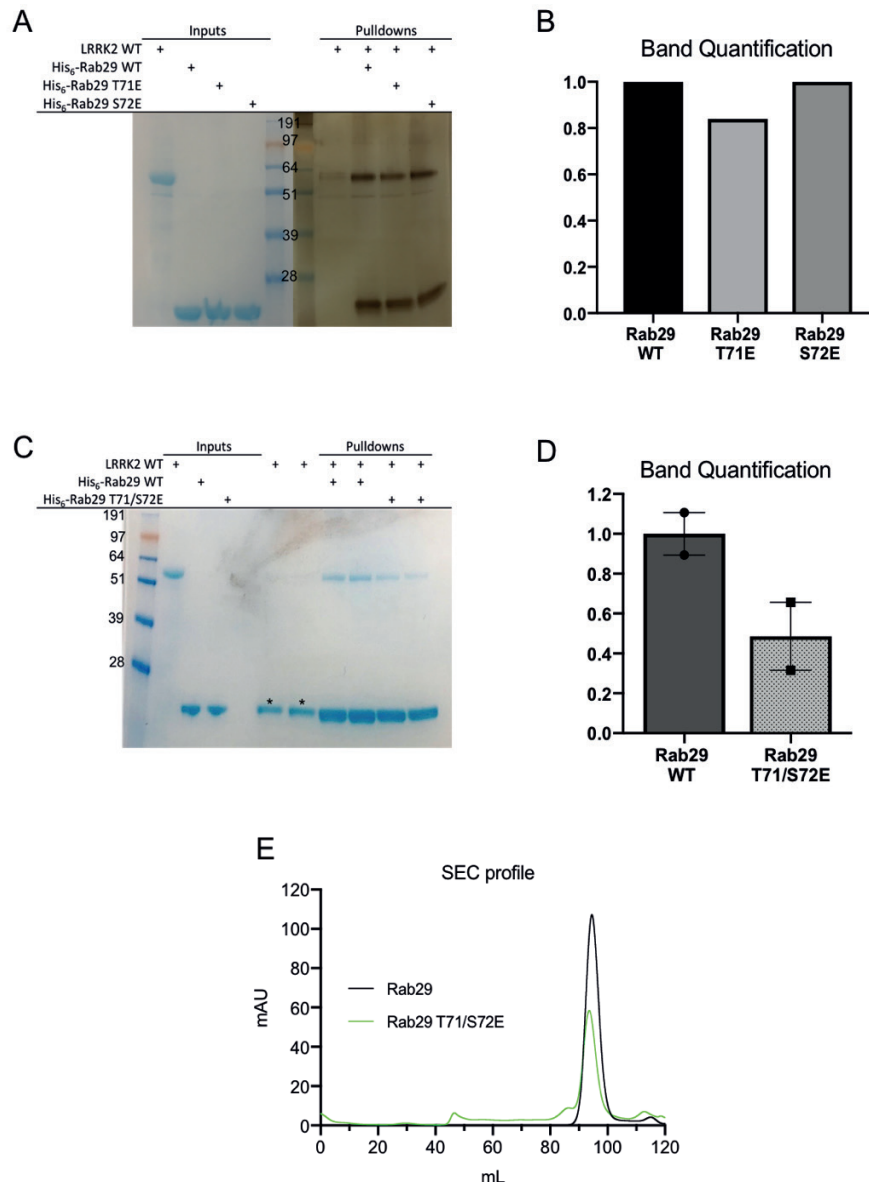


Figure 5.10. Impact of Rab29 Switch 2 phosphorylation on interaction with the LRRK2 ARM domain.

Rab29 Switch 2 phosphorylation sites were mutated to glutamate to mimic the properties of phosphorylated residues. A) Affinity pulldown analysis of Rab29^{T71E} and Rab29^{S72E} bind to LRRK2¹⁻⁵⁵² similarly to the Rab29 WT protein. Pulldown samples were silver-stained to improve visualisation. B) Band quantification of the single phosphomimetic pulldowns. Pulldowns were quantified normalising Rab29 WT control to 1. C) Introduction of the Rab29^{T71/S72E} double phospho-mutant causes a reduction in binding to LRRK2¹⁻⁵⁵². Bands in negative control samples arising from dirty beads are indicated by an asterisk. D) Band quantification of the double phosphomimetic pulldowns. Contaminant bands were subtracted from total Rab intensities. Pulldowns were quantified normalising Rab29 WT control to 1. Individual data points are plotted and error bars represent the range. E) SEC overlay of Rab29 WT and Rab29^{T71/S72E} constructs show similar elution profiles indicating introduction of phospho-mutants does not disrupt Rab29 tertiary structure.

