UNIVERZITA KARLOVA V PRAZE
PŘÍRODOVĚDECKÁ FAKULTA
Katedra experimentální biologie rostlin

MASTER'S THESIS

CHARACTERIZATION OF SELECTED RAB GTPASE ACTIVATING PROTEIN (RAB GAP) OF *ARABIDOPSIS THALIANA*

Charakterizace vybraného proteinu
aktivujícího RAB GTPázy (RAB GAP)
v *Arabidopsis thaliana*

Bc. Jáchym Metlička
Tutor: RNDr. Michal Hála, Ph.D.
Consultant: doc. RNDr. Viktor Žárský, CSc.

Praha 2016

I hereby declare that the work presented here is, to my best knowledge and belief, original and the result of my own investigations and consultations with my tutor, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other university.

Formulations and ideas taken from other sources are cited as such. This work has not been published.

In Prague, August 10th, 2016 Jáchym Metlička

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor RNDr. Michal Hála, Ph.D. for his support and guidance. He was always willing to discuss the thesis matters, provided good insight and answered sometimes stupid questions with patience – this made my work in the lab much easier. I would very much like to thank to all members of lab 202 for providing a good work environment and jolly atmosphere. Their willingness to advise, teach and assist helped me to overcome my shyness and significantly expanded my skill set. Members of Laboratory of Cell Biology at IEB also deserve the same gratitude.

I would also like to thank Alexandra Elbakyan for her indirect assistance. Thanks to you I could jump over the walls instead of looking for the keys. I hope things go well for you.

My thanks go to Madla for managing to listen to my endless rants about everything and for bearing with me in general.

Last but not least, I would like to thank my family for their continuing psychological care and material support. Hopefully something good comes out of it.

CONTENTS

LIST OF ABBREVIATIONS

Please note that plurals of the shortcuts are routinely used in this thesis – e.g. singular GOI, plural GOIs.

Abbreviation:	**Meaning:**
ABA	abscisic acid
AGE	agarose gel electrophoresis
Arabidopsis	*Arabidopsis thaliana*
ARF	ADP-ribosylation factor
ARL	ARF-like
BD	binding domain
BFA	brefeldin A
cAMP	cyclic adenosine monophosphate
CC	coiled-coil
CDS	coding sequence
ddH_2O	deionized water
DMSO	dimethyl sulfoxide
ER	endoplasmatic reticulum
FM4-64	styryl dye commonly used for membrane staining
FRET	Förster resonance energy transfer
FTase	farnesyltransferase
GA	Golgi apparatus
GAP	GTPase activating protein
GDF	GDI displacement factor
GDI	guanine dissociation inhibitors
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
(sec)GFP	(secretory) green fluorescent protein
GGTase	geranylgeranyltransferase
GOI	gene of interest
GTP	guanosine triphosphate
HMMER	Hidden Markov Model-based sequence alignment tool
HVD	hypervariable C-terminal domain
IAA	Indole-3-acetic acid
IM	infiltration medium
LECA	last eukaryotic common ancestor
LUCA	last universal common ancestor
MPA	meat peptone agar
MPB	meat peptone broth
MS	Murashige & Skoog
MUSCLE	multiple sequence comparison by log-expectation
NLS	nuclear localization signal

Abbreviation:	Meaning:
NPC	nuclear pore complex
OD	optical density
PCR	polymerase chain reaction
PH	pleckstrin homology
PM	plasmatic membrane
PMSF	phenylmethylsulfonyl fluoride
PoQ	PAL OF QUIRKY
PPT	phosphinothricin
PVC	prevacuolar compartment
RabGGT	Rab geranylgeranyl transferase
RE	restriction endonuclease
REP	Rab escort protein
ROI	region of interest
RT	room temperature
SD	synthetic defined
SMART	Simple Modular Architecture Research Tool
TGN	trans-Golgi network
TM	transmembrane
TRAPP	transport protein particle
VHA-a1	Vacuolar H(+)-ATPase subunit a isoform 1
WARBM	Warburg Micro syndrome
WT	wild type
XVE	estradiol-inducible promoter system
Y2H	yeast two-hybrid
yeast	*Saccharomyces cerevisiae*
(E)YFP	(enhanced) yellow fluorescent protein
YPAD	yeast extract peptone dextrose medium + adenine
2,4-D	2,4-dichlorophenoxyacetic acid

ABSTRACT

Rab GTPases (Rabs) are the most populous branch of eukaryotic Ras GTPase superfamily. In active GTP-binding conformation, they serve as key instruments in defining transient membrane identity and through various effectors regulate formation, transport, conversion, and fusion of membrane vesicles. This is important for upkeep of compartmentalized structure of eukaryotic cells and for facilitating both endo- and exocytic processes. Rabs are converted into GDP-binding conformation by interactions with Rab GTPase activating proteins (Rab GAPs) that possess ability to significantly speed up weak intrinsic GTP hydrolytic activity of Rabs. Through this process, Rab GAPs can limit scope of the Rabs' activity and lay out spatiotemporal boundaries for varying Rab populations. In this thesis, I tried to characterize a Rab GAP, GAP2, seemingly necessary for standard development of thale cress plants. Besides TBC catalytic domain, GAP2 (product of At2g39280 gene) possesses a C-terminal coiled-coil motif, which was previously found to interact with Rab GTPases. Experiments aiming to complement T-DNA insertion mutant in GAP2, elucidate GAP2 intracellular localization, novel interacting partners, and character of interaction with the Rabs discovered in the pilot study were undertaken. The results suggest that GAP2 is primarily cytoplasmic and its interaction with RabA1a and RabE1d is solely through the coiled-coil motif. Y2H screen for novel interactors uncovered coiled-coil motif from a closely related Rab GAP (At3g55020) and lead to realization that GAP2 on its own can dimerize through the C-terminal coiled-coil motif. Efforts to complement the mutant were ultimately unsuccessful.

Key words: GTPase-activating protein (GAP), membrane traffic, Rab effector, Tre-2/Bub2/Cdc16 (TBC) protein, Rab GTPase, Ras superfamily, coiled-coil dimerization, exocytosis, GAP cascade, Rab cascade

ABSTRAKT

Rab GTPázy (Raby) jsou nejpočetnější rodinou eukaryotické super-rodiny Ras GTPáz. V aktivní GTP-vázající formě slouží jako nástroje vymezující proměnlivou identitu membrán a skrz rozličné efektory regulují utváření, transport, proměnu a fúzi membránových váčků. To je zásadní pro udržení kompartmentalizované struktury charakteristické pro eukaryotické buňky a pro zajištění endo- a exocytózy. K deaktivaci Rabů dochází skrz vazbu Rab GAPů, tj. proteinů disponujících schopností výrazně urychlit hydrolýzu GTP vázaného v Rabech. Tímto procesem mohou Rab GAPy omezovat rozsah aktivity Rabů a vytyčovat časoprostorové ohraničení odlišných populací Rab GTPáz. V této práci jsem se pokusil popsat Rab GAP, označený jako GAP2, který je zřejmě nutný pro standardní vývoj rostlin huseníčku rolního. Kromě charakteristické katalytické TBC domény obsahuje GAP2 (produkt genu At2g39280) C-terminální coiled-coil strukturní motiv, který byl už dříve pozorován v interakcích s konkrétními Rab GTPázami. Provedl jsem experimenty, jejichž cílem mělo být komplementování T-DNA inzerčního mutanta v genu GAP2, zjištění vnitrobuněčné lokalizace GAP2 proteinu, nalezení dosud nepopsaných interaktorů a popis interakce s Raby objevenými v pilotních experimentech. Výsledky nasvědčují, že GAP2 je primárně cytoplasmatický protein a jeho interakce s RabA1a a RabE1d probíhá pouze skrz coiled-coil motiv. Dvouhybridní kvasinkový screen jako nového interaktora odhalil coiled-coil motiv z blízce příbuzného Rab GAPu (At3g55020) a vedl ke zjištění, že GAP2 může skrz C-terminální coiled-coil motiv dimerizovat sám se sebou. Snahy o komplementaci mutanta byly přes veškeré úsilí neúspěšné.

Klíčová slova: GTPázy-aktivující protein (GAP), membránový tranport, efektor Rabu, Tre-2/Bub2/Cdc16 (TBC) protein, Rab GTPáza, super-rodina Ras, dimerizace skrz coiled-coil, exocytóza, GAP kaskáda, Rab kaskáda

1. LITERATURE REVIEW

1.1. SMALL GTPASES

Small GTPases are a fairly heterogeneous group of enzymatically active proteins 20 to 40kDa in size. Their common characteristic is the ability to bind and hydrolyze guanosine triphosphate (GTP) molecule, resulting in bound guanosine diphosphate (GDP) and a free phosphate (Pi). Both binding and hydrolysis take place in the structurally conserved G-domain and once the latter occurs, it is accompanied by a significant conformational change of the domain. Small GTPases are often called "molecular switches", thanks to different populations of downstream interactors associated with each of the two conformations. Small GTPases regulate many key processes in eukaryotic cells, such as cellular growth, proliferation and differentiation, signal transduction, vesicular transport (including all major steps of endo- and exocytosis) and nucleocytoplasmic transport (Takai et al. 2001). Many small GTPases are associated with membranes in at least some part of their functional cycle.

1.1.1. G-DOMAIN

The G domain is an 18-20kDa domain present in all G proteins, such as trimeric G proteins, translation factors, dynamins, signal recognition particles (SRP), SR receptors and others. The sequence of the 160-180 residue-long domain is variable across the numerous G-proteins but certain amino acid residues are conserved – These are responsible for interactions with GTP and for the canonical hydrolytic activity. Primary structure identity across the most diverse of small GTPases of Ras superfamily is quite high at ~30%. The secondary structural homology of the domain is much higher - shared core is composed of 5 α-helices interspersed with 6 β-strands, all connected by loops (Figure 1-A). The β-strands form a β-sheet with the α-helices distributed on both of its sides (Figure 1-B). 5 hydrophobic loops, which are located near the GTP binding site bear the 5 conserved residue motifs. From N- to C-, these are numbered G1 to G5 and have the following consensus sequence: G1 = GXXXXGKS/T; G2 = T; G3 = DXXGQ/H/T; G4 = T/NKXD; G5 = C/SAK/L/T.

G1 (also called Walker A motif or P-loop) connects β1-strand and α1-helix and wraps around the negatively charged phosphates in GTP by interacting with the main chain nitrogen atoms. G2 (so-called switch I) is localized between α1-helix and β2-strand and with its single conserved threonine residue interacts with the γ-phosphate through its main chain NH group. G3 (so-called switch II) between β3-strand and α2-helix facilitates a water-mediated binding of

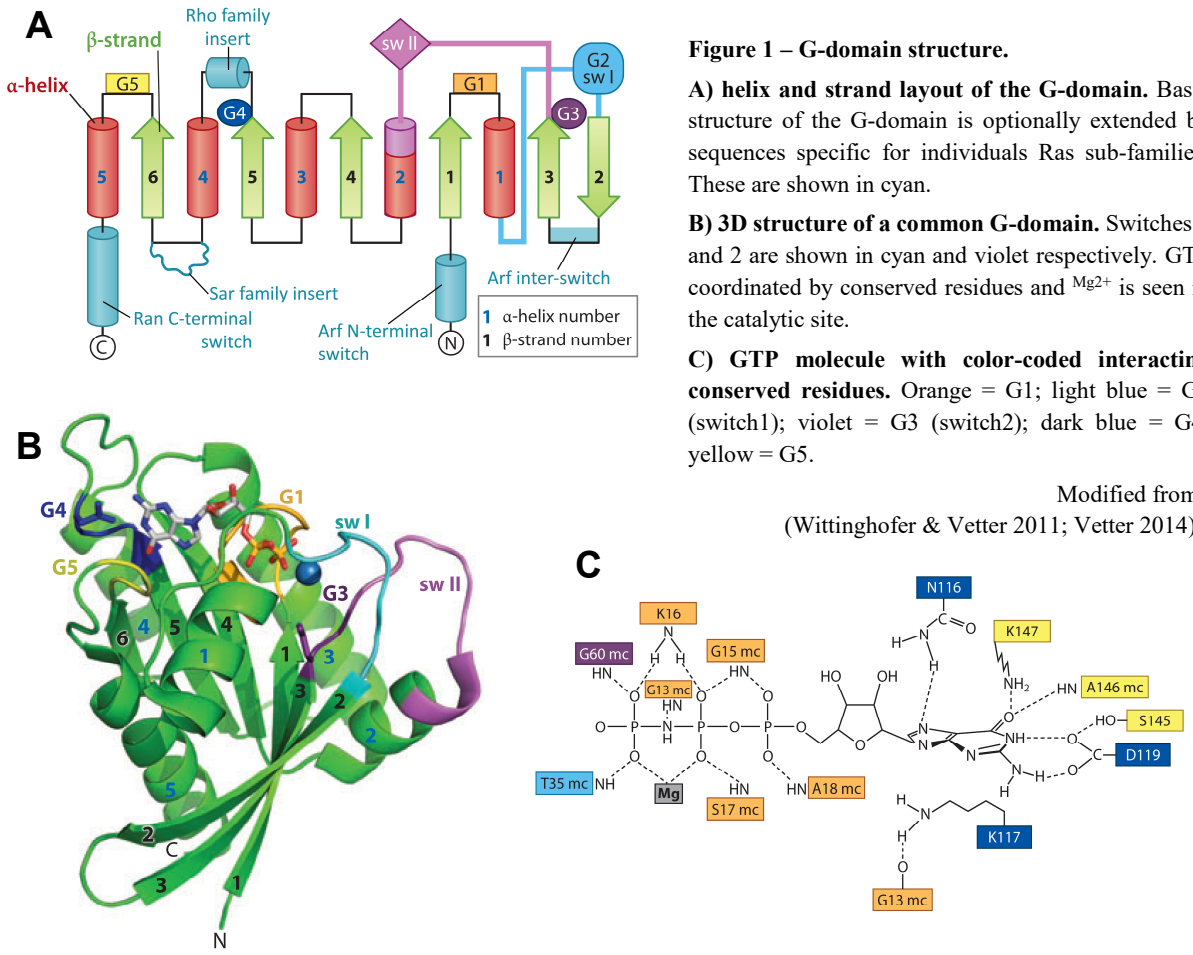

Figure 1 – G-domain structure.

A) helix and strand layout of the G-domain. Basic structure of the G-domain is optionally extended by sequences specific for individuals Ras sub-families. These are shown in cyan.

B) 3D structure of a common G-domain. Switches 1 and 2 are shown in cyan and violet respectively. GTP coordinated by conserved residues and $^{Mg2+}$ is seen in the catalytic site.

C) GTP molecule with color-coded interacting conserved residues. Orange = G1; light blue = G2 (switch1); violet = G3 (switch2); dark blue = G4; yellow = G5.

Modified from (Wittinghofer & Vetter 2011; Vetter 2014)

Mg^{2+} through its aspartate residue. Main chain NH group of semi-conserved glycine interacts with the γ-phosphate of GTP. Mg^{2+} is critical for high-affinity binding of negatively charged phosphates of GTP and its hydrolysis. G4 and G5 loops define G domain's selectivity for guanine – most G domains have a conserved alanine residue in the G5 motif , which interacts with O6 oxygen of guanine by means of its main chain NH group (Figure 1-C) (Wittinghofer & Vetter 2011; Paduch et al. 2001).

G2 threonine's hydroxyl and G3 glycine's main-chain interactions with the GTP γ-phosphate are responsible for the conformational switch that occurs after hydrolysis to GDP and Pi. The arrangement can be thought of as a spring-loaded mechanism - As long as bound GTP is intact, both switches are held in close proximity of the γ-phosphate oxygens. This stable "closed" conformation is suitable for binding effectors. However, after it is cleaved off the switches become disordered and are thought to dynamically fluctuate between both conformations very rapidly. This disfavors effectors without high-enough binding energy, since for lasting interaction the flexible region first has to be fixed in place at the effectors' expense (Vetter 2014).

Inquiry into purpose of small GTPases and their relationships with other proteins has been greatly helped by utilization of following amino acid substitutions within the G-domain. These are:

- Q-to-L substitution in the G3 motif - Allows for permanent activation of the G-domain by disrupting the GTP hydrolysis ability. Resultant protein is GTP-stabilized.
- S-to-N substitution in the G1 motif – Decreases G-domain's affinity for GTP, leading to GDP-bound, deactivated GTPase. Resultant protein is GDP-stabilized. It can potentially sequester significant part of upstream activators and result in dominant-inhibitory effect
- N-to-I substitution in the G4 motif – Decreases G-domain's affinity for all guanine nucleotides. Predicted outcome is very swift exchange of hydrolyzed GTP possibly resulting in: permanent activation, sequestration of upstream or downstream interactors, inhibitory effect.
- D-to-N substitution in the G4 motif – G-domain binds xanthosine 5'-triphosphate (XTP) and GTP/GDP affinity is significantly lowered. Resultant behavior is: Inhibitory effect similar to N-to-I. Addition of XTP results in its binding and eventual hydrolysis, releasing the block.

(Olkkonen & Stenmark 1997)

G domain can contain additional inserts or flanking sequences as is the case of Rho, Arf and Ran families of Ras superfamily (Figure 1-A).

1.1.2. RAS SUPERFAMILY OF SMALL GTPASES

Defining characteristics of the superfamily are the aforementioned G-domain and a hypervariable C-terminal domain (HVD). HVD can be posttranslationally prenylated or palmitoylated to facilitate attachment to membranes (Vetter 2014).

Ras GTPase superfamily can be phylogenetically divided into 5 distinct families. These are Ras, Rho, Arf/Sar, Ran, and Rab. Wuichet and Sogaard-Andersen (2014) performed phylogenetic analysis of prokaryotic small GTPases and elucidated their relation to eukaryotic Ras superfamily. They propose that last universal common ancestor (LUCA) contained 2 elementary small GTPases, each with characteristic sequence in its G3 motif: Rup-relative with DxPGQ and MglA-relative with GTPGQ. Bacteria and archaea contain descendants from both of these ancestors: Rup1 (DxxGx), MglA1 (TVPGQ), and MglA2, -3, -5 (GTPGQ) in bacteria; Rup2 (DxPGQ), and MglA4 (GTPGQ) in archaea. Eukaryotes differ in this regard. G3 motif in all eukaryotic small GTPases contains invariable aspartate in the first position, identifying their LUCA ancestor as Rup-relative and indicating that their ancestor lost the MglA-descended sequence. The analysis also shows that eukaryotic Ras GTPase superfamily almost certainly is not monophyletic but actually originates from 2 different sources. It seems that whereas eukaryotic Arf group ancestor was truly inherited from LUCA's Rup-relative, a bacterial Rup ancestor was later horizontally transferred into eukaryotic lineage and after diversification gave rise to second eukaryotic group comprising Ras, Rho, Ran, and Rab (Figure 2). A good source of genomic material for such horizontal transfer might have been mitochondrion.

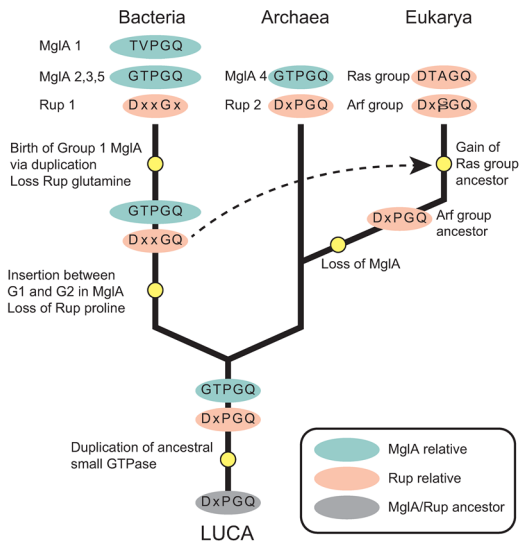

Figure 2 – Small GTPase clade in LECA is not monophyletic. Whereas Arf group has been directly inherited from LUCA, ancestor of other Ras subfamilies was probably acquired in a horizontal gene transfer.
(Wuichet & Søgaard-Andersen 2015)

Another recent phylogenetic study concentrated solely on eukaryotic Ras superfamily members' G-domains. Input data was sourced from 11 proteomes ranging from *Plasmodium falciparium* (*Alveolata*) to *Homo sapiens* (*Vertebrata*). Resulting phylogenetic tree suggests, that closest to the root is a signal recognition particle receptor subunit β. This protein is a part of signal recognition receptor responsible for targeting nascent secretory proteins into ER. Arf GTPase family, responsible for membrane trafficking is second closest to the origin. Next diverging clade is Ras GTPase family –

responsible for transduction of extracellular sensory input. Then a Rho GTPase family, facilitating cytoskeleton reorganization. Even further from the root lay sequences belonging to Ran family, responsible for regulation of nuclear trafficking. Rab GTPases are the latest evolutionary step. All these clades, however were already present in the last common eukaryotic ancestor (LECA) (Rojas et al. 2012).

Following text tries to very quickly surmise purpose of subfamilies within the Ras superfamily in yeast, animal, and plant cell. It is nowhere near exhaustive as its purpose is more to illustrate the multitude of functions. Rab subfamily is explored more thoroughly, since it is closely related to topic of this thesis. However, due to its great divergence attention is mainly paid to mechanisms shared by all Rab GTPases and role of Rab GTPases in exocytosis.

1.1.2.1. Arf GTPases

ADP-ribosylation factors (ARFs) are present in all eukaryotic lineages. They are most known as regulators of vesicle budding in both exocytic and endocytic pathways. Their G-domain has a specific N-terminal amphipathic helix that is mostly myristoylated. In GTP-binding form, the helix allows for close association with membranes and the ARFs then trigger assembly of vesicular coat complexes. True ARF GTPases recruit COPI and clathrin while SAR GTPases, a clade within the ARF GTPase family, recruit COPII. Both groups also recruit lipid modifiers, such as phospholipases or lipid kinases (Donaldson & Jackson 2011). In addition to these groups, ARF-like (ARL) proteins exits. These generally are not N-terminally myristoylated and possess diverse functionality such as regulation of microtubule dynamics, lipid trafficking or mitochondrial morphology (Kahn et al. 2014).

ARF and SARI proteins are present in all eukaryotic cells in varying numbers. Yeast genome encodes 3 ARFs and 1 SAR1, while mammalian genome encodes 6 and 2. Plant cells possess genes for 12 ARFs and 4 SAR1 proteins. The increased number of ARF and SAR1 genes points at functional specialization in plant vesicular trafficking (Yorimitsu et al. 2014).

1.1.2.2. Ras GTPases

The first representatives of the superfamily - Rat sarcoma (Ras) proteins - were discovered around year 1980. They were detected as oncogenes in rat-derived sarcoma viruses and their orthologs have since been found in all eukaryotic lineages with the exception of plants. Ras GTPases function as signaling nodes at plasma membrane. The association with plasma membrane is facilitated by either geranylgeranylated or prenylated cysteine residue at C-terminal CAAX box as well as palmitoylated upstream cysteine. CAAX box is a conserved

motif targeted by either farnesyltransferase (FTase) of geranylgeranyltransferase I (GGTase I) enzymes[1] (Lane & Beese 2006). Some Ras family members do not have this upstream cysteine – these instead rely on a region rich in basic amino acids (Hall 1990). Ras GTPases are activated by extracellular stimuli and through their effectors launch signaling cascades resulting in gene expression changes.

S.cerevisiae has 4 paralogs – 3 of them experimentally confirmed. Their role is in determination of budding site (Park et al. 1997) and glucose-induced cAMP signaling (Conrad et al. 2014).

39 paralogs encoded by 36 genes have been discovered in *H.sapiens* genome (Wennerberg 2005). They regulate cell proliferation, differentiation, morphology, and apoptosis. Mutations in genes coding for Ras GTPases and related regulators are involved in many human cancers and are important focus of therapeutic research (Takai et al. 2001).

Surprisingly, no Ras orthologs have been found in plants, even though it was present in the LECA. It is possible that their function is compensated for by greatly expanded Rho GTPase family (Winge et al. 2000).

1.1.2.3. Rho GTPases

Rho GTPases are present in all eukaryotic cells. They are often associated with plasmalemma and/or other endomembranes and are implicated in cellular signaling, cytoskeletal and membrane reorganization and polarized growth. Just like Ras GTPases, they are prenylated on their C-terminal cysteines by FTases or GGTases.

S.cerevisiae has 6 Rho GTPase paralogs (Cdc42, Rho1 - Rho5), of which only 2 are essential: Cdc42 is involved in controlling cell polarity. It functions as a master regulator, accumulating in presumptive bud site, organizing the actin cytoskeleton and activating polarized secretion. It has been proven to interact with exocyst subunit Sec3. Rho1 is the second essential yeast Rho GTPase. It is present in areas of polarized growth and is necessary for cell-wall synthesis and actin cytoskeleton regulation (Perez & Rincón 2010).

