L'RRK de Triomphe: a solution for LRRK2 GTPase activity?

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Keywords

LRRK2; Parkinson's disease; Neurodegeneration; GTPase; Protective; Wnt

Abbreviations

COR, C-terminal of Roc; GAD, GTPase activated by dimerisation; GAP, GTPase activating protein; GEF, Guanine nucleotide exchange factor; LRRK2, Leucine-rich repeat kinase 2; PD, Parkinson's disease; Roc, ras of complex proteins.

Abstract

Leucine-rich repeat kinase 2 (LRRK2) is a central protein in the pathogenesis of Parkinson's disease (PD), yet its normal function has proven stubbornly hard to elucidate. Even though it remains unclear how pathogenic mutations affect LRRK2 to cause PD, recent findings provide increasing cause for optimism. We summarise here the developing consensus over the effect of pathogenic mutations in the Roc and COR domains on LRRK2 GTPase activity. This body of work has been greatly reinforced by our own study of the protective R1398H variant contained within the LRRK2 GTPase domain. Collectively, data point toward the pathogenicity of GTP-bound LRRK2, and strengthen a working model for LRRK2 GTPase function as a GTPase-activated by dimerisation. Together with the identification of the protective R1398H variant as a valuable control for pathogenic mutations, we have no doubt that these triumphs for the LRRK2 field will accelerate research towards resolving LRRK2 function, and towards new treatments for PD.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide and is characterised by resting tremor, akinesia, bradykinesia and postural instability, although additional non-motor symptoms are common. Parkinsonian symptoms coincide with the death of dopaminergic neurons in the

substantia nigra *pars compacta*, with degeneration eventually spreading to other brain regions [1].

Mutations have been identified in several genes causing familial PD [2]. Of particular interest is *LRRK2*, which encodes Leucine-rich repeat kinase 2 (LRRK2). *LRRK2* mutations account for 1-40% of PD cases in different populations, and present symptoms and brain pathologies that closely resemble idiopathic PD [2,3]. However, the precise function of LRRK2 remains a mystery.

LRRK2 consists of a catalytic core incorporating Ras of complex proteins (Roc), C-terminal of Roc (COR), and kinase domains, which is flanked by several protein-protein interaction domains (Figure 1) [4]. Seven *LRRK2* missense mutations segregate with PD, namely N1437H, and R1441C/G/H in the Roc domain, Y1699C in the COR domain, and G2019S and I2020T in the kinase domain [4]. Additional variants that increase PD susceptibility have been identified in the COR (R1628P) and WD40 (G2385R) domains [5,6].

Here we review data relating to the effect of hereditary mutations on LRRK2 GTPase function. We argue that there is an overwhelming consensus about the effect of PD-causing mutations on GTPase activity that is strengthened by our own study of the R1398H protective variant. Thus in contrast to the view that the LRRK2 field is largely in disagreement, the relationship between GTPase activity and disease susceptibility may be resolved.

LRRK2 GTPase Domain: GAD or small GTPase?

The clustering of pathogenic mutations within the Roc domain, has established this domain as central to LRRK2 pathology. The Roc domain is a bona-fide GTPase [7-10], and is important for the rest of the molecule, since LRRK2 kinase activity is dependent on the binding of guanine nucleotides [11]. Nonetheless, the precise mechanism

underpinning GTPase activity is the subject of debate, with two competing models in existence. These are summarised below.

The Ras Small GTPase Model. Based on homology to Ras-like GTPases, the LRRK2 Roc domain has been suggested to function analogously to small GTPases. Small GTPases act as molecular switches, with binding to effector proteins dictated by whether they are in the 'inactive' (GDP-bound) or 'active' (GTP-bound) states. For Ras itself, the best-described effectors are Raf kinases. These preferentially bind GTP-bound Ras, triggering Raf activation, with the hydrolysis of GTP to GDP terminating the signal [12].