5.3 Discussion

5.3.1 Isothermal titration calorimetry

While the interaction between the Rab32 subfamily and the LRRK2 ARM domain has been demonstrated by several independent means, no quantitative methods had been employed to date. Isothermal titration calorimetry was initially chosen to further investigate binding and the thermodynamics of the complex formation, and experiments were carried out for LRRK2 and Rab38 in various nucleotide-bound states. It is evident from the ITC data that LRRK2 requires the active form of Rab38 as a binding partner, shown in Figure 5.1. This is the first time a clear nucleotide dependency for the interaction has been demonstrated. Various kinetic parameters measured for each experiment are represented in Table 5.1. Favourable binding enthalpy is indicated by the negative ΔH values, and suggest polar interactions between LRRK2 and active Rab38 are responsible for binding. Positive entropy (ΔS) values indicate a spontaneous reaction is favoured. The dissociation constant (K_d) is measured between 1-2 μM , indicating a moderate binding affinity. ITC can also provide the reaction stoichiometry of two proteins in solution, however in these experiments the precise stoichiometry of the LRRK2:Rab38 interaction remains unclear, with measured N -values ranging from 0.6 – 0.9 (Table 5.1). It is important to note that the N -value equals the stoichiometry only if the concentrations used for the fit are accurate and the proteins are 100% active. It may be noteworthy that the wild-type Rab38 containing the non-hydrolysable GTP analogue GppNHp provides a stoichiometry close to 1, while in the case of the Rab38 Q69L ‘activating’ mutation, which shifts the equilibrium to the GTP bound state, the measured stoichiometry is reduced. It is possible this mutation may still allow some delayed hydrolysis to GDP. Since Rab38 Q69L had not been exchanged with GppNHp in this circumstance, it is possible a portion of inactive protein could be present.

A disadvantage of ITC lies with the high concentrations of protein and large volumes needed to carry out titrations. Both Rab32 and Rab29 were not amenable to ITC under the same experimental conditions due to protein precipitation, and so an alternative method to determine binding affinity was required.

5.3.2 Fluorescence anisotropy

Anisotropy is the general term used to describe the directional dependence of a physical property, and fluorescence anisotropy is commonly used method to study protein-protein interactions. In this experiment, polarised light is applied to a sample containing a fluorescently tagged molecule of interest and an untagged interaction partner. Only those fluorophores that are in proper alignment will be excited by the incident beam. In the case of a static object the light subsequently emitted from the fluorophore is also polarised. However, an excited fluorophore conjugated to a small molecule that tumbles rapidly in solution will quickly depolarise the emitted light and the sample will have a low anisotropy value. Larger molecules, on the other hand, tumble more slowly and therefore retain a greater emission polarisation. A schematic representation highlighting the principles of a fluorescence anisotropy experiment is given below (Figure 5.11). An increase in anisotropy due to the slower rotational diffusion of larger protein complexes can be directly related to the binding of a fluorescently labelled protein to its interaction partner. Given the availability of fluorescent nucleotides derivatives, Rab proteins can be easily labelled for this technique, and the mant (N-Methylanthraniloyl) modified guanine nucleotides were chosen for this experiment. The fluorescent mant reporter group is found on either the 2' or 3' oxygen of the ribose sugar, and Rab proteins were exchanged with mant-GppNHp or mant-GDP using the standard protocol. This mant moiety is small and surface exposed, thereby not disrupting the structure or biochemical properties of the Rab proteins [452]. The mant group has previously been shown to have no impact on effector binding in a Ras GTPase system [453]. Fluorescence anisotropy gives the most robust results if the size ratio between the components of the complex is high. The mant-labelled Rab GTPases are approximately three times smaller than LRRK2¹⁻⁵⁵². While this difference in size is not optimal, it was sufficient for any change in anisotropy to be accurately measured (Figure 5.2). Binding affinities for all three members of the Rab32 subfamily were in the low micromolar range, and are in good agreement with the previous ITC results obtained for Rab38. Thus, in this work we have quantified binding of the Rab32 family to LRRK2 for the first time, and determined all members interact with equal affinities to the LRRK2 armadillo domain.

