Studying the interaction between Parkinson's disease related proteins Roco4 and Rab1D

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ABSTRACT

Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene are the cause of late-onset autosomal Parkinson's disease (PD). LRRK2 protein belongs to the Roco family of proteins and has both kinase and GTPase activity; mutations in such domains have been associated with familial PD. This protein has been identified as a regulator of a wide variety of cellular processes, playing a very important role in membrane trafficking pathways. Despite all the efforts to describe and functionally characterize LRRK2, its physiological role and protein structure remain not fully understood, which limits the possibilities of developing drugs to target and inhibit the PD mutated LRRK2. Here, we use *Dictyostelium discoideum* Roco4 protein, a LRRK2 homologue, and focus in its downstream effector Rab1D in order to understand the role of phosphorylation in this signaling pathway. We show that Rab1D is a good model to study kinase activity in vitro and we also provide some insight into the protein localization in the cell, which is closely related to the ER and vesicle trafficking and might therefore be playing a role in the development of Parkinson's disease.

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INTRODUCTION

LRRK2 and Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder that affects around 10 million people all over the world, with a mean onset age of 60 years. Patients present symptoms such as strong tremors, motor impairment, rigidity, bradykinesia or hypokinesa, which derive from the loss of dopaminergic neurons in the subtantia nigra[1]. PD has an age-associated penetrance in which genetic susceptibility contributes to risk. During the past years, several genes and mutations have been found to contribute to this disease, being *PARK* and *LRRK2* the predominant genetic risk factors[2].

LRRK2 is a large cytosolic multidomain protein which consists of 2527 amino acids, has a molecular weight of 285 kDa and belongs to the Roco family of proteins. As such, it contains multiple functional domains; starting in the N-terminal region, LRRK2 has two protein-protein interaction domains, ankyrin domain and leucine rich repeat (LRR) domain, which are followed by a Roc GTPase domain and a linker region named COR domain. A serine/threonine kinase domain is also present after the Roc-COR part of the protein and finally a WD40 domain is found at the C-terminus of LRRK2 for protein-protein interaction[3].

More than 40 mutations in LRRK2 have been identified as risk factors for PD, but those which have been verified as pathogenic are mostly found in the core of the protein; LRR, Roc, COR and kinase domains. One of this is the G2019S mutation and it represents the most common pathogenic mutation, which causes an increased phosphorylation activity in LRRK2. However, it remains unclear whether the pathogenic effects are mediated by the kinase activity only. Data also suggest that mutations in the Roc and COR domains cause a decreased GTPase activity of LRRK2 and recent studies show that both enzymatic activities are involved in the onset of Parkinson's disease[4].

Although the molecular mechanisms underlying PD are still poorly understood, it seems clear that autophagy and mitochondrial homeostasis are key in this disease and both are regulated through pathways that involve LRRK2 and other PD related proteins[4,5]. Studies have shown a relation between mutations in LRRK2 and altered mitochondrial function[6–8], but further research is needed in order to fully understand the pathways that take part in the development of the disease and eventually come up with the best therapeutic approach.

Dictyostelium discoideum Roco4 as a model to study LRRK2

LRRK2 being such a big protein presents a drawback when it comes to purifying large amounts of the full length mammalian protein. Problems with stability and solubility, together with strong binding to chaperons limit the possibilities of working with this protein [9]. Therefore, related proteins purified from other organisms are commonly used to study structure, activation mechanism and phenotypes of LRRK2. Remarkably, the social amoeba *Dictyostelium discoideum* contain 11 Roco family members, being Roco4 the most interesting one due to its very similar domain topology[10].

Roco4 turns out to be an essential protein in *Dd* and its depletion causes a strong phenotype in the cells, which are no longer able to complete a full developmental cycle[11]. The structure of the Roco4 kinase domain, both wild type and PD mutation

G1179S (G2019S in LRRK2) has been solved and has proven to be a good model to study LRRK2[10]. The availability of Roco4 knockouts and purified domains makes this protein a great tool to study PD related proteins both in vivo and in vitro, as we will do in this project.

Rab proteins

As mentioned before, the understanding of how mutations in LRRK2 cause Parkinson's disease is still far from complete and most of the research is focused in finding out the intramolecular regulation of the protein and how mutations affect this process. In the pharmacological field, kinase inhibitors have been developed and they successfully disrupt phosphorylation. However, these drugs have an insufficient brain uptake or brain activity and they also have toxic effects in liver, lungs and kidney, where LRRK2 is also expressed[12].

In a recent study, using a phospho-proteomics approach, a subset of Rab GTPases was identified as LRRK2 substrates. Mammalian Rab10 and Rab8a were found to be the main Rabs regulated via LRRK2 kinase[13]. Rab10 phosphorylation by LRRK2 was also shown in a different study and with a different approach[14], confirming that this protein is indeed a substrate of LRRK2 and thus might be involved in PD.

Given this background, several experiments were performed in our laboratory in order to find *Dd* Rab proteins which are phosphorylated by LRRK2 homologous protein Roco4. Kinase assays were performed by K. Rosenbusch using Roco4 kinase domain and a subset of Rab proteins. Rab1D was found to be the most phosphorylated protein among the whole subset of Rabs. In addition, genomic analysis revealed the Thr residue in the switch II region to be conserved in *Dd* Rab1D (unpublished data), making this protein an interesting target to study Roco4 downstream effectors and translate results back to mammalian LRRK2.

In this study, we aim to determine the kinetics of Rab1D phosphorylation by Roco4. In order to confirm the Thr residue as a phosphorylation site, this amino acid will be replaced by Ala or Glu, creating phospho-dead and phospho-mimic variants of Rab1D respectively. This set of proteins will also be used to analyze whether phosphorylation in the switch II region of the protein has an effect in the G-protein cycle. Moreover, we will also investigate Roco4-Rab1D binding both in vivo and in vitro to stablish whether there is direct interaction between these proteins and where in the Roco4 multidomain structure Rab1D is binding.

On the other hand, using *Dictyostelium discoideum* cells, we aim to determine Rab1D localization and analyze the effect of phosphorylation in localization of the protein. This way, we might be able to get an indication of Rab1D function in the cell and how Roco4 kinase activity modifies such function.

Taken together, these experiments should provide sufficient information to clarify whether Rab1D is somehow involved in the pathway that leads to mitochondrial damage via LRRK2 hyperactivity and if so, this protein could be a good target for pharmaceutical development of Parkinson's disease drugs.

MATERIALS AND METHODS

Culture and transformation of cells

Dictyostelium discoideum Ax2 cells, in vegetative state, were grown at 21°C, in petri dishes or shaking suspension at 150 rpm, in HL5-C medium containing 10 μg/mL of the required selection marker for plasmid expression. When starvation experiments were performed, cells were grown in non-nutrient agar plates until aggregation was observed (typically 5-6 hours).

For cloning purposes, *E. coli* cells were transformed by heat shock and grown at 37°C in LB medium (tryptone, yeast extract, NaCl) containing antibiotics. pGEX4T1 plasmids, containing N-terminal GST and ampicillin resistance, were used for protein expression in *E. coli* Rosetta cells and pDM317 plasmids, containing G418 resistance, were used to make GFP-tagged fusion proteins for further expression in *Dictyostelium* cells.

In order to obtain different mutants of Rab1D, quick-change PCR was performed to introduce point mutations in the gene. Primers used for this purpose are indicated in Table 1. These PCR products were expressed in pBluescript plasmid and replicated in *E. coli* DH5α cells.

Rab1D	5'- GCGGATCCATGTCTATGGCACCAGAAACG
Rab1D T220A	5'- GGACAAGAAAGATTTAGAGCTATTACATCATCATAC
Rab1D T220D	5'- GGACAAGAAAGATTTAGAGATATTACATCATCATAC

Table 1. Quick change primer sequences to obtain Rab1D and its mutants.

Protein purification

Rab1D and the mutant versions of the protein (Rab1D T220A and Rab1D T220D) were expressed as N-terminal GST fusion proteins in E. coli. Cells were grown at 37°C to an OD of 0.5-0.8 in Terrific Broth Medium (Formedium) and then protein production was induced with 0.1 mM IPTG at 22°C overnight. Cells were pelleted at 18°C (6000 rpm for 12 minutes), washed with 0.9% NaCl and lysed by sonication in low-salt buffer (50mM Tris, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT). Lysate was clarified by centrifugation at 4°C (12000 rpm for 1 hour).

Proteins were purified by gel filtration chromatography in a GSH column and eluted in low salt-buffer containing 20 mM glutathione. Further purification was performed by size exclusion chromatography in low salt buffer with 1 mM ATP and fractions containing the protein of interest were collected and concentrated using Amicon Ultra-15 10K centrifugal filter devices.