Small GTPases are switched between active and inactive states by protein-protein interactions with guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs overcome the slow dissociation of GDP while GAPs enhance GTP hydrolysis (Figure 2) [13,14]. Thus, GAPs promote the GDP-bound state and are considered inhibitory, whereas GEFs promote the GTP-bound state and are considered activators. However, since GAPs and GEFs are both required for small GTPase function, these proteins have the effect of increasing GTPase activity *in vitro.*

Supporting this model, several publications report candidate LRRK2 GAPs and GEFs. These include ArfGAP1, which binds LRRK2 in mammalian cells and increases GTP hydrolysis *in vitro* [15,16], and RGS2, which is also reported to increase GTP hydrolysis [17]. The only suggested LRRK2 GEF is ARHGEF7, which enhances GTP hydrolysis and promotes GTP exchange *in vitro* [18,19]. These observations come with the caveat that LRRK2 interacts with a number of small GTPases that could be targets of ArfGAP1, RGS2 and/or ARHGEF7 and may be present in the assays as contaminants [20]. More concerningly, none of these proteins bind directly to the Roc domain. Taking these observations together, ArfGAP1, RGS2 and ARHGEF7 may modulate LRRK2 GTPase activity, but their mechanism of action is distinct from the GEFs and GAPs of small GTPases.

The GTPase Activated by Dimerisation (GAD) Model. Advocates of the GTPase activated by dimerisation (GAD) model propose a mechanism for LRRK2 GTPase function that is based around homology with prokaryotic Roco proteins. This model differs from the small GTPase model in three key aspects (Figure 2). Firstly, the GAD model requires the LRRK2 Roc and COR domains to act as a single functional unit: a so-called RocCOR tandem domain. This idea is quite persuasive since these domains are found together throughout nature [21]. Secondly, the GAD model requires LRRK2 to function as a homodimer, with dimerisation mediated by the COR domain [22]. And thirdly, the GAD model does not require GAPs or GEFs.

Some interesting observations support this model. Most notably, a number of reports indicate that LRRK2 is largely dimeric *in vivo*, and dimerisation appears crucial for kinase activity [23-25]. In addition, the prokaryotic Roco structure predicts that the intramolecular Roc-COR interface is particularly important. Residues at this location are highly conserved across evolution, while a number of pathogenic *LRRK2* mutations are predicted to reside at this interface. Consistently, these mutations have the same effects on GTPase activity in prokaryotic Roco proteins and human LRRK2 [22,26].

All PD-causing RocCOR mutations increase the proportion of GTP-bound LRRK2, whilst the protective R1398H mutation has the opposite effect

A number of genetic linkage studies have identified an R1398H LRRK2 Roc domain mutant as a protective variant [27-29]. R1398H is the only variant of this type identified to date but has the potential to revolutionise the study of LRRK2 biology, since it can be expected to have the opposite effect to PD-causing mutations in disease-relevant processes.

We have recently reported the effects of R1398H on LRRK2 GTPase function, and found data consistent with its status as a protective variant [30]. In particular, R1398H displayed decreased binding to GTP and increased GTP hydrolysis. Thus, R1398H appears to shift LRRK2 towards the GDP-bound state. Remarkably, this is the opposite to all reports for pathogenic RocCOR tandem domain mutations (Table 1). Taken together, these observations are consistent with GTP-bound LRRK2 being pathogenic, and raise the possibility of targeting LRRK2 GTP-binding for PD treatment. Intriguingly, a LRRK2 GTP-binding inhibitor has been reported to reduce neurodegeneration in mouse models [31]. How such compounds transfer to human PD patients remains to be seen, but their potential is promising.

We also found R1398H to increase interaction between RocCOR tandem domain constructs in yeast two-hybrid assays [30]. This is in contrast to the pathogenic R1441C/G/H and Y1699C mutations, which weaken interaction. We do not know which of the many potential *inter* or *intra*molecular interactions within a RocCOR dimer are affected, although it is unlikely to be COR-COR dimerisation, since the data are largely recapitulated in assays that use the RocCOR tandem domain as bait, but use an isolated Roc domain as prey [30]. In any case, the changes in interaction strength seen in this assay correlate with altered GTPase function: R1398H increases LRRK2 dimerisation and decreases GTP binding; pathogenic mutants have the opposite effect.