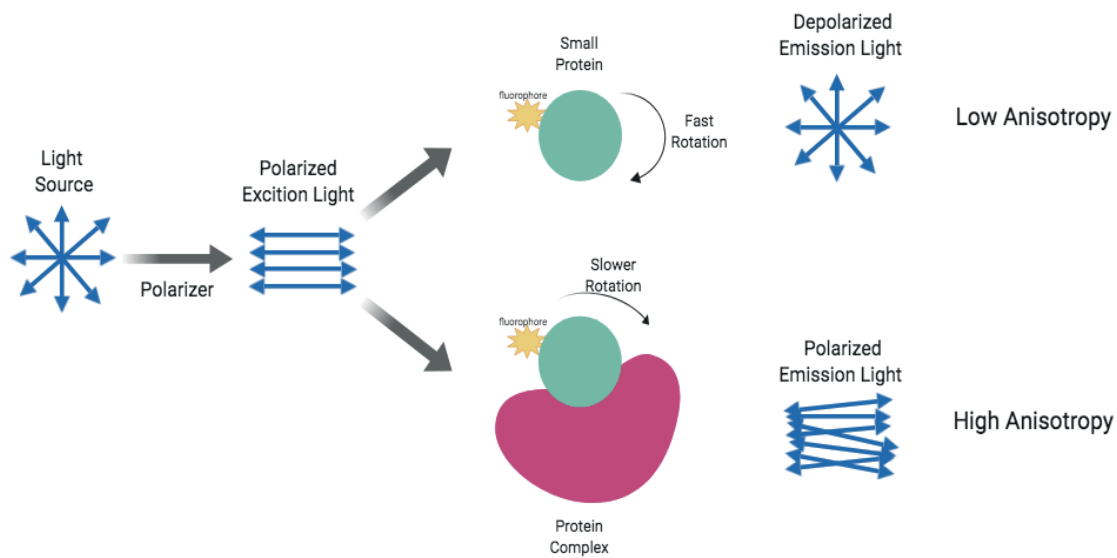


Figure 5.11. Basic principles of fluorescence anisotropy

Diagram illustrating the effect of rotational diffusion rate on the anisotropy of a sample. Emitted light from large complexes that tumble more slowly in solution remains polarised or anisotropic.

5.3.3 Mapping the Rab binding domain of LRRK2 1-552

To investigate the potential LRRK2:Rab binding interfaces identified from structural studies in Chapter 4, candidate residues were probed using site-directed mutagenesis and the impact on binding was addressed through pulldowns and fluorescence assays. Severe mutations, such as the replacement of residues of interest with alanine, are frequently observed in the literature. In this circumstance, it was critical to ensure that all observations made could be attributed to the ablation of charge rather than from a disruption of local or global protein structure. As such, mild substitutions were chosen that lack charge but maintain polar character at the protein surface. For LRRK2, substituting aspartate (D) residues with asparagine (N) and glutamate (E) with glutamine (Q) results only in the replacement of the negatively-charged oxygen atom with an amino group. In the case of the Rab arginine or lysine mutations, minimizing the reduction in side chain length was an additional consideration, and thus a glutamine substitution was chosen as a result.

Homology modelling of the truncated LRRK2 ARM domain identified 3 distinct clusters of negative charge predicted on the surface of the protein (Figure 5.3A). Interestingly, the Parkinson's disease risk-associated mutant E334K is found within the extended loop between residues 317-342. However, this loop had previously been deleted for crystallisation trials without any perturbation of Rab binding. Although the effects of the E334K mutation are unknown, it appears unrelated to the binding of the Rab32 subfamily to LRRK2. For our mutational analyses, we first investigated the double glutamate negative charge towards the N-terminus of LRRK2 (E192, E193). A LRRK2 E193K variant was previously identified that alters binding to Drp1, an important mediator of mitochondrial fission [454]. Pulldown analysis in this work using the 'Nmut' (E192Q, E193Q) construct showed no observable difference compared with LRRK2 wild-type binding to Rab38 (Figure 5.3B). This is in keeping with previously reported results that show Rab32/38 binding occurs between residues 265-552 of the LRRK2 ARM domain [300]. We therefore concluded these negatively-charged glutamate residues were not directly involved in the interaction with the Rab32 subfamily.

A second potential interface was then examined closer to the C-terminus of the truncated armadillo repeats. This region contains a cluster of four negatively-charged amino acids between residues 386-392. Initially, aspartate residues at positions 390 and 392 were tested,

denoted as LRRK2 '2mut' (D390N, D392N), and a modest reduction in binding was observed for both Rab38 and Rab29 compared to wild-type LRRK2 (Figure 5.3C&D). Introduction of further mutations within this region resulted in increased proteolysis during purification, as seen by degradation bands on SDS-PAGE in Figure 5.3E, however sufficient amounts of intact protein were present to test the impact of these mutants on binding to Rab38. Pulldowns of these '3mut' and '4mut' constructs clearly illustrates that elimination of the negative charge in this cluster abolishes the interaction with Rab38.

Since LRRK2 '2mut' was stably expressed and purified, fluorescence assays were carried out to further support the pulldown data. The SEC profiles using crude sample of both proteins show LRRK2 '2mut' behaves identically to wild-type protein (Figure 5.4B). A fluorescence titration test was carried out with mantGppNHp-bound Rabs and wild-type LRRK2 to examine the change in fluorescence intensity with increasing concentrations of LRRK2. As shown in Figure 5.4C, binding of LRRK2 to the Rab proteins resulted in a sufficient change in the environment of the mant fluorophore to increase the fluorescent signal. A concentration of 10 μ M LRRK2 was chosen to compare intensity change between wild-type and mutant proteins for all experiments as the signal begins to plateau at this concentration. Rab29 was chosen to examine the effect of LRRK2 '2mut' versus wild-type as this GTPase showed the highest overall response in relative fluorescence units (RFU). A reduction in fluorescence signal is observed upon introduction of LRRK2 '2mut', suggesting a disruption in binding to Rab29(mant-GppNHp) (Figure 5.4D). The change in signal was also measured for Rab29(mant-GDP) as a control. Interestingly, the 'DED' motif found within this stretch of amino acids is highly conserved among mammalian LRRK2 sequences, but not within the wider animal kingdom. Overall, these data suggest the negatively-charged residues in the region 386 – 392 within the ARM domain make important contributions to the LRRK2 binding interface with Rab GTPases.