To cleave GST tag, 100 mg of purified protein were incubated with TEV protease in the same GSH gel filtration column.

Protein pull-down

In order to pull-down GST-tagged proteins, Glutathione Sepharose 4B beads (GE Healthcare) were used. First, beads were washed with low-salt buffer to remove ethanol

(preservative) and then 20 μg of GST-Rab1D were incubated for 3 hours at 4°C on a rotor, together with 20 μg of the different Roco4 domains; LRR, kinase domain and WD40. Finally, beads were washed and samples were run on a 12% SDS-Page gel, which was stained with Comassie solution to make proteins visible.

Furthermore, Protein A magnetic beads (Bio-Rad) were used to pull-down Rab1D-GFP from *Dictyostelium* cell lysate expressing. Cells were grown to a concentration of $1x10^7$ in shaking cultures, harvested and lysed in low-salt buffer containing 2% Triton X-100 and a phosphatase inhibitor mix. After the beads were washed, 1 μL of α-GFP antibody was bound to the beads by incubation at 4°C on a rotor during 2 hours. Subsequently, both the lysate and the different Roco4 GST-tagged domains were incubated overnight at 4°C. Samples were eventually run on a 12% SDS-Page gel and western blot was performed to detect GST proteins bound to Rab1D-GFP.

Radioactive assays

Kinase assay

Radioactive kinase assay was performed using ^{32}P labelled ATP. Rab proteins (100 μM) were incubated at 30°C together with 0.05 mg/ml Roco4 kinase domain in buffer containing 25 mM Tris, 15 mM MgCl2, 20 mM b-glycerol phosphate, 1 mM NaF, 1 mM EGTA, 1 mM Na_3VO_4 and 2 mM DTT. As a substrate, a mix of ATP was added to the samples, containing 190 μM ATP and 10 μM ^{32}P ATP. Samples were taken at different time points, reaction was stopped using 100 mM EDTA and unbound ATP was washed off using 50 mM phosphoric acid. A scintillation counter was used to measure the amount of incorporated ^{32}P.

GTPase Assay

Radioactive GTP labelled with ^{32}P was used as a substrate to perform GTPase assays. The experiment was performed using both ^{32}P-γ-GTP and ^{32}P-α-GTP separately in order to differentiate the phosphates that were released specifically by G-proteins (Rabs).

As a previous step to the assay itself, proteins were pre-loaded with GTP; 50 μM of Rab proteins were incubated at 30°C for 30 minutes together with 100 μM ^{32}P GTP and 20 mM EDTA. To stop the reaction, 40 mM MgCl2 was added.

In order to start the experiment, 50 μM of Roco4 kinase domain and 100 μM of ATP were added to the GTP-loaded proteins. Samples were taken at different time points and amount of ^{32}P was measured using a scintillation counter.

Proteomics

To analyze Rab1D binding interactions, samples were prepared for mass spectrometry. AX2 *Dictyostelium discoideum* cells were grown in shaking cultures, $1x10^9$ cells were harvested and lysed as previously described. Again, a GSH gel filtration column was used to bind GST-Rab1D, which was then incubated with the cell lysate overnight at 4°C. Proteins were eluted in the same buffer described before, concentrated and sent for mass spectrometry analysis. The experiment was performed with wild-type Rab1D, as well as the phospho-dead and phospho-mimic mutants.

Moreover, Rab1D was analyzed with a phospho-proteomics approach to find phospho-sites within the protein. Using a buffer containing 25 mM Tris, 15 mM $MgCl_2$, 150 mM

NaCl, 2 mM DTT and 50 μM ATP, purified Rab1D was incubated together with Roco4 kinase domain for 1 hour at 30°C. 1 μM of each protein was used and further phosphorylation was prevented by addition of 60 mM EDTA at the end of the experiment. Samples were then sent away for analysis.

Microscopy

Dictyostelium discoideum cells were imaged using LSM800 Zeiss microscope and GFP proteins were excited with a 488 nm laser.

For experiments in vegetative state, cells were simply harvested from a petri dish and put on a glass slide for analysis in the microscope. Rab1D and its phospho-dead mutant were analyzed in untreated vegetative cells and also in cells treated with different drugs to study localization to internal structures in the cell. Latrunculin A (5 μM) was used to inhibit actin polymerization and Nocodazole was used in a concentration of 10 μg/mL to prevent microtubule formation.

When analyzing Rab1D localization in starved cells, cells were first grown in petri dishes containing HL5 medium, then harvested from the plates, washed two times with phosphate buffer and finally plated on non-nutrient agar plates. After 5-6 hours of starvation, cells were collected and placed on a slide to be imaged with the microscope.

Immunostaining was also performed to label certain structures inside the cell and analyze co-localization with Rab1D. To do so, cells were harvested from the plates and transferred to MatTek chambers, where they were allowed to settle for 1 hour. Cells when then fixed using 4% paraformaldehyde, permeabilized with 0.1% Triton-X and blocked with 2% BSA. All these solutions were prepared in 100 mM phosphate buffer to ensure proper preservation of GFP signal. In order to label the endoplasmic reticulum, mouse α-calreticulin (1:5) was used. Furthermore, mouse α-p25 (1:10) antibodies were used to label the perinuclear recycling complex. Both antibodies were then labelled with α- mouse Texas Red (Santa Cruz Biotechnology) as a secondary antibody.

Phenotype of Rab1D overexpression

To study the effect that overexpressing Rab1D has on *Dictyostelium discoideum* cells, different experiments were performed. First, we looked into aggregation of cells overexpressing Rab1D. Cells were starved in non-nutrient agar plates and images were taken every 10 minutes to analyze how fast cells stream and aggregate.

Also, to study whether overexpression of Rab1D causes the cells to already starve in HL5 medium, cAR1 (cAMP receptor) expression was analyzed. Cells were grown to a concentration of 2.5×10^7 cells/mL in HL5 medium and then shifted to phosphate buffer to induce starvation. Samples were taken every 30 minutes, cells were lysed by boiling in SDS buffer and western blot was performed to detect cAR1 levels at different time-points.

RESULTS

Rab1D and Roco4 biochemistry

Rab1D wild type and phospho-mutants were successfully purified

First, in order to perform different in vitro assays and compare Rab1D with its phospho-mutants, point mutations were introduced in the wild type version of the protein and subsequently all three proteins were purified as described before. In Figure 1, purity of the samples obtained is shown. Except for Rab1D T220D, which showed some contamination bands below the protein itself, Rab1D and Rab1D T220A were purified without any background after TEV cleavage.

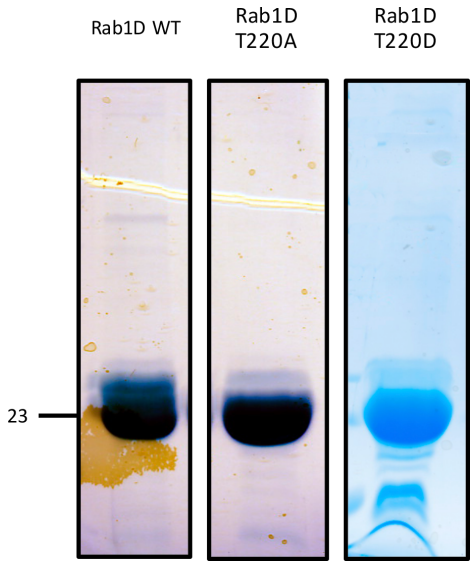


Figure 1. Coomassie staining of 14% SDS-Gel with purified proteins, concentration obtained was the following: 9,4 mg/mL Rab1D, 12,6 mg/mL Rab1D T220A and 17,68 mg/mL Rab1D T220D. Protein size indicated in kDa.

Phosphorylation by Roco4 is decreased in Rab1D T220 mutants

Kinase assays were performed to study the phosphorylation reaction between Roco4 and Rab1D. As expected, we found that both mutants are less phosphorylated than the wild type version of the protein (Figure 2); Ala residue cannot be phosphorylated and Glu residue resembles the phosphorylated form of the protein, so it cannot be phosphorylated any further. However, phosphorylation was only partially decreased instead of completely impaired, suggesting that other sites in the protein are being phosphorylated.

Unfortunately, some experimental mistakes were made and we were unable to stablish kinetics for these reactions. Once counts are obtained, the amount of incorporated phosphate can be calculated and then using kinetic equations it is possible to get further information from this type of assay. To do so, it is necessary to measure the total amount of radioactive phosphate before the assay, which we didn't do.

Phosphorylation state of Rab1 does not affect G-protein cycle

In order to study whether Rab1D phosphorylation via Roco4 would affect Rab1D G-protein cycle, we performed radioactive GTPase assays to see whether or not there was any difference in GTP hydrolysis between wild-type and phospho-dead mutant. Although GTP hydrolysis was slightly enhanced in the phospho-dead mutant, the effect was not significant enough, so no further analysis was performed (Figure 3).