Mammalian Rho GTPases are a diverse family, encompassing 22 paralogs, which can be divided into 8 subgroups: Cdc42, Rac, Rho, Rnd, RhoD, RhoH, RhoBTB and Miro. Mammalian Cdc42 is very homologous to the one in yeast and in GTP-binding state participates in filopodia formation. Members of Rho subgroup trigger stress fiber formation, when

[1] C stands for cysteine. A for aliphatic amino acid. X for either Ala, Met, Ser, or Gln (resulting in farnesylation); or Leu, and Phe (geranylgeranylation)

activated. Rnd subgroup, on the other hand, causes stress fiber loss. (Sorokina & Chernoff 2005).

A.thaliana's 11 Rho GTPases are titled ROPs (Rho-related GTPase of plants). Just as in Metazoa and Yeast, ROP GTPases are involved in signal transduction and regulation of cell polarity and morphogenesis. They have no clear homologs to subfamilies reported in mammals but their sequence bears closest resemblance to that of animal Rac subgroup. They sometimes lack the prenyl group and can be palmytoilated (Yalovsky 2015). The 11 members have various expression/localization profiles with some being expressed only in flowers and pollen, or roots and stem only, and others with fairly ubiquitous expression (Vernoud et al. 2003). They have been found to control tip growth in development of root hairs and pollen tubes and formation of epidermal cell shape, all through regulated assembly of actin (Fu et al. 2001; Molendijk et al. 2001; Fu et al. 2002). ROP GTPases signaling is also implicated in negative regulation of ABA signaling and the other way around (Miyawaki & Yang 2014).

1.1.2.4. Ran GTPases

Ran GTPases (Rans) are commonly known as regulators of transport into the nucleus through nuclear pore complexes (NPCs). Unlike other Ras superfamily members, they do not possess prenyl anchors and do not associate with membranes in any part of their GTPase cycle. Rans indirectly facilitate import of cargo tagged with nuclear localization signal (NLS) and export of cargo to cytosol, both by association with specific effectors in each nucleotide conformation. Nuclear localized GDP-bound Ran is converted to GTP-bound form by Ran GEF protein. GTP-Ran then facilitates disassembly and outward recycling of adaptor proteins required for import of the NLS-cargo. Outside the nucleus, it is swiftly converted to GDP-Ran by Ran GAP proteins and imported back to the nucleus by association with its exclusive carrier and the cycle starts anew. Each NPC is capable of facilitating transport of ~100 to 1000 cargoes per minute by this mechanism (Stewart 2007). Export functions on the same basis. Outward bound cargo binds to exportins, which associate with GTP-Ran and translocate together through the NPC. After GTP hydrolysis, the complex disassembles.

Other functions of Ran GTPase are regulation of spindle assembly and reconstitution of nuclear envelope. In both of these, Ran functions through the same effector as in nuclear import, importin β (Dasso 2002).

All eukaryotes with the exception of plants carry only one Ran GTPase isoform. Most plant species have three isoforms.

1.1.2.5. Rab GTPases

Rab GTPases (Rabs) are present in all eukaryotic organisms and are the most populous branch of Ras superfamily. They consist of two domains – conserved catalytic G-domain and C-terminal hypervariable domain (HVD). In addition to being almost universally double prenylated at their C-terminal cysteine residues, they can be reliably discerned from other small GTPases by five conserved motifs in their G-domain (RabF1–RabF5)(Pereira-Leal & Seabra 2000). It was originally thought, on basis of experiments with targeting of hybrid Rabs with swapped HVDs, that the HVD serves as an addressing signal and is responsible for targeting into specific membranes (Chavrier et al. 1991), but it was later found that multiple regions within Rab GTPases play role in recruitment to the correct membrane (B. R. Ali et al. 2004).

Rabs are known to be regulators of intracellular vesicular transport and have been implicated in all steps of both secretory and endocytic pathways. There is evidence for their involvement in regulation of vesicle formation, transport to a target membrane, vesicle docking and fusion. They regulate this wide array of steps through recruitment of various effector complexes, when activated by GEFs. Amount of Rab GTPases differs significantly across organisms – Yeasts have just 11, human cells more than 70, and Arabidopsis 57 members (Pereira-Leal & Seabra 2001; Lazar et al. 1997).

Rabs are first synthesized as soluble proteins in the cytosol. They are afterwards modified by Rab geranylgeranyl transferase (RabGGT) typically by two sequential prenylations of C-terminal cysteine residues via thioether bond. The RabGGT does not depend on Rabs' specific C-terminal amino acid motif, as is the case with modification of Ras and Rho GTPases by GGTase-I or FTase. It instead recognizes a Rab GTPase:Rab escort protein (REP) heterodimeric complex that forms in cytoplasm after Rabs' translation (Durek et al. 2004). REPs belong to the REP/GDI superfamily. Both REPs and Guanine dissociation inhibitors (GDIs) share specific 2-domain composition: Larger domain I mediates interaction with the GDP-bound Rab, while the smaller domain II contains a hydrophobic pocket for the geranylgeranyl anchors. REPs, unlike GDIs, have intrinsic affinity for RabGGT, thanks to 2 amino acid substitutions in domain II. REP has different attraction towards non-, mono-, and double-prenylated Rab GTPases with high, highest, and lowest affinity respectively. After second prenylation, separation of Rab:REP from RabGGT occurs and the REP deposits the double-prenylated Rab into the membrane (Hutagalung & Novick 2011). Experiments with Rabs modified in their C-terminal domains (deletion and/or rearrangement of cysteines)

showed, that omission of 1 geranylgeranyl moiety disrupts Rabs' targeting and they then by default accumulate in ER membrane (Gomes et al. 2003).

After Rabs' incorporation into the target membrane, Guanine nucleotide exchange factors (GEFs) swap bound GDP for GTP and thus facilitate their activation and prevent extraction by GDIs. Activated Rabs can then recruit various effectors, such as promoters of coat assembly, molecular motors, tethering complexes, etc., and through them trigger budding, transport, tethering, or fusion of the membranes they reside in. Necessity for high spatiotemporal specificity of Rab activity dictates a need for means by which Rabs can be deactivated and recycled back into appropriate membrane and protein context after their job is done. GTPase activating proteins (GAPs) are responsible for the deactivation by accelerating hydrolysis of GTP to GDP. Doing this disrupts interactions between effectors and Rabs. Mechanism of Rab GAP-assisted hydrolysis is described later in the text.

GDIs with GDI displacement factor (GDF) proteins facilitate the recycling phase of the functional cycle. Unlike REPs, which can only deposit into the membrane, GDIs have very high affinity for both mono- and double-prenylated Rabs and are capable of extracting the two geranylgeranyl anchors from membranes. They can then mask the hydrophobic moieties from aqueous environment and move as a complex away from the membrane (Goody et al. 2005). GDIs are capable of reinserting the Rabs in their target membranes as well. However, GDFs are required to overcome GDIs' promiscuous affinity towards Rabs and to enable separation of the complex on the specific membrane. Mechanism of this process is not yet established, but

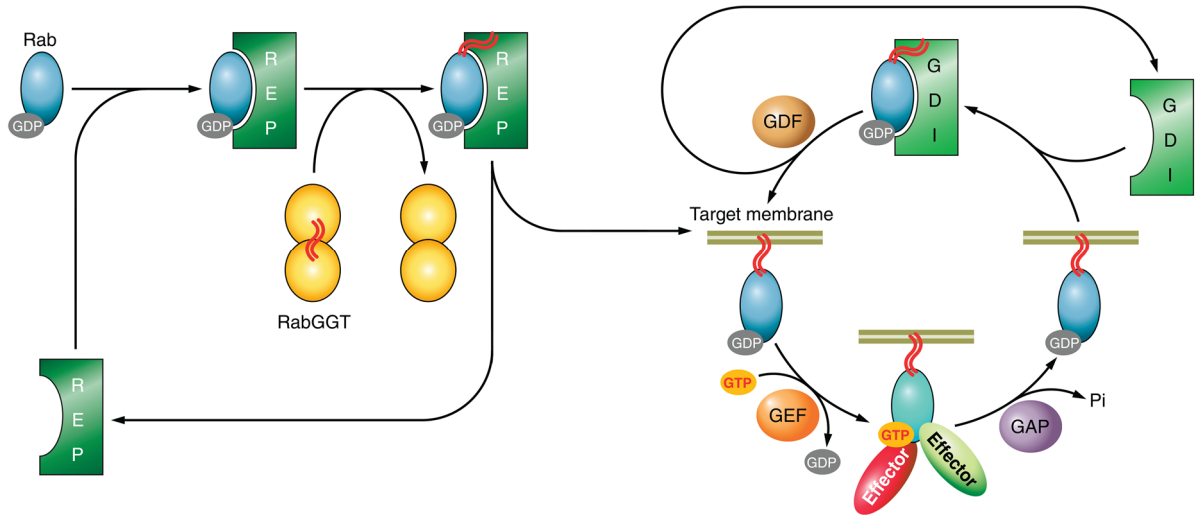

Figure 3 - Rab GTPase cycle - Newly synthesized GDP-bound Rabs are associated with REP and presented to RabGGT. This enzyme facilitates their prenylation and REP then deposits them to a target membrane. Functional cycle of Rab GTPase involves its cycling between cytosol and membrane. GEF can activate the GTPase by swapping the bound GDP for GTP. Active Rab then recruits various effectors. The GTPase can be deactivated by GAP. This is usually followed by its extraction from the membrane by GDI. Reinsertion into the membrane is facilitated by GDF, capable of releasing the Rab from the GDI. Modified from (Hutagalung & Novick 2011)

mammalian Yip3/PRA1, integral membrane protein, and its rice ortholog OsPRA1 have been uncovered as GDFs in the endocytic pathway (Bahk et al. 2009; Sivars et al. 2003). After reinsertion, and GDI dissociation, they can be once more activated by GEFs and the cycle starts anew (Figure 3) (Hutagalung & Novick 2011). This is closely related to a central idea of suggested GEF-dependent targeting of Rabs: GDIs let Rabs sample various membranes by cyclic insertions and removals, until they place the Rab into vicinity of its respective GEF. It then activates it and prevents further interaction with GDI (Barr 2013).

It was thought in the past, that each specific Rab GTPase facilitates only a particular step on their designated membrane, such as transport from Golgi, or fusion of vesicles (Pfeffer 1996), but current knowledge advocates multi-functional roles for Rab GTPases. First, they can direct their associated vesicles to different target membranes depending on the context of their upstream GEF. Second, a GTPase specific for a certain pathway can facilitate more than a single step by sequential recruitment of different effectors. An example of the former is yeast Rab Ypt1. It can be activated by 2 different versions of a TRAPP(I or III) complex and thus switch between exocytic, or autophagic pathway from ER (Kim et al. 2016). The latter is true for many Rabs and is critical for execution of Rab cascades, which are described later in the text.

1.1.2.5.1. Exocytosis in Yeast

First Rab GTPase was discovered in yeast genetic screens for mutants defective in protein secretion. Temperature sensitive *Sec4-8* cells suffered from arrested bud growth and were found to accumulate secretory vesicles at non-permissive temperatures. Sec4p was determined to be thus required in late stage of exocytosis, but since the mutant phenotype related to plasma membrane, it was simply considered to be of the Ras or Rho GTPases (Salminen & Novick 1987). Another positive exocytic regulator GTPase Ypt1p, however, was found to be required for ER to GA vesicle transport and pointed to existence of a novel branch (Segev et al. 1988). Two more exocytic Ypts were found in coming years – Ypt31p and Ypt32p. These two GTPases exhibited 81% of sequence identity and 90% similarity. Mutants in one or the other did not exhibit any phenotype. However, double mutants were not viable. Conditional-lethal *ypt31-1* mutants in an *ypt32* null background suffered from accumulation of Golgi-derived membranes (Benli et al. 1996; Jedd et al. 1997). These 4 proteins – Ypt1p in ER-to-Golgi; Ypt31/32p in Golgi to early secretory vesicles; and Sec4p in interaction with tethering complex exocyst, together form the Rab GTPase set facilitating secretory pathway (Guo et al. 1999).

1.1.2.5.2. Plant Rab GTPases

The 57 Rabs present in *A.thaliana* segregate into 8 clades (Rab A to Rab H) which cover all 6 basic yeast clades plus 2 from animals (Rab2 and Rab18). 33 of 41 mammalian Rab GTPase subclasses have no homologs in *Arabidopsis* genome, but some of the *Arabidopsis* clades are much more populous and diverse than their analogous groups in animals. Those diversified clades are: RabA with 6; RabC, RabD and RabF with 2; and RabG with 3 subclasses. This diversification seems to be well-conserved among angiosperms (Rutherford & Moore 2002). I will give very brief digest on known functions of each clade with the exception of RabA and RabE. These two will be analyzed separately further below because of their relation to my thesis' experimental section.

RabB clade is homologous to animal Rab2 and is probably involved in transport between ER and *cis*-Golgi, since dominant negative NtRab2 mutants in *Nicotiana tabacum* suffered from arrested transport from ER to Golgi and slowed growth of tobacco pollen tubes. Fluorescently marked NtRab2 also localized to Golgi (Cheung et al. 2002). RabC is homologous to animal Rab18, which facilitates endocytosis, but sequences responsible for subclass specificity differ between the animal and plant homologs, so similar function cannot be definitely expected (Rutherford & Moore 2002). Function of RabD is similar to the one of its yeast and mammalian homologs, Ypt1 and Rab1 respectively – transport from ER to Golgi. This was confirmed by experiments with dominant negative AtRabBD2a, which caused retention of Golgi-targeted GFP markers in the ER (Batoko et al. 2000). RabF is related to mammalian Rab5 and 22 and yeast Ypt51/52/53, which are all involved in endocytosis. Both F1 and F2 Rabs localize to putative endosomal or prevacuolar structures. RabF1 is an interesting exception to the rule of C-terminal prenylation, instead being N-myristoylated and palmitoylated at its N-terminus with large part of the common HVD missing. It is implicated in transport from endosomes to PM (Ueda et al. 2001). *Arabidopsis* RabG3f is homologous to Ypt7 and Rab7 and

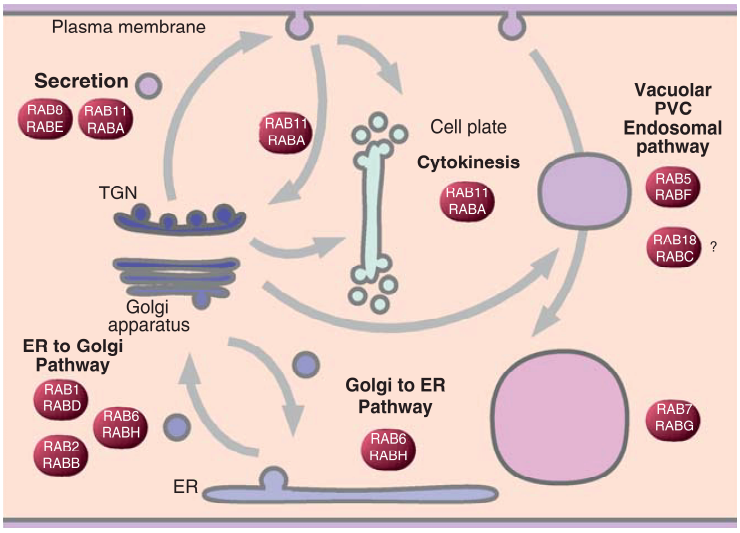

Figure 4 – Localization of Rab GTPase clades in plant cell. Upper title belongs to mammalian homologous clade, bottom is the plant one.
Modified from (Saito & Ueda 2009)

localizes to prevacuolar compartments (PVC) and tonoplast. Expression of dominant negative RabG3f leads to enlarged PVC (Cui et al. 2014). RabH clade is homologous to Ypt6p and Rab6. It might therefore facilitate Rab6-like anterograde transport through Golgi and from Golgi to ER. Or, since RabH1b can complement ypt6-deficient yeast, it is possibly responsible for recycling from early endosomes to TGN (Bednarek et al. 1994) (Figure 4).

1.1.2.5.3. Arabidopsis RAB GTPases in Exocytosis

RabA sub-family of plant GTPases is functionally homologous to animal Rab11 and yeast Ypt31/32. In general it functions between TGN and plasmatic membrane. But RabA is greatly diversified in plants – in Arabidopsis its members are encoded by 26 genes (of total 57 plant Rabs) suggesting need for greater control over protein and membrane trafficking processes.

RabA1 clade in *Arabidopsis* consists of 9 members. Expression profiles for member RabA1a – RabA1d show whole-plant abundance. Rabs A1f to A1i are primarily expressed in flowers and only transcriptomic data exists. RabA1e is root specific.

RabA1a expression was found to be induced by auxin. It co-localized with FM4-64 stained endocytic vesicles. RabA1a seems to be necessary for auxin signaling, since raba1a mutants did not increase transcript levels for auxin-responsive genes, whereas RabA1a over-expressing line increased the transcript levels even in absence of IAA or 2,4-D. The *raba1a* mutant exhibited increased primary root elongation and branching of lateral roots compared to WT, whereas RabA1a over-expressing line suffered from decreased root elongation and only very limited branching when cultivated on 0.01µM IAA medium (Koh et al. 2009).

RabA1b localizes to mobile punctate structures near TGN and its constitutive active form was shown to also decorate plasma membrane. RabA1b-labeled vesicles were shown to be dependent on actin filaments for their movement to PM. The vesicles near TGN appeared in close arrangement to endosomes labeled with RabF1 (ARA6) - Rab responsible for transport from multi-vesicular endosomes to the plasma membrane. Expression of dominant negative RabA1b led to increase in size of ARA6 endosomes. Members of RabA1 clade seem to be important for salt stress tolerance, since quadruple *raba1a* - *raba1d* mutants, as well as dominant-negative mutants in RabA1b, suffered from hypersensitivity to salt-induced stress (Asaoka et al. 2013).

RabA1d localizes to early endosomes and TGN and is involved in cell plate formation. During early stages of cytokinesis, it is present at the entire cell plate, later it becomes restricted to margins of the expanding cell plate. Areas of presence were determined to be those with

active vesicle fusion. RabA1d was also observed in tips of growing root hairs labeling moving vesicles. These were found to be dependent on actin cytoskeleton (Berson et al. 2014).

RabA2 subclass contains 4 homologs. Expression profiles in roots show activity in wide range of cell types for RabA2c and A2d, whereas A2a and A2b are restricted to specific locations: A2a is upregulated in root meristem, A2b in columella. All RabA2 members decorate the same post-Golgi membrane domain. The labeled compartments were found to partly overlap with *trans*-Golgi, marked by VHA-a1 and were defined as early endosomal for their quick intake of FM4-64 dye. RabA2 and A3 proteins label cell plates between phragmoplast microtubules in either disc structure in early stages or expanding ring appearance in later stages of cytokinesis. RabA2a accumulation at the division plane started at telophase. GDP-binding mutant RabA2a was more localized in cytoplasm and Golgi, whereas GTP-binding hydrolysis-deficient RabA2a decorated mainly plasma membrane besides also increasing presence in cytoplasm. Expression of dominant negative RabA2a resulted in inhibition of cytokinesis (Chow et al. 2008).

RabA3 intracellular localization in root was identical to that of RabA2, including the behavior during cytokinesis. Its expression, however, was limited to epidermis (Chow et al. 2008).

RabA4 clade is represented by 5 gene homologs in Arabidopsis. RabA4a has not been studied in any detail besides expression patterns from microarrays and RabA4e is considered a pseudogene.

RabA4b is expressed ubiquitously in plant tissue. Fusion of the protein with fluorophore localized it to punctate randomly distributed post-Golgi compartments. Fractionation experiments determined that the compartments are distinct from TGN. Specific localization of the compartments was observed in tips of growing root hair cells. Mature root hairs lacked this localization and mutant plants with defective root hair morphology had either improper or no localization of EYFP-RabA4b. Same was true for plants with disturbed actin organization. T-DNA insertional mutants for RabA4b did not suffer from polarized growth issues – probably because of redundancy within the RabA4 subclass (Preuss et al. 2004). GTP-bound RabA4b was found to interact with two members of phosphatidylinositol 4-OH kinase family, PI-4Kβ1 and PI-4Kβ2 in Y2H and biochemical assays. Co-localization of RabA4d with the PI-4Kβ1 was also confirmed by fluorescence microscopy. (Preuss et al. 2006). Electron tomography of RabA4b and PI4Kβ1 showed, that they co-localize with budding secretory vesicles on Golgi-associated and free TGN cisternae, but not with *trans*-Golgi. RabA4b secretory vesicles were also observed proximal to and merging with PM (Kang et al. 2011). Another RabA4b

interacting protein was discovered in 2015. PLANT U-BOX13 (PUB13) important for salicylic acid-mediated defense interacts with both the Rab and the PI4Kβ1/β2 lipid kinases. It seems that PUB13 functions as a negative regulator of SA-mediated defense, since *Pub13* mutants have constitutively activated defense against pathogens (Antignani et al. 2015).

RabA4c is implicated in stress induced callose deposition in *Arabidopsis* through PMR4 as its effector. RabA4c upregulation is induced by infection with pathogenic fungi *Golovinomyces cichoracearum* (Powdery Mildew). It was found that over-expression of RabA4c under 35S constitutive promoter prevents infection by the pathogen because of accelerated callose deposition by callose synthase PMR4. Over-expression of dominant negative RabA4c led to no such effect. Physical interaction between the Rab and the stress-induced callose synthase was confirmed by FRET (Ellinger et al. 2014). RabA4c in *Arabidopsis* has also been implicated in membrane trafficking during recovery from chilling stress (Einset et al. 2007).

RabA4d is very similar to RabA4b, but its expression is limited to flowers and pollen. It was observed in similar tip-localized manner in *Arabidopsis* pollen tubes. T-DNA insertion lines for RabA4d exhibited shortened tubes with bulging in the tip region. Time-lapse microscopy found the *raba4d* pollen tubes to grow substantially slower than in WT plants. Complementation of RabA4d with RabA4b partially rescued the growth defects. As in root hairs, PI4Kβ1 was determined as the effector protein and was required for normal growth. Deposition of pectin, but not of callose was affected in *raba4d* mutant in comparison with WT plants (Szumlanski & Nielsen 2009).

RabA5 and RabA6 have not been studied so far and only transcriptomic data exist, showing that RabA5a to RabA5e isoforms each have different expression profiles (Winter et al. 2007).

RabE clade is homologous to yeast Sec4 and mammalian Rab8 and Rab10, and is believed to promote post-Golgi secretory trafficking. In *A.thaliana* it is represented by 5 isoforms a – e sharing 86% sequence identity and ubiquitous tissue and developmental expression. The exceptions are RabE1d and RabE1e - the former upregulated in rosette leaves and both downregulated in pollen. Transient transformation experiments in *N.tabacum* revealed that RabE1d was shown to act downstream of RabD and dominant-inhibitory (NI) mutant caused accumulation of secretory GFP (secGFP) in ER and Golgi (Zheng et al. 2005).

Localization of RabE1d was determined to be mainly on plasma membrane and partially on Golgi as well. GTP-restricted form did not associate with Golgi, apparently targeting tonoplast instead. 4 out of 4 tested isoforms (-a, -b, -d, -e) were found to interact in Y2H with

a pathogen effector protein AvrPto of *Pseudomonas syringae* (*P.st*) in *Arabidopsis,* confirming an interaction previously observed in tomato (*Solanum lycopersicum*) as well. AvrPto interacted with only WT or constitutively active RabE1d. No interaction was observed for GDP-restricted form. Infection of plants with *P.st* resulted in focused accumulation of RabE1d at the PM of mesophyll cells. Plants with expressed constitutively active form gained resistance against the infection. Plants suffering from RabE1d co-suppression had wavy leaves caused by shortened midrib and normal leaf lamina and their overall size was reduced when compared to WT (Speth et al. 2009).

Phosphatidylinositol-4-phosphate 5-kinase 2 (PIP5K2) was identified as an interactor of all RabE members. The strongest interaction in Y2H screen with RabE1d was observed with GTP-restricted form, weaker with WT form, and the weakest with GDP-restricted form. MORN (membrane occupancy and recognition nexus) domain of PIP5K2 is necessary and sufficient for the interaction. *In vitro* kinase activity of PIP5K2 against PtdIns(3)*P* and PtdIns(4)*P* was 5x higher when interacting with RabE1d than alone. PIP5K2 turned out to be PM localized, when expressed in full-length, and cytosol localized when missing the MORN domain. RabE1d was found to localize at PM in cells over-expressing PIP5K2 and at Golgi in cells not transformed with PIP5K2. MORN domain was found to be responsible for this interaction as well (Camacho et al. 2009).

Despite the large degree of sequence identity between the isoforms, different members of RabE clade might facilitate different functions within a cell. Such is apparently the case for RabE1c. Its GTP-binding form has been recently detected as a binding partner of PEX7. PEX7 is a cytosolic receptor responsible for peroxisomal import of proteins containing PTS2 sequence motif. RabE1c has been implicated in targeting PEX7 for degradation in presence of overexpressed GFP-PEX7. Such effect was not replicated in WT plants and thus physiological meaning of this remains uncertain (Cui et al. 2013).