5.3.4. Identification of Rab residues critical for LRRK2 binding

Two regions of surface-exposed positive charge were identified in the switch regions of the active Rab32 and Rab38 structures that may be important for mediating the interaction with the negatively-charged LRRK2 interface (Figure 5.5A). Using pulldown assays, Rab38 was

initially used to test the impact of mutations at both the Switch 1 and Switch 2 sites. In this case, substitution of the single Switch 1 arginine (R39Q) caused a visible reduction in LRRK2 binding compared to wild-type Rab38, while the interaction was not affected by the introduction of the double Switch 2 mutations (R77/81Q) (Figure 5.5C). Band intensities were also quantified and the ratio of LRRK2 bound to bait Rab38 protein was plotted on a bar graph for visualisation purposes. A hydrophobic isoleucine residue found within the RabF1 motif at the end of Switch 1 is frequently exploited for Rab-effector interactions. This residue was also examined to further probe the LRRK2:Rab interface. No impact on LRRK2 binding was observed, and lends support to the hypothesis that surface electrostatics are important for the LRRK2:Rab interaction (Figure 5.5B).

An overlay of the Rab38 SEC profiles show a slight shift of the R39Q Switch 1 mutant to a lower retention volume, which typically suggests the presence of a higher molecular weight species (Figure 5.6A). A possible explanation may be that the protein subjected to SEC had previously undergone a freeze/thaw cycle, and it is unlikely to imply the properties of this Rab have been modified. The SEC profiles of the equivalent Switch 1 mutants in Rab32 and Rab29 are identical to their wild-type counterparts, indicating that protein misfolding or aggregation is not the cause of reduced binding (Figure 5.6B&C). Fresh protein was used in all binding assays. Fluorescence assays using 10 μ M LRRK2 show reduced binding of Rab38 upon removal of the Switch 1 charge, with a response similar to background backing of inactive Rab38(mantGDP). Rab11(mant-GppNHp/GDP) were tested as additional controls and show no change in fluorescence upon incubation with LRRK2 (Figure 5.7A). The same effect is observed with the Rab32^{R55Q} Switch 1 mutant (Figure 5.7B). Importantly, for all Rab mutants tested by fluorescence, a disruption in nucleotide binding can be ruled out as a cause for the observed effects. The background fluorescence for each Rab construct in the absence of LRRK2 was measured to ensure equal loading of mant nucleotides. Since excess nucleotide was removed prior to the experiment, this also provided confidence in the activity state of the Rabs being tested.

Curiously, the substitution of the equivalent Switch 1 lysine residue of Rab29 does not invoke the same effect as with Rab32/38. Pulldown analysis shows no impact of Rab29^{K37Q} binding to LRRK2 compared with Rab29 wild-type (Figure 5.8A&B). The change in fluorescence

intensity upon incubation with LRRK2 is also not significantly affected (Figure 5.8C). While the general properties of positive charge are conserved at this Switch 1 position within the Rab32-subfamily, the substitution of Rab29 to lysine may act as an exquisite example of the subtleties dictating Rab specificity. Upon consideration of the side chain properties of both amino acids, arginine contains a guanidinium group capable of forming multiple hydrogen bonds. The lysine side chain, however, contains only a single amino group and is thus more limited in the number of bonds it can form. While mutational studies of surface residues are a useful tool to begin dissecting the molecular basis of specificity, the active structure of Rab29 will need to be determined to help fully decipher the nuances of Rab29 interactions. It could be the case whereby differences in the functional properties of Rab29 versus Rab32/38 are reflected in differences in the binding mode of Rab29.

5.3.5 Impact of Rab29 phosphorylation

Rab29 function is unique from Rab32/38 and can, by an unknown mechanism, activate the LRRK2 kinase domain, thereby regulating its activity [306]. Studies of the positive charge found within Switch 1 previously discussed have suggested there may be a distinction in the binding mode of Rab29 to the shared Rab32-subfamily binding site on LRRK2. Here we also set out to examine an additional distinguishing feature of Rab29 which, unlike Rab32/38, is also a downstream substrate of LRRK2. LRRK2 phosphorylates a subset of Rab GTPases including Rab8A, Rab10, Rab12 and Rab29 at a conserved Ser/Thr site in Switch 2 that can modify their interactions with effectors and regulators. Rab29 possesses an adjacent threonine and a serine residue (Figure 5.9), both of which have been shown to be phosphorylated by LRRK2. A doubly-phosphorylated peptide identified by mass-spectroscopy indicates it is possible both residues can also be found phosphorylated simultaneously [149]. To mimic phosphorylation these Switch 2 sites were mutated to glutamate and it was shown that Rab29^{T71/S72E} was unable to activate LRRK2. The ability of single site phosphomimetics, Rab29^{T71E} and Rab29^{S72E}, to activate LRRK2 was unaffected [306]. Here, we also generated these Rab29 Switch 2 mutants to investigate whether Rab29 phosphorylation could interfere with binding to the LRRK2 ARM domain. Pulldown analysis of the Rab29 site single phosphomimetic mutants shows no observable impact upon binding to LRRK2¹⁻⁵⁵² (Figure 5.10 A&B). This is in keeping with previously published results showing the ability of Rab29^{T71E}