Again, due to experimental errors, we were not able to calculate kinetics of the reaction.

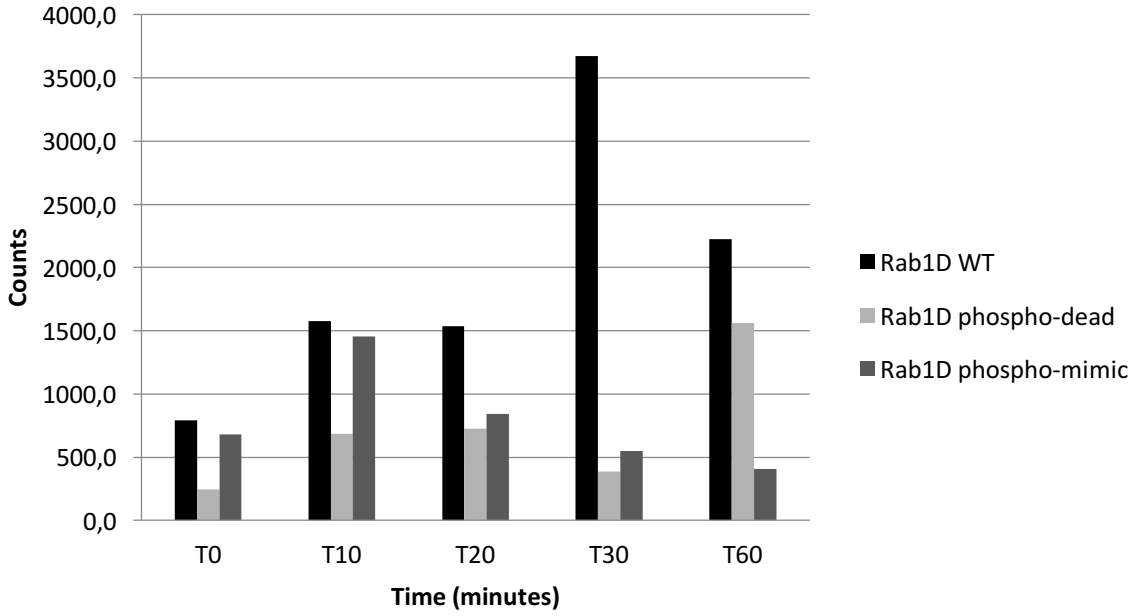


Figure 2. Kinase assay with wild type Rab1D, Rab1D T220A (phospho-dead) and Rab1D T220D (phospho-mimic).

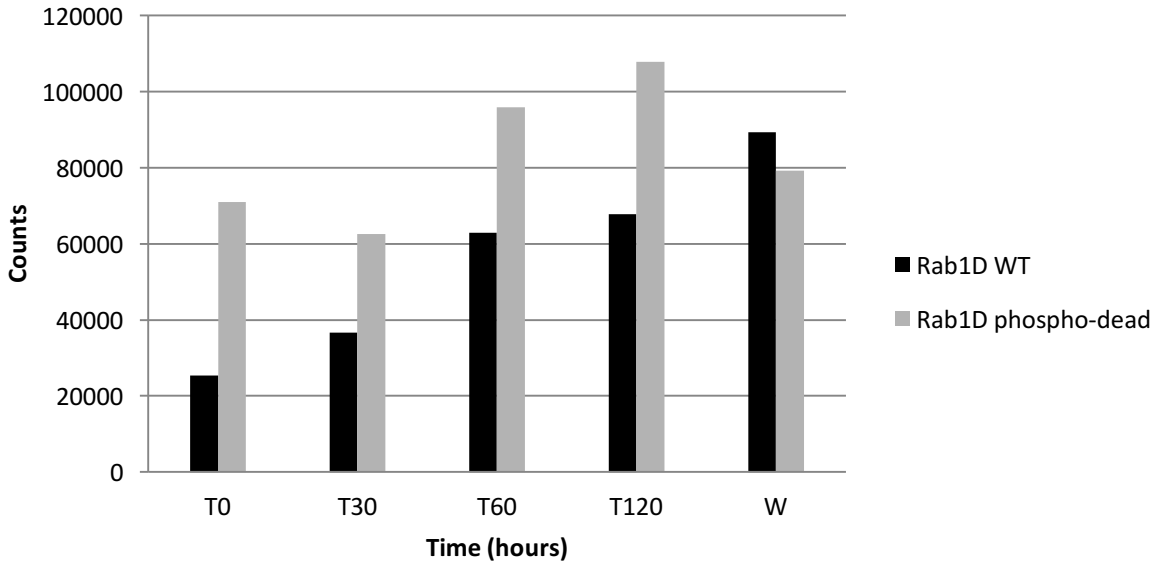
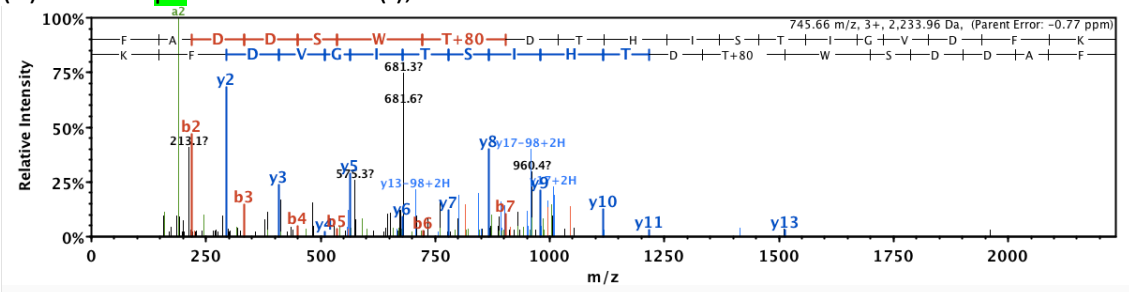


Figure 3. GTPase assay Kinase assay with wild type Rab1D, Rab1D T220A (phospho-dead).

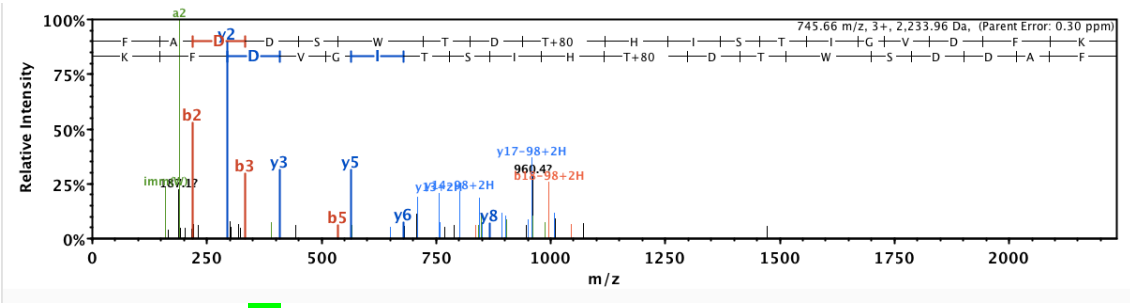
Phospho-proteomics reveal new potential phosphorylation sites

Since both Rab1D mutants were still being phosphorylated in radioactive kinase assays, we wondered whether there was other Thr residues within the protein that were also being phosphorylated. As shown in Figure 4, mass spectrometry confirmed T220 to be a phosphorylation site, which was consistent with genomic analysis and kinase assays. On top of that, another 8 peptides containing Thr residues were found, explaining why Roco4 was still able to phosphorylate each mutant.