1.2. GAP PROTEINS

Although small GTPases possess everything necessary to form a GTP hydrolytic site and demonstrably can perform this catalytic reaction by themselves, they are not very quick about it. Rate of hydrolysis among small GTPases varies in orders of magnitude from 5×10^{-6}/s for Rab6a to 1×10^{-3}/s for Cdc42. Insufficient rigidity of the catalytic site means that chance of proper arrangement of necessary residues is quite low. Nevertheless, it is generally faster than spontaneous GTP hydrolysis in water (Vetter 2014). Interaction with multi-domain proteins termed GAPs greatly accelerates this process. GAPs' primary structure is not universally conserved, but each GTPase subfamily's GAP proteins share a conserved interacting domain accompanied by an array of other domains. Functionally, the underlying principle of GTPase activation remains the same for all GAPs. Interaction of GAP with the GTP-bound enzyme stabilizes the structure of the catalytic site and allows for nucleophilic attack by a water molecule to the γ-phosphate of the bound GTP (Figure 5). Ras- and RhoGAPs utilize an arginine residue, termed arginine finger, which displaces other water molecules from the G-domain active site and in co-operation with Mg^{2+} co-factor changes conformation of the GTP to one favoring the reaction. It also stabilizes a glutamine residue in switch II-located G3-motif of Ras/Rho GTPase. The stabilized glutamine is responsible for orienting of the water molecule responsible for the attack (Scheffzek 1997; Rittinger et al. 1997; te Heesen et al. 2007; Rudack et al. 2012). Arf GTPase family utilizes different GAPs for its Arf/Arl/Sar clades. GAPs for Arf and Arl again utilize a combination of their arginine finger and G3-motif glutamine (Ismail et al. 2010; Veltel et al. 2008). GAPs for Sar also utilize the finger, but instead of glutamine, Sar employs a histidine residue to stabilize the attacking water molecule (Bi et al. 2002). RanGAP stabilizes switch II domain and uses an asparagine residue to stabilize the G3-motif glutamine, but it does not directly interact with the GTP molecule as is the case in the previous complexes (Seewald et al. 2002). Just as other subfamilies' GAPs have their conserved catalytic cores, so do Rab GAPs. Their conserved α-helical TBC1 domain (Named after proteins Tre-2, Bub2 and Cdc16 where it was observed for the first time) is ~200 amino acid long and contains 6 conserved motifs (A to F). Motifs A (RxxxW), B (IxxDxxR), and C (YxQ) are almost

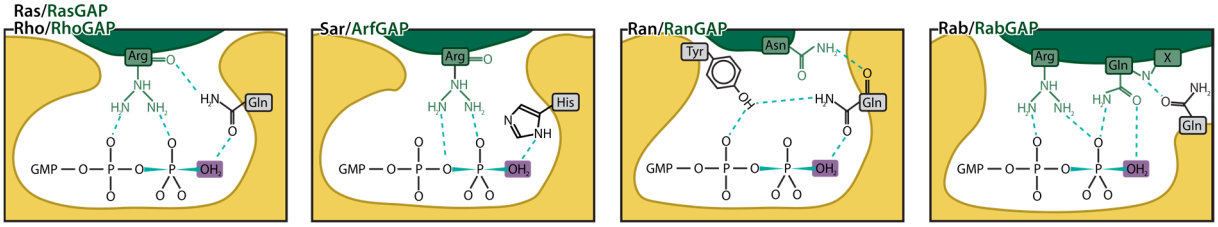

Figure 5 – Interactions within the catalytic site between GTPases and their respective GAPs during transition-state of GTP hydrolysis. retraced from (Bos et al. 2007)

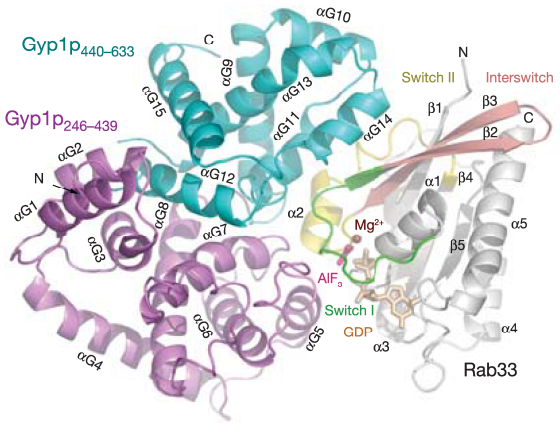

Figure 6 – Gyp1p TBC domain in complex with Rab33. AlF_3 is used to simulate transition state before GTP hydrolysis. (Pan et al. 2006)

universally conserved (Neuwald 1997). Crystal structure of Gyp1p, a yeast YptGAP, revealed that it is composed of 16 α-helices that together form V-shape with apparent Ypt binding site in the groove. The structure does not superimpose with other subfamilies' known α-helical GAP domains (Rak et al. 2000). Co-crystalization or Gyp1p with Rab33a (Figure 6) elucidated the mechanism of interaction: Rab GAPs function similarly to Ras- and RhoGAPs. The catalytic arginine finger is located in TBC motif B within the GAP groove and inserts into Rab's catalytic site. Most Rab GTPases possess the conserved G3-motif glutamine, but it serves different purpose than in Ras or Rho GTPases. Here it helps to stabilize interaction in the Rab:Rab GAP heterodimer. GAP's TBC domain provides its own glutamine in motif C and utilizes it during the transition-state of GTP hydrolysis for coordination of the water molecule (Pan et al. 2006).

Arginine finger in the TBC domain is not absolutely conserved. There are examples in mammalian cells of TBC-bearing proteins with the finger missing. TBC1D3 substitutes the arginine with glycine. It, however, still possesses weak GAP activity towards RAB5A (Hodzic et al. 2006) but only at 10-fold molar excess of the GAP, so it probably operates more as Rab effector *in vivo* (Frittoli et al. 2008). Similar to this is probable effector Tre2 with missing arginine and glutamine catalytic residues. It is very similar to RN-Tre, the functional GAP of Rab5, but even restoring mutations in the catalytic site did not reinstate its potential GAP activity (Bizimungu & Vandenbol 2005). The fact, that the loss of arginine finger effectively turns the TBC-protein from GAP to putative effector is commonly utilized in interaction studies. Substitution of the arginine for e.g. alanine prevents hydrolysis of GTP and thus often results in prolonged interaction of GTP-bound Rab:Rab GAP complex, which is detectable in yeast two hybrid tests (Itoh et al. 2006).

Non-canonical Rab GAPs exist as well. Rab3GAP activates GTP hydrolysis in Rab3 family, while lacking anything resembling TBC domain. It functions as a heterodimeric complex of p130 catalytic, and p150 non-catalytic subunit (Fukui et al. 1997; Nagano et al. 1998). Despite dissimilarity to traditional Rab GAPs, it employs catalytic arginine finger (Clabecq et al. 2000).

Placement of TBC domain within Rab GAPs' is not universally constrained to certain part of the protein. In different representatives of the group, the domain can exist on its own, or be flanked on either side by sequences providing specificity towards a certain Rab, facilitating interaction with lipids, or possessing additional enzymatic ability (Bos et al. 2007).

Untangling of Rab GAP enzyme:substrate specificity is tricky. First issue stems from the fact that detection of accelerated GTP hydrolysis through *in vitro* biochemical assays between potential GAP and established Rab GTPase does not mean that the pair interacts *in vivo* as well. The most one can infer from the result is that the GAP is capable of accelerating GTP hydrolysis. And even that might not be physiologically relevant, if molar ratio of GAP to Rab crosses certain threshold (Frittoli et al. 2008). A good example of a broad *in vitro* substrate specificity is *S.cerevisiae*'s Gyp1p. In assays it was found to accelerate hydrolytic activity of Ypt1p, Sec4p, Ypt7p, Ypt51p and even hydrolysis-deficient Sec4p(Q79L) (Du 1998). However, *in vivo* activity of this Rab seems to be specific towards Golgi-located Ypt1p (Du & Novick 2001). Misleading negative or positive results can also arise from experimenting with partial GAPs, both in Y2H screens as well as GTP hydrolysis assays since the absence of amino-acids flanking the catalytic TBC domain impacts both binding specificity and rate of hydrolysis (Fuchs et al. 2007; Albert 1999).

1.2.1. Rab GAPs in exocytosis

In yeast, multiple GAPs taking part in secretory pathway have been identified. Previously mentioned Gyp1p is GAP for Ypt1p – the Rab facilitating transport between ER and Golgi (Du & Novick 2001). Gyp5p and Gyp8p are another GAPs potentially implicated in regulation of Ypt1p (De Antoni et al. 2002). Gyp6p is active near Golgi – It stimulates hydrolytic activity of Ypt6 (Suda et al. 2013). Already mentioned Gyp5p with its closest non-catalytic homolog Gyl1p take part in polarized exocytosis and were observed at PM and post-Golgi membranes (Chesneau et al. 2004). Msb3p/Gyp3p and its homolog Msb4p/Gyp4p probably serve as GAPs for Sec4p, the Rab implicated in late steps of secretory pathway (Gao et al. 2003).

In mammals, the non-canonical Rab3GAP interacts with Rab3a, a GTPase implicated in exocytosis through neuronal and endocrine secretory vesicles (Fukui et al. 1997). Another TBC-GAP acting on this Rab *in vivo* near PM is FLJ13130, which also seems to interact there with other Rabs, among them Rab27a responsible for secretion of exosomes and Rab35 involved in recycling of endosomes to PM (Ishibashi et al. 2009; Itoh & Fukuda 2006). Rab11, a GTPase participating in final stages of secretory pathway is stimulated by EVI5 (Dabbeekeh et al. 2007). Rab8, implicated besides other pathways in trafficking from TGN to PM interacts

with GAP TBC1D17 (Vaibhava et al. 2012). Rab1, responsible for trafficking from ER to Golgi is negatively regulated by TBC1D20 (Sklan et al. 2007).

1.2.2. Plant GAPs

Scientific knowledge regarding plant Rab GAPs is extremely limited. OsGAP1, GAP in rice with TBC domain close to C-terminus and predicted transmembrane domain near N-terminus, was found to interact with RabA homologs OsRab8a, OsRab9b, OsRab8c, and RabE;Ypt31p/32p homolog OsRab11 in Y2H screen. In GTP hydrolysis assay, OsGAP1 significantly increased intrinsic activity of OsRab11 and OsRab8a and mutation of its arginine finger to alanine disabled this ability. N- and C-terminal truncated versions were tested against OsRab11 and OsRab8a in Y2H screen and GTP hydrolysis assay. TBC domain with the entire C-terminal sequence were necessary for interaction and activity. Omission of the N-terminal sequence posed no problem. OsGAP1 was found to facilitate recycling of OsRAB11 through OsGDI3 in yeast expression system. Cellular localization of OsGAP1 in *Arabidopsis* protoplasts points at TGN and PVC, since it was not affected by BFA. Overexpression of OsGAP1 without arginine finger (OsGAP1RA) with GFP-PM marker exhibited arrested transport to the PM and accumulation of the marker in TGN. Overexpression also blocked transport from Golgi to the vacuole. Overexpression of OsRab11 (but not of OsRab8a) relieved the inhibitory effect (Heo et al. 2005). RabGAP22 (At5g53570) was implicated in defense of *Arabidopsis* against fungal pathogen *Verticillium longisporum*, since *rabgap22-1* mutants lacked proper resistance response. It is expressed in root meristem, vascular tissue, guard cells of stomata, and trichomes. RabGAP22 was found to interact with serine:glyoxylate aminotransferase (AGT1) and to localize to peroxisomes during the pathogen infection. Stomata-closure response was impaired in the null mutant. During no-stress conditions, the protein distinctly localized into the nucleus. (Roos et al. 2014). A study in *N.benthamiana* plants identified a putative TBC-domain protein, which possibly assists with intercellular transport of Bamboo mosaic virus. The study does not include any pointers towards the identity of the protein (Huang et al. 2013).

2. HYPOTHESES AND GOALS

In this work, my interest was focused on one of 22 Arabidopsis GAPs - At2g39280 (*GAP2*). This particular GAP was chosen based on preliminary data collected by Dr. Hála showing potential interaction with RAB GTPases (unpublished data). Moreover, one intron and one exon T-DNA insertion lines are available. Homozygotes of the 15th exon insertion line (*GAP2*-E) are conditionally lethal, whereas homozygotes of the 2nd intron insertion line (*GAP2*-I) have no visible phenotype.

- **Hypothesis**: T-DNA mutation in 15th exon of Rab GAP-encoding gene *GAP2* is responsible for conditional lethality of seedlings and altered phenotype.
    - **Goal 1**: Verify presence of *GAP2* transcript in phenotype-less *gap2*-i mutant
    - **Goal 2**: Complement the *gap2*-e mutant with GAP2 CDS under estradiol-inducible promoter.
    - **Goal 3**: Cultivate complemented *gap2*-e plants on estradiol-treated MS plates, transfer to estradiol-free medium and observe plant response.

Besides well-conserved Rab GTPase binding TBC domain, GAP2 also contains a novel C-terminal coiled-coil motif. Yeast two hybrid library screens with constitutively active Rab GTPases as baits identified this domain as an interactor.

- **Hypothesis:** GAP2 interacts with some Rab GTPases through its TBC domain.
    - **Goal 4**: Test GAP2 against various Rab GTPases in Y2H screen. As a bait, use full-length GAP2 and truncated GAP2 missing the C-terminal domain.
    - **Goal 5**: Transform *Col-0* plants with fluorescently marked GAP2 protein and observe localization
    - **Goal 6**: Transiently co-transform *N.benthamiana* leaves with fluorescently marked GAP2 and Rab GTPase proteins and observe whether co-localization occurs.
- **Hypothesis:** GAP2-encoded Rab GAP interacts with Rab GTPases not only through the conserved TBC domain, but also through its novel C-terminal coiled-coil domain. The C-terminal domain is sufficient for these interactions.
    - **Goal 7**: Test the GAP2 C-terminal domain against various Rab GTPases in Y2H.
- **Hypothesis:** GAP2 can interact with other proteins besides Rab GTPases.
    - **Goal 8**: Perform Y2H screen against Arabidopsis cDNA library with GAP2 as bait.

3. METHODS AND MATERIALS

Following methods and materials have been used to obtain the results presented in this thesis. All centrifugation steps were performed either in Eppendorf MiniSpin Plus (Max. RCF 14 100 × *g*) and Eppendorf Centrifuge 5415 R (Max. RCF 16 100 × *g*) for microcentrifugation tubes, or Hettich Universal 32 R Centrifuge for 14 and 50mL Falcon tubes, unless otherwise noted. Cultivations of bacterial liquid cultures were performed in GFL 3032 or Certomat H shaking incubators. Yeast and bacterial plates were cultivated in Q-cell incubator (Poll Lab). Yeast liquid cultures were cultivated in Q-cell incubator with addition of KM-2 shaker (Edmund Bühler).

3.1. GROWTH MEDIA

Following table lists composition of all media used for cultivation of organisms.

Medium:	Components:	Amount (per liter):	Notes:
MPB *(for bacteria)*	Nutrient broth N° 2 (Biolife) ddH_2O	25 g Add to 1 L	*Mix well. Autoclave. Add required antibiotics before use.*
MPA *(for bacteria)*	Nutrient agar N° 2 (Biolife) NaCl (Lach-Ner) ddH_2O	40 g 5 g Add to 1 L	*Mix well. Autoclave. Allow to cool to 60°C and add antibiotics.*
½ MS *(for plants)*	MS salts + B5 vitamins (Duchefa) Sucrose (Lach-Ner) Plant agar (Duchefa) 8 g for horizontal; 16 g for vertical plates ddH_2O	2.2 g 10 g Add to 1 L	〃
YPAD *(for yeasts)*	Yeast extract (Oxoid) Peptone (Oxoid) 50% filtered glucose (Duchefa) in ddH_2O Adenine sulfate dihydrate (Bio Basic) Plant agar (Duchefa) (add for plates) ddH_2O	11 g 22 g 40 ml 22 mg 20 g Add to 0.96 L	*Mix all constituents besides glucose. Adjust pH to 5.8 with NaOH. Autoclave. Add filtered glucose in a laminar flow cabinet.*
SD *(for yeasts)*	Yeast nitrogen base powder (Sigma) -W; -L; -WL; -WLHA drop-out powder (Sigma, Clontech) 50% filtered glucose (Duchefa) in ddH_2O Plant agar (Duchefa) (add for plates) ddH_2O	6.7 g 1.92 g; 1.6 g; 1.54 g; 0.6 g 40 ml 20 g Add to 0.96 L	〃

Table 1 - Composition of growth media

3.2. PLANTS

Following Arabidopsis plant lines were used in this thesis:

Name	Line	Ecotype	Mutated Gene	Type of mutation	Source
Col-0	Col0 WT	Columbia 0	N/A	N/A	NASC
GAP2-I	SAIL_1207_E12	Columbia 0	AT2G39280	T-DNA insertional (intron)	NASC
GAP2-E	SALK_131980	Columbia 0	AT2G39280	T-DNA insertional (exon)	NASC

Table 2 – Arabidopsis lines used in the experiments

3.2.1. SEED STERILIZATION:

Arabidopsis seeds sown onto sucrose-containing MS plates needed to be surface-sterilized beforehand. A combination of diluted ethanol and bleach wash steps was used. Required amount of seeds was transferred from storage to a fresh 2mL microcentrifuge tube. ~1.5 mL of 70% ethanol was added and the tube was placed onto a shaker for 3 minutes. The tube was then quickly spun in a microcentrifuge and majority of the liquid removed from the tube by either decanting or aspiration. Next, 10% bleach solution was added and the tube was once again shaken for 3 minutes and then spun and liquid removed. This step was repeated 2 times in total. The tube with sedimented seeds and remaining bleach solution was then transferred to a laminar-flow cabinet to ensure sterile environment for the remaining steps. The tube was filled with sterile deionized and distilled water (ddH_2O) to total volume of 2 mL and agitated to ensure dilution of the bleach. It was then spun and the liquid was aspirated. This was repeated 5 times.

3.2.2. PLANT CULTIVATION:

Arabidopsis seeds were stratified inside a fridge at 4°C for 2 days. Seeds already on MS plates or soil were stratified in a cold chamber under identical conditions.

Plants were grown either on MS agar plates or Jiffy peat pellets. When screening for transformed plants, pellet nettings were removed and peat was pooled into a large continuous surface for easier application of an herbicide. Plants were grown under long-day conditions (16h light / 8h dark) at 100 $\mu m/m^2/s$ PAR.

3.2.3. PLANT DNA ISOLATION:

Plant DNA was isolated for the purpose of either genotyping or Southern blot analysis.

For the former, a small excision or a whole leaf was removed from each plant and placed into a 1,5mL microcentrifuge tube. 400µL of DNA extraction buffer (200mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added and the tissue was homogenized with a micropestle. 300 µL of chloroform was added and the mixture was vortexed on shaker (Scientific Industries Vortex-genie 2) for 5 minutes to precipitate proteins. The tubes were then centrifuged for 3 minutes at 14 100 × *g*. 300 µL of upper phase of supernatant was carefully transferred to a clean tube and 300 µL of isopropanol was added and mixed well by repeated inversion. Tubes were placed on ice for 10 minutes to facilitate DNA precipitation and then were centrifuged once more for 5 minutes at 14 100 × *g*. Supernatant was decanted and DNA remained in an opalescent pellet at the bottom. Tubes were placed upside down on a paper towel to drain remaining liquid and let dry for 10 - 30 minutes. DNA was dissolved in 100 µL 2mM

Tris (pH adjusted to 8.5) by gentle tapping or leaving it at 4°C overnight. For genotyping from smaller tissue mass all reagent volumes were halved and only 50 µL of DNA solution was obtained.

For southern-blot analysis a DNAzol Reagent (Invitrogen) was used to obtain necessary amount of genomic DNA. Procedure was carried out according to a supplied manual.

3.2.4. PLANT RNA ISOLATION:

Young seedlings were grown on 1% agar MS plates, harvested and pooled to acquire ~150 mg of tissue. This was transferred into a pre-chilled mortar and ground into fine powder, which was then processed with Qiagen RNeasy Plant Mini Kit according to a supplied manual. RNA solution was stored at -80°C to prevent degradation.

3.2.5. STABLE TRANSFORMATION OF *A. THALIANA*:

A. tumefaciens transformed with chosen binary plasmid was cultivated overnight in 100 ml of MPB medium. The culture was then centrifuged in 50mL Falcon tubes at 3700 × *g* for 15 min at 4°C and resuspended in a 50mL Falcon tube by addition of 50 ml of 5% sucrose with 0.05% Silwet L-77 surfactant (Ambersil Ltd. UK). Roughly 6 weeks old plants had their existing siliques and open flowers removed. The remaining inflorescences were then dipped into the solution for ~30 seconds. The plants were then covered with a plastic bag and cultivated in darkness for 24 h. The bag was removed and plant was cultivated under standard conditions in the chamber. This process was optionally repeated after about a week to improve yield of transformed seeds, with the obvious exception of the siliques removal step. Potential transformed seeds were selected by resistance to herbicide or antibiotic when cultivated. Presence of the transformation was further verified by genotyping resultant plants.

3.2.6. TRANSIENT TRANSFORMATION OF *N. BENTHAMIANA*:

A. tumefaciens carrying desired binary construct was cultivated overnight in 2 mL of MPB medium and spun at 2 200 × *g* for 5 min at room temperature (RT). The bacterial pellet was then resuspended in 1 mL of infiltration medium (IM)(50mM MES pH 5.6, 2mM Na_3PO_4, 0.5% glucose, and 100 µM acetosyringon), spun again, and resuspended in 0.5 mL of IM. A small aliquot of the IM suspension was diluted 50× and its OD_{600} measured on a spectrophotometer BioMate 5 (Thermo Spectronic). A calculated volume of IM suspension was then mixed with additional fresh IM to obtain 0.5 mL of 0.1 OD_{600} IM inoculum. In case of co-transformation, supplementary Agrobacterium cultures were prepared and measured as above, but the

calculated volumes were added to the previous inoculum. Prepared IM suspension was aspirated into 1mL syringe. Inoculum was slowly injected into a leaf mesophyll through opened stomata by gently pushing the tip of the syringe against abaxial side of the leaf, while providing light counteracting pressure by index finger from the adaxial side. Opening of the stomata was ensured by placing the plants under intensive light-source for 1 hour beforehand. Two days after transformation, 10 mm × 10 mm regions of the leaf surrounding the inoculum's point of entry were cut out and observed under epifluorescence or confocal microscope.

3.3. BACTERIA:

Four different strains belonging to two different bacterial species were used: DH5α, TOP10, and DB3.1 of *E.coli* and GV3101 of *A.tumefaciens*. DH5α was used mostly for amplifications of isolated plasmids, whereas TOP10 was the preferred choice for transformation of ligation mixtures. DB3 was used for amplification of Gateway entry and destination vectors. All cultivation steps were performed at 37°C for *E.coli* and 28°C for *A.tumefaciens*, unless otherwise noted.

3.3.1. TRANSFORMATION OF BACTERIA:

Electroporation cuvettes were pre-chilled on ice. 50 µL aliquots of competent bacteria frozen in glycerol were removed from -80°C and placed on ice. Plasmids were added to the bacteria – 1 µL for ligation mixtures; 0.1-0.5 µL for amplifications. Thawed mixtures of bacteria and DNA were transferred into the cuvettes. Electroporation was performed in Eporator instrument (Eppendorf) at 2500 V for *E.coli* and 2000 V for *A.tumefaciens* strains. The cuvettes were placed back on ice and 0.5 mL of MPB medium was added to each one. Volume of each cuvette was then transferred into a 1.5mL microcentrifuge tube and was cultivated for 1 hour with shaking. It was afterwards poured on agar plates with appropriate antibiotic selections (Table 3) and cultivated until discernible colonies appeared.

3.3.2. OVERNIGHT CULTIVATION OF BACTERIAL CULTURE:

Necessary antibiotics were added to MBP medium in proper concentrations (Table 3). The medium was then distributed into glass cultivation tubes with loose fitting caps – 1-4 mL per tube. A single bacterial colony from an agar plate was picked with a sterile wooden toothpick and dropped into the MBP medium in a tube. The tubes were then cultivated overnight in a shaking incubator. For larger volumes of bacterial culture (e.g. 100 mL for floral dip), a smaller

volume of 1 mL was cultivated overnight as per above and then added into an Erlenmeyer flask with sterile MBP medium and antibiotics for another night of cultivation.