and Rab29^{S72E} to activate LRRK2 was unaffected [306]. However, introduction of the Rab29 double phosphorylation mutant (T71/S72E) notably reduced its ability to interact with LRRK2¹⁻⁵⁵² (Figure 5.10 C&D). Size exclusion chromatographs of Rab29 wild-type and Rab29^{T71/S72E} show the same characteristic retention volume expected for soluble monomeric Rab GTPases, thus, protein misfolding or aggregation can be ruled out as the cause for the reduction in LRRK2 binding (Figure 5.10E). Given that Thr71 and Ser72 are both surface exposed and oriented away from the nucleotide binding pocket, it is unlikely substitution to glutamate would impact upon the activity state of Rab29. Nevertheless, HPLC analysis should be carried out to confirm these results are not due to the inactivation of the protein. Dual phosphorylation at adjacent sites has been observed in other proteins, and may allow for precise fine-tuning of protein-protein interactions [455, 456]. Previous studies have shown a marked preference for threonine over serine residues by LRRK2 [148, 406]. It could be speculated that the additional phosphorylation of Ser72 may act as an inhibitory mechanism to dampen the pathway. These preliminary results might point to a potential feedback system whereby the extent of Rab29-mediated LRRK2 activation is stringently controlled by the degree of kinase activity, with high levels of Rab29 phosphorylation preventing further recruitment via ARM domain interactions, and thus conferring protection from aberrant signalling. Further biochemical and cellular data are required to thoroughly test this hypothesis.

5.3.6 Conclusion

In this chapter, binding affinities for the LRRK2:Rab32-subfamily are reported for the first time, and show all members of the subfamily bind with equivalent affinities measured in the low micromolar range. Mutational studies have identified a patch of negatively charged amino acids between residues 386-392 within the LRRK2 ARM domain that contribute to complex formation. Investigations of surface-exposed positive charge within the Rab switch regions pinpoint an arginine residue in Switch 1 that is important for mediating the interaction of Rab32/38 with LRRK2. Remarkably, the equivalent Switch 1 lysine residue in Rab29 is not critical for binding to the same site. Preliminary pulldown analysis suggests Rab29 may utilise Switch 2 more extensively, with a reduction in LRRK2 binding found upon introduction of a double glutamate mutation (T71E, S72E) that mimics phosphorylation by the LRRK2 kinase

domain. Overall, here we identify some of the features and key determinants of the Rab32 subfamily of GTPases binding the LRRK2 armadillo domain.

Chapter 6: Final Discussion

6.1 Overview

Parkinson's disease is the second most common neurodegenerative disorder, and clinical diagnosis arises from the presence of classical motor symptoms, particularly bradykinesia and resting tremor. Current available therapies target and alleviate symptoms of the disease, however they do not slow down or stop progression. Levodopa is a precursor to dopamine and is used as a replacement agent to compensate for the loss of endogenous dopamine in the brain. Since its development in the late 1960's, levodopa remains one of the most commonly used and most effective treatment methods. However, long term complications such as fluctuations between good and reduced motor/non-motor control, dyskinesia, and drug-induced psychosis frequently arise with continued use of dopaminergic therapies [239]. Furthermore, treatment-resistant features become more prominent in later stages of the disease, and include postural instability, freezing of gait, and speech dysfunction [457]. As a result, a major goal in the treatment of Parkinson's disease is to identify preventative strategies that will slow down or stop the neurodegenerative process.

The underlying causes of PD are heterogeneous in nature, and can arise from a combination of genetic and environmental risk factors. Since its discovery in 2004, there has been much interest in elucidating the mechanisms of LRRK2 signalling in the context of PD, given that mutations in this protein are the most frequent cause of familial forms of the disease [244]. These pathogenic mutations typically result in the over-activation of the kinase domain, and so inhibitors of LRRK2 kinase activity represent a promising class of drug targets [458]. Some challenges still remain however, with safety concerns arising from adverse effects observed in the kidney and lung of animal models [459-461].

Multiple LRRK2 variants have also been identified as risk factors in the development of sporadic PD, and so it is hypothesized both inherited and idiopathic forms may share common pathogenic mechanisms [462, 463]. LRRK2 has been associated with a large number of cellular processes, however despite intensive research the specific molecular pathways involved in normal and pathophysiological LRRK2 function remain largely unknown. Recently, Rab GTPases have emerged as key players in LRRK2 function, as both downstream substrates of

the kinase, and upstream regulators of catalytic activity. Rabs are master regulators of vesicle trafficking events, and the coupling of membrane association and release with the GTP/GDP cycle is a salient feature of these proteins. In their active, GTP-bound conformation Rabs recruit effectors to specific subcellular membranes to elicit downstream effects. Post-translational modifications of Rab GTPases can occur in the nucleotide-sensitive switch regions and can alter their interactions with both regulators and effectors. LRRK2 phosphorylates a subset of Rab GTPases including Rab8, Rab10 and Rab12 on a conserved Thr/Ser residue within Switch 2, allowing for precise modulation of transport and trafficking pathways.