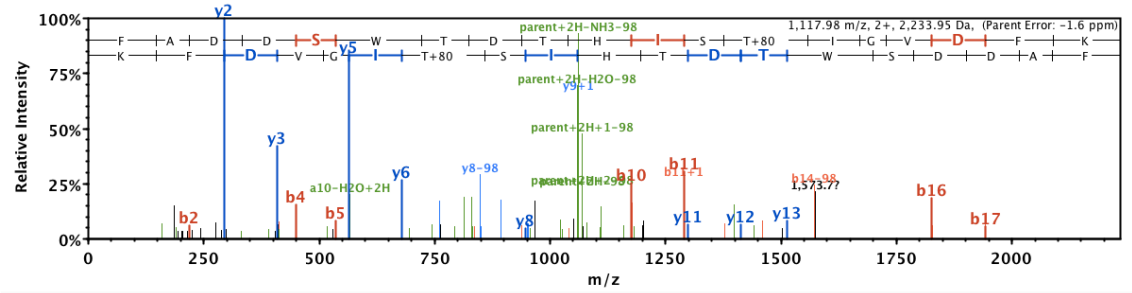
(R)FADDSW**pT**DTHISTIGVDFK(I), Thr36



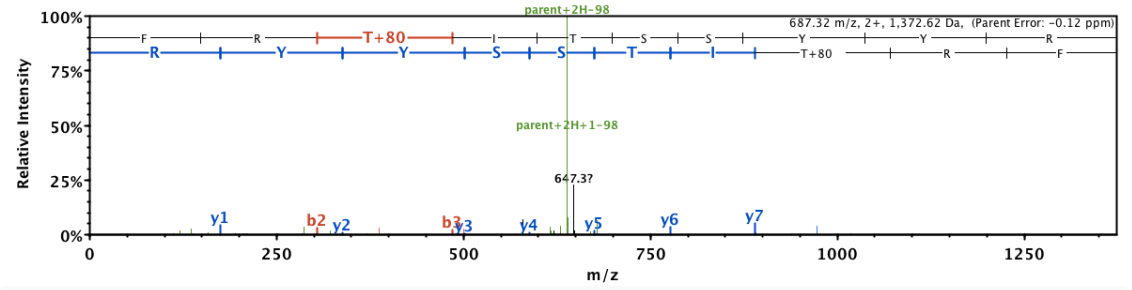
(R)FADDSWTD**pT**HISTIGVDFK(I), Thr38



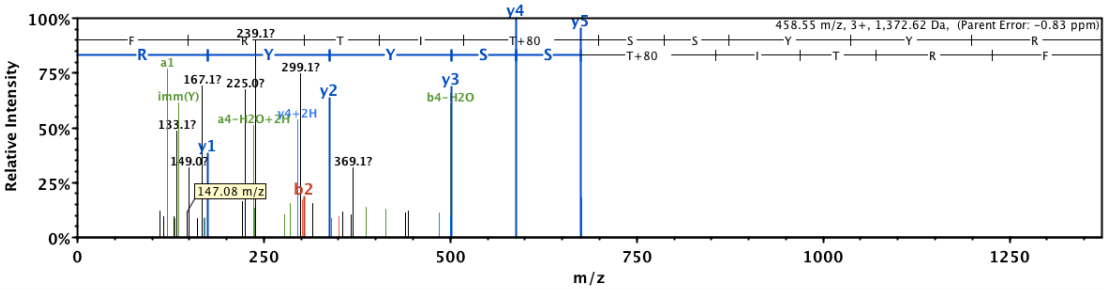
(R)FADDSWTDTHIS**pT**IGVDFK(I), Thr42



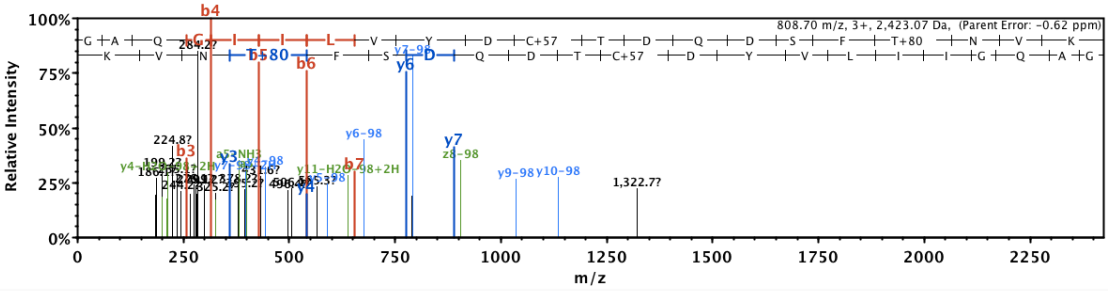
(R)FR**pT**ITSSYYR(G), Thr74



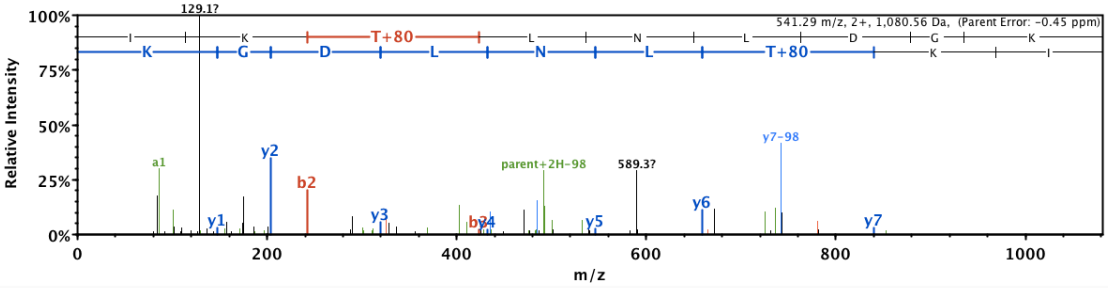
(R)FRTIpTSSYYR(G), Thr76



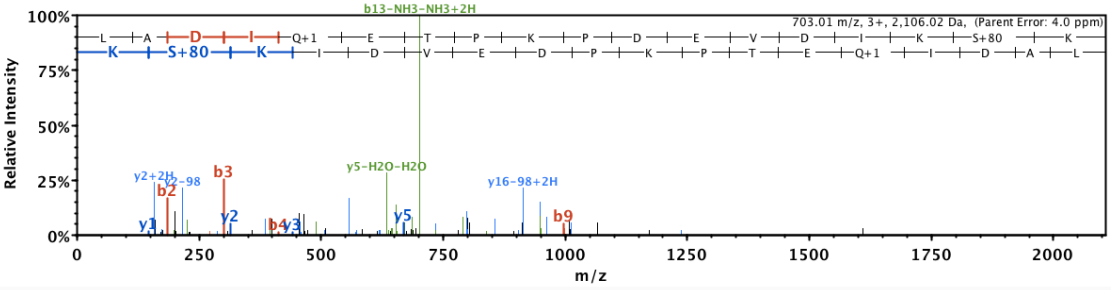
(R)GAQGIILVYDCTDQDSFpTNVK(Q), Thr99



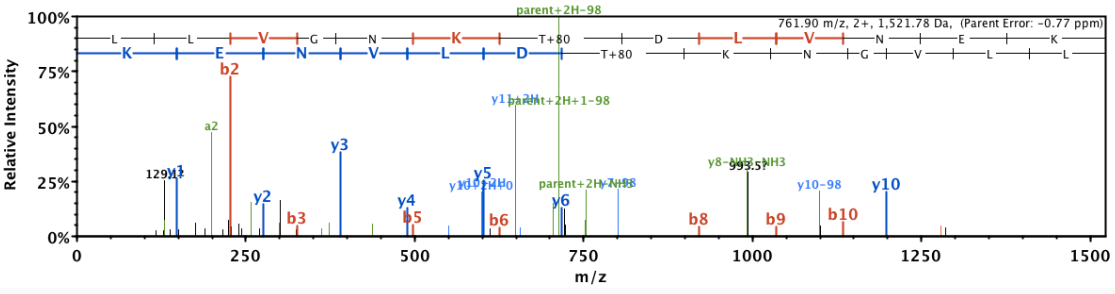
(K)IKpTLNLDGK(T), Thr51



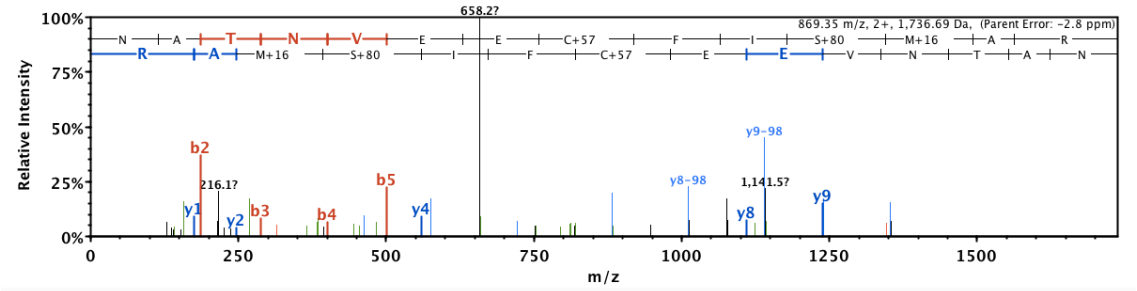
(R)LADIQETPKPDEVDIKpSK(N), Ser191



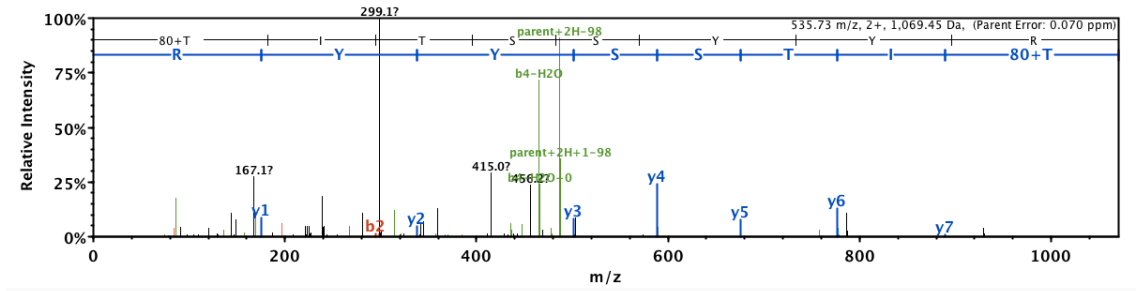
(K)LLVGNKpTDLVNEK(V), Thr125



(K)NATNVEECFIpSMAR(D), Ser166



(R)pTITSSYYR(G), Thr74



(R)TIpTSSYYR(G), Thr76

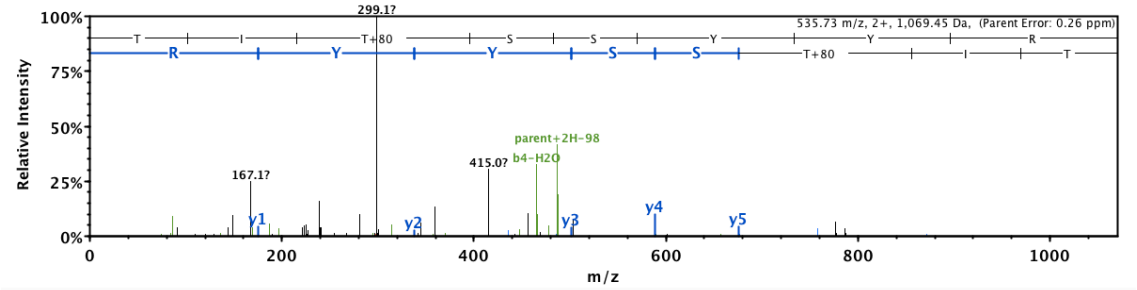


Figure 4. Phosphorylation sites of Rab1D: mass spectrometry data of 8 new peptides found in Rab1D with phosphorylated Thr residues (green). The spectra were displayed using Scaffold software and corresponding trypsinized peptides are shown.

Rab1D does not directly bind to Roco4

As an initial approach to studying Rab1D-Roco4 interaction, we performed several pulldown experiment to analyze whether Rab1D is binding to any of the Roco4 domains; LRR (N-terminus), Kinase or WD40. As shown in Figure 5, Rab1D did not bind to either of those domains, even when the experiment was repeated with different incubation times. In order to check if Rab1D needs binding interactors to bind to Roco4, pulldown experiments were also performed with *Dictyostelium discoideum* cells expressing Rab1D-GFP. However, this didn't reveal any further information, since we didn't find interactions with any of the Roco4 domains (Figure 6).

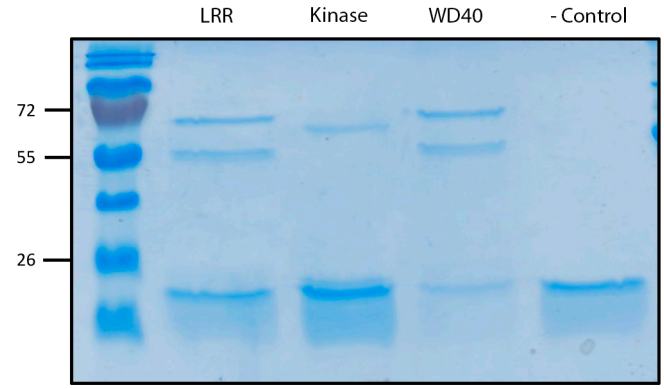


Figure 5. Coomassie staining of 14% SDS-Page gel containing pull-down experiment samples, molecular weight indicated in kDa. Purified GST-Rab1D and GST-tagged Roco4 domains were used and Rab1D did not directly bind to any of the domains. As a negative control, GST was also incubated with Rab1D and we could also find free GST in the rest of the samples (lower band below 26 kDa).

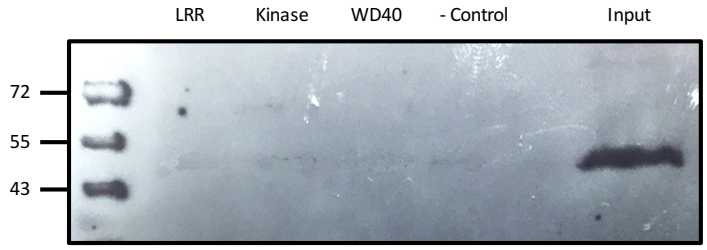


Figure 6. Western blot of Roco4 domains pulled down in AX2 cell lysate expressing Rab1D-GFP. Cell lysate was also loaded as input. Detection was done with α-GFP 1:5000. Rab1D did not bind to any of the Roco4 domains.

Dd Roco4 does not belong to the Rab1D interactome