Antibiotic	Stock concentration	Concentration in medium per mL
Ampicillin	100 mg / mL ddH_2O	0.1 mg
Kanamycin	50 mg / mL ddH_2O	0.05 mg
Chloramphenicol	34 mg / mL methanol	0.034 mg
Gentamycin	25 mg / mL ddH_2O	0.025 mg
Rifampicin	15 mg / mL methanol	0.015 mg
Hygromycin	50 mg / mL ddH_2O	0.050 mg
Spectinomycin	50 mg / mL ddH_2O	0.050 mg
Cefotaxim	100 mg / mL ddH_2O	0.050 mg

Table 3 – Concentrations of commonly used antibiotics

3.3.3. PLASMID ISOLATION FROM BACTERIA BY ALKALINE LYSIS:

Two different isolation methods were used - High Pure Plasmid isolation Kit (Roche) with glass fiber filter tubes for DNA binding or alkaline lysis with ethanol DNA precipitation. The kit method was performed per manufacturer's instructions. The non-kit method is described below.

2 to 4 mL of overnight culture was centrifuged in a 2mL tube at 14 000 × *g* for 2 minutes and the supernatant decanted. When volume exceeded 2 mL, the tube was re-filled with the rest of the culture and spun again. Resultant pellet was resuspended in 100 µL of GTE (50mM glucose and 25mM Tris/Cl – pH adjusted to 8.0) and left for 5 min at RT. 200 µL of NaOH/SDS (200mM NaOH, 1% SDS) was added to precipitate proteins. Tubes were inverted 6 to 8 times to ensure proper mixing and left on ice for 5 min. Subsequently, 150 µL of KOAc/HOAc (3M KOAc, 2M HOAc) was added to each tube and they were inverted 6 to 8 times. The samples were then spun at 14 000 × *g* for 5 minutes and resultant supernatants were carefully transferred into fresh set of tubes. 1 mL of 96% chilled EtOH was added to each tube and vigorously vortexed. The tubes were then placed on ice for 2 min to speed up DNA precipitation before centrifugation at 14 000 × *g* for 3 min. Supernatants were poured off, tubes re-spun for 10 sec and remaining EtOH was removed by aspiration and evaporation. Pellet was dissolved in 40 µL of TE buffer (10mM TRIS, pH 8, 1mM EDTA).

3.4. YEAST:

Two different *Saccharomyces cerevisiae* yeast strains were used – AH109 (Clontech) and NMY51 (DUAL Systems) because of plasmid compatibility requirements. Yeasts were cultivated at 30° (with shaking for liquid medium) unless otherwise noted. Yeasts were obtained from our lab's stock deep-frozen in glycerin by streaking onto an YPD streak plate. Single colony from the plate was then transferred onto a fresh plate and used for inoculations of liquid YPD media.

3.4.1. TRANSFORMATION OF YEAST:

YPD or SD-W/L medium was inoculated a day beforehand with selected yeast strain – 3 mL per planned transformation – and cultivated in a shaking Erlenmeyer flask for 16 to 18 hours. The culture was then spun down in a sterile Falcon tube at 700 × *g* for 5 minutes at RT. The medium was decanted, the yeasts resuspended in sterile ddH_2O (1.2 mL per planned transformation) and the suspension equally distributed into separate 1.5mL microcentrifuge tubes and spun at 700 × *g* for 5 minutes. Liquid above sedimented yeasts was pipetted away. 12 µL of denaturated carrier DNA (herring sperm – conc. 10 mg/mL) as well as 0.5 to 2 µL of a plasmid DNA was added to each tube. Contents were briefly vortexed and 0.5 mL of PEG/LiAc solution (40% PEG 3350, 0.1M LiAc, 10mM Tris-Cl pH 7.5, 1mM EDTA) was added. The tubes were cultivated on a vortex shaker for 15 min and then placed into 42°C heating block for 15 minutes. 60 µL of EtOH was added after 10 minutes in the heating block. The tubes were spun at 700 × *g* for 5 minutes, washed in sterile ddH_2O, resuspended in ddH_2O – 200 µL for half-plate; 500 µL for full plate – and spread plated onto SD-W/L medium plates for selection of transformants. Plates were cultivated until 2 mm colonies appeared. These could then be inoculated into liquid SD medium for transformation with a second plasmid or protein isolation.

3.4.2. YEAST TWO-HYBRID SYSTEM (Y2H):

Y2H was used to test interactions between proteins. Pairs of shuttle vectors carrying chosen CDSs were consecutively transformed into suitable yeast strains - pGBKT7 and pGADT7 into AH109; pLexA and pGADT7 into NMY51. Two colonies of each secondary transformant were picked from SD-WL plates and resuspended in 100 µL ddH_2O. The suspension was serially diluted to 1:30, 1:900 and 1:27 000 samples. 15 µL of each was pipetted onto SD-WL and SD-WLHA agar plates and let dry. The plates were then cultivated in a sterile plastic bag for 2 to 3 days. Growth of a transformant on both SD-WL and SD-WLHA plates implied an interaction between vector's gene products.

3.4.3. YEAST TWO-HYBRID SCREEN AGAINST CDNA LIBRARY:

Bait-expressing yeasts were inoculated into 10 mL of SD-W medium and cultivated for 8 hours. The culture was then poured into 100 mL of fresh SD-W and grown overnight. Next day, 1 mL aliquot was taken, spun at 2500 × *g* for 5 minutes and resuspended in 1.5mL tube with 1 mL ddH_2O. Its OD_{546} was measured on a spectrophotometer. Volume of culture representing 30

OD units[2] was transferred into 50mL Falcon tubes and spun down at 700 × *g* for 5 minutes. The pellet was resuspended in 1L Erlenmeyer flask into 200 mL of 2 × YPAD pre-warmed to 30°C. 1 mL starting aliquot was taken, spun, resuspended in ddH_2O and its OD_{546} measured. The culture was cultivated and aliquots periodically taken and measured until their OD_{546} quadrupled compared to the starting aliquot, indicating that 2 cellular divisions occurred. The culture was divided into 4 50mL Falcon tubes and spun at 700 × *g* for 5 min. Each pellet was then resuspended in 30mL of ddH_2O and centrifuged again. Water was decanted and each pellet resuspended in 1 mL of LiAc/TE solution (100mM LiAc, 10mM TRIS, pH 8, 1mM EDTA) and transferred to a 1.5mL tube. The tubes were spun at 700 × *g* for 5 minutes, supernatants pipetted away and each pellet resuspended in 600 µL of LiAc/TE. Four 50mL Falcon tubes were set up. Following was added to each tube: 600 µL of the yeast suspension, 100 µL of carrier DNA (containing 200 µg of herring sperm DNA), 2.5 mL of PEG/LiAc (40% (w/v) PEG 4000 in LiAc/TE) and 20 µL of cDNA library. All constituents were mixed by vortexing for 1 min. Falcon tubes were incubated for 45 min at 30°C, with short vortexing after 15th and 30th minute. 160 µL of DMSO (Sigma) was added to each tube and mixed by shaking. The suspensions were then incubated at 42°C for 20 minutes, with occasional shaking by hand, and afterwards pelleted at 700 × *g* for 5 minutes. Each pellet was resuspended in 3 mL of 2×YPAD medium and all were pooled into one Falcon tube. The cells were then incubated at 30°C for 90 minutes with shaking. Next, they were spun at 700 × *g* for 5 min and resuspended in 4.5 ml of 0.9% NaCl solution. 10 µL aliquot of the suspension was taken and diluted to 1:500, 1:50 000, and 1:500 000 solutions. These were plated onto SD-WL plates to determine transformation efficiency. Rest of the suspension was plated onto SD-WHLA – 300 µL per plate. All plates were let to dry and then placed into a plastic bag and cultivated at 30°C for 3 to 4 days.

3.4.4. PLASMID ISOLATION FROM YEAST BY ALKALINE LYSIS:

The process used for plasmid isolation was nearly identical to the one used for bacteria. Yeasts were grown overnight in 2 mL of SD selection medium. The culture was then centrifuged in a 2mL microcentrifuge tube at 14 000 × *g* for 2 min and the supernatant decanted. The pellet was resuspended in 100 µL of GTE and 300 µL of glass beads was added to the suspension. It was then vigorously agitated for 5 min on a vortex shaker. The rest of the procedure was identical to the bacterial alkaline lysis.

[2] $V(\text{mL}) = \frac{30}{\text{measured } OD_{546}}$

3.4.5. PROTEIN ISOLATION FROM YEAST:

A single transformed-yeast colony was inoculated into 5 mL of SD selection medium and grown overnight. The same was done with an untransformed strain as a control, but 10 mL of YPAD medium was used for cultivation. The cultures were vortexed for 30 s, poured into 25 mL aliquots of YPAD medium and incubated with shaking (240 rpm) until the OD_{600} reached 0.4 to 0.6. Number of OD_{600} units in the cultures was established[3]. The cultures were quickly chilled by pouring into Falcon tubes filled halfway with ice and spun at 100 × *g* for 5 min at 4°C. Supernatant with ice was decanted and the cell pellet resuspended in 50 mL of ice-cold ddH_2O. The centrifugation step was repeated, supernatant decanted, and the cell pellets frozen in liquid nitrogen. Cracking buffer (8M urea, 5% SDS, 40mM TRIS pH 6.8, 0.1mM EDTA, 0.4mg/ml bromphenol blue, 2mM mercaptoethanol, 1mM PMSF), pre-warmed to 60°C, was added to the frozen cells – 100 µL per 7.5 OD_{600} units. Cells were resuspended, 80 µL of glass beads added per 7.5 OD_{600}. The samples were heated at 70°C for 10 min, vortexed for 1 min, and centrifuged at 14 000 × *g* for 5 min at RT. The first supernatants were transferred to fresh 1.5mL screw cap tubes and placed on ice. The pellet-containing tubes were boiled in a water bath for 5 min, 100 µL of cracking buffer added, vortexed, and centrifuged as before. The second supernatants were combined with the first ones, and briefly boiled. The samples were stored in -80°C.

3.5. DNA MANIPULATION:

DNA was stored in -20°C in ddH_2O or 200mM Tris-HCl.

3.5.1. POLYMERASE CHAIN REACTION (PCR):

PCR was used for 3 purposes: cloning, genotyping, and site-directed mutagenesis. TPersonal, TGradient, or TProfessional TRIO thermal cyclers from Biometra were used. PCR reactions were set up on ice. Q5 High-Fidelity DNA Polymerase (New England Biolabs) was used for cloning and directed-mutagenesis purposes because of its proofreading activity. Genotyping was performed with DreamTaq DNA polymerase (Thermo Scientific) where only size and presence of a product were of concern. PCR products were separated by agarose gel electrophoresis (AGE), cut out, and purified with PCR Product Purification Kit (Roche), if necessary for future use.

[3] $\mathrm{OD}_{600}\ \mathrm{units} = \mathrm{OD}_{600}\ \mathrm{of\ 1mL\ sample} \times \mathrm{total\ V(mL)}$

No.	1		2		3		4		5		6		7		8		
	1st GAP2 cloning		2nd GAP2 cloning		GAP2-I genotyping		GAP2-E genotyping		GAP2-E v.2 genotyping		pUBN-GFP GAP2-CDS		pMDC7 GAP2-CDS		GAP1 genotyping		
	°C	s	°C	s	°C	s	°C	s	°C	s	°C	s	°C	s	°C	s	
1 -	98	60	98	60	92	60	92	60	92	60	92	60	92	60	98	60	
2 -	98	15	98	15	92	10	92	15	92	15	92	15	92	15	98	15	
3 -	62	20	60	20	60	20	60.5	20	60.5	20	54	20	56	20	60.2	20	29 ×
4 -	72	90	72	90	72	75	72	60	72	60	72	60	72	60	72	90	
5 -	72	180	72	180	72	300	72	300	72	300	72	300	72	300	72	300	
Primers used:	GAP2-Fw2 → ← GAP2-Rv2		GAP2-Fw → ← GAP2-Rv		G-A2SILwt → ← G-A2SILin Sail LB3 →		← G-A2SLKwt G-A2SLKin → ← Salk Lbb1.3		← GAP2-Rv2 G-A2SLKin → ← Salk Lbb1.3		GFP for → ← GFP rev		RIN4_1A_for_seq → ← GAP2-R303A-RP		GAP1-Fw → ← GAP1-IR1		

Table 4 – Commonly used PCR programs for cloning, genotyping, etc.

Name of primer	5'-Sequence-3'	Restriction site
GAP2-Fw2	TAGTATAGTATTGCTTCTTGCTCCACCGG	
GAP2-Rv2	**GTCGAC**AATCTAATCGGCAATTTGTAT	SalI
GAP2-Fw	TCCCGGGTATGATCGCCGACGCCGTTTCTAAG	XmaI / SmaI
GAP2-Rv	TGTCGACCTAGGGACTGACCATGACCGGC	SalI
G-A2SILwt	CCCGGGATGATCGCCGACGCCGTTTCTA	XmaI / SmaI
G-A2SILin	CTCAGAGCCATAGGTGCACCGCC	
Sail LB3	CATCTGAATTTCATAACCAATCTCG	
G-A2SLKwt	ATTCTAGGGACTGACCATGACCG	
G-A2SLKin	AGATGGTCAAACAAGACAATCGGCG	
Salk Lbb1.3	ATTTTGCCGATTTCGGAAC	
GAP2-R303A-RP	GACCTGGAAATGTC*GC*GGGTAAATCCTTTT	
GAP2-IF2	GAATTCGTTACTTTGCTACAATCAATGAC	EcoRI
GAP2-IF1	GAATTCCTGAAGTTCTTATTCATGGCG	EcoRI
RIN4_1A_for_seq	CAACTTTGTACAAAAAAGCAG	
pGAD424 for seq	AGATACCCCACCAAACCCA	
GFP for	ATGGTGAGCAAGGGCG	
GFP rev	CTTGTACAGCTCGTCCATGC	
GAP1-Fw	ATGAGGAGCGTTGCCGCCGCCG	
GAP1-IR1	CAGCAACAGGGATACCAGAGACACCTGT	

Table 5 - PCR programs –Primers used for PCR and sequencing. GREEN marks 5' extension of primers; *RED* marks site-mutation-inducing bases. **BOLD** marks presence of a restriction site.

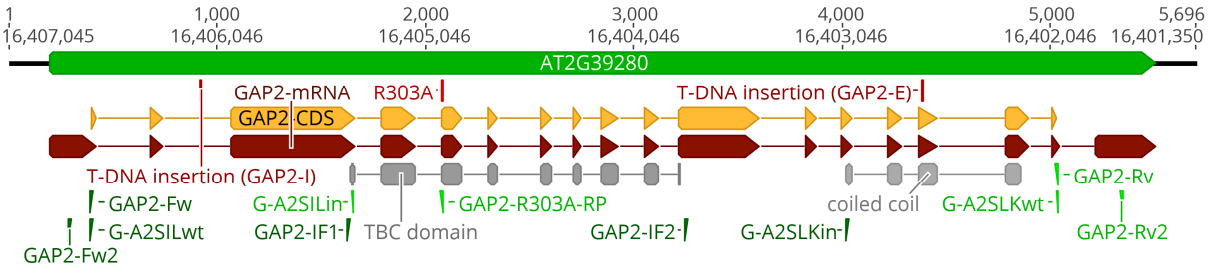

Figure 7 - GAP2 gene with primers and important domains

3.5.1.1. Amplification from cDNA:

20µL reaction contained 14.25 µL of ddH_2O, 4 µL of Q5 reaction buffer, 1 µL of cDNA, 0.25 µL of dNTPs (2.5mM each), 2x 0.25 µL of primers (100µM), and 0.1 µL of Q5 polymerase. Reaction volume was scaled up or down as necessary.

3.5.1.2. Site-directed mutagenesis:

Two subsequent PCR reactions were used to obtain a mutated CDS from a plasmid carrying GOI. First reaction was identical to classic cloning reaction, but only a part of the CDS was amplified. A forward primer binding to the sequences flanking the undesired bases was designed with desired bases in place and used together with a reverse primer binding to the end of CDS for first round of PCR. The product (so-called *megaprimer*) was separated by AGE, isolated from gel and added as a primer in the second reaction.

3.5.1.3. Genotyping:

20 µL PCR reaction consisted of 16.25 µL of ddH_2O, 2 µL of DreamTaq buffer, 1 µL of gDNA, 0.25 µL of dNTPs (2.5mM each), 2 × 0.25 µL of primers (100µM), and 0.1 µL of DreamTaq DNA polymerase. Two combinations of 3 primers were used for genotyping of T-DNA insertion mutants. One pair flanked the T-DNA insertion site and successfully amplified the region only when no insertion was present. Second pair was similar, but one of the two primers actually annealed to the T-DNA sequence border and a product amplified only when T-DNA was present. Presence of the PCR products was checked by AGE.

3.5.2 DNA RESTRICTION AND FRAGMENT ISOLATION:

Type II restriction endonucleases (REs) were used for DNA restriction – either for downstream application or just for diagnostic digest. High-Fidelity REs (New England Biolabs) were used preferentially to non-HF REs (Thermo Scientific), because of their fast processivity, shared CutSmart buffer and lack of star activity. REs were used to excise a region of interest (ROI) from either a plasmid or a PCR fragment. Destination vector was also processed with REs to produce compatible overhangs or blunt ends on both ROI and the vector. Typical 20µL restriction reaction contained the following: 2 µL of 10×RE buffer, 0.2 µL of each RE, up to 15 µL of DNA, and ddH_2O added to a total of 20 µL. The reaction volume was scaled up, when required. The reaction mixtures were incubated at 37°C - 30 minutes for verification, 1 hour to overnight for other applications. Both ROIs and the linearized destination vectors were separated from unwanted DNA fragments by AGE, excised with a sterile scalpel blade and

isolated with MinElute Gel Extraction Kit (Qiagen). DNA concentration of resulting elutes was measured with Nanodrop 2000 spectrophotometer (Thermo Scientific) to calculate proper molar amounts for use in ligation reaction.

3.5.3. DNA LIGATION:

Both blunt and sticky end ligations were performed with T4 DNA Ligase (Thermo Scientific). 10µL ligation mixture was set up in 0.2mL tube on ice and contained the following: 1 µL of T4 reaction buffer, 0.5 µL of T4 DNA Ligase, up to 50 ng of linear vector DNA, and insert DNA[4]. ddH_2O was added up to total reaction volume. Extra ligation mixture with insert DNA swapped for ddH_2O was used as a control. Ligation was performed in a thermoblock at 22°C for 30 min. 10 µL of chloroform was added to the tube and it was vigorously vortexed for 1 min. The ligation mixture was then centrifuged at 10 000 × g for 3 minutes. 8 µL of upper water phase was carefully transferred into clean 0.2mL tube and stored at -20°C or used for electroporation into bacteria.

3.5.4. GATEWAY CLONING:

ROI was excised and ligated into an entry vector. The entry vector was transformed into compatible bacteria strain, amplified, and isolated. LR reaction was set up in 0.2mL tube at RT with following constituents: 0.5 – 3.5 µL of entry vector (max. 150 ng), 0.5 µL of destination vector, and TE to total of 4 µL. 1 µL of LR Clonase II enzyme mix was added to the reaction, quickly mixed by vortexing and the tube briefly centrifuged. The mixture was incubated at 25°C for 1 hour. 0.5 µL of Proteinase K was added, briefly vortexed, and incubated at 37°C for 10 minutes. 1 µL of the LR reaction was transformed into compatible cells and plated onto MPA plates with appropriate antibiotics.

3.5.5. AGAROSE GEL ELECTROPHORESIS (AGE):

Electrophoresis was used to verify presence and size of a PCR product, restriction pattern, and for isolation of DNA fragments for further processing. 1% agarose gel was routinely used and was prepared as follows: 1 g of UltraPure Agarose (Invitrogen) was mixed with 100 mL of TBE (10 mM Tris, 20 mM boric acid, 1 mM EDTA) buffer in an Erlenmeyer flask. The suspension was dissolved by heating to boiling point in a microwave oven for brief moments. Water lost

[4] Volume of insert DNA was such that a molar ratio of insert DNA to vector DNA was 3:1.
$$\left(\frac{conc.(ng/\mu L)_{vector}}{length(bp)_{vector}}\right) \times V_{vector} \times 3 = \left(\frac{conc.(ng/\mu L)_{insert}}{length(bp)_{insert}}\right) \times V_{insert}$$

by evaporation was compensated for with ddH_2O to keep the agarose concentration at 1% (w/v). 10 µL of GelRed DNA stain (Biotium) was added to the liquid and mixed by subtle circular shaking motion to prevent bubbles from forming. Liquid agarose solution was poured into casting tray with well combs and allowed to solidify. The tray was then submersed into the TBE buffer-filled electrophoresis unit and the combs removed. Samples for analysis were mixed with DNA loading dye (Fermentas) in 5:1 ratio and loaded into wells along a GeneRuler DNA Ladder Mix (Thermo Scientific). AGE was run at 90 – 140 V until sufficient separation of bands was achieved.

3.5.6. DNA SEQUENCING AND CHROMATOGRAM ANALYSIS:

Maximum of 300 ng of plasmid or linear DNA was mixed with 1 µL of sequencing primer (5µM concentration) and ddH_2O added to 8 µL. Sequencing was done by Faculty of Science sequencing facility. Sanger sequencing with capillary electrophoresis was used. Resulting chromatograms were hand-trimmed and aligned to reference sequence in Geneious 7 (Biomatters) bioinformatics software.

3.6. RNA MANIPULATION:

RNA was stored in -80°C and handled in sterile gloves. RNAse-free microcentrifuge tubes and pipette tips were used for setting up reactions involving RNA.

3.6.1. DNASE TREATMENT:

1 µL of DNAse buffer was added to volume representing 1000 ng of RNA. ddH_2O was added to 9 µL and 1 µL of DNAse enzyme was added. Mixture was incubated at 37°C for 30 min. 1 µL of 50 mM EDTA was added and the mixture incubated at 65°C for 10 min.

3.6.2. RT-PCR:

Transcriptor First Strand cDNA Synthesis Kit (Roche) was used for reverse transcription per the supplied manual.

3.7. PROTEIN MANIPULATION:

3.7.1. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE):

SDS-PAGE was utilized to verify presence and size of expressed proteins. 12% acrylamide gel (2 mL 30% Acrylamide-Bis, 1.25 mL Tris pH 8.8, 1.75 mL ddH_2O, 20 µL 10% APS, 5 µL

TEMED) was used with stacking gel (0.325 mL 30% Acrylamide-Bis, 0.625 mL Tris pH 8.8, 1.525 mL ddH_2O, 12.5 µL 10% APS, 2.5 µL TEMED) on top. Protein samples were boiled for ~10 min and loaded into wells. PageRuler protein ladder (Thermo Fisher) was added into the first lane. The gel was run for ~60 min at 180V.

3.7.2. WESTERN BLOT:

Electroblotting was used to transfer proteins from within the polyacrylamide (PA) gel onto a nitrocellulose (NC) membrane. The PA gel was trimmed, the NC membrane and 6 pieces of thick filter paper cut to slightly exceed its dimensions. All were soaked in a transfer buffer (25mM Tris, 190mM glycine, 20% methanol) and stacked from bottom to top onto a graphite anode of an electro-blotting apparatus in the following arrangement: three filter papers, NC membrane, PA gel, three filter papers. Cover of the apparatus containing a graphite cathode was placed on top of the stack. The blotting was performed for 1 – 1.5 hours at current of 1.5 mA per cm^2 of the top filter paper area. Afterwards, the NC membrane was stained by Ponceau S dye for 1 min and subsequently washed in water. Next the NC membrane was washed in 1 × PBS (137mM NaCl, 10mM Na_2HPO_4, 2.7mM KCl, 1.8mM KH_2PO_4) solution and incubated overnight in a blocking solution (1 × PBS, 5% non-fat dry milk, 0.5% Tween 20) at 4°C to prevent non-specific interactions. Next day the membrane was incubated in a blocking solution with 1000 × diluted primary antibody for 1 hour at RT. The membrane was then washed 3 × for 10 min in PBS with 0.5% Tween 20 and then incubated for 1 hour at RT in blocking solution with 15 000 × diluted secondary antibody. It was then again washed 3 × in PBS with Tween 20. The secondary antibody was visualized as luminescence with Amersham ECL Prime Western Blotting Detection Reagent (GE healthcare Life Sciences) and documented with exposure and development of MEDIX XBU radiographic film (Foma).