The Rab32 subfamily were found to function upstream of LRRK2 kinase activity, and interact with the N-terminus, comprising of armadillo (ARM) repeats followed by an ankyrin (ANK) domain [300]. A limited number of cellular and biochemical studies have been conducted on the LRRK2 N-terminus to date, with most intensive research efforts focussed on the catalytic domains. LRRK1 is a homologue of LRRK2 in humans, and both proteins share a similar domain architecture. However, LRRK1 does not contain N-terminal armadillo repeats or a C-terminal WD40 domain. Interestingly, mutations in LRRK1 do not confer any known risk for the development of PD [303]. Thus, the role and importance of the surrounding accessory domains must not be overlooked or undermined. In this work we carried out investigations into the interaction of the Rab32 subfamily with the LRRK2 N-terminus.

6.2 Present findings

All members of the Rab32 subfamily are involved in the organisation of the *trans*-Golgi network. Rab32/38 are best characterised for their role in melanogenesis and the regulation of lysosome-related organelles, while the related Rab29 is implicated in retromer-mediated trafficking [176, 179]. Rab32/38 bind to the ankyrin domain of their shared effector VARP, which regulates endosomal dynamics [182]. However, in the case of LRRK2, Rab32/38 was shown to interact with the N-terminal armadillo repeats as opposed to the adjacent ankyrin domain [300]. Herein lies an example of the plasticity of the Rab switch interface in recognising and interacting with structurally unique binding partners, while also exquisitely

distinguishing between similar domains within different proteins. Through binding with the ARM domain, Rab32/38 were shown to localise LRRK2 to transport vesicles and recycling endosomes [300]. The biological significance of this has not yet been established.

The related Rab29 GTPase recruits LRRK2 to the *trans*-Golgi network, and was reported to interact via the ankyrin domain. This was inferred from the introduction of mutations within the ankyrin repeats that prevented Rab29-mediated kinase activation, but no direct interaction with the LRRK2 ankyrin domain had been demonstrated [306]. In this work, we have clarified that all three members of the subfamily bind within the first 552 residues of the LRRK2 armadillo domain, and no interaction with the ankyrin repeats is observed. The ankyrin domain is found in close proximity to the kinase in the full length LRRK2 structure [330], and so the disruption of intra-domain interactions from the introduction of ankyrin mutations could potentially explain the elimination of kinase activity.

From competition assays it appears the Rab32 family share the same or an overlapping binding site within the LRRK2 armadillo repeats. Structural studies were then undertaken to identify molecular determinants of the interaction. Human LRRK2 has proven notoriously difficult to crystallise, with extensive efforts being carried out to overcome this hurdle [464]. Attempts by other groups have focused primarily on LRRK2 residues 1327 – 2527, comprising the Roc-COR domains, the kinase domain and the WD40 repeats. In our hands, crystallisation of the LRRK2 N-terminus was also unsuccessful. In place of an experimental structure, a homology model of LRRK2¹⁻⁵⁵² was generated and a negatively charged surface towards the C-terminus of this construct was identified as the putative Rab binding domain. Mutational studies demonstrated a reduction in Rab binding at this site following the removal of surface charge, and suggests these negatively-charged residues contribute to complex formation.

The structures of Rab32(GTP) in complex with VARP, and Rab32(GDP) bound to the Salmonella protease GtgE are published and deposited in the Protein Data Bank [182, 207]. The uncomplexed structure of Rab32(GTP) is also available in our lab (unpublished). Here, two novel X-ray structures of active Rab38 and inactive Rab29 are reported at high resolution. Thus, structural information is now available for all members of the Rab32 subfamily. Examination of the active Rab38 and Rab32 proteins identified positively charged residues in

the nucleotide-dependent switches that may be important for mediating the interaction. The structure of Rab29 GTPase was solved in its inactive conformation, and reveals an unusual open Switch 1 conformation. Additionally, the position of the glutamate 68 residue pointing into the nucleotide-binding cavity is also found in the structure of Rab32(GDP) and may play a role in conferring GtgE specificity to the Rab32 subfamily.

Biophysical analyses give a mechanistic insight into the LRRK2:Rab interaction, and it was determined that all three members of the Rab32 subfamily bind with equal affinities to LRRK2. Dissociation constants were measured in the low micromolar range, and to the best of our knowledge, this is the first time binding affinities have been reported for the interaction. We also provide clear evidence of dependency on GTP for binding. This data hints that LRRK2 can be considered a classical effector of the Rab32 subfamily. However, evidence for a direct biological function arising as a consequence of the interaction must be identified *in vivo* for the definition to strictly apply.