In order to get an insight into Rab1D binding interactions within the cell, Rab1D, Rab1D T220A and Rab1D T220D were pulled down in AX2 cell lysates and analyzed via mass spectrometry. Table 2 shows interesting hits found after data analysis; we selected proteins that bind to either one of the mutants but not to the wild-type protein or vice versa and we also picked some proteins that bind only to the phosphorylated forms of Rab1D. It must be noted that Roco4 did not show up as a Rab1D binding partner and interestingly, several mitochondrial proteins were found as Rab1D binding partners together with some ER protein, which gives an indication of Rab1D possible localization in the cells.

Furthermore, we present some proteins in Table 2 that were also found to bind to the N-terminus of Roco4 in previous experiments and again, interestingly some of them are known to have a function in mitochondria.

GENE	RAB1D WT	RAB1D T220A	RAB1D T220D	N-TER ROCO4	DESCRIPTION
VATM	0	1	2	Yes	Transmembrane subunit of vacuolar ATP synthase
PURH	1	0	4	Yes	Bifunctional purine biosynthesis
ATP5B	5	8	18	Yes	ATP synthase beta subunit; MITOCHONDRIAL
MHSP70	1	2	2	Yes	Stress-70 protein; MITOCHONDRIAL
PDHB	0	0	2	Yes	Pyruvate deshydrogenase beta subunit
MCFZ/Q	0	2	3	Yes	Belongs to substrate protein carrier protein family; MITOCHONDRIAL
CTXB	1	1	7	Yes	Cortexilin; ER marker
FIME	1	2	7	Yes	Calcium-regulated actin bundling protein
DPP3	0	1	4	Yes	Dipeptidyl-peptidase III
THRS1	1	1	3	Yes	Threonyl-tRNA synthetase
ARFA	1	0	3	No	ADP-ribosilation factor 1
ARC	1	0	3	No	Actin related protein 2/3
THRXE	1	0	3	No	Putative thioredoxin
CYC1	0	1	3	No	Cytochrome C
TUBB	1	0	3	No	Tubulin beta chain
ARPB	1	0	3	No	Actin related protein 2
CRTA	0	2	2	No	Calreticulin, ER Marker
ACPA	1	0	2	No	f-actin capping protein beta subunit
SEVA	1	0	2	No	Severin; actin filaments

Table 2. Interesting hits found in Rab1D mass spectrometry analysis. Numbers indicate the amount of unique peptides found.

Rab1D localization in *Dictyostelium discoideum*

Rab1D localizes to the membrane and some internal structures

In order to have a first look at Rab1D localization, live cells expressing Rab1D-GFP were imaged in vegetative state. In such conditions, Rab1D was clearly localizing to the cell membrane, but also patches inside the cells were present. Moreover, bright dots were observed too, both on the membrane and inside the cells (figure 7).

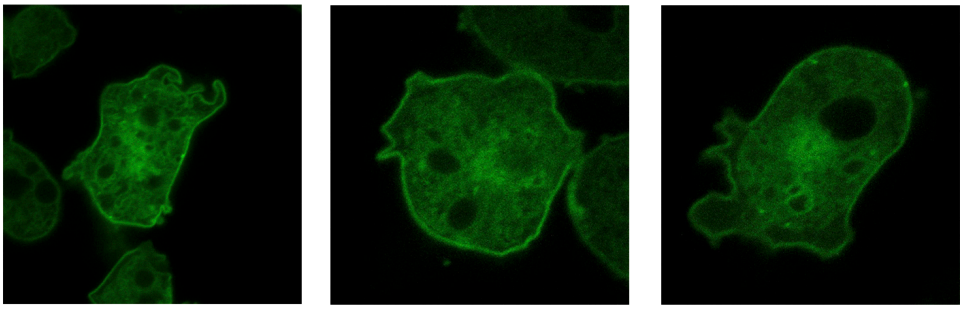


Figure 7. Rab1D-GFP localization in *Dictyostelium discoideum* live cells in vegetative state.