3.8. CONSTRUCTION OF PLASMIDS:

Following plasmids were created for purpose of this thesis. Ligated plasmids were transformed into compatible competent bacteria and cultivated on MPA plates with antibiotic selection. 2 to 5 colonies were cultivated in MPB medium for minipreps. Isolated plasmids were checked by a diagnostic digest, unless otherwise noted.

pJET1.2-GAP2(wt)

AT2G39280 (GAP2) CDS was amplified from cDNA with PCR in two steps. First, outer primers GAP2-Fw2 and GAP2-Rv2 were used (Table 4-1). A ~2 500 bp product was purified with AGE, isolated from gel and used as a template for the second round of PCR. Inner primers GAP2-Fw and GAP2-Rv, both with restriction-site extensions, were used (Table 4-2) and after AGE and isolation from gel, a ~2250 bp product was obtained. This blunt-ended DNA was ligated into a linear pJET1.2 vector, transformed into TOP10 *E.coli* strain, isolated, and verified by sequencing. The cloning had to be repeated 3 times, because of unexpected frame-shifting insertions on intron-exon borders within the CDS. Eventually, two serviceable GAP2 sequences in pJET1.2 were obtained – each with one frame-shifting insertion (11bp and 5bp respectively). Fortunately a BamHI restriction site was present between the two insertions, so both plasmids were combined by cloning via restriction digest (BamHI + SalI). The incorrect region in one CDS was patched with the correct sequence from the other (Figure 8). This way a full-length pJET1.2-GAP2 was obtained.

pJET1.2-GAP2(R303A)

NotI was used to cut the backbone of pJET1.2-GAP2(wt). The linearized plasmid was then used as a template for site-directed mutagenesis with GAP2-R303A-RP and GAP2-Fw primers. 303rd codon of GAP2 was modified to code for alanine instead of arginine. Second round of

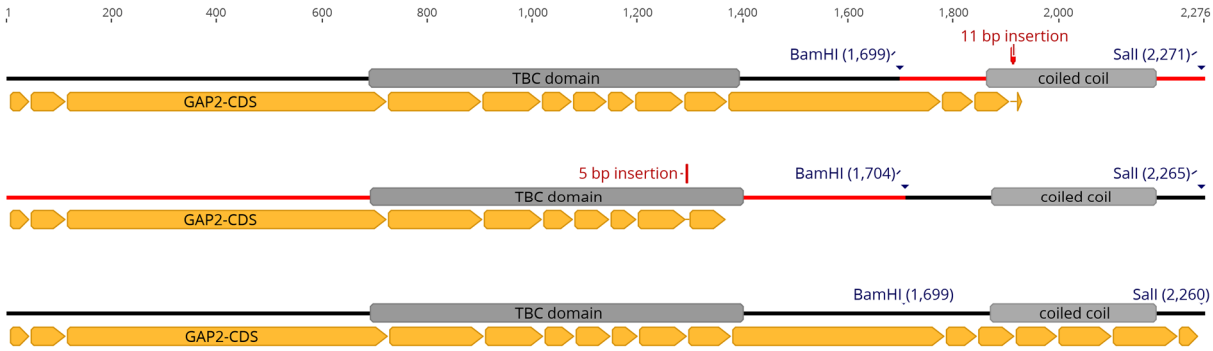

Figure 8 - Repairing pJET1.2-GAP2 CDS. Sequences 1 and 2 contained insertions, resulting in premature stop codon. Sequence 3 was produced by digestion of both plasmids and substitution of the 11 bp insert in first plasmid with corresponding sequence from the second. pJET1.2 backbone is not shown.

PCR was performed with the megaprimer and GAP2-Rv (Figure 9). The PCR product was ligated into pJET1.2. Presence of the mutation was confirmed by sequencing.

pGBKT7-GAP2(wt) / (R303A)

Empty pGBKT7 plasmid and both pJET1.2-GAP2(wt) and pJET1.2-GAP2(R303A) plasmids were digested with XmaI and SalI-HF restrictases to produce compatible overhangs. Fragments were separated by AGE and purified from gel – 7296 bp linear pGBKT7 and 2261 bp GAP2(wt) / GAP2(R303A). Both GAP2 sequences were ligated into pGBKT7.

pGADT7-GAP2(wt) / (R303A)

Empty pGADT7 plasmid was digested with XmaI and XhoI restrictases. Linearized 7959 bp plasmid was separated by AGE and purified from gel. Previously obtained 2261 bp GAP2(wt) and GAP2(R303A) fragments were ligated into pGADT7.

pJET1.2-GAP2-coil-only

Primers GAP2-IF2 and GAP2-Rv were used in PCR with pJet1.2-GAP2(wt) as a template (Figure 9). The 859 bp product was ligated into pJET1.2 (Figure 10-3).

pGBKT7-GAP2-coil-only

Empty pGBKT7 was digested with EcoRI and SalI-HF. The same was done with pJET1.2-GAP2-coil-only plasmid. Both plasmids' fragments were separated by AGE and 7291 bp pGBKT7-backbone and 856 bp GAP2-coil-only were purified from gel and ligated together.

pGADT7-GAP2-coil-only

Empty pGADT7 was digested with EcoRI and XhoI, fragments separated by AGE and purified from gel. The 856 bp GAP2-coil-only fragment obtained previously was ligated into pGADT7.

pGBKT7-GAP2(wt)-coil-less / (R303A)-coil-less

XmaI and BamHI were used to open empty pGBKT7 and to excise 1 700 bp coil-less fragment (Figure 10) from pGBKT-GAP2(wt) and pGBKT-GAP2(R303A). Fragments were separated by AGE and purified. Both coil-less fragments were ligated into pGBKT7 (Figure 10-2).

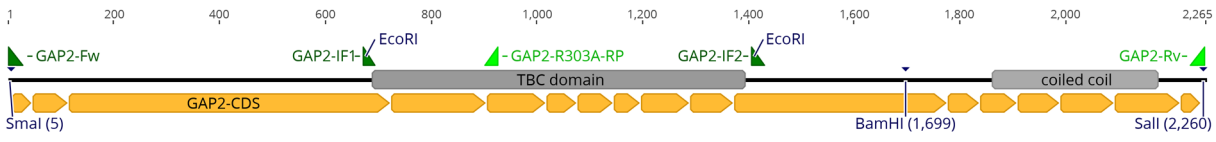

Figure 9 - Primers aligned to pJet1.2-GAP2(wt). GAP2-R303A-RP with GAP2-Fw were used for first round of in-situ mutagenesis**.** The megaprimer was then used with GAP2-Rv primer**..** Vector backbone is not shown.

pGADT7-GAP2(wt)-coil-less / (R303A)-coil-less

Empty pGADT7 was opened with XmaI and BamHI restrictases. Previously obtained coil-less GAP2(wt) and GAP2(R303A) fragments were ligated into pGADT7.

pENTR3C-GAP2(wt) / (R303A)

DraI and XhoI enzymes were used to digest pENTR3C plasmid into 2 246 bp backbone and 481 bp ccdB region. pJET1.2-GAP2(wt) and pJET1.2-GAP2(R303A) were digested with DraI and SalI into 2 259 bp CDS region and 2 984 bp backbone. Fragments were separated by AGE and CDS of both GAP2(wt) and GAP2(R303A) was ligated into the PENTR3C vector.

pUBN-GFP-GAP2(wt) / (R303A); pGWB6-GAP2(wt) /GAP2(R303A); pMDC7-GAP2(wt) / (R303A)

Gateway cloning was used to create these plasmids. pENTR3C-GAP2(wt) / (R303A) plasmids were used as entry clones, pUBN-GFP-DEST, pGWB6, and pMDC7 as destination vectors.

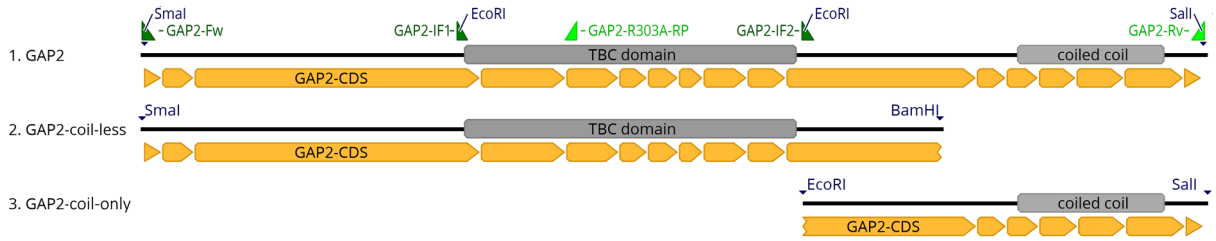

Figure 10 - Partial forms of GAP2-CDS used in Y2H plasmids.

4. RESULTS

4.1. PHYLOGENETICS OF TBC-PROTEINS IN SELECTED ARCHAEPLASTIDS

A.thaliana encodes 23 putative Rab GAP genes. For 22 of those, matching ESTs have been recovered, suggesting their active transcription and potential translation into actual proteins.

A rudimentary phylogenetic analysis has been performed on Rab GAPs from following species representing distinct branches of archaeplastids: *Arabidopsis thaliana* (thale cress), *Oryza sativa japonica* (rice), *Zea mays* (maize), *Physcomitrella patens* (spreading earthmoss), *Selaginella moellendorffii* (spikemoss), *Sorghum bicolor* (sorghum), and *Volvox carteri*. *Saccharomyces cerevisiae* (budding yeast) was added because of its small and relatively well analyzed Rab GAP family.

Protein sequences were acquired from UniProt (UniProt Consortium 2015) proteome database for each organism and cross-referenced with Simple Modular Architecture Research Tool (SMART)(Letunic et al. 2015). Only 1 protein sequence was included per gene. If multiple alternatives were predicted, the longest one for the given gene was picked. TBC domains, as predicted by SMART were extracted from all sequences, aligned with MUSCLE (Edgar 2004) and all columns with gaps removed in BioEdit program. 74 residues long gapless alignment was obtained from the TBC domains. Maximum-likelihood phylogenetic analysis was performed by online tool Phylogeny.fr (Dereeper et al. 2008) and the unrooted tree visualized in Geneious software. TBC and other SMART-recognized domains' positions were illustrated on full-length proteins next to the tree (Figure 11).

Resulting tree uncovered good correlation between TBC domain conservation and similarity of overall arrangement of recognized domains among plants. Distribution of the included plant species among the branches was fairly uniform, suggesting that significant diversity of Rab GAP proteins was already present in their common ancestor. Unlike in mammalian GAPs, no specific catalytic or interacting domains were identified besides the TBC domain. Coiled-coil motifs, and transmembrane (TM) regions were predicted for multiple Rab GAPs, but never in the same protein. TM region in Rab GAPs seems quite rare, but has been observed before - TBC1D20, the GAP for Rab1, is associated with membrane through such N-terminal transmembrane region (Sklan et al. 2007). GAP2 (F4IUX9_ARA) protein with its coiled-coil motif near the C-terminus is located in a clade with close homologs that share the conserved coiled-coil motif.

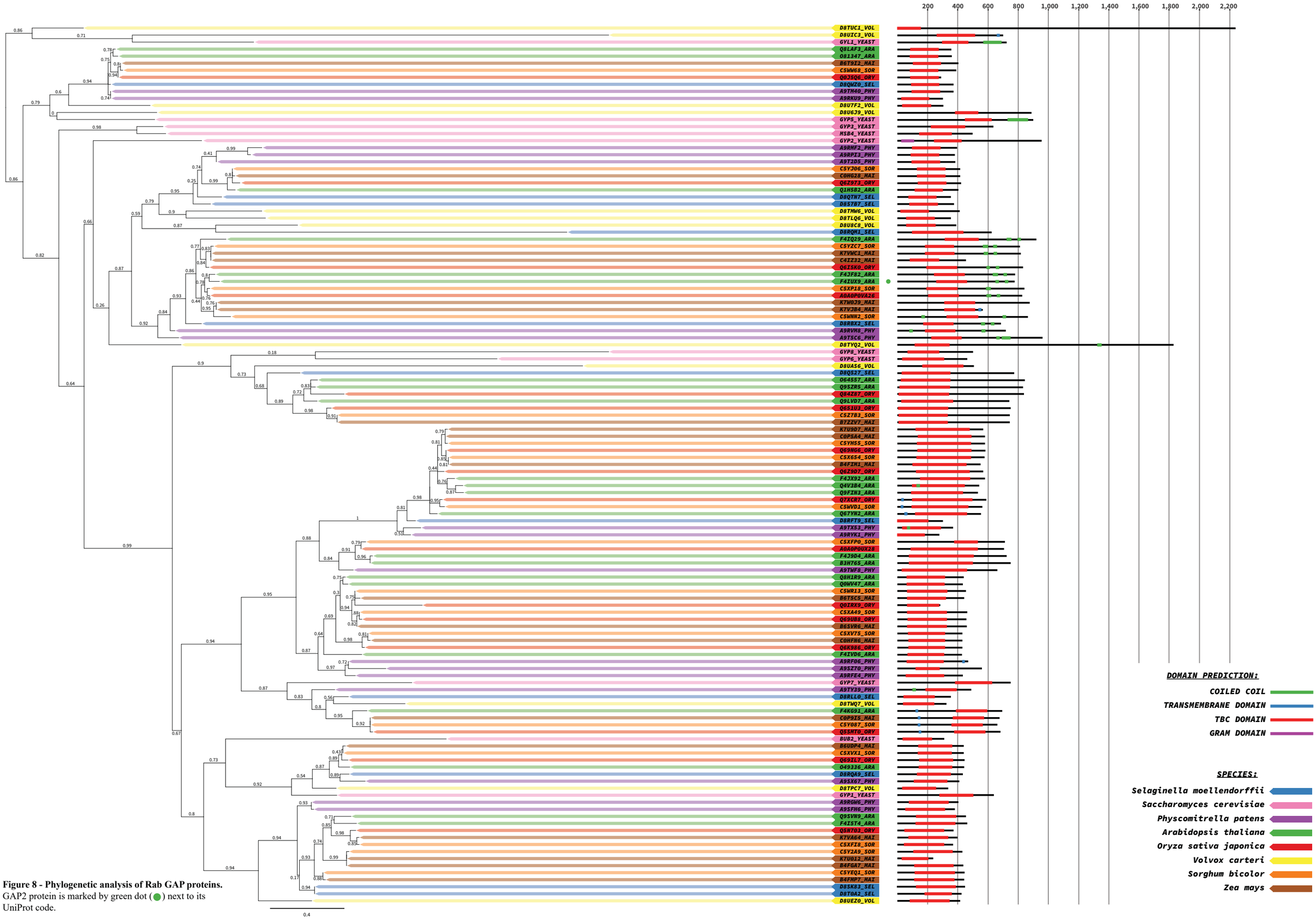

Figure 8 - Phylogenetic analysis of Rab GAP proteins.
GAP2 protein is marked by green dot (●) next to its UniProt code.

4.2. SEARCH FOR POTENTIAL GAP2 INTERACTORS:

Yeast two-hybrid screening was utilized to test interactions of GAP2 with various Rab GTPases. The following section describes this in logically ordered structure and does not reflect the real order in which the tests were carried out.

4.2.1. Y2H SCREEN AGAINST CDNA LIBRARY

pGBKT-GAP2(R303A) was used as bait in Y2H screen against *A.thaliana* cDNA library. Transformation efficiency could not be established, since no colonies formed on SD -WL plates. However, colonies on SD -WLHA eventually appeared. First 18 colonies to form were transferred onto separate SD -WLHA plates in groups of 6, grown for 3 days, and each transferred into liquid SD –WLHA medium for overnight cultivation. Next day, DNA was isolated from the yeasts and electroporated into TOP10 *E.coli* cells. The bacteria were cultivated on MPA-Amp plates suitable for the cDNA library vector. Bacterial colonies were transferred into liquid MPB-Amp medium, grown overnight and the plasmid DNA was isolated with a kit. Before sending samples for sequencing, a diagnostic digest with BamHI and PstI (known to cut between the vector backbone and the cDNA insert) was performed and fragments were separated by AGE. Only samples with bands matching the vector backbone length were submitted for sequencing. In the meantime, more colonies appeared on the SD –WLHA selection plates. Pink colonies were preferentially picked and treated as before. In total 250 colonies were transferred, of those 30 had their DNA isolated and transformed into bacteria. 23 samples were sequenced in total. The pGAD424 sequencing primer binding to activation domain was used.

Sequences from chromatograms were blasted against *A.thaliana* transcriptomic subset of NCBI Reference Sequences database and 22 of them returned hits.

Plasmids with interesting sequences were re-tested in a standard Y2H screen against pGBKT7-GAP2(R303A) and pGBKT7-∅ to rule out auto-activation. These sequences coded for fragments of following genes (Figure 12):

AT2G24600 - Ankyrin repeat family protein (Ank)
AT3G55020 - RabGAP/TBC domain-containing protein (GAP1)
AT5G16220 - protein PAL OF QUIRKY (PoQ)
AT5G59210 - myosin heavy chain-related protein (Myo)

AT3G55020 (GAP1) is highly homologous to GAP2 and the sequence covered very similar to the C-terminal coiled-coil motif in GAP2. I therefore decided to test GAP2 for possible homodimerization as well. GAP2 (R303A) interacted with GAP1- c-terminal fragment and also with itself. Ankyrin, Myosin heavy and Pal of Quirky all tested negative for interaction (Figure 13).

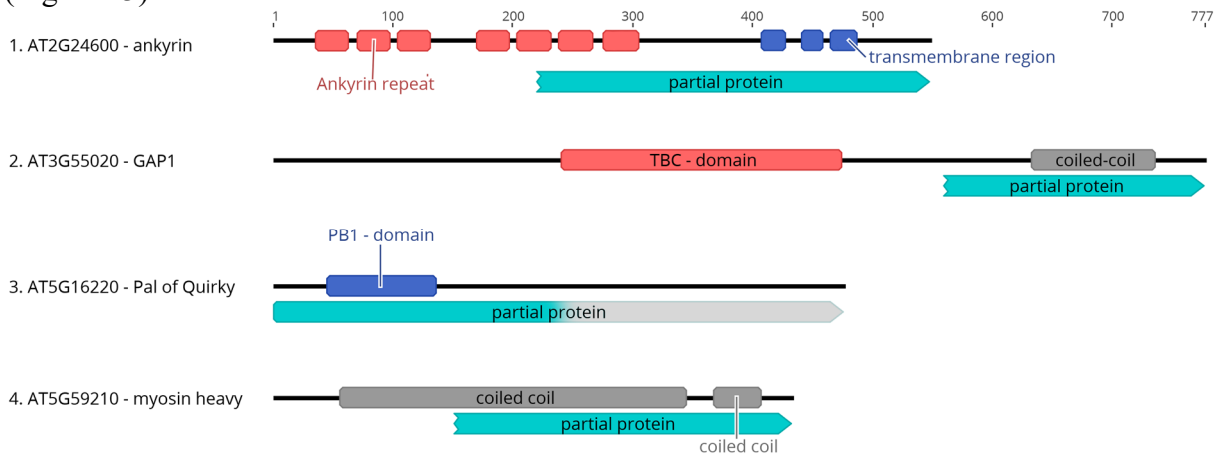

Figure 12 - Potential interactors found in the Y2H screen against cDNA library – Turquoise annotation illustrates partial proteins encoded by the cDNA library plasmids. In Pal of Quirky, only part was sequenced (illustrated by full-color segment), so the real length of the protein is not known. Domains recognized by SMART are included for context.

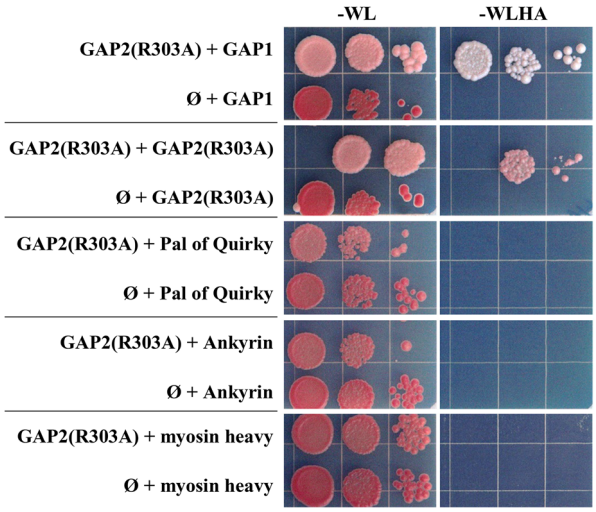

Figure 13 - Y2H screen of potential GAP2 interactors GAP2(R303A) as a bait interacted both with GAP2(R303A) and GAP1-fragment as prey. Other interactions were not observed.

4.2.2. TESTING OF GAP2 AGAINST RAB GTPASES IN Y2H SCREEN

Y2H screen was performed with combinations of full-length and partial GAP2s against Rab GTPases. (Table 6). GAP2s were tested in both WT and R303A versions. GAP1-coil fragment from previous screen was also tested. All GTPases carried Q-to-L mutation in their GTP binding site to disable GAP-assisted GTP hydrolysis.

		pGADT7-				
		∅	RabA1a	RabA4a	RabA5b	RabE1d
pGBKT7-	∅	-	-	-	-	-
	GAP2(wt)	-	-	-	-	-
	GAP2(R303A)	-	-	-	-	-
	GAP2(wt)-coil-less	-	-	-	NT	-
	GAP2(R303A)-coil-less	-	-	-	NT	-
	GAP2-coil-only	NT	±	-	NT	+

		pLexA-				
		∅	RabA2a	RabA3	RabA5c	RabE1d
pGADT7-	∅	NT	-	-	-	-
	GAP2(wt)	NT	-	-	-	-
	GAP2(R303A)	NT	-	-	-	-
	GAP2(wt)-coil-less	NT	NT	NT	NT	NT
	GAP2(R303A)-coil-less	NT	-	-	-	NT
	GAP2-coil-only	NT	±	±	-	NT
	GAP1-coil-fragment	NT	-	-	-	-

Table 6 – Results of Y2H combinations performed with GAP2 against Rabs
(+) = interacts; (±) = interacts weakly (-) = does not interact; (NT) = not tested.
pGADT7-based plasmids were used as prey, pGBKT7- and pLexA-based plasmids as bait.

First two repetitions of the screen were performed only with full-length variants of GAP2 and gave no positive results (not shown). To rule out possibility of problems stemming from use of somewhat old pGBKT7 plasmid stock, GAP2(wt) and GAP2(R303A) were re-cloned into fresh pGBKT7, expressed in yeast side by side with the old plasmids and empty pGBKT7 and proteins extracted. Presence of the GAL4 binding domain in the proteins was verified by western blot in both old and new plasmids (Figure 14) with Anti-GAL4 DNA-BD antibody. Bands matched predicted size of 106 kDa, but another smaller band around 75 kDa appeared in both GAP2-transfected yeast cultures. No further testing of the BD domain was carried out, as at this time, functionality of the interactors was concurrently proven by the screen against cDNA library and homodimerization of GAP2. Y2H screens were performed against Rab GTPases in pGADT7 and pLexA plasmids with GAP2 in pGBKT7 and pGADT7 respectively (Table 6). Some GAP2 constructs have not been included, since they

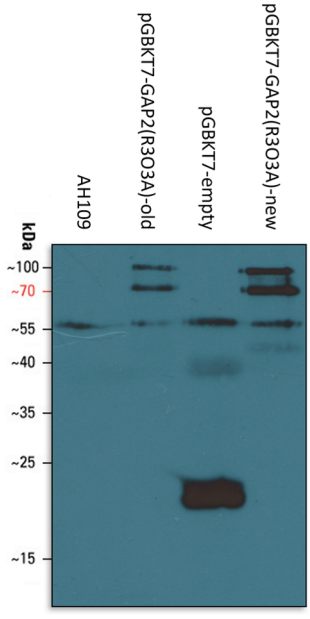

Figure 14 – Western blot of proteins expressing the GAL4 binding domain.
Predicted sizes:
GAP2 with GAL4 binding domain - 106 kDa
GAL4 binding domain - 27 kDa
Untransfected AH109 culture was used as a control.

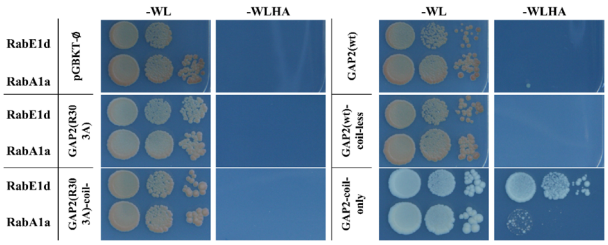

Figure 16 (up) - Result of Y2H screen of full-length and partial GAPs in pGBKT7 against Rab GTPases in pGADT7.

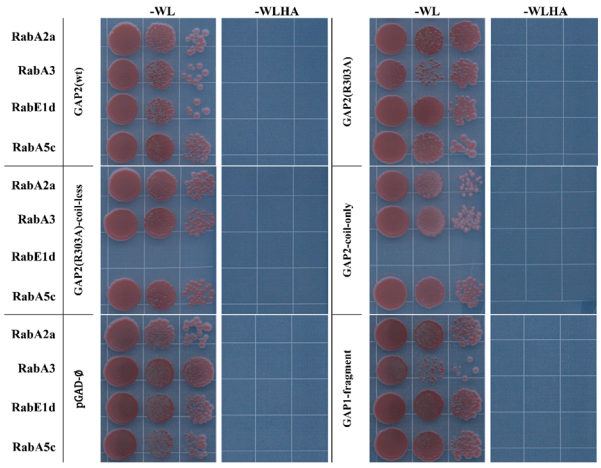

Figure 15 (right) – Result of Y2H screen of full-length and partial GAPs in pGADT7 against Rab GTPases in pLEX.

were not ready in both variants at the time of testing due to issues with their cloning. pLexA-based RabA2a, -A3, -E1d, and -A5c did not interact with any tested GAP (Figure 15). Possible weak interaction was later observed for RabA2a and RabA3, both with GAP2-coil-only (not pictured). Some combinations with RabE1d did not grow succesfully on the –W/-L agar plates. pGADT7-based RabE1d and RabA1a interacted with GAP2-coil-only in pGBKT7. Interaction with RabE1d was markedly stronger one of the two. Other results were negative (Figure 16).