Despite high sequence identity, a shared binding site and equivalent affinities documented *in vitro*, an important biological distinction exists between Rab29 and Rab32/38. Rab29 is capable of activating LRRK2 kinase while Rab32/38 do not possess this ability. Mutational studies of Rab32/38 revealed an arginine residue in Switch 1 that is critical for mediating the interaction with LRRK2. A surprising result emerged when it was found that mutation of the equivalent Switch 1 lysine in Rab29 did not elicit the same effect. In this case, removal of the positive charge had no observable impact on binding LRRK2. While Rab specificity is multifactorial, preliminary results from the substitution of this single residue implies a unique mode of Rab29 binding. While proteins interact with one another through a combination of hydrogen bonding, electrostatic interactions, hydrophobic and Van der Waals forces, the identification of charged, solvent-exposed residues is a convenient starting point for experimentally mapping a binding site in the absence of an available complex structure. It will be fascinating to further dissect the subtle differences that distinguish Rab29 from Rab32/38 going forward.

LRRK2-mediated phosphorylation of Rab29 in Switch 2 is another discriminating feature of this GTPase within the subfamily. Rab32/38 do not possess this conserved phosphorylation

site, and are not downstream substrates of LRRK2. Unlike other Rab substrates, Rab29 possesses two adjacent phosphorylation sites, Thr71 and Ser72, and both residues have been shown to be phosphorylated [149]. It was previously determined that double phosphorylation within Switch 2 prevented Rab29-mediated activation of LRRK2 kinase, while phosphorylation of either individual site had no effect [306]. Following the generation of phosphomimetic Rab29 constructs, we also observe a similar trend in this study. Here, introduction of the double phosphorylation mutant reduced Rab29 interaction with the LRRK2 ARM domain, while the single site mimetics remained comparable to wild-type. It will be important to confirm these observations hold true for the intrinsic phosphorylation of Rab29 Switch 2, as phosphomimetic substitutions have rendered Rab GTPases non-functional in some circumstances [408]. Nonetheless, these initial findings may link phosphorylation-mediated kinase inhibition directly to a disruption in Rab29 binding the LRRK2 ARM domain. This in turn may elude to a robust intrinsic regulatory mechanism whereby a high or sustained level of kinase activity resulting in the dual phosphorylation of Rab29 dampens further any activation via the ARM domain interactions.

Finally, a fascinating observation was made by Kalogeropoulou *et al.* (2018) when they showed a LRRK2 truncation missing the N-terminus (LRRK2⁹⁷⁰⁻²⁵²⁷) could no longer phosphorylate Rab29, yet the phosphorylation of Rab8 and was Rab10 was unaffected [297]. This suggests a distinction between substrates and lends further credence to the importance of the LRRK2 N-terminus is the regulation of kinase activity. One could speculate from this data that LRRK2 might phosphorylate active Rab29 when associated with the ARM domain at the membrane, and the resulting Switch 2 modifications result in a weakened affinity and dissociation of the complex. It will be exciting to see how the precise mechanisms of this interplay are unravelled.

6.3 Future directions and perspectives

While we have identified a variation in Rab29 binding to the LRRK2 armadillo repeats, obtaining the structure of active Rab29(GTP) will be necessary to directly pinpoint subtle conformational differences between the Rab32 subfamily, and aid in explaining the observed differences in binding of Rab29 and Rab32/38 to LRRK2. How Rab29 can function to activate the kinase domain of LRRK2 also requires further explanation. Future work will need to clarify

whether Rab29 can directly activate the LRRK2 kinase domain by inducing a structural rearrangement, or if Rab29 interacts with a unique effector at the Golgi not shared by Rab32/38, and indeed if the differences in binding LRRK2 identified here result in a slightly different surface presented for subsequent binding of additional factors following recruitment.

Three potentially pathogenic LRRK2 variants in an Indian population have recently been identified in the C-terminal half of the armadillo domain: A397T, G472R, L550W. Each of these substitutions are predicted to have a destabilising effect on the armadillo domain structure [465]. Given the proximity of A397 to the Rab binding domain identified in this study, it will be interesting to test the effect of this variant on interactions with the Rab32 subfamily. Critically, the impact of all mutations investigated here need to be validated by cellular experiments to determine their biological relevance *in vivo*.

A structure of the LRRK2 ARM domain in complex with these Rab GTPases is the ultimate goal for understanding structure-function relationships and to aid in elucidating the underlying mechanisms of PD. It is also particularly relevant given the growing interest in developing inhibitors that target kinase activation rather than kinase activity [466]. There is particular focus on Rab29 as a consequence of its known regulatory function, however further investigations into the functions of Rab32/38 should be not overlooked. While Rab32 and Rab38 exhibit more specific cell expression profiles and are best characterised in melanocytes, both these GTPases have also been implicated in various neurological disorders [234, 411]. Critically, the dopaminergic neurons lost in the substantia nigra pars compacta are melanin-positive. The potential role of neuromelanin in PD is currently not well understood since commonly used animal models lack neuromelanin, however age-dependent accumulation of neuromelanin is hypothesized to interfere with normal cell function [467]. In addition, while evidence suggests PD patients have a lower risk for the development of most types of cancer, a positive association between PD and cutaneous melanoma (CM) has been established. Both an increased risk in the development of PD was linked with a family history of melanoma, and a higher prevalence of melanoma is found in patients diagnosed with PD [468, 469]. One study found the most commonly mutated *PARK* gene observed in CM was *PARK8* (LRRK2) [470], suggesting common pathways and shared disease mechanisms.