These observations are consistent with LRRK2 functioning as a dimeric GTPase that involves both Roc and COR domains, rather than as a monomeric Roc domain. Strikingly, molecular modelling of pathogenic and protective RocCOR mutations using the prokaryotic Roco structure makes this case even stronger, since the equivalent residues to R1398, N1347, R1441 and Y1699 are in remarkably close spatial proximity at the intramolecular Roc-COR interface [22,30]. In the case of R1398, mutation to histidine may prevent the formation of a Roc-COR hydrogen bond [30]. Based on this structural information, we and others have speculated that pathogenic and protective variants within the LRRK2 RocCOR domain all affect

GTPase function by altering intramolecular Roc-COR interactions [22,30,32]. Interestingly, this has been demonstrated for the Y1699C mutation which is located within the COR domain but still affects GTPase activity, further supporting the GAD model [32]. Indeed, mutations at residues equivalent to R1441 and Y1699 in prokaryotic Roco proteins recapitulate the impaired GTP hydrolysis seen in human LRRK2 [7,22,32].

The GAD model also has serendipitous support from other studies of the R1398 site. Based on homology to Ras proteins, an artificial R1398L mutation was generated to mimic the tumorigenic Q61L substitution that renders Ras proteins constitutively GTP-bound. Contrary to expectation however, R1398L behaved like R1398H by decreasing GTP-binding and increasing GTP hydrolysis (Table 1) [15,30,33,34]. It is self-evident that behaviour opposite to that predicted from Ras is a strong argument against the small GTPase model, but the fact that R1398L would also prevent the same putative Roc-COR hydrogen bond as R1398H makes a tantalizing case for the GAD model.

Pathomechanisms for mutations outside the RocCOR tandem domain

The above data make a compelling argument for PD-causing RocCOR mutants shifting LRRK2 to the GTP-bound state. But what about mutations outside the RocCOR tandem domain? It has long been suggested that LRRK2 kinase activity may govern GTPase function through autophosphorylation mechanisms, and in agreement with this, Roc domain autophosphorylation is reported to modulate GTPase function [35,36]. Based on this, one would expect mutations that alter kinase activity to indirectly affect GTPase function. However, the G2019S kinase domain mutation, which increases LRRK2 kinase activity, has been demonstrated repeatedly to have no affect on GTP-binding or GTP hydrolysis [10,15,33,34,37-39]. At the other extreme of kinase activity, an artificial kinase-dead mutation also has no effect [34]. It is perhaps surprising that the effects of altered kinase activity and autophosphorylation should

be in conflict, but we note that autophosphorylation levels for LRRK2, even G2019S, are very low [35,40]. Thus, whilst the behaviour of hyper- and hypo-phosphorylated LRRK2 may be different in *in vitro* GTP-binding and hydrolysis assays, the range of Roc domain autophosphorylation achievable in cells may be insufficient to affect GTPase function. As such we would argue that "kinase activity controls GTPase activity" models appear unlikely (for this reason the possibility that the two enzymatic activities are connected is not included in Figure 3). The only other publication describing non-RocCOR mutations in GTPase assays is a surprising recent report of increased GTPase activity with the pathogenic I2020T kinase domain and G2385R WD40 domain mutations [41]. These observations cannot be ignored, but since they are contrary to every single publication for pathogenic RocCOR mutations, these experiments need replicating. In any case, it is clear that alternative pathomechanisms to increased GTP-binding must exist. So what could they be (Figure 3)?

The most obvious pathomechanism for non-RocCOR mutations is increased substrate phosphorylation. Substrate phosphorylation is a distinct concept to kinase activity, since the phosphorylation of substrate protein is dependent on more than just kinase activity. This is an important distinction, since G2019S remains the only mutation for which elevated kinase activity is agreed; even the adjacent I2020T mutation seems unable to elicit a reproducible effect [9,42-44] Perhaps more tellingly, the WD40 domain G2385R risk variant impairs kinase activity [44,45]. It would be surprising if both increased *and* decreased LRRK2 kinase activity were able to cause PD, but G2019S and G2385R could still have the same effect on substrate phosphorylation, for example by differentially affecting kinase-substrate binding. With this in mind, we note a recent report of a group of Rab GTPases (Rabs 8/10/12) as LRRK2 kinase substrates [46]. Remarkably, all pathogenic LRRK2 mutations examined increased Rab phosphorylation in cells. These included mutations that do not affect kinase activity, as well as the kinase-enhancing G2019S and kinase-impairing G2385R mutations [46]. It is worth contrasting this with LRRK2 autophosphorylation at serine-1292, which is also increased by multiple PD-causing mutations [47,48]. In