To get a better insight into these structures, vegetative cells were also fixed and imaged with longer exposure times so that higher resolution images could be acquired (Figure 8). DAPI staining revealed that the patch observed inside the cells was always in close proximity with the nucleus.

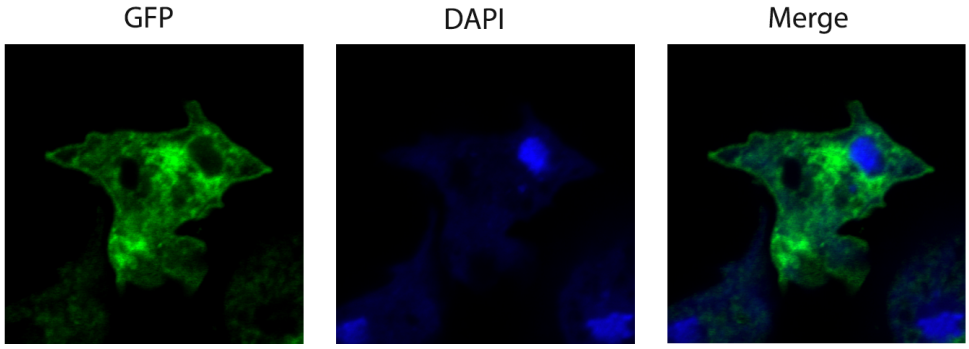


Figure 8. Localization of Rab1D in cells fixed with 4% paraformaldehyde. DAPI (1 μg/mL) was used to stain the nucleus.

Localization of Rab1d changes upon phosphorylation, but is independent of Roco4

We next looked into the effect of phosphorylation on Rab1D localization and whether or not this was happening upon Roco4 kinase. To do so, both Rab1D and its phospho-dead mutant, Rab1D T220A, were expressed as GFP fusion proteins in wild type *Dictyostelium* cells (AX2), as well as in mutant cells with a Roco4 knockout (Figure 9). We found that protein localization does indeed change when Rab1D cannot be phosphorylated; in cells expressing Rab1D T220A mutant, the protein was not localizing to the membrane anymore, therefore the GFP signal became more cytosolic. However, patches inside the cells were still observed, indicating that the protein is still localizing to some structures within the cell.

This pattern was the same in both strains, suggesting that it is not Roco4 that has an effect on Rab1D localization, but phosphorylation itself, which might occur through other kinase proteins.

In Figure 10, western blot with cell lysates from the same cells used for microscopy is shown; Rab1DGFP was successfully expressed in all strains.

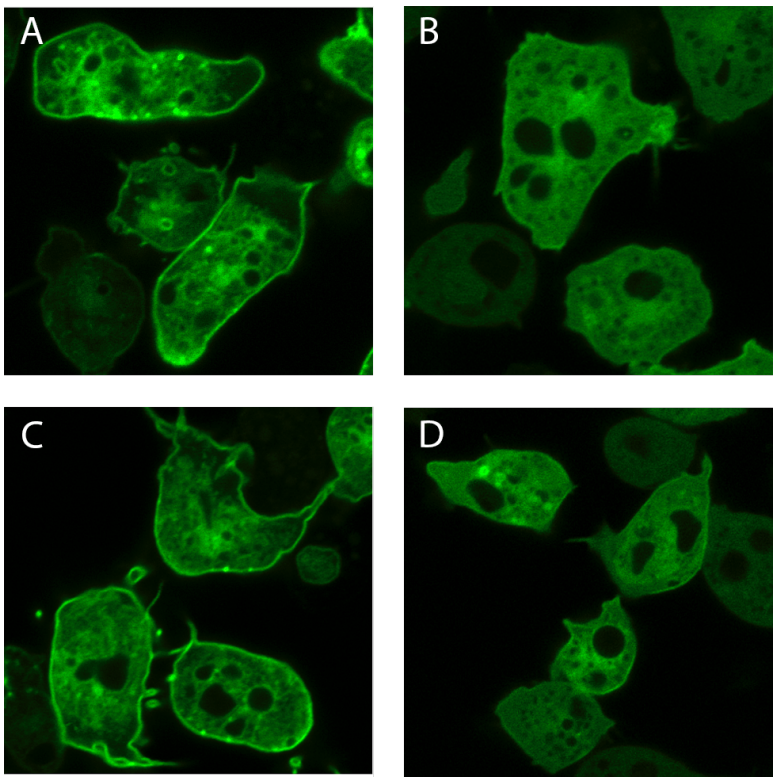


Figure 9. Rab1D-GFP localization in vegetative cells. In AX2 cells (A, B) Rab1D localizes to the membrane, to patches inside the cell and to bright spots in the cytosol and the membrane, whereas Rab1D T220A is mainly cytosolic, although some patches are still visible. In Roco4 null cells, same localization in observed (C, D).

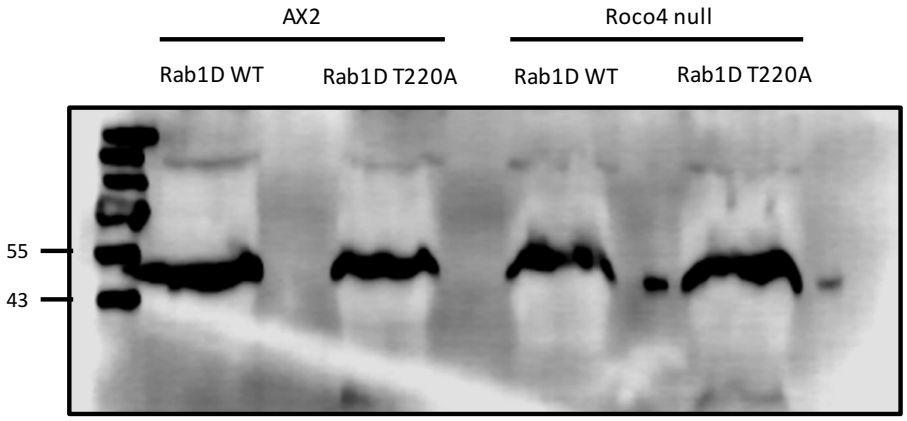


Figure 10. Western blot of cells expressing Rab1D-GFP. Detection was performed with α-GFP at a concentration of 1:5000.

Microtubule inhibition disrupts Rab1D localization inside the cell

To further investigate to which internal structures Rab1D was localizing, we treated cells with different drugs. First, Latrunculin A was used to inhibit actin polymerization. This didn't have any effect in Rab1D localization, since the protein was still at the membrane and patches inside the cell looked the same as in untreated cells (Figure 11). Subsequently, since patches seemed to always localize next to the nucleus, Nocodazole was used to inhibit microtubule formation and therefore disrupt structures such us endoplasmic reticulum or Golgi.

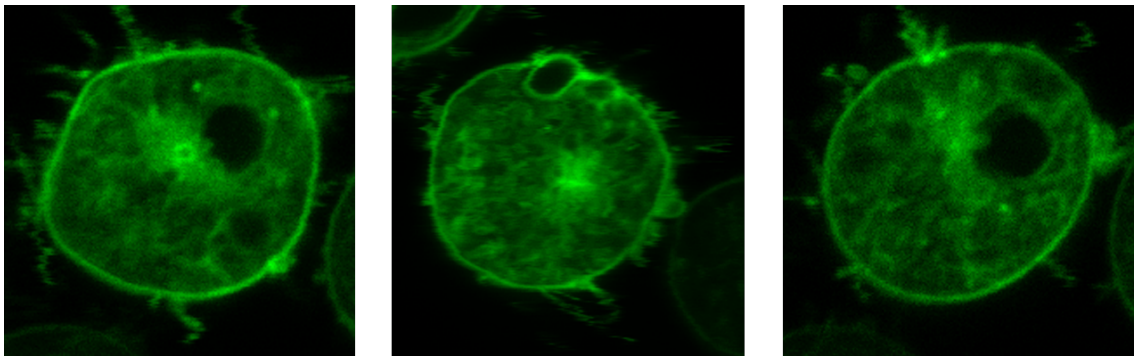


Figure 11. Rab1D-GFP localization in AX2 cells after 30 minutes of Latrunculin A (5 μM) addition. Localization is the same as observed before, so it is not affected by inhibition of actin polymerization.

We found that after treatment with Nocodazole, Rab1D still localized to the membrane, as expected, but the patches inside the cells look different to those observed previously in non-treated cells (Figure 12). Instead of a uniform patch around the perinuclear area, several smaller patches were distributed around the cytosol, as in small stacks of membrane structures. This gave an indication of Rab1D localization being closely related to microtubule.