LRRK2 is also homologous to B-RAF kinase, a known driver of malignant melanoma [249, 471]. Rab38 has been implicated in the metastatic transformation of melanoma [472], thus understanding the molecular basis of the LRRK2 interaction with Rab GTPases may therefore be critical to deciphering the link between these diseases.

Overall, this study highlights the importance of *in vitro* work. This approach allows us to clarify and validate the relationship between two molecules in detail. We can also discriminate between a functional contact whereby two proteins are involved in the same biochemical pathway or complex, and a direct interaction between two molecules [473, 474]. An updated working model of LRRK2:Rab interactions is provided in Figure 6.1. However, it will be essential to couple structural and biophysical studies with biological work to fully evaluate the role of LRRK2 and Rab GTPases in a cellular context. Further knowledge of Rab-mediated LRRK2 signalling may culminate with the identification and development of novel strategies for therapeutic intervention.

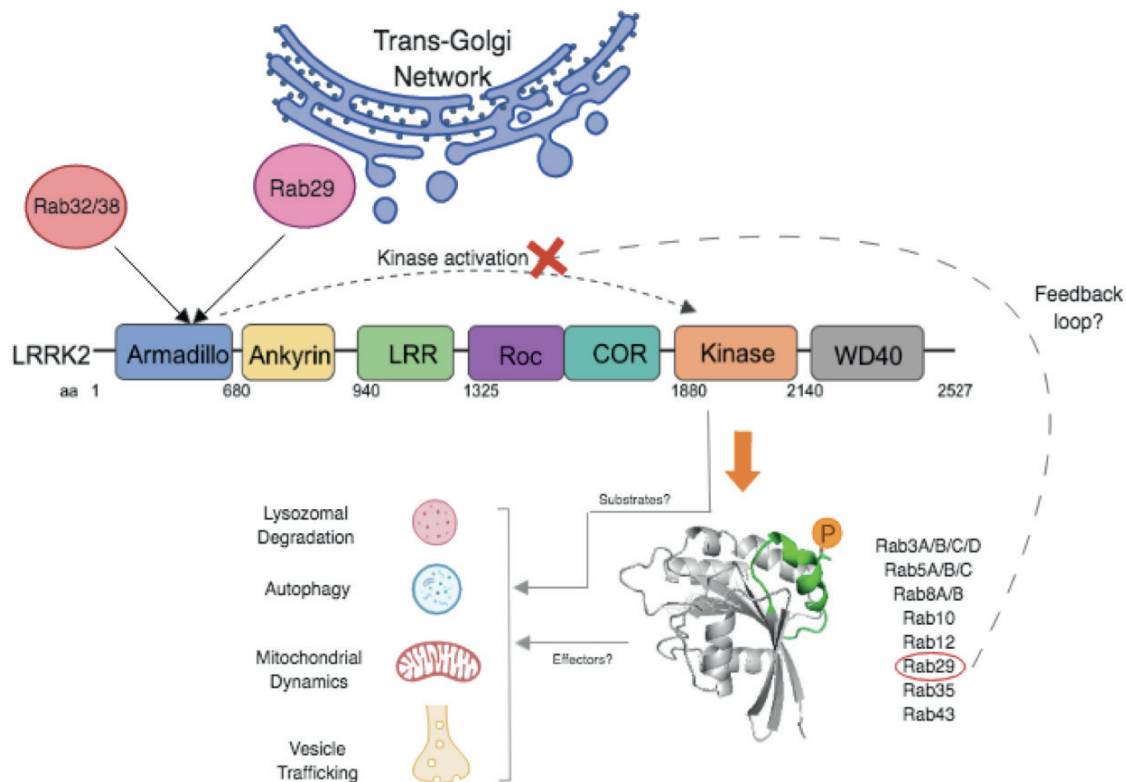


Figure 6.1. Working model of LRRK2 as a Rab GTPases signalling hub

Rab32, Rab38 and Rab29 interact with LRRK2 via the N-terminal armadillo repeats. No direct interaction is observed between Rab29 and the ankyrin domain. Rab32/38 localise LRRK2 to recycling endosomes and transport vesicles. Rab29 recruits LRRK2 to the *trans*-Golgi network and activates the kinase domain by an unknown mechanism. LRRK2 kinase phosphorylates a subset of Rab GTPases on a conserved Ser/Thr residue in Switch 2 that modifies Rab binding to regulators and effectors. The phosphorylation of Rab29 may disrupt its interaction with the ARM domain and represent an inhibitory mechanism. Through its kinase activity LRRK2 can modulate numerous cellular processes.

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