both cases, mutations outside the kinase domain enhance substrate phosphorylation at least as well as G2019S [46-48], thereby demonstrating a lack of correlation between kinase activity and substrate phosphorylation, and illustrating the need to dissociate these concepts. Presumably non-kinase domain mutations increase serine-1292 autophosphorylation by making this residue more accessible to the kinase domain. Of the two, we would argue that Rab8/10/12 phosphorylation is more likely to have pathological relevance, since the stoichiometry of *in vitro* Rab8/10/12 phosphorylation compares favourably to LRRK2 autophosphorylation [46]. Perhaps more importantly, serine-1292 autophosphorylation appears to be unaffected by G2385R [47]. But in any case, both are exciting stories and we await the next developments: it will be intriguing to examine how R1398H affects these phosphorylation events.

Besides its enzymatic activities, LRRK2 has a remarkable number of reported interactors (currently 151 on BIOGRID, http://thebiogrid.org/125700/summary/homo-sapiens/lrrk2.html) and likely functions as a scaffold protein, nucleating multi-protein complexes through protein-protein interactions [49,50]. It is self-evident that mutations in protein-protein interaction domains could alter binding to LRRK2, while RocCOR mutations have also been shown to affect binding to certain interactors [51-54]. How mutations in the kinase domain might do the same is less intuitive, although altered ATP binding and autophosphorylation are likely to affect the topology of LRRK2. In any case, there are numerous reports of altered protein binding to pathogenic LRRK2 mutants, including deregulated interactions with the G2019S and I2020T kinase domain mutants (e.g. FADD, GSK3β, Rac1 [52,55,56]). It is easy to envision how changes to any one of the individual protein-protein interactions within a multi-protein complex could elicit the same functional outcome. Thus, altered protein-protein interaction is a persuasive explanation for both RocCOR and non-RocCOR mutations.

Concluding remarks

Data from the study of pathogenic RocCOR mutations and the R1398H protective variant allow two conclusions to be drawn. Firstly, increased GTP-binding is a common pathomechanism for RocCOR mutations; and secondly, the GAD GTPase model for LRRK2 appears likely. It therefore seems reasonable to infer that GTP-bound LRRK2 is a pathogenic species, and the GDP-bound form protective. This raises the possibility of treating *LRRK2* PD – and perhaps idiopathic PD – with compounds that disrupt GTP binding. As mentioned, this therapeutic strategy is already under development, with encouraging results [31].

It should be reiterated that pathogenic mutations outside the RocCOR domain do not all affect LRRK2 GTPase function. Nonetheless, it is logical to assume that the effects of all pathogenic LRRK2 mutations converge at some point downstream. This point of convergence is perhaps the Holy Grail of LRRK2 biology. Currently, LRRK2 has been linked to a multitude of cellular processes, but those with pathological relevance are not agreed. However, we believe the R1398H mutant has the potential to greatly expedite LRRK2 research, since this mutant represents a powerful control that gives greater confidence to experiments producing small changes. When studied in cellular assays relevant to *LRRK2* PD, R1398H can be expected to behave oppositely to PD-causing mutants. With this in mind we note that R1398H increases canonical Wnt signalling activity [30], which is in direct contrast to pathogenic variants throughout LRRK2 [30,53]. Given the established importance of properly regulated Wnt signalling for the normal function of neurons, these observations could have great implications. Of course, R1398H has yet to be studied in many of the other processes in which LRRK2 has been implicated, and may present opposite effects in some of these assays too.

A cure for LRRK2 Parkinson's disease remains a long way off, but advances are being made. In particular the data supporting a model for LRRK2 GTPase function as described herein appear overwhelming. As such, determining the role of LRRK2

GTPase activity in PD pathogenesis may be one of the first triumphs for the LRRK2 field.

Acknowledgements

We would like to thank Simone Grannó, Vicky Spain and Craig Blackstone for their work on the R1398H project, and to acknowledge the valuable contributions of other members of the Kirsten Harvey lab, past and present, and of our collaborators in the LRRK2 field.