Rab1D is localizing to the perinuclear ER

Since Rab1D was clearly localizing to some structure around the nucleus, immunostaining was performed using α-Calreticulin to stain the endoplasmic reticulum (ER). As shown in Figure 13, calreticulin staining showed a clear ring around the nucleus, which corresponds to perinuclear ER, and ER was also visible next to the cell membrane. Rab1D was also observed in such a ring around the nucleus, co-localizing with calreticulin. In this case, the GFP ring was not as clear as in the calreticulin image, probably because Rab1D is also localizing to other structures around the nucleus, such as Golgi and therefore that ring is merged with different patches. It must also be noted that Rab1D localization to the membrane is not as clear as in live cells, which is probably due to membrane damage during fixation and permeabilization of the cells.

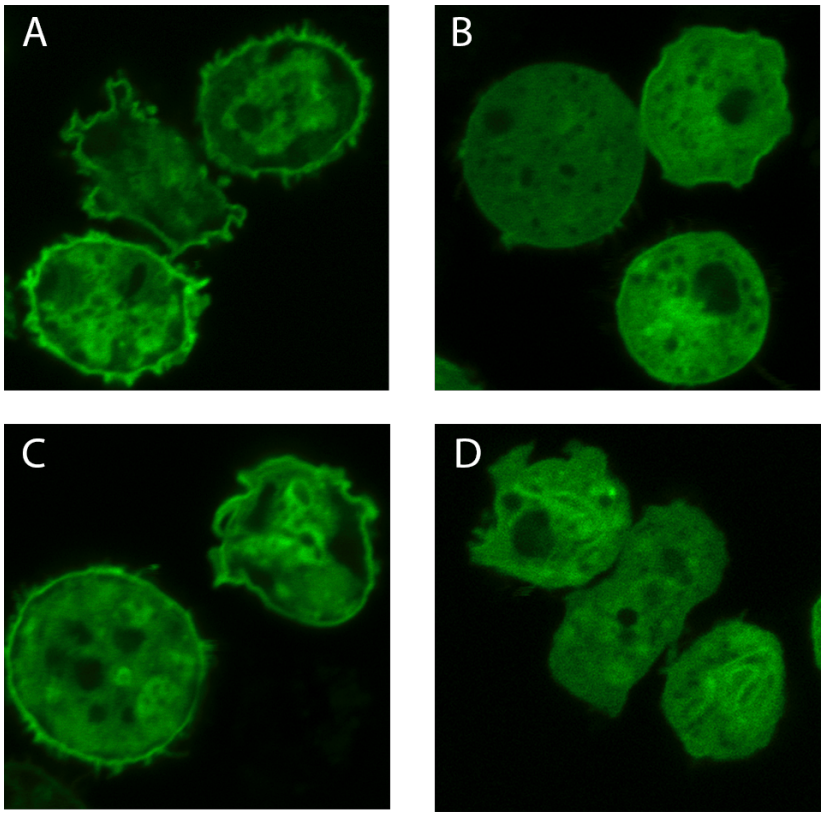


Figure 12. Localization of Rab1D in cells treated with 10 μg/mL Nocodazole for 45 minutes.

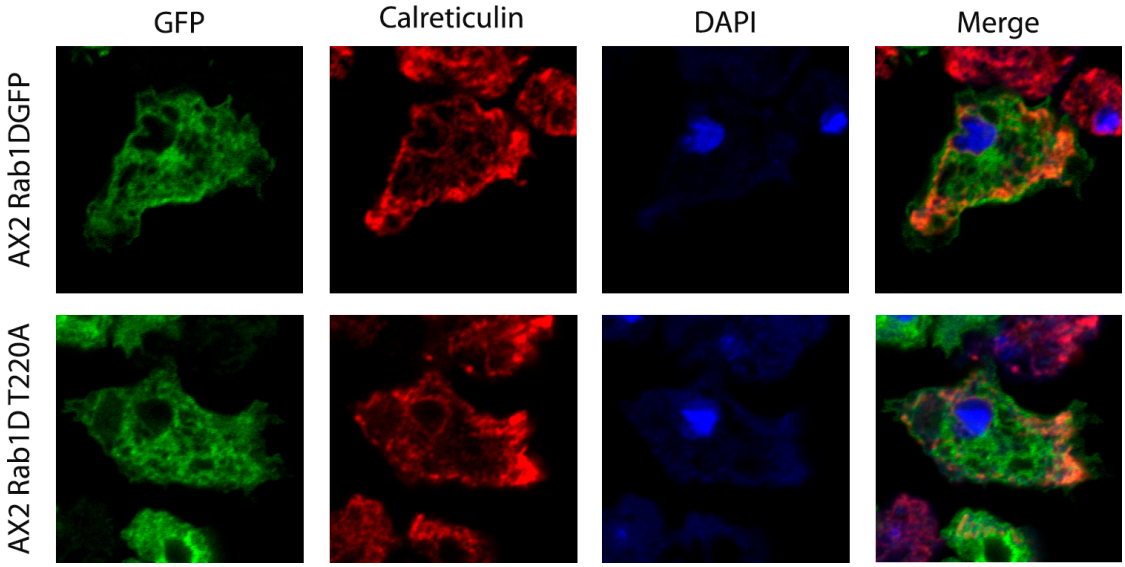


Figure 13. Immunostaining of cells expressing GFP-tagged Rab1D, wild type and phospho-dead. α-Calreticulin (1:5) was used to stain the endoplasmic reticulum.

Rab1D overexpression phenotype

Overexpressing Rab1D makes cells aggregate faster

When starving cells for confocal microscopy, we noticed early aggregation of cells overexpressing Rab1D. therefore, we performed development experiments to compare aggregation between these cells and a wild type strain. In typical AX2 cells (Figure 14), aggregation started after 4 hours and clear aggregates were observed after 5 hours of starvation, as expected. However, in the Rab1D overexpression cells, we observed cells starting to aggregate in about 3 hours of starvation (Figure 15) and after 4 hours aggregates were already visible, confirming that indeed overexpressing Rab1D has an effect on aggregation.

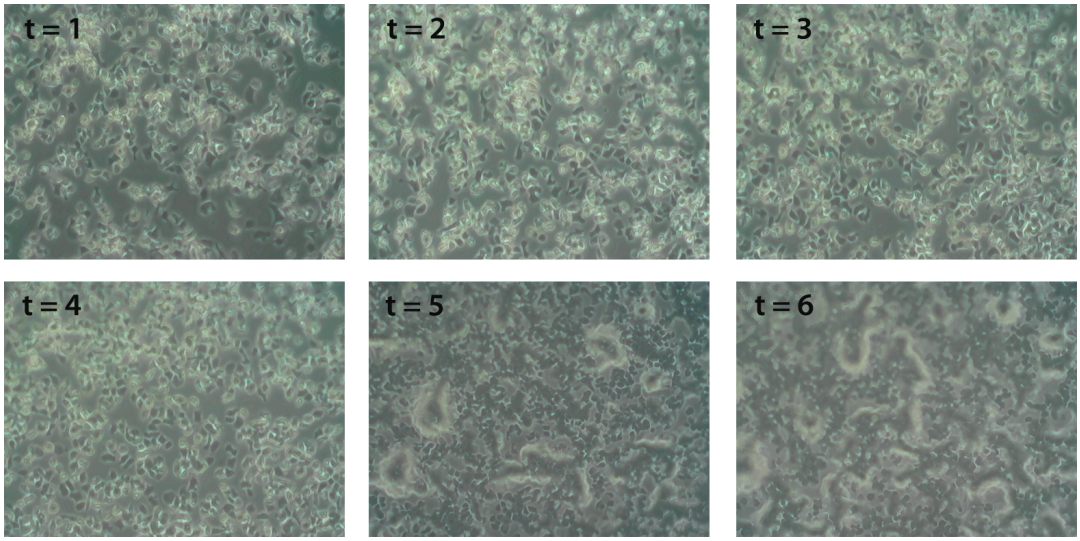


Figure 14. Cell aggregation in Dictyostelium discoideum AX2 cells starved in non-nutrient agar plates. Starvation time (t) is indicated in hours.