4.2.3. TESTING OF GAP2 AGAINST ITSELF AND GAP1 IN Y2H SCREEN

The partial and full-length GAP2 proteins (Table 7) were also tested for dimerization and for interaction to determine whether the proteins dimerize through the C-terminal coils or differently. GAP1 fragment recovered from the library screen was included in the screen as well because of its shared C-terminal coil. As a bait, GAP2-coil-only strongly interacted with multiple prey: full-length GAP2(wt) and GAP2(R303A), coil-less GAP2(R303A), GAP2-coil-only, and the homologous GAP1-coil-fragment. Full-length GAP2(R303A) bait interacted strongly with GAP2-coil-only and GAP1-coil-fragment. Weaker interaction was detected against full-length GAP2(R303A) and GAP2(WT). This was unlike in the previous GAP2(R303A) homodimerization test, which resulted in much stronger interaction (Figure 17).

		pGADT7-						
		∅	GAP2(wt)	GAP2(R303A)	GAP2(wt)-coil-less	GAP2(R303A)-coil-less	GAP2-coil-only	GAP1-fragment
pGBKT7-	∅	-	-	-	NT	-	-	-
	GAP2(wt)	-	-	-	NT	-	-	-
	GAP2(R303A)	-	±	±	NT	-	+	+
	GAP2(wt)-coil-less	-	-	-	NT	-	-	-
	GAP2(R303A)-coil-less	-	-	-	NT	-	-	-
	GAP2-coil-only	NT	+	+	NT	+	+	+

Table 7 – Combinations of baits and plasmids used in Y2H screen of full-length and partial GAPs
(+) = interacts; (±) = interacts weakly (-) = does not interact; (ND) = not tested.

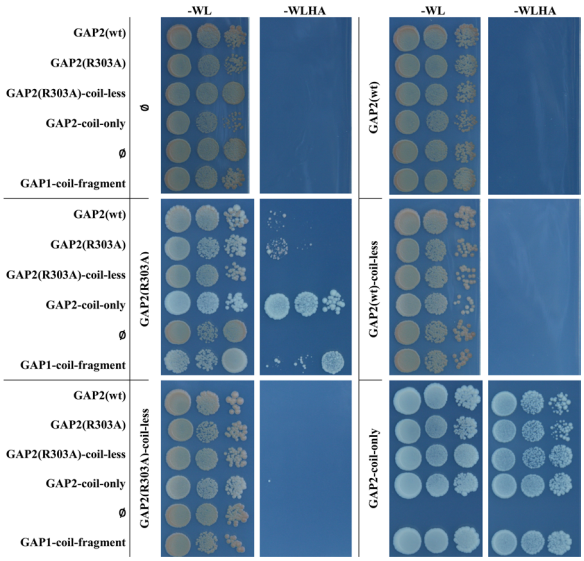

Figure 17 Results of GAP2 dimerization Y2H screen

4.3. INTRACELLULAR LOCALIZATION OF GAP2

To visualize localization of GAP2 in plant cell as well as to complement its mutation in one step, pUBN-GFP-GAP2(wt) vector was chosen for stable transformation of *GAP2-E* heterozygotes. The plasmid contained GAP2(wt) fused to N-terminal GFP under constitutive ubiquitin promoter. The plants were transformed by floral-dip and ~ T1 seeds were sown onto peat soil and cultivated under long-day conditions. Once the cotyledons reached ~1 mm in diameter, plants were sprayed with BASTA herbicide (120mg / L). Application of the herbicide was then 2 × repeated in 4-day interval. 3% of seedlings, a suspiciously high ratio, exhibited BASTA-resistance. None of them, however, displayed green fluorescence when examined under epifluorescence stereo microscope. Randomly selected resistant plants also tested negative in genotyping for GFP sequence (Table 4-6). Plants were disposed of and the cultivation was repeated with T1 seeds on horizontal ½ MS plates with stronger selection (PPT 20 µg/ml, Cefotaxim 300 µg/ml). Surviving plants were transplanted onto peat pellets two weeks later. None of them, however, displayed GFP signal when examined under fluorescence stereo microscope. There were no further attempts to work with pUBN-GFP-GAP2 in *Arabidopsis*. Instead my attention was turned to different plasmid.

pGWB6-GAP2(wt), a GFP-GAP2(wt) encoding plasmid with stronger 35S promoter, was prepared and transformed by floral-dip into *GAP2-E* heterozygotes and Col-0. T1 Seeds were sown onto horizontal ½ MS plates with selection (Hygromycin B 25µg/mL, Cefotaxim 300µg/mL) and cultivated for 10 days. Non-bleached seedlings were then transferred into Magenta vessels with identical medium and after 10 days transplanted into soil and cultivated for collection of seeds. T2 seeds were planted onto peat pellets and checked for GFP signal after 10 days. No signal was observed and the plants were discarded.

Stable transformation was not the only approach used to determine localization – transient transformations by infiltration of *N.benthamiana* with *Agrobacterium* were performed as well. pGWB6-GAP2(wt) was successfully transformed into the leaves and observed in epidermal cells. Results from epifluorescence microscopy suggested possible accumulation in convex lobes (Figure 18). Because of limiting lateral resolution delivered by epifluorescence microscopy, next observations were performed on confocal fluorescence microscope.

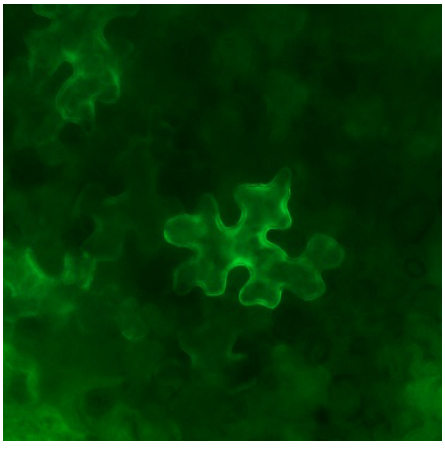

Figure 18 – Expression of GFP-GAP2(wt) in *N.benthamiana*. Epidermis cells observed under epifluorescence exhibited suspicious accumulation of green signal in convex lobes.

To verify the results from epifluorescence microscopy, pGWB6-GAP2(wt) was co-infiltrated with a plasmid carrying RFP fused to microtubule marker. Free YFP with RFP-tubulin was used for comparison of localization. Accumulation into the lobes was observable on the confocal microscope in epifluorescence mode for both GFP-GAP2 and free YFP, but was not present in confocal mode and therefore dismissed as an optical artifact. Just as free YFP, GAP2(wt) was present mostly in cytoplasm. Unlike free YFP (Figure 19-B), only minimal presence was observed in the nucleus (Figure 19-A).

pGWB6-GAP2(wt) was also co-transformed with plasmids expressing mRuby-RabA1a, mRuby-RabA2a, mRuby-RabA4a, and mRuby-RabA4c fusion proteins. The same was done for pGWB6-GAP2(R303A). Every Rab was co-transformed in two versions – one wild type, and one with the Q-L mutation to lower its intrinsic GTPase activity. GTP-bound RabA GTPases are thought to predominantly localize to the PM. I hoped to observe increased GFP-GAP2 decoration of PM in case of its interaction with a Rab. No clear occurrence of this was observed for any combination of GAP2 : RabA Q-L. In some samples, both Rabs and GAP2s were observed near the PM, but whether they were on the PM or just in the cytoplasm adjacent to it could not be determined. However, some cells for each co-expression showed obvious

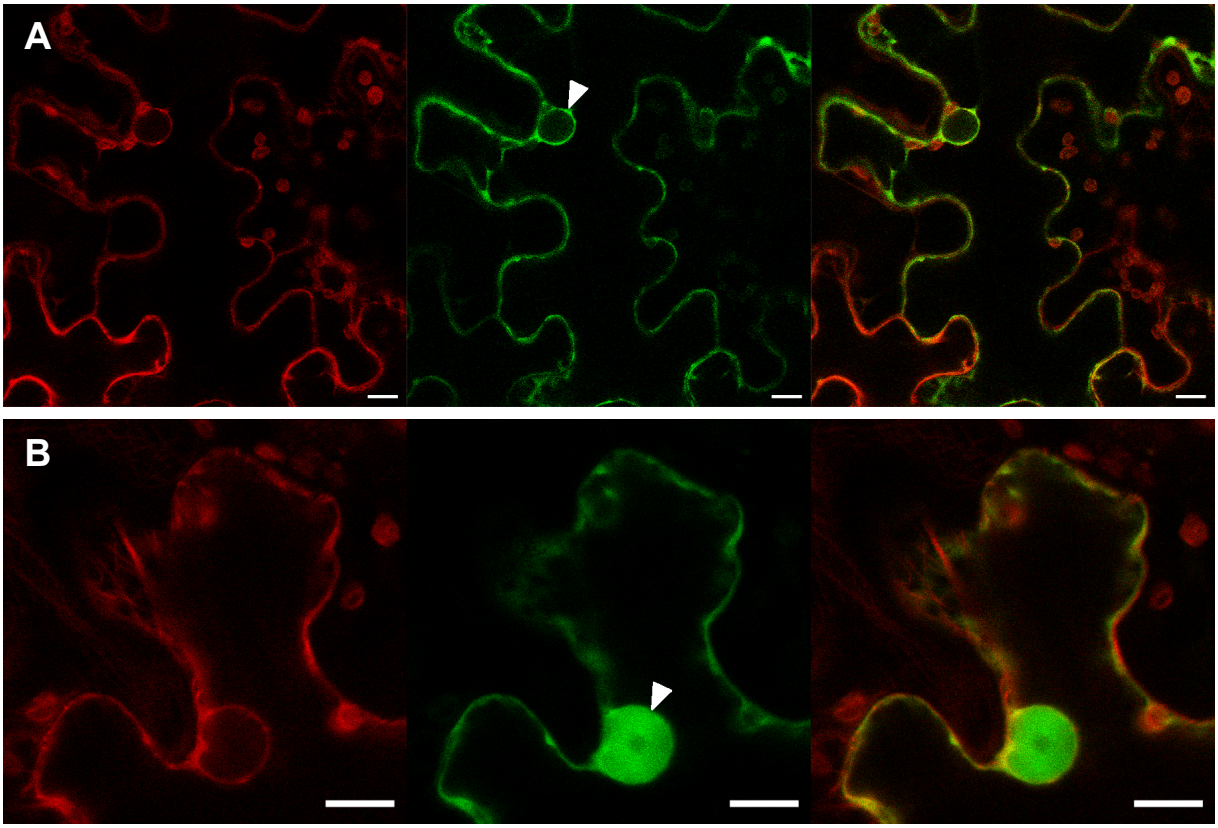

Figure 19 – Transient transformations of *N.benthamiana*: A) Intracellular localization of GFP-GAP2(wt) fusion protein and mRFP-tubulin; B) Intracellular localization of free YFP protein and mRFP-tubulin. No co-localization of GFP-GAP2(wt) with microtubules or accumulation in the lobes were observed, but there was a clear difference in nuclear localization between GFP-GAP2(wt) and free YFP (white arrows). Scale bar = 10 µm.

cytoplasmic localization of the Rabs and GAPs (Figure 20-A, -C). In all Rab : Rab GAP combinations, in some cells, punctate GFP bodies could be observed. In cells expressing RabA1a-QL with GAP2(wt) the mRuby signal strongly co-localized with many GFP bodies (Figure 20-B). Much weaker co-localization was also observed for RabA1a-QL + GAP2(R303A). Other combinations of RabA1a and GAP2 did not exhibit such co-localization.

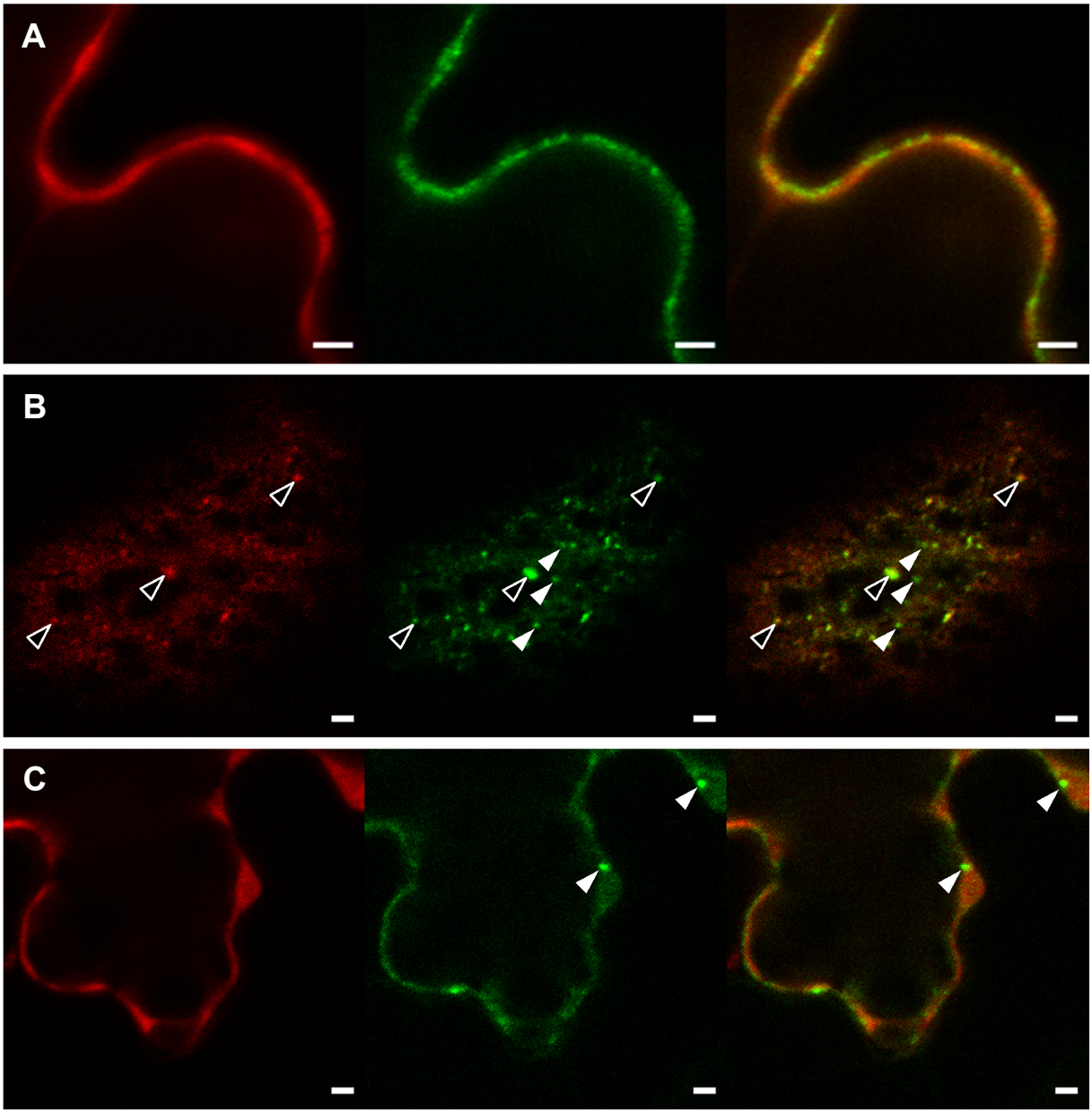

Figure 20 – Transient expression of GAPs with Rabs in *N.Benthamiana* epidermal cells. (Scale bar = 2 µm)

A) GFP-GAP2(wt) + mRuby-RabA4a(wt) – detail of convex and concave lobe.
Punctate bodies can be seen dotting the cytoplasm close to the membrane.

B) GFP-GAP2(wt) + mRuby-RabA1a(QL) – detail of cortex.
Punctate bodies can be observed in the cortex. Some have clear co-localization of both Rab and GAP2 (black arrow), other seems to be GAP only (white arrow)

C) GFP-GAP2(R303A) + mRuby-RabA4a(wt) – accumulation of cytoplasm in concave lobe.
Cell exhibits clear cytoplasmic localization of both RabA4a and GAP2(R303A). GAP forms punctate bodies in the cytoplasm accumulation (white arrow).

4.4. VERIFICATION OF GAP2 TRANSCRIPT IN PHENOTYPE-LESS *GAP2-I*

In the third part of my work, I dealt with two mutant alleles of GAP2 derived from T-DNA insertion. The first line, GAP2-I, has the insertion in the second intron, while the second line, GAP2-E has the insertion in the 15th exon. Preliminary data (Hála, unpublished) show, that while *gap2-i* homozygous mutant has no obvious phenotype changes, *gap2-e* homozygous mutants occur with decreased frequency in progeny of heterozygous plants (9% to expected 25%), do not germinate on soil and die within one week of germination on agar media. To verify whether the difference between the phenotype-less *gap2-i* and knock-out *gap2-e* homozygous lines stems from difference in GAP2 expression, *gap2-i* homozygous plants, verified by genotyping (Table 4-3, Figure 22-B), were checked for presence of the gene transcript. Lack of the transcript in *gap2-e* homozygous mutants has been in the past verified at the occasion of their successful cultivation (Hála, personal communication)

Total RNA was isolated from homozygous *GAP2-I* plants cultivated on vertical plates with MS medium. The RNA was transcribed into cDNA. PCR was set-up with 3 different pairs of primers against *GAP2* (Figure 21-A):

- GAP2-IF2 + GAP2-Rv2 (T_a=48.2°C)
- GAP2-Fw2 + GAP2-R303A-RP (T_a=55.5°C)
- GAP2-Fw + GAP2-R303A-RP (T_a=55.5°C)

A pair of primers against gene AT3G55020 (GAP1) was used as a positive control:

- GAP1-Fw + GAP1-IR1 (T_a=60.3°C)

PCR products were loaded onto AGE, which was run for 1h at 140V. The gel was photographed (Figure 21-B). The cDNA was contaminated by gDNA but because the amplified area spanned

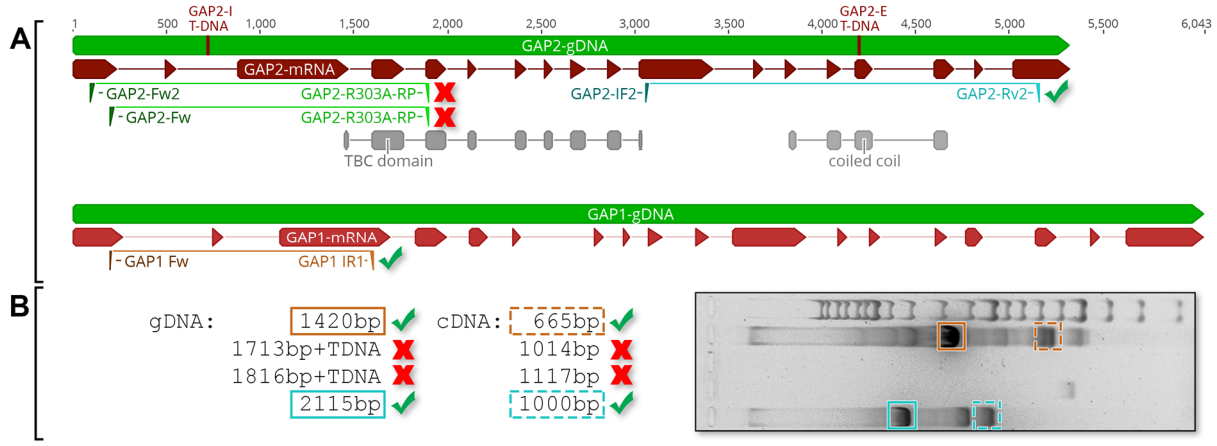

Figure 21 – A) primers aligned to GAP2 and GAP1 – Potential PCR transcript spans multiple introns. It should therefore be possible to discern cDNA from possible gDNA contamination. **B) Results of GAP2(I)-cDNA PCR** – PCR amplified C-terminal region of GAP2 is present in both gDNA and cDNA template forms. The same can be said about the GAP1 control. Primers flanking the GAP2(I) T-DNA insertion did not amplify a product.

multiple introns, the gel could be resolved. For GAP1 positive control, both cDNA and gDNA amplified correctly with bands 665 bp and 1420 bp long. For GAP2, region spanning the T-DNA insertion could not be amplified, because the runtime was not long enough to let the polymerase process the entire T-DNA insertion. However, C-terminal half of the cDNA could be detected. That meant that the *GAP2-I* homozygous plants are not total knock-outs and produce at least partial GAP2 mRNA.

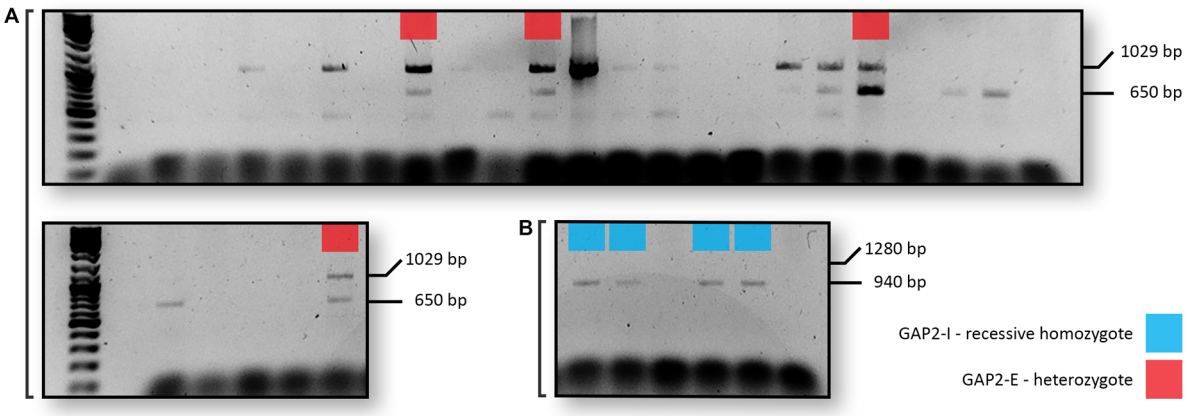

Figure 22 – Genotyping of GAP2-E and GAP2-I plants. A) For GAP2-E, WT band is ~1030 bp long and insertion band is ~650bp. Heterozygous plants were chosen for subsequent experiments. B) For GAP2-I, WT band is ~1280 bp and insertion band is ~940 bp. Recessive homozygous plants were chosen.

4.5. COMPLEMENTATION OF GAP2-E MUTANT

GAP2-E seeds were sown onto pellets, cultivated under long day conditions and genotyped for the exon T-DNA insertion (Table 4-4, Figure 22-A). Heterozygous plants were grown to maturity and their seeds collected. These seeds were used for all subsequent experiments.

Heterozygous GAP2-E plants were transformed by floral dip with pMDC7-GAP2(wt) plasmid. In this plasmid, GAP2(wt) is expressed under estrogen-inducible promoter. ~2000 of T_1 seeds were sown onto horizontal MS plates with selection (Hygromycin B 25µg/mL, Cefotaxim 300µg/mL). After 10 days, ~40 non-bleached seedlings were transferred into Magenta vessels with identical selection medium and cultivated under long day conditions. Two weeks later, 39 surviving plants were transplanted onto peat pellets, cultivated, and genotyped for GAP2-E heterozygosity. Instead of G-A2SLKwt primer, GAP2-Rv2 primer was used to avoid amplification of the inserted GAP2-CDS (Table 4-5). 17 heterozygous lines were detected and their seeds collected. 2 lines (L9, L13) were chosen at random for further work. ~350 T_2 seeds from each of the two lines were planted onto 2 × 3 horizontal MS plates with 5µM β-estradiol and cultivated for 10 days. Genotyping for recessive homozygotes among the seedlings on plates was performed - 52 were tested for lack of non-disturbed *GAP2* allele (Table 4-5). No recessive homozygote was detected.

To check for expression of β-estradiol-inducible GAP2, RNA was isolated from both lines, treated with DNAse and reverse-transcribed into cDNA. PCR was used to verify the expression with primer pair RIN4_1A_for_seq + GAP2-R303A-RP (Table 4-7, Figure 23-C). A gDNA from one of the seedlings was used with the same primers as a positive control. Primers GAP1-Fw + GAP1-IR1 were used to check for gDNA contamination, because the β-estradiol-inducible GAP2(wt) gene gives identical product length for both cDNA and gDNA. Expression of the inducible GAP2 was not detected in either one of the lines (Figure 23-A).

To address a possibility of position effect, 2 more batches of T2 seeds from other heterozygous lines were sown onto 2 × 3 horizontal MS plates with β-estradiol and cultivated (to improve physical contact of the seedlings with the inducer). 10 days later, RNA from one plate for each line was isolated, treated with DNase and transcribed into cDNA. PCR was performed as before and induced expression in both lines was detected (Figure 23-B). 50 seedlings were then genotyped for recessive homozygosity. Only one plant tested positive and

it exhibited the mutant phenotype and did not survive. I conclude that it is probably impossible to complement *gap2-e* homozygote this way.

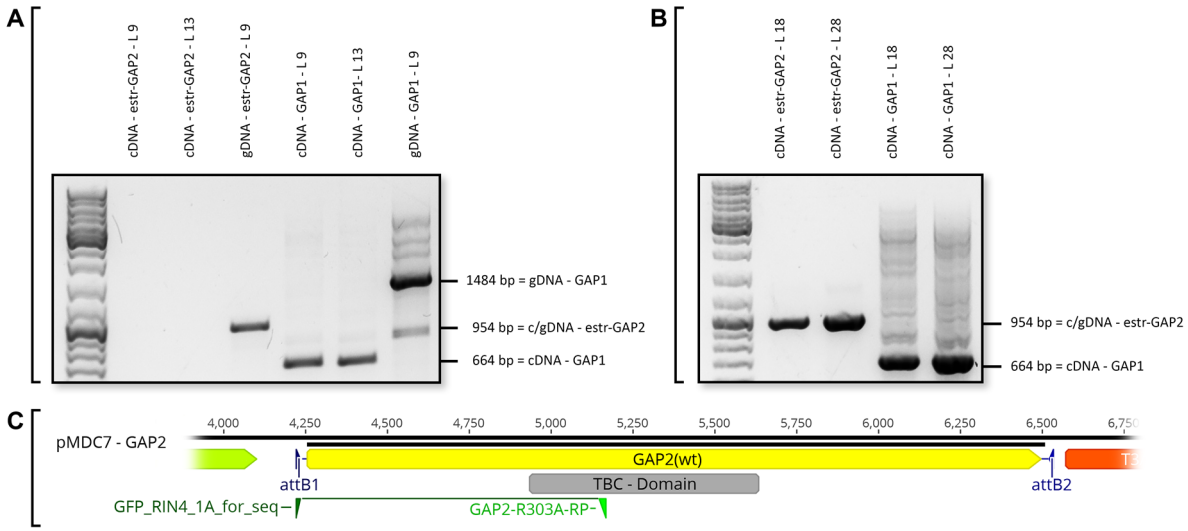

Figure 23 – Verification of estradiol-inducible GAP2 expression. A) First two lines (L9 and L13) did not express the gene, but the CDS was present in gDNA (lane 3). Genotyping for GAP1 was used as a control of cDNA purity – it was considered not contaminated, since only spliced CDS could be detected. B) Second two lines (L18 and L28) expressed the gene. Genotyping for GAP1 was used as a control of cDNA purity. Since no 1484 bp band could be detected, the cDNA was considered not contaminated. C) Primers aligned to pMDC7 T-DNA insert. Genotyping exploits the attB site specific for the inserted GAP2-CDS. The site is present in both gDNA and RNA transcript / cDNA.

5. DISCUSSION

An Y2H screen against *Arabidopsis* cDNA library, a pilot experiment aimed at discovering potential interactors for RabA and RabE, plant orthologs of mammalian exocytic Rab11 and Rab8, was performed by Dr. Hála as a logical continuation of his research into small GTPases and their connection to exocyst-mediated secretion in plants. The screen yielded 2 almost identical C-terminal coiled-coil (CC) motif fragments of 2 putative Rab GAPs encoded by genes AT3g55020 (GAP1) and AT2g39280 (GAP2). The fragments interacted with constitutively active GTP-binding Q-L forms of RabA2a, -A2d, -A3, and RabE1d GTPases, while having no detectable affinity for GDP-bound deactivated forms or other tested Rabs (-B1b, -D2a, -A5c). Sequence for very similar coiled-coil motif was found in another gene encoding closely related GAP protein in *Arabidopsis* genome: At2g37290 (GAP2). From the available T-DNA insertional mutants for all 3 genes, an exon-disrupting line for GAP2 exhibited conditionally lethal phenotype, which, when exceptionally avoided, still resulted in very reduced speed of growth and disturbed root system (unpublished data).

5.1. OBSERVED Y2H INTERACTIONS

My work with the GAP2 protein was supposed to verify whether the interaction with the detected Rabs is facilitated exclusively through the C-terminal coiled-coil domain or whether it just supplements a TBC domain-mediated bind between GAP2 and its effector. It was long expected that the TBC domain is the only Rab-interacting part of GAPs. However, arising amount of evidence has been provided that there are also other domains interacting with Rabs outside the TBC domain. Interaction solely through the C-terminal coiled-coil domain would provide the means for the Rab GAP's participation in a GAP cascade, a signaling mechanism partly responsible for progression of compartments along the exocytic and endocytic pathways. Transition between different compartment identities is connected with, among others, exchange of Rab population. Both exocytic and endocytic pathways include progression through multiple distinct Rab-defined compartments. The cell must possess a mechanism that enables timely replacement of consecutive Rab populations. Participation of Rab GAP and Rab GEF proteins in this process can be logically expected, since GDIs cannot extract activated GTP-bound Rabs and inserted non-activated GDP-bound Rabs would be under constant threat of removal by GDIs. Exchanges of Rabs could be elegantly achieved by GEF and GAP signaling cascades. In GEF cascade, a GTP-bound Rab recruits the GEF, which then proceeds to activate the Rab downstream in the pathway from the previous one. The newly activated Rab can then again

recruit another GEF, which activates next downstream Rab, etc. Counter to the activating steps facilitated by the GEFs operate GAP cascades. In them, the recently activated Rab recruits a GAP capable of deactivating the Rab upstream in the pathway. When the next Rab downstream gets activated by recruited GEF, it in turn recruits another GAP and effectively shuts down the Rab that indirectly turned it on (Figure 24)(Rivera-Molina & Novick 2009). Both sequential activation and deactivation of Rabs by GEFs and GAPs respectively have already been described in both yeast and mammalian cells.

Ypt32p-Sec4p GEF cascade has been observed in late yeast secretory pathway. Ypt31p/32p GTPases, markers of late Golgi, regulate budding of secretory vesicles, and their transport to the PM periphery by association with their effector Myo2 - a myosin V motor (Lipatova et al. 2008). During transport, they transition from Ypt31p/32p-defined to Sec4p-defined compartments. Sec2p is a GEF that facilitates GDP/GTP exchange of Sec4p Rab. It was found to interact with GTP-bound Ypt32p through a binding site located outside of its catalytic domain. Ypt32p and Sec4p can bind to GEF at the same time and Ypt32p is necessary for vesicular Sec2p localization (Ortiz et al. 2002). After Sec4p recruitment, Ypt31p/32p is eventually replaced with Sec15p – an exocyst subunit, during vesicle maturation. Myo2 binds to Sec4p and Sec15p, full exocytic apparatus eventually assembles and the vesicle is tethered to the exocytic site (Jin et al. 2011; Guo et al. 1999). Another yeast example of something akin to a GEF cascade is the transition from Ypt1p to Ypt32p during maturation of Golgi. TRAPPI is a 7-subunit protein complex that functions as GEF for Ypt1p at *cis*-Golgi. It can be altered to TRAPPII complex by addition of three subunits and it then loses affinity for Ypt1p and instead acquires GEF activity towards Ypt31p/32p. However, simultaneous binding of Ypt1p, TRAPPII and Ypt32p has not been observed (Wang & Ferro-Novick 2002; Morozova et al. 2006). Analogous cascade has been observed in mammalian cells. Rab11, ortholog of Ypt32p, stimulates GEF activity of its effector Rabin8, ortholog of Sec2p, which can then activate Rab8, a Sec4p ortholog (Knödler et al. 2010). Surprisingly, no Sec2p/Rabin8 ortholog was detected in archaeplastids (Elias 2008). Endocytic GEF cascades exist as well – In mammalian cells, the Rab22-Rab5 cascade was observed in membrane

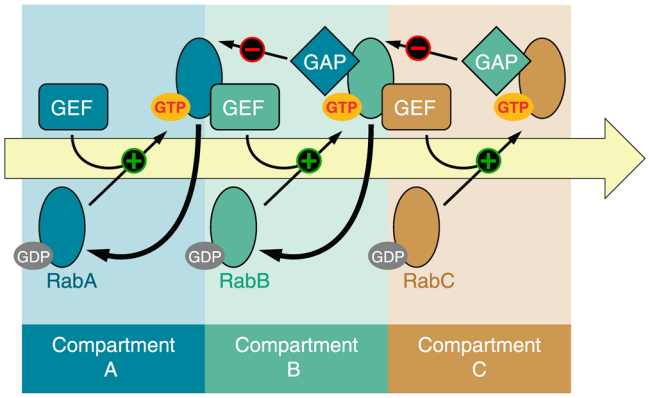

Figure 24 - Rab cascade – Upstream Rabs recruit GEFs, which in turn activate downstream Rabs. In the opposite direction function the GAPs – they get recruited by the downstream Rabs and shut down the upstream Rabs.

Modified from (Hutagalung & Novick 2011)

of early endosomes. Rab22 interacts in non-substrate manner with Rabex-5, a GEF for Rab5, and recruits it to early endosomal membrane. There the GEF activates GDP-bound Rab5 and promotes endosomal fusion (Zhu et al. 2009). Rabex-5 is implicated in another event as well – a positive feedback loop that could be considered a Rab5-Rab5 cascade. GDP-bound Rab5 delivered to early endosomal membrane gets activated by Rabex-5 complexed to 2 units of another protein, Rabaptin-5. After the activation, Rabex-5 no longer has affinity for Rab5 and Rabaptin-5 binds to it instead. Rabaptin-5 remains in complex with Rabex-5 and Rabex-5 can activate other nearby Rab5 GTPases, resulting in a positive feedback loop. This stabilizes a Rab5 patch on the membrane, which is necessary for endosomal fusion (Lippé et al. 2001).

As said before, activation/stabilization of downstream Rab populations solves only half of the problem. Without removal of the upstream Rab populations, the membrane would simply accumulate Rabs from multiple steps and that's demonstrably not the case. In yeast, GAP cascade between Ypt31p/32p, Rabs of late Golgi and early secretory vesicles, and Ypt1p, Rab responsible for fusion of ER vesicles with Golgi and assisting inner-Golgi transport, has been possibly elucidated. Ypt1p is a marker of early Golgi and it is replaced with Ypt31p/32p during cisternae maturation to late Golgi. Gyp1p, GAP for Ypt1p interacts with Ypt32p outside its TBC domain. *Gyp1*-deficient cells exhibit slower conversion from Ypt1p to Ypt32p, compartments with both Rab populations colocalizing were more frequent than in WT, and Ypt1p effectors were found in late Golgi. Mizuno-Yamasaki et al. (2012) propose, that Ypt32p recruits Gyp1p to the compartments, which then deactivates Ypt1p and allows its removal by a GDI. Another potential GAP cascade has been described between typically discreetly localized Ypt32p and Ypt6p Rabs. Normally, Ypt6p localizes to medial Golgi, and Gyp6p, its GAP, is recruited to *trans*-Golgi/TGN by Ypt32p as its effector. Amino acid substitution of Gyp6p's catalytic arginine finger with lysine disrupted discreet localization and resulted in Gyp6p being localized not just in medial Golgi, but *trans*-Golgi, TGN and secretory vesicles at bud neck as well (Suda et al. 2013). So far, no GEF or GAP coordinated Rab cascades have been described in plants.

As seen in the examples mentioned for both GEFs and GAPs, the interaction facilitated outside their primary domains is a common motif of the cascades, since it allows for the GEF/GAP to function simultaneously as an effector and stimulator of hydrolytic activity. The fact, that I was unable to detect interaction between full-length or partial TBC-only form of the GAP2 and RabA or RabE, but was able to repeat the interactions through the C-terminal coiled-coil motif implies that the interaction occurs strictly outside the catalytic domain and these Rabs are not substrates for GAP2 but might instead serve as the upstream Rabs in GAP cascade.

Interactions between GAP and Rab outside the TBC domain were observed also in other context. Rab binding to C-terminal CC motif of a GAP was observed in Y2H screen of mouse embryo cDNA library with Rab6 Q-L as bait. Biochemical assay performed with full-length RabGAPCenA and a shorter coil-less version showed that presence of the coil significantly increases hydrolysis rates of Rab6 and RabGAPCenA (Cuif et al. 1999). The increase in hydrolysis activity or specificity of this particular GAP for this particular Rab has been later disputed by Fuchs and her team (2007), who showed that the GAP's CC motif readily interacts with Rabs from many different families and the full-length GAP is specific towards other GTPases. However, the question of possible GAP cascade implications was never addressed in this work. Actual CC motif interaction in GAP cascade between downstream Rab GTPase and GAP with hydrolytic ability for the upstream Rab was recently discovered in *C.elegans* by Liu & Grant (2015). RAB-5 and RAB-10 are GTPases associated with early endosomes and endosomal recycling of proteins and lipids to PM respectively. TBC-2 is a GAP with known catalytic function towards RAB-5 and it possesses multiple CC motif stretches located N-terminally from the TBC domain. One of these stretches was found to be responsible for non-catalytic interaction with GTP-bound RAB-10 and was required for the GAP's recruitment from cytoplasm to the endosomes. Another CC motif stretch on TBC-2 is utilized by AMHP-1/Amphiphysin, a BAR- and SH3-domain containing peripheral membrane protein. Just as RAB-10, this protein known for association with recycling endosomes was required for proper recruitment of TBC-2. Loss-of-function *tbc-2* mutants typically accumulate human transferrin receptor in enlarged endosomal structures instead of recycling them back to the PM, but complementation with TBC-2 including the coiled-coil motif rescued the phenotype. TBC-2 without the CC motif, however, was unable to associate with the endosomes and stayed in cytoplasm.

There are only 2 Rab GAPs in yeast that possess C-terminal CC domains. These are Gyp5p and its close homolog Gyl1p. These two GAPs seem to be capable of dimerization both *in vitro* and *in vivo* and the coils have been proposed and partially elucidated as the region responsible for this interaction (Friesen et al. 2005). Furthermore in biochemical assay, hydrolytic activity of Gyp5p with Ypt1p was accelerated by addition of Gyl1p, even though Gyl1p does not actually have the catalytic arginine finger and on its own did not accelerate Ypt1p-mediated hydrolysis. Both of these proteins also interact with yeast Amphiphysin homolog Rvs167, implicated in exocytosis and are responsible for its correct intracellular localization. Dimeric GAP setup such as this could potentially function as a signaling unit in

GAP cascades, with the non-catalytic Gyl1p providing effector functionality towards a downstream Rab GTPase and Gyp1p activating the upstream Rab.

My observation of heterodimerization of GAP2 with GAP1-fragment through their CC motifs is similar to this situation, but I also observed the homodimerization of GAP2 mediated by the CC motif. However, some authors are of the opinion that Gyp5p might possibly form a homodimer through the coiled-coil as well, since it is well conserved between Gyp5p and Gyl1p and interaction of C-terminal region of Gyp5p with full-length Gyp5p was observed in Y2H screen (Talarek et al. 2005). Homodimerization has also been proposed for human EVI, GAP for Rab11, possessing substantial coiled-coil motif in C-terminal region (Faitar et al. 2005). Sadly, no more work related to the possible dimerization has been performed. Other homodimerization in GAPs have not been observed.

Dimerization has been also observed in an *Arabidopsis* GEF. VPS9a, responsible for exchange of GDP for GTP in plant Rab5 homologs RabF1, -F2a, and -F2b was found to dimerize through a C-terminal region containing a sequence conserved only among plant Rab5 GEFs. The region was also necessary for *in vitro* interaction with RabF1, but VPS9a without the region actually resulted in accelerated RabF1-mediated GTP hydrolysis. The conserved region was present even in species lacking in RabF1, so the purpose might be primarily for the dimerization (Sunada et al. 2016). Other Rab5 GEFs in yeast and animals have been observed in dimerized form as well. ALS2 in human cells was found to effectively displace GDP only as a homo-oligomer (Kunita et al. 2004), and yeast VPS9p also exists in dimeric form (Prag et al. 2003).

The argument for GAP2 as a part of GAP cascade would be much stronger, had an interaction with another Rab through the TBC domain been obtained. This, in my very limited testing, was not the case.

Even though the other 3 potential interactors, transmembrane ankyrin repeat protein, Pal of Quirky, and Myosin-heavy chain, from the Y2H screen of cDNA library did not interact in subsequent Y2H test, it is worthwhile to look at their functions and find whether analogous proteins exist as GAP interactors in other organisms besides plants. Ankyrin-repeat is a very common protein-protein interaction motif and according to SMART can be found in more than 170 *Arabidopsis* proteins. No interaction of a GAP with Ankyrin-repeat motif has been described in plants, but human non-calatytic TBC protein USP6/TRE17 interacts with ACAP1, an ankyrin-repeat containing ArfGAP for GTPase Arf6 (Rueckert & Haucke 2012). However, given the sheer number of proteins with Ankyrin-repeat motif, relevance of this similarity is very dubious. Pal of Quirky (PoQ) is a plant specific protein containing an N-terminal PB1

domain, which is characteristic for mediation of hetero- or homodimerizations with other PB1 proteins. In Arabidopsis more than 40 proteins possess this domain. PoQ was observed either unaccompanied in intracellular compartments or at the PM in protein complex containing a membrane anchored C2-domain protein QUIRKY and receptor-like kinase STRUBBELIG. This multiprotein unit seems to function as a signal transduction complex in regulation of cell growth anisotropy (Trehin et al. 2013). Surprisingly as a part of her master's thesis on genes SCRAMBLED and QUIRKY in development of *Arabidopsis* fruit, Nguyen (2008) performed an Y2H screen against cDNA library with QUIRKY as bait. As potential interactors, she uncovered GAP1 (At3g55020) and GAP2 (AT2g39280). Interaction with protein similar to PoQ has not been observed in either yeast of animal Rab GAPs. Retrieved sequence of myosin heavy chain-related protein suggested interaction through its predicted coiled-coil domain. Again, Nguyen (2008) detected a protein with identical name (no accession code was provided) as a putative QUIRKY interactor. In HMMER, homologs of this protein were found only among Viridiplantae, where it is present in both chloro- and streptophyta (Finn et al. 2011).

Outside the plant kingdom, interaction between yeast GAP and proteins other than Rab GTPases has been described by Tcheperegine et al. (2005). Msb3 and Msb4, both implicated in polarized exocytosis were found to interact with Spa2, protein required for their localization in the bud tip. Msb4 was also established as interactor of GDP-bound Cdc42, a small GTPase belonging to Rho family. Gyp6p co-localizes with endosomal Na^+/H^+ exchanger Nhx1 and interacts with its C terminus (R. Ali et al. 2004). Following interactions have been observed between mammalian Rab GAPs and various proteins. EVI5, GAP for Rab11, was found to interact with γ-tubulin, β-tubulin, INCENP, aurora B kinase and survivin (Faitar et al. 2006). TBC1D3, Rab5 GAP, was also found to interact with α- and β-tubulin (He et al. 2014). Lamb et al. (2016) found that TBC1D14 binds to TRAPPC8 subunit of TRAPPIII complex, a GEF for Rab1. TBC1D15, GAP for Rab7, interacts with mitochondria-bound Fis1 and assists with formation of autophagosome around damaged mitochondria (Yamano et al. 2014). TBC1D17 interacts with Rab8 effector, optineurin, protein rich in CC motifs, which recruits it to for deactivation of Rab8 (Vaibhava et al. 2012). TBC1D22, a homolog of yeast gyp1p, competes with phosphatidylinositol 4-kinase class III beta (PI4KB) for interaction with Golgi adaptor acyl coenzyme A binding domain protein 3 (ACBD3) (Greninger et al. 2013). TBC1D5, GAP for Rab7, binds to retromer subunit VPS29, ATG9, clathrin and AP2 complex (Popovic & Dikic 2014). RN-Tre, a GAP for Rab5 interacts with F-actin, its bundling protein actinin-4 and with Eps8 through its SH3 domain (Lanzetti et al. 2000; Lanzetti et al. 2004).

The listed interactions demonstrate versatility of Rab GAPs and their involvement in a number of cellular processes. Some of the interactions are similar for more Rab GAPs regardless of their origin, while other interactions are probably unique for a given species.

5.2. Intracellular localization of GAPs

My observations of GFP-GAP2 were limited to transient transformations in *N.benthamiana*, since the efforts for expression of GFP-fused protein inside *Arabidopsis* were not successful. GFP signal of GAP2 in *N.benthamiana* epidermal cells suggested cytoplasmic localization of the protein. Even though Rab GAPs are generally cytoplasmic by default, they can associate with membrane-bound proteins through conserved domains or otherwise, and thus decorate defined regions within the cell. An example of this is TBC1D2, a GAP for mammalian Rab7a. Its sequence includes pleckstrin homology (PH) domain, which allows it to bind to tight junctions in keratinocytes through association with cadherin complexes (Frasa et al. 2010). Another such domain is phosphotyrosine-binding (PTB) domain of GAPCenA, which interacts with both GDP- and GTP-bound Rab36 and together with it localizes to perinuclear vesicles or GA (Kanno et al. 2010). Other localizing interactions have been observed for TBC1D4, which localizes to GLUT4-endosomal vesicles by binding to insulin-regulated aminopeptidase (IRAP) (Larance et al. 2005). Mammalian EVI5 localizes to nucleus and pericentriolar region of interphase cells (Faitar et al. 2006)

OATL1 is a GAP for Rab33B and resides on autophagosomal membrane thanks to its N-terminally mediated binding of mammalian homologs of Atg8 (Itoh et al. 2011). Previously mentioned RabGAP22 of *Arabidopsis* localizes to either nucleus in no-stress conditions or onto peroxisomes during pathogen infection (Roos et al. 2014). As mentioned in the previous section concerning the GAP cascades, recruitment of Rab GAPs can also be non-permanent, depending on conformation of their recruiting partner. GAP2 might depend on similar mechanism for specific localization because of the previously observed interaction between coiled-coil motif and only GTP-bound Rabs. Interesting in this regard is my observation of punctate bodies containing constitutively active RabA1a-QL and GAP2(wt) and to a lesser degree of those containing RabA1a-QL and GAP2(R303A). Koh et al. (2009) found RabA1a-marked vesicles to co-localize with FM4-64. Since I did not perform co-infiltrations with this or any other organelle marker, I cannot deduce real character of the bodies. They might be just non-specific aggregates resulting from the over-expression, but the fact that they co-localize possibly confirms the interaction observed in the Y2H screen with RabA1a-QL and GAP2-coil-only

region. Expression of GAP2 under its natural promoter was not performed, because of the heterologous system of *N.benthamiana*.

5.3. GAP2-E MUTANT AND ITS COMPLEMENTATION

Deletion of a gene encoding a Rab GAP does not always result in a loud and clear phenotypic trait, but absence of the gene product can affect the organism in a deleterious way, varying from slight decrease in the organism's fitness to nonviability of the cell. Sometimes all that occurs is a slow-down of a pathway or overlap of otherwise temporally separated Rab populations, with the former being caused by absence of timely GDP-Rab recycling by GDIs and the latter attributable to the loss of function in GAP cascade signaling. Often, loss of function of a GAP results in a phenotype similar to that obtained by expression of QL form of its substrate Rab GTPase. Suda et al. (2013) found that yeast *gyp1*Δ *gyp6*Δ mutants suffered from mixing of Ypt1p, Ypt6p, and Ypt32p Rabs, that otherwise occupy distinct *cis*-, *medial*-, and *trans*-Golgi respectively. Recycling between post-Golgi organelles did not function effectively and resulted in accumulation of internal structures. VPS10, which normally cycles between late-Golgi and pre-vacuolar compartment, accumulated on vacuolar membrane and glycosylation function of the Golgi was negatively affected. The cells were viable. Lafourcade et al. (2003) worked with yeast cell with disrupted Gyp2p implicated in deactivation of recycling Ypt6p. Deletion of *gyp2* did not result in any recycling defect, suggesting either redundancy or sufficient intrinsic GAP activity of Ypt6p. According to Bi et al. (2000) and Gao et al. (2003), simultaneous disruption of MSB3 and MSB4, genes encoding GAPs for Sec4p resulted in altered, more round shape of the yeast cell. Partial disruption of actin cytoskeleton and intense accumulation of secretory vesicles were other strong phenotypic traits of the mutant. *gyp5*Δ *gyl1*Δ yeast strain exhibited symptoms similar to that of constitutively active Sec4p-QL – slowed down growth, slow secretion of invertase and accumulation of secretory vesicles. However, the cells were viable (Chesneau et al. 2004). Deleterious mutation of GYP7, a GAP for Ypt7p implicated in homotypic vacuolar fusion, did not affect yeast viability or vacuolar morphology (Vollmer et al. 1999). Null mutant in gyp8p, GAP for Ypt1p, did not suffer from mixing of Ypt1p and Ypt32p in the Golgi as was the case with *gyp1*Δ and *gyp6*Δ mutants (Rivera-Molina & Novick 2009).

Thanks to complexity of multicellular organisms and their dependence on both exo- and endocytosis for signaling, nutrition, etc., loss of Rab GAP genes that results in only benign or slightly deleterious phenotypes in yeast, can have much bigger implications in animals. The compounding effect of the issues with vesicular transport may result in manifestation of

diseases. In HeLa cells, RNA interference against TBC1D20, ER-residing GAP for Rab1 (Ypt1 homolog), does not cause especially pronounced phenotype besides increased presence of Rab1 effector p115, more compact Golgi, and cytoplasm-scattering of COPII-positive structures (Haas et al. 2007). However, disruption of the gene in context of the whole human organism results in Warburg Micro syndrome (WARBM). WARBM is characterized by eye abnormalities such as congenital cataract and optic nerve atrophy, microcephaly accompanied by severe intellectual impairment and seizures, microgenitalia, and quadriplegia. In mice, the phenotype of *tbc1d20*Δ mutant is very similar, with exception of brain abnormalities (Park et al. 2014). The non-canonical RAB3GAP1 + RAB3GAP2 pair is responsible for deactivation of Rab3 GTPase associated with synaptic vesicles. Null mutation in any of the two subunits again causes WARBM and on the intracellular level affects synaptic homeostasis (Müller et al. 2011). Depletion of EVI5 mimics phenotype of constitutively active Rab11 and results in appearance of Sec15-marked vesicles near the PM (Laflamme et al. 2012). Truncation of gene coding for TBC1D4, GAP required for transfer of glucose transporter 4 (GLUT4) vesicles to the PM, was reported in an insulin-resistant patient (Dash et al. 2009). Besides worsened resistance of *rabgap22*Δ mutants to fungal pathogens (Roos et al. 2014), no other loss-of-function Rab GAPs were described in *Arabidopsis*.

Homozygous *gap2-e* plants could not be well characterized because of their nearly unavoidable early lethal phenotype. On soil, they were unable to germinate, while on MS medium, they usually did not survive past emergence of 4 true leaves. They suffered from limited root growth and were much smaller than WT plants of the same age. Complementation efforts were undertaken with GAP2 CDS under constitutive and inducible promoters, but none were successful. With the constitutive promoters, the intent was to obtain both WT and *gap2*-e homozygous plants expressing GFP-fused GAP2 for observation and complementation respectively. It is not very clear why in case of pUBN-GFP-GAP2 plasmid, despite successful herbicide selection of T1 plants, none expressed the protein. Conceivable reason for the lack of expression is gene silencing. The pGWB6-based constructs with stronger 35S promoter suffered from the same problem. Selection of the transgenic plants was successful with non-suspicious ratio of resistant to non-resistant seedlings, but none exhibited GFP signal under epifluorescence microscope. T2 generation of transformed plants was grown and no complementing *gap2*-e homozygote could be detected. This situation is more puzzling, since the identical constructs were successfully used for the *N.bethamiana* infiltrations. It is possible that the transformed *Arabidopsis* plants silenced the gene. It is possible to use *rdr6*Δ mutants

instead of *Col-0* for transformations to avoid the silencing problem. It will be attempted in the future.

Estradiol-inducible lines of *GAP2-E* plants were obtained and presence of the transcript was verified in T2 generation, but no complementing plants were found. The issue might stem from characteristic of the inducing agent. According to Borghi (2010), XVE system is not suitable for whole-plant induction past seedling stage, since 17β-estradiol used for the induction does not propagate well through the plant and the expression is limited to cells close to source of the inducer. Dexamethasone inducible system would have probably been a better choice, since the inducer is supposedly systematically transported throughout the plant. It will be used in the future. Complementation under natural promoter will be attempted as well.

6. CONCLUSIONS

Only some of the goals set forth to test my hypotheses were successfully attained:

Hypothesis: T-DNA mutation in 15th exon of Rab GAP-encoding gene GAP2 is responsible for conditional lethality of seedlings and altered phenotype.

- At least a partial GAP2 transcript was detected in the phenotype-less *gap2-i* mutant, showing that the intron T-DNA insertion does not result in full knock out of the gene.

- GAP2-E heterozygous plants were successfully transformed with GAP2-CDS under inducible promoter and the gene transcribed, but complementation of the *gap2-e* mutant was ultimately unsuccessful.

- Experiments with the inducible complemented *gap2-e* mutant could not be performed.

Hypothesis: GAP2 interacts with some Rab GTPases through its TBC domain.

- No interactions were detected between the full-length or coil-less GAP2 and the RabA or RabE GTPases.

- *Col-0* and *GAP2-E* plants were transformed with T-DNA encoding GFP-fused GAP2 under constitutive promoters, but no fluorescence could be observed.

- Expression of the GAP2 protein in *N.benthamiana* suggested cytoplasmic and non-nuclear localization. Potential co-localization in putative bodies has been observed for RabA1a-QL with GAP2(wt) or GAP2(R303A).

Hypothesis: GAP2-encoded Rab GAP interacts with Rab GTPases not only through the conserved TBC domain, but also through its novel C-terminal coiled-coil domain. The C-terminal domain is sufficient for these interactions.

- GAP2 C-terminal domain with the CC motif was the sole part implicated in interactions with RabA and RabE GTPases.

Hypothesis: GAP2 can interact with other proteins besides Rab GTPases.

- The coiled-coil motif of closely related GAP1 protein was uncovered as a novel interactor of GAP2. GAP2 was found to homodimerize through the aforementioned motif.

REFERENCES

Albert, S. (1999) Identification of the catalytic domains and their functionally critical arginine residues of two yeast GTPase-activating proteins specific for Ypt/Rab transport GTPases. *The EMBO Journal* **18**, 5216–25.

Ali, B.R. et al. (2004) Multiple regions contribute to membrane targeting of Rab GTPases. *Journal of Cell Science* **117**, 6401–12.

Ali, R. et al. (2004) Inhibition of sodium/proton exchange by a Rab-GTPase-activating protein regulates endosomal traffic in yeast. *The Journal of Biological Chemistry* **279**, 4498–506.

Antignani, V. et al. (2015) Recruitment of PLANT U-BOX13 and the PI4Kβ1/β2 phosphatidylinositol-4 kinases by the small GTPase RabA4B plays important roles during salicylic acid-mediated plant defense signaling in Arabidopsis. *The Plant cell* **27**, 243–61.

De Antoni, A. et al. (2002) Significance of GTP hydrolysis in Ypt1p-regulated endoplasmic reticulum to Golgi transport revealed by the analysis of two novel Ypt1-GAPs. *The Journal of Biological Chemistry* **277**, 41023–31.

Asaoka, R. et al. (2013) Arabidopsis RABA1 GTPases are involved in transport between the trans-Golgi network and the plasma membrane, and are required for salinity stress tolerance. *The Plant journal : for cell and molecular biology* **73**, 240–9.

Bahk, J.D., Bang, W.Y. & Heo, J.B. (2009) Plant PRA plays an important role in intracellular vesicular trafficking between compartments as GDF. *Plant signaling & behavior* **4**, 1094–5.

Barr, F.A. (2013) Review series: Rab GTPases and membrane identity: causal or inconsequential? *The Journal of Cell Biology* **202**, 191–9.

Batoko, H. et al. (2000) A rab1 GTPase is required for transport between the endoplasmic reticulum and golgi apparatus and for normal golgi movement in plants. *The Plant cell* **12**, 2201–18.

Bednarek, S.Y. et al. (1994) A small GTP-binding protein from Arabidopsis thaliana functionally complements the yeast YPT6 null mutant. *Plant physiology* **104**, 591–6.

Benli, M. et al. (1996) Two GTPase isoforms, Ypt31p and Ypt32p, are essential for Golgi function in yeast. *The EMBO Journal* **15**, 6460–75.

Berson, T. et al. (2014) Trans-Golgi network localized small GTPase RabA1d is involved in cell plate formation and oscillatory root hair growth. *BMC plant biology* **14**, 252.

Bi, E. et al. (2000) Identification of novel, evolutionarily conserved Cdc42p-interacting proteins and of redundant pathways linking Cdc24p and Cdc42p to actin polarization in yeast. *Molecular Biology of the Cell* **11**, 773–93.

Bi, X., Corpina, R. a & Goldberg, J. (2002) Structure of the Sec23/24-Sar1 pre-budding complex of the COPII vesicle coat. *Nature* **419**, 271–7.

Bizimungu, C. & Vandenbol, M. (2005) At least two regions of the oncoprotein Tre2 are involved in its lack of GAP activity. *Biochemical and biophysical research communications* **335**, 883–90.

Borghi, L. (2010) Inducible Gene Expression Systems for Plants. In L. Hennig & C. Köhler, eds. *Plant Developmental Biology: Methods and Protocols*. Methods in Molecular Biology. Totowa, NJ: Humana Press, 65–75.

Bos, J.L., Rehmann, H. & Wittinghofer, A. (2007) GEFs and GAPs: critical elements in the control of small G proteins. *Cell* **129**, 865–77.

Camacho, L. et al. (2009) Arabidopsis Rab-E GTPases exhibit a novel interaction with a plasma-membrane phosphatidylinositol-4-phosphate 5-kinase. *Journal of Cell Science* **122(Pt 23),**, 4383–92.

Clabecq, A., Henry, J.-P. & Darchen, F. (2000) Biochemical Characterization of Rab3-GTPase-activating Protein Reveals a Mechanism Similar to That of Ras-GAP. *The Journal of Biological Chemistry* **275**, 31786–91.

Conrad, M. et al. (2014) Nutrient sensing and signaling in the yeast Saccharomyces cerevisiae. *FEMS Microbiology Reviews* **38**, 254–99.

Cui, S. et al. (2013) Proteomic analysis reveals that the Rab GTPase RabE1c is involved in the degradation of the peroxisomal protein receptor PEX7 (peroxin 7). *The Journal of Biological Chemistry* **288**, 6014–23.

Cui, Y. et al. (2014) Activation of the Rab7 GTPase by the MON1-CCZ1 Complex Is Essential for PVC-to-Vacuole Trafficking and Plant Growth in Arabidopsis. *The Plant cell* **26**, 2080–97.

Cuif, M.H. et al. (1999) Characterization of GAPCenA, a GTPase activating protein for Rab6, part of which associates with the centrosome. *The EMBO Journal* **18**, 1772–82.

Dabbeekeh, J.T.S. et al. (2007) The EVI5 TBC domain provides the GTPase-activating protein motif for RAB11. *Oncogene* **26**, 2804–8.

Dash, S. et al. (2009) A truncation mutation in TBC1D4 in a family with acanthosis nigricans and postprandial hyperinsulinemia. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 9350–5.

Dasso, M. (2002) The Ran GTPase: theme and variations. *Current Biology* **12**, R502-8.

Dereeper, A. et al. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic acids research* **36**, W465-9.

Donaldson, J.G. & Jackson, C.L. (2011) ARF family G proteins and their regulators: roles in membrane transport, development and disease. *Nature reviews. Molecular cell biology* **12**, 362–75.

Du, L.-L. (1998) Identification of a Sec4p GTPase-activating Protein (GAP) as a Novel Member of a Rab GAP Family. *The Journal of Biological Chemistry* **273**, 3253–56.

Du, L.L. & Novick, P. (2001) Yeast rab GTPase-activating protein Gyp1p localizes to the Golgi apparatus and is a negative regulator of Ypt1p. *Molecular Biology of the Cell* **12**, 1215–26.

Durek, T. et al. (2004) Synthesis of fluorescently labeled mono- and diprenylated Rab7 GTPase. *Journal of the American Chemical Society* **126**, 16368–78.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research* **32**, 1792–7.

Einset, J. et al. (2007) Membrane-trafficking RabA4c involved in the effect of glycine betaine on recovery from chilling stress in Arabidopsis. *Physiologia Plantarum* **130**, 511–8.

Elias, M. (2008) The guanine nucleotide exchange factors Sec2 and PRONE: candidate synapomorphies for the Opisthokonta and the Archaeplastida. *Molecular biology and evolution* **25**, 1526–9.

Ellinger, D. et al. (2014) Interaction of the Arabidopsis GTPase RabA4c with its effector PMR4 results in complete penetration resistance to powdery mildew. *The Plant cell* **26**, 3185–200.

Faitar, S.L. et al. (2005) EVI5 is a novel centrosomal protein that binds to alpha- and gamma-tubulin. *Genomics* **86**, 594–605.

Faitar, S.L. et al. (2006) EVI5 protein associates with the INCENP-aurora B kinase-survivin chromosomal passenger complex and is involved in the completion of cytokinesis. *Experimental cell research* **312**, 2325–35.

Finn, R.D., Clements, J. & Eddy, S.R. (2011) HMMER web server: interactive sequence similarity searching. *Nucleic acids research* **39**, W29-37.

Frasa, M.A.M. et al. (2010) Armus Is a Rac1 Effector that Inactivates Rab7 and Regulates E-Cadherin Degradation. *Current Biology* **20**, 198–208.

Friesen, H. et al. (2005) Interaction of the Saccharomyces cerevisiae cortical actin patch protein Rvs167p with proteins involved in ER to Golgi vesicle trafficking. *Genetics* **170**, 555–68.

Frittoli, E. et al. (2008) The primate-specific protein TBC1D3 is required for optimal macropinocytosis in a novel ARF6-dependent pathway. *Molecular Biology of the Cell* **19**, 1304–16.

Fu, Y., Li, H. & Yang, Z. (2002) The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during Arabidopsis organogenesis. *The Plant cell* **14**, 777–94.

Fu, Y., Wu, G. & Yang, Z. (2001) Rop GTPase-dependent dynamics of tip-localized F-actin controls tip growth in pollen tubes. *The Journal of Cell Biology* **152**, 1019–32.

Fuchs, E. et al. (2007) Specific Rab GTPase-activating proteins define the Shiga toxin and epidermal growth factor uptake pathways. *The Journal of Cell Biology* **177**, 1133–43.

Fukui, K. et al. (1997) Isolation and characterization of a GTPase activating protein specific for the Rab3 subfamily of small G proteins. *The Journal of Biological Chemistry* **272**, 4655–8.

Gao, X.-D. et al. (2003) The GAP activity of Msb3p and Msb4p for the Rab GTPase Sec4p is required for efficient exocytosis and actin organization. *The Journal of Cell Biology* **162**, 635–46.

Gomes, A.Q. et al. (2003) Membrane targeting of Rab GTPases is influenced by the prenylation motif. *Molecular Biology of the Cell* **14**, 1882–99.

Goody, R.S., Rak, A. & Alexandrov, K. (2005) The structural and mechanistic basis for recycling of Rab proteins between membrane compartments. *Cellular and molecular life sciences : CMLS* **62**, 1657–70.

Greninger, A.L. et al. (2013) ACBD3 interaction with TBC1 domain 22 protein is differentially affected by enteroviral and kobuviral 3A protein binding. *mBio* **4**, e00098-13.

Guo, W. et al. (1999) The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. *The EMBO Journal* **18**, 1071–80.

Haas, A.K. et al. (2007) Analysis of GTPase-activating proteins: Rab1 and Rab43 are key Rabs required to maintain a functional Golgi complex in human cells. *Journal of Cell Science* **120**, 2997–3010.

Hall, A. (1990) The cellular functions of small GTP-binding proteins. *Science (New York, N.Y.)* **249**, 635–40.

He, Z. et al. (2014) Cytoplasmic Retention of a Nucleocytoplasmic Protein TBC1D3 by Microtubule Network Is Required for Enhanced EGFR Signaling Y.-H. Song, ed. *PLoS ONE* **9**, e94134.

te Heesen, H., Gerwert, K. & Schlitter, J. (2007) Role of the arginine finger in Ras.RasGAP revealed by QM/MM calculations. *FEBS letters* **581**, 5677–84.

Heo, J.B. et al. (2005) OsGAP1 functions as a positive regulator of OsRab11-mediated TGN to PM or vacuole trafficking. *Plant & cell physiology* **46**, 2005–18.

Hodzic, D. et al. (2006) TBC1D3, a hominoid oncoprotein, is encoded by a cluster of paralogues located on chromosome 17q12. *Genomics* **88**, 731–6.

Huang, Y.-P. et al. (2013) A putative Rab-GTPase activation protein from Nicotiana benthamiana is important for Bamboo mosaic virus intercellular movement. *Virology* **447**, 292–9.

Hutagalung, A.H. & Novick, P.J. (2011) Role of Rab GTPases in membrane traffic and cell physiology. *Physiological reviews* **91**, 119–49.

Chavrier, P. et al. (1991) Hypervariable C-terminal domain of rab proteins acts as a targeting signal. *Nature* **353**, 769–72.

Chesneau, L. et al. (2004) Gyp5p and Gyl1p are involved in the control of polarized exocytosis in budding yeast. *Journal of Cell Science* **117**, 4757–67.

Cheung, A.Y. et al. (2002) Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. *The Plant cell* **14**, 945–62.

Chow, C.-M. et al. (2008) Rab-A2 and Rab-A3 GTPases define a trans-golgi endosomal membrane domain in Arabidopsis that contributes substantially to the cell plate. *The Plant cell* **20**, 101–23.

Ishibashi, K. et al. (2009) Identification and characterization of a novel Tre-2/Bub2/Cdc16 (TBC) protein that possesses Rab3A-GAP activity. *Genes to Cells* **14**, 41–52.

Ismail, S.A. et al. (2010) The structure of an Arf-ArfGAP complex reveals a Ca2+ regulatory mechanism. *Cell* **141**, 812–21.

Itoh, T. et al. (2011) OATL1, a novel autophagosome-resident Rab33B-GAP, regulates autophagosomal maturation. *The Journal of Cell Biology* **192**, 839–53.

Itoh, T. et al. (2006) Screening for target Rabs of TBC (Tre-2/Bub2/Cdc16) domain-containing proteins based on their Rab-binding activity. *Genes to Cells* **11**, 1023–37.

Itoh, T. & Fukuda, M. (2006) Identification of EPI64 as a GTPase-activating Protein Specific for Rab27A. *The Journal of Biological Chemistry* **281**, 31823–31.

Jedd, G., Mulholland, J. & Segev, N. (1997) Two new Ypt GTPases are required for exit from the yeast trans-Golgi compartment. *The Journal of Cell Biology* **137**, 563–80.

Jin, Y. et al. (2011) Myosin V transports secretory vesicles via a Rab GTPase cascade and interaction with the exocyst complex. *Developmental cell* **21**, 1156–70.

Kahn, R.A., East, M.P. & Francis, J.W. (2014) ARF-Like (ARL) Proteins. In *Ras Superfamily Small G Proteins: Biology and Mechanisms 2*. Cham: Springer International Publishing,, 215–51.

Kang, B.-H. et al. (2011) Electron tomography of RabA4b- and PI-4Kβ1-labeled trans Golgi network compartments in Arabidopsis. *Traffic (Copenhagen, Denmark)* **12**, 313–29.

Kanno, E. et al. (2010) Comprehensive Screening for Novel Rab-Binding Proteins by GST Pull-Down Assay Using 60 Different Mammalian Rabs. *Traffic* **11**, 491–507.

Kim, J.J., Lipatova, Z. & Segev, N. (2016) TRAPP Complexes in Secretion and Autophagy. *Frontiers in cell and developmental biology* **4**, 20.

Knödler, A. et al. (2010) Coordination of Rab8 and Rab11 in primary ciliogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 6346–51.

Koh, E.-J. et al. (2009) Altered ARA2 (RABA1a) expression in Arabidopsis reveals the involvement of a Rab/YPT family member in auxin-mediated responses. *Plant molecular biology* **70**, 113–22.

Kunita, R. et al. (2004) Homo-oligomerization of ALS2 through its unique carboxyl-terminal regions is essential for the ALS2-associated Rab5 guanine nucleotide exchange activity and its regulatory function on endosome trafficking. *The Journal of Biological Chemistry* **279**, 38626–35.

Laflamme, C. et al. (2012) Evi5 promotes collective cell migration through its Rab-GAP activity. *The Journal of Cell Biology* **198**, 57–67.

Lafourcade, C., Galan, J.-M. & Peter, M. (2003) Opposite roles of the F-box protein Rcy1p and the GTPase-activating protein Gyp2p during recycling of internalized proteins in yeast. *Genetics* **164**, 469–77.

Lamb, C. a et al. (2016) TBC1D14 regulates autophagy via the TRAPP complex and ATG9 traffic. *The EMBO Journal* **35**, 281–301.

Lane, K.T. & Beese, L.S. (2006) Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *Journal of lipid research* **47**, 681–99.

Lanzetti, L. et al. (2004) Rab5 is a signalling GTPase involved in actin remodelling by receptor tyrosine kinases. *Nature* **429**, 309–14.

Lanzetti, L. et al. (2000) The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5. *Nature* **408**, 374–7.

Larance, M. et al. (2005) Characterization of the Role of the Rab GTPase-activating Protein AS160 in Insulin-regulated GLUT4 Trafficking. *The Journal of Biological Chemistry* **280**, 37803–13.

Lazar, T., Götte, M. & Gallwitz, D. (1997) Vesicular transport: how many Ypt/Rab-GTPases make a eukaryotic cell? *Trends in biochemical sciences* **22**, 468–72.

Letunic, I., Doerks, T. & Bork, P. (2015) SMART: recent updates, new developments and status in 2015. *Nucleic acids research* **43**, D257-60.

Lipatova, Z. et al. (2008) Direct Interaction between a Myosin V Motor and the Rab GTPases Ypt31/32 Is Required for Polarized Secretion. *Molecular Biology of the Cell* **19**, 4177–87.

Lippé, R. et al. (2001) Functional synergy between Rab5 effector Rabaptin-5 and exchange factor Rabex-5 when physically associated in a complex. *Molecular Biology of the Cell* **12**, 2219–28.

Liu, O. & Grant, B.D. (2015) Basolateral Endocytic Recycling Requires RAB-10 and AMPH-1 Mediated Recruitment of RAB-5 GAP TBC-2 to Endosomes J. G. Donaldson, ed. *PLOS Genetics* **11**, e1005514.

Miyawaki, K.N. & Yang, Z. (2014) Extracellular signals and receptor-like kinases regulating ROP GTPases in plants. *Frontiers in plant science* **5**, 449.

Mizuno-Yamasaki, E., Rivera-Molina, F. & Novick, P. (2012) GTPase networks in membrane traffic. *Annual review of biochemistry* **81**, 637–59.

Molendijk, A.J. et al. (2001) Arabidopsis thaliana Rop GTPases are localized to tips of root hairs and control polar growth. *The EMBO Journal* **20**, 2779–88.

Morozova, N. et al. (2006) TRAPPII subunits are required for the specificity switch of a Ypt-Rab GEF. *Nature cell biology* **8**, 1263–9.

Müller, M. et al. (2011) Rab3-GAP controls the progression of synaptic homeostasis at a late stage of vesicle release. *Neuron* **69**, 749–62.

Nagano, F. et al. (1998) Molecular cloning and characterization of the noncatalytic subunit of the Rab3 subfamily-specific GTPase-activating protein. *The Journal of Biological Chemistry* **273**, 24781–5.

Neuwald, A.F. (1997) A shared domain between a spindle assembly checkpoint protein and Ypt/Rab-specific GTPase-activators. *Trends in biochemical sciences* **22**, 243–4.

Nguyen, J.P.A. (2008) Finding Interactions of SCRAMBLED by Using the Yeast Two- Hybrid System. Master's thesis. University of California, San Diego.

Olkkonen, V.M. & Stenmark, H. (1997) Role of Rab GTPases in Membrane Traffic. In *Physiological Reviews*., 1–85.

Ortiz, D. et al. (2002) Ypt32 recruits the Sec4p guanine nucleotide exchange factor, Sec2p, to secretory vesicles; evidence for a Rab cascade in yeast. *The Journal of Cell Biology* **157**, 1005–16.

Paduch, M., Jeleń, F. & Otlewski, J. (2001) Structure of small G proteins and their regulators. *Acta Biochimica Polonica* **48**, 829–50.

Pan, X. et al. (2006) TBC-domain GAPs for Rab GTPases accelerate GTP hydrolysis by a dual-finger mechanism. *Nature* **442**, 303–6.

Park, A.K. et al. (2014) Targeted disruption of Tbc1d20with zinc-finger nucleases causes cataracts and testicular abnormalities in mice. *BMC Genetics* **15**, 135.

Park, H.-O. et al. (1997) Two active states of the Ras-related Bud1/Rsr1 protein bind to different effectors to determine yeast cell polarity. *Proceedings of the National Academy of Sciences* **94**, 4463–8.

Pereira-Leal, J.B. & Seabra, M.C. (2001) Evolution of the Rab family of small GTP-binding proteins. *Journal of molecular biology* **313**, 889–901.

Pereira-Leal, J.B. & Seabra, M.C. (2000) The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. *Journal of molecular biology* **301**, 1077–87.

Perez, P. & Rincón, S.A. (2010) Rho GTPases: regulation of cell polarity and growth in yeasts. *Biochemical Journal* **