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Mutation	LRRK2 Construct (expression in)	GTP Binding (type of assay)	GTP Hydrolysis (type of assay)	Prediction	Reference
I1371V	Full Length (M)	↑ (Pd)	ns	↑ GTP-bound	[11]
"I1371"V	*C.tepidum* Roc + COR (B)	— (F)*	↓↓↓ (Pu)	↑ GTP-bound	[22]
"I1371"A	*C.tepidum* Roc + COR(B)	— (F)*	↓↓↓ (Pu)	↑ GTP-bound	[22]
N1437H	Full Length (M)	↑↑↑ (Pd)	ns	↑ GTP-bound	[39]
R1441C	Full Length (M)	↑ (Pd)	ns	↑ GTP-bound	[11]
R1441C	Roc (M)	— (R)	↓↓ (Pu)	↑ GTP-bound	[9]
R1441C	Full Length (M)	— (Pd)	↓ (Pu)	↑ GTP-bound	[8]
R1441C	Roc (B)	ns	↓ (Pu)	↑ GTP-bound	[57]
R1441C	Full Length (M)	↑↑↑ (M)	ns	↑ GTP-bound	[39]
R1441C	Full Length (M)	ns	↓ (IP)	↑ GTP-bound	[33]
R1441C	Roc + COR +kinase (Y)	— (Pd)	— (IP)**	No change	[33]
R1441C	LRR + Roc + COR +kinase + WD40 (I)	ns	↓ (Pu)	↑ GTP-bound	[36]
R1441C	Full Length (M)	↑↑ (Pd)	ns	↑ GTP-bound	[16]
R1441G	Full Length (M)	↑ (Pd)	ns	↑ GTP-bound	[11]
R1441G	Roc (M)	— (R)	↓↓ (Pu)	↑ GTP-bound	[9]
R1441G	Full Length (M)	ns	↓ (IP)	↑ GTP-bound	[33]
R1441H	Roc (B)	↑ (F)	↓↓ (Pu)	↑ GTP-bound	[58]
"R1441"A	*C.tepidum* Roc + COR (B)	— (F)*	↓↓↓ (Pu)	↑ GTP-bound	[22]
Y1699C	Full Length (M)	↑ (Pd)	ns	↑ GTP-bound	[11]
Y1699C	Full Length (M)	ns	↓ (IP)	↑ GTP-bound	[33]
Y1699C	Full Length (M)	— (Pd)	↓↓ (Pu)	↑ GTP-bound	[32]
Y1699C	Full Length (M)	↑↑ (Pd)	ns	↑ GTP-bound	[16]
"Y1699"C	*C.tepidum* Roc + COR (B)	— (F)*	↓↓↓ (Pu)	↑ GTP-bound	[22]
R1398H	Full Length (M)	↓↓ (Pd)	↑ (Pu)	↓ GTP-bound	[30]
T1343G	Full Length (M)	— (Pd)	— (IP)	—	[34]
D1994A	Full Length (M)	— (Pd)	— (IP)	—	[34]
R1398L	Roc + COR + kinase (Y)	↓ (Pd)	↑ (IP)	↓ GTP-bound	[33]
R1398L	Full Length (M)	ns	↑↑ (IP)	↓ GTP-bound	[16]
R1398L	Full Length (M)	— (Pd)	↑↑ (IP)	↓ GTP-bound	[34]
R1398L/T1343V	Full Length (M)	— (Pd)	↓↓ (IP)	↑ GTP-bound	[34]
R1398L/G2019S	Full Length (M)	— (Pd)	↑↑ (IP)	↓ GTP-bound	[34]
R1398L/D1994A	Full Length (M)	↓↓ (Pd)	↓↓ (IP)	?	[34]
R1398L/T1343V/G2019S	Full Length (M)	— (Pd)	— (IP)	—	[34]
R1398L/T1343V/D1994A	Full Length (M)	↓↓ (Pd)	↓↓ (IP)	?	[34]
R1398Q	Full Length (M)	— (Pd)	— (IP)	—	[34]
R1398Q/T1343G	Roc + COR + kinase (Y)	↓ (Pd)	↑ (IP)	↓ GTP-bound	[33]
R1398Q/T1343G	Full Length (M)	— (Pd)	— (IP)	—	[34]

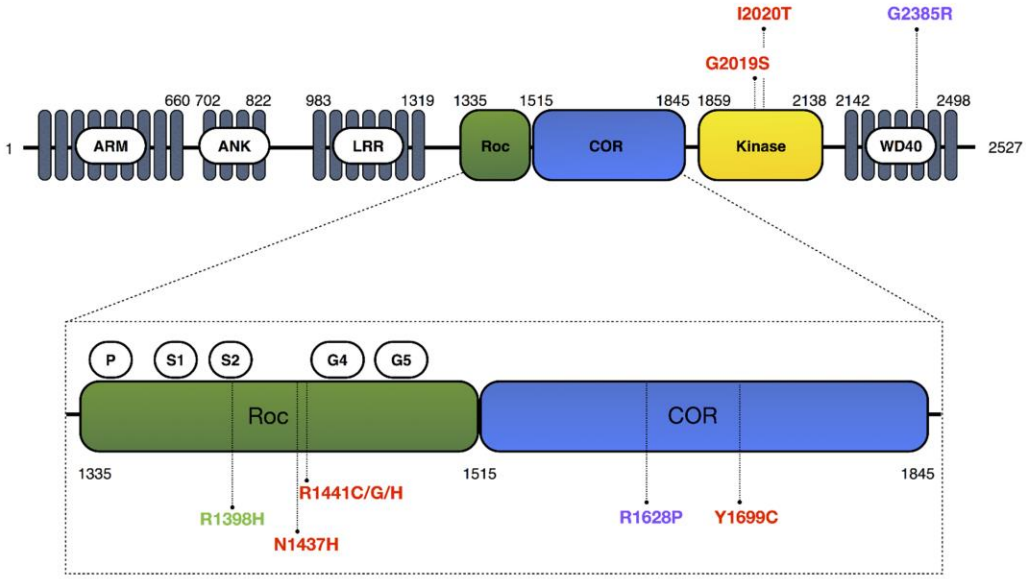
Figure 1 – A schematic model of LRRK2 structure. LRRK2 contains N-terminal armadillo (ARM), ankyrin (ANK) and leucine-rich repeats (LRR), with WD40 repeats at the C-terminus. The enzymatic core is comprised of Ras-of-complex (Roc) and C-terminal of Roc (COR) domains (shown in magnified box), and a kinase domain. PD-causing mutations are shown in red, risk variants in purple, and the protective R1398H variant in green. Motifs conserved with small Ras-like GTPases are shown as white boxes and illustrate the guanine nucleotide phosphate-binding motif (P-loop - P), switch I (S1), switch II (S2) conformational switch regions, and G4 and G5 loops.

Figure 2 – Possible models of the LRRK2 GTPase. (**A**) A Ras small GTPase model of LRRK2 when GDP-bound (top) and GTP-bound (bottom). The action of suggested LRRK2 GEFs and GAPs is depicted. (**B**) A GAD-based model of LRRK2, obligate COR-mediated dimeric form (top) shifting to a GTP-dependent *cis*-dimerisation of the Roc domains (bottom).

Figure 3 – Possible LRRK2 pathomechanisms. (**A**) A substrate that interacts with multiple domains of wildtype LRRK2 (black arrows), and undergoing basal levels of phosphorylation by the LRRK2 kinase domain (red arrow). (**B**) Substrate phosphorylation is increased directly by the G2019S mutation, or (**C**) indirectly by other PD-causing mutations that enhance enzyme-substrate interaction. (**D**) Alternatively, pathological mutations throughout LRRK2 might affect individual protein-protein interactions within a common multiprotein complex.

Table 1 – Summary of data from all publications investigating the effects of RocCOR domain mutations on LRRK2 GTPase activity. Expression systems are indicated: M = mammalian cells, B = bacteria, Y = yeast, I = insect cells. Up- and down-arrows represent statistically significant increases and decreases relative to wild-type protein. Approximate strength of change is represented by number of arrows, where one arrow indicates ≤1.5-fold, two arrows between 1.5- and 3-fold, and three arrows greater than 3-fold; where numerical values are not reported comparisons are estimated. Types of GTP-binding experiment are represented as: Pd = pulldowns of

LRRK2 protein from cellular lysates with GTP-coupled beads; R = radiolabelled nucleotide binding assays using purified protein; F = fluorescently labelled nucleotide binding assays using purified protein. The use of purified protein (Pu) or immunoprecipitates (IP) in GTP hydrolysis experiments is also shown. * Indicates data referred to in text but not shown. ** Clear but non-significant trend towards decreased GTPase activity. Note mutations at T1343 and D1994A are artificial Roc domain and kinase-dead mutations.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1
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ANK
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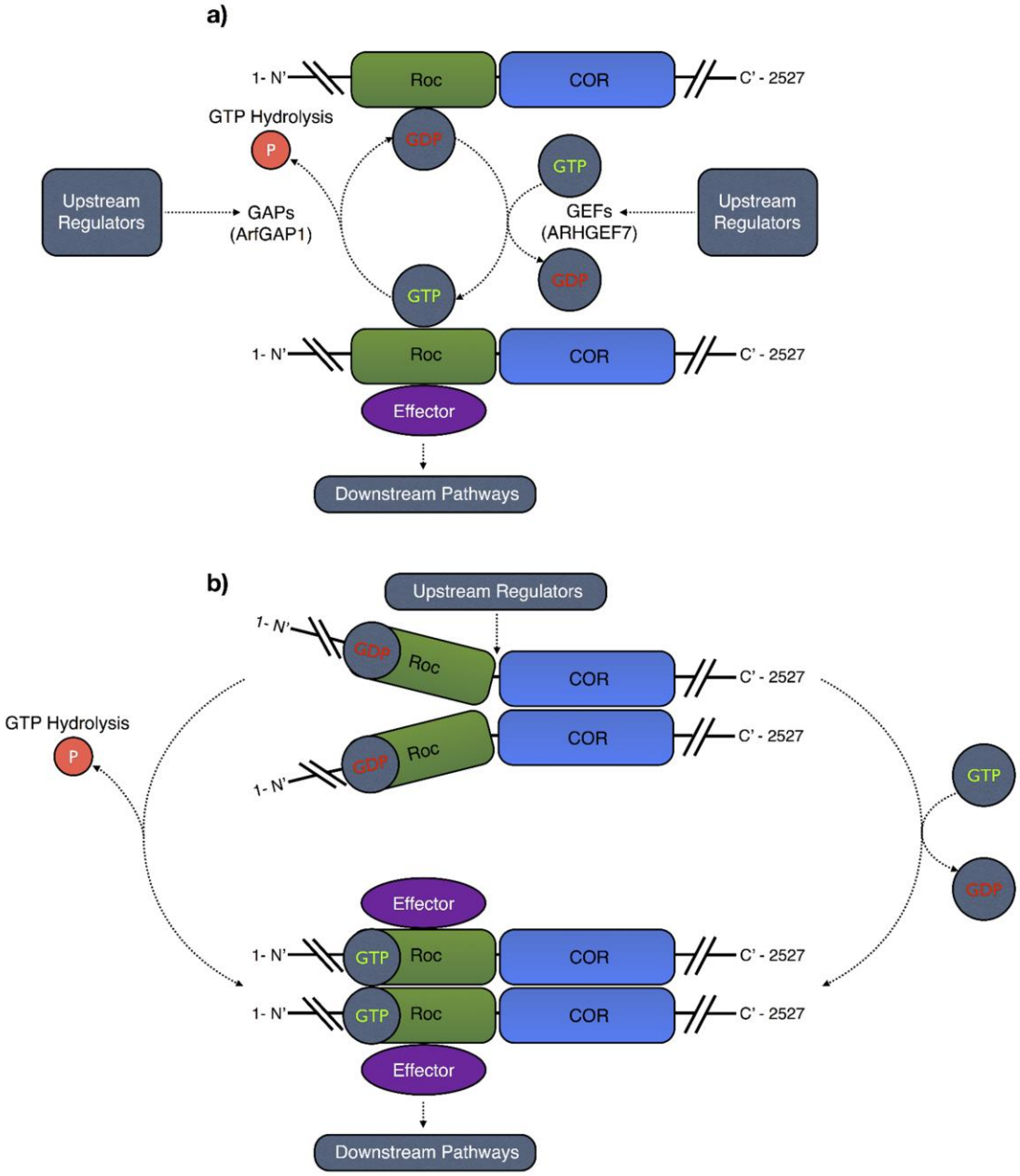
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Roc
COR
C' - 2527
GTP Hydrolysis
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GDP
GTP
Upstream Regulators
GAPs (ArfGAP1)
GEFs (ARHGEF7)
Upstream Regulators
GDP
GTP
1- N'
Roc
COR
C' - 2527
Effector
Downstream Pathways
b)
Upstream Regulators
1- N'
GDP
Roc
COR
C' - 2527
GTP Hydrolysis
P
COR
C' - 2527
GDP
Roc
1- N'
GTP
GDP
Effector
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1- N'
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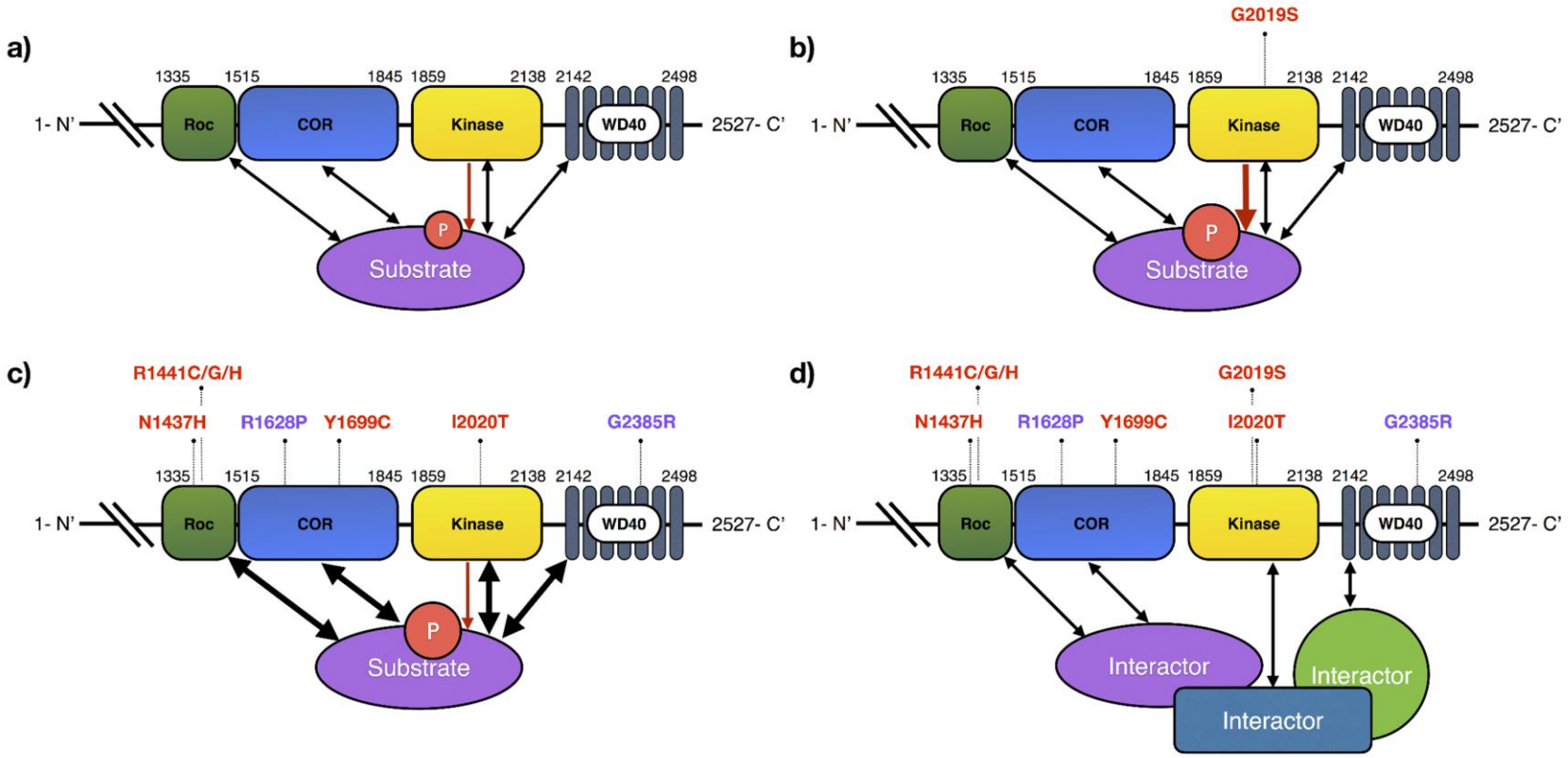
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G2385R
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Interactor
Interactor
Interactor