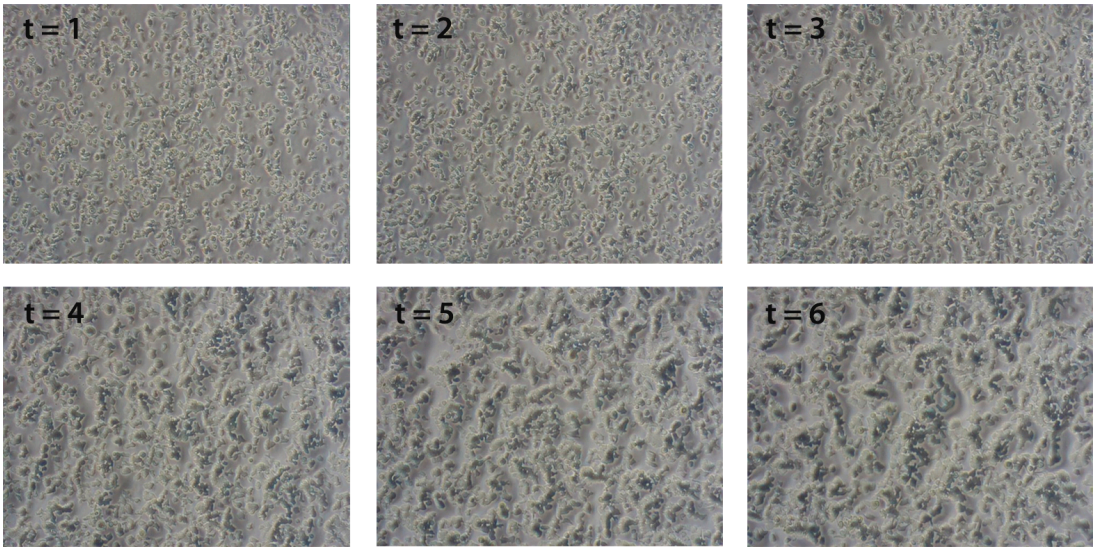


Figure 15. Cell aggregation in Dictyostelium discoideum AX2 cells starved in non-nutrient agar plates and overexpressing Rab1D. Starvation time (t) is indicated in hours.

DISCUSSION

Recently, Rab GTPases have been put on the spotlight of Parkinson's disease research, since they are involved in vesicle trafficking and these events have been identified as key players in the development of the disease. As they are LRRK2 downstream effectors, it is of great interest to find out the link between increased kinase activity and mitochondrial and autophagy defects, which might very likely be happening through Rab mediated pathways[15]. In this study we analyze how these proteins interact using *Dictyostelium discoideum* as a model organism and we show that *Dd* Roco4 and Rab1D are a good model to study this pathway both in vivo and in vitro, allowing further translation of results to the mammalian homologue LRRK2 and its Rab substrates.

Taken together, our *in vitro* biochemical assays show that Roco4 and Rab1D do no interact in a strong, stable manner. Even though Rab1D is a confirmed substrate, pulldown experiments and immunoprecipitation do not show interaction with any of the Roco4 domains. Mass spectrometry analysis is consistent with this data and proves that Roco4 is not present in Rab1D interactome, indicating that such interaction must be very short or indirect. This is consistent with what is known for other kinases; substrate specificity is not so high and sometimes need other scaffolding proteins to form a stable complex in vivo that helps phosphorylation[16,17]. Mass spectrometry experiments show some proteins that interact with wild-type Rab1D but not with Rab1D phospho-dead mutant and at the same time bind to the N-terminus of Roco4, reinforcing the idea that a scaffolding structure might be needed for phosphorylation to happen in a stable manner and therefore it is not possible to show direct binding of Roco4 and Rab1D in vitro. The fact that this interaction is weak and transient might also explain why Rab1D is not highly phosphorylated when performing *in vitro* kinase assays with Roco4 kinase. It is also important to consider that Roco4 reaches its highest levels of expression in *Dictyostelium discoideum* after 12 hours of development. This could also be the reason why we didn't find Roco4 in mass spectrometry analysis with cell lysates.

The construction of Rab1D T220A mutant results in a decreased phosphorylation by Roco4 kinase *in vitro*, as expected, but doesn't completely impair phosphorylation. Same is true for the T220D mutant, which resembles the phosphorylated form of Rab1D. This is consistent with the finding of 8 new peptides containing Thr residues via phospho-proteomics, which could still be getting phosphorylated. It would be interesting to create new constructs in order to mutate these other residues, creating Rab1D mutants in which phosphorylation is completely impaired and allowing a better understanding of the kinetics of this reaction.

Regarding GTPase activity, we haven't been able to show any difference between Rab1D wild-type and phospho-mutants. However, this might be due to experimental errors and we cannot consider these results conclusive. The assay used in this study should be optimized and repeated so that we can make a final statement about the effect of phosphorylation in the G-protein cycle of Rab1D.

It would also be interesting to get more insights into some of the proteins found in the Rab1D interactome via mass spectrometry. Here we show some proteins that bind to the wild-type version of Rab1D and also to its phospho-mimic variant but not to phospho-dead Rab1D. Interestingly, some of these proteins were also found to bind to the N-terminus of Roco4 in previous experiments from our group and furthermore, they

have a known mitochondrial function. Further analysis of this proteins related to Rab1D and Roco4 may reveal an interesting link between them and therefore shed light into the biochemical pathways that lead to mitochondrial malfunction in Parkinson's disease.

We also had a deep look into Rab1D localization in *Dictyostelium discoideum*, aiming to understand the role of this protein in the cell. We show that Rab1D localizes to the perinuclear ER, as it colocalizes with the known ER protein calreticulin. Also, the use of different drugs shows a possible localization to Golgi, although more experiments need to be performed to confirm this hypothesis. These results are consistent with the cellular function known for other Rabs. It has been shown that a variety of Rabs, such as human Rab1 and Rab8a, localize to the ER and play a role in the late secretory pathway between ER and Golgi; they are thought to regulate ER tubulation rather than vesicle budding and cells where Rab proteins are not expressed show a much simpler ER structure[18]. On top of that, we also observe a very clear localization to the cell membrane, suggesting a possible role in vesicle trafficking between the cell and its environment.

On the other hand, the analysis of the T220A mutant reveals a clear effect of phosphorylation in Rab1D localization in the cell. We show here how Rab1D localization to different membranes in the cell is happening upon phosphorylation; Rab1D is present at the cytosol and shifts to ER, plasma membrane and other membrane structures when it gets phosphorylated. Such mechanism has already been shown for other Rabs and it is known that phosphorylation by LRRK2 in the switch II inhibits interactions with GDI proteins, increasing stability of Rab proteins in the membranes [19]. Moreover, it has also been found that the GDP-bound form of Rabs is present at the cytosol, where it interacts with REPs (Rab escort proteins) allowing insertion in its target membranes. Once inserted, they are activated by GEF proteins that allow the conversion of GDP to GTP[15]. This background is also an indication that phosphorylation has an effect on the G-protein cycle, making the protein stay in the GTP-bound state and avoiding its return to the cytosol. Thus, it supports the previous idea that our in vitro GTPase assays are not correct and must be repeated in other to show a difference in GTP hydrolysis upon phosphorylation.

Furthermore, we show here that such change in localization via phosphorylation doesn't seem to be mediated by Roco4 activity. Our results with cells in which Roco4 has been knocked out show the same localization of the protein as in wild-type cells. Again, a reason for this might be the fact that cells were not used at the appropriate developmental stage, when Roco4 is highly expressed. Another possible explanation is that phosphorylation is happening via other kinases in the cell and therefore we don't see a difference between wild-type cells and Roco4 knockouts, but only between the phosphorylated and non-phosphorylated form of the protein. It would be interesting to use LRRK2 inhibitors to inhibit other kinases in the cell and see if there is an effect in Rab1D localization. We could then rule out the hypothesis that Rab1D phosphorylation by Roco4 is affecting on its localization to membranes.

Finally, we also show a phenotype of Rab1D overexpression in the cells. When starved, *Dictyostelium discoideum* cells secrete cAMP which serves as a chemoattractant and makes cells aggregate until they form slugs. Then they produce spores that spread around the environment, which is the mechanism of this cells to move to areas that are more rich in nutrients. Our results suggest a possible role of Rab1D in nutrient exchange, since cells overexpressing Rab1D begin to aggregate faster than wild type cells. A

hypothesis is that these cells might have an increased or decreased uptake of nutrients that makes them starve faster when they are grown in normal media and therefore also aggregate faster when transferred to non-nutrient media. We only prove this with a simple aggregation experiment, but the effect is very clear so it would be interesting to analyze development markers to confirm this effect of overexpressing Rab1D in *Dictyostelium discoideum*. There is reason enough to have a more detailed look into this process, as it has already been shown that overexpression of other Rabs result in a phenotype with increased growth and aggregation rates, which suggest a role of some Rab proteins in endocytic processes[20]. Also, localization of Rab1d closely resembles the localization of a protein called SibC, which is also present at the plasma membrane and the perinuclear area. This protein is internalized by macropynocitic cups, which also play a role in fluid uptake, so it would be interesting to show colocalization of Rab1D and SibC in order to get further insight into Rab1D function in the cell. Eventually, the construction of a Rab1D knockout would help figure out the role of this protein and clarify whether or not it is an interesting target to further investigate the development of Parkinson's disease.

All of our results reveal that Rab1D is a good model to study the Roco4 pathway since it shows some promising data that might be interesting to continue to investigate. The availability of both proteins and the ease to work with them, both in vitro and in vivo, makes them a great tool for PD research and further experiments may reveal important information that could be later translated back to the human model of the disease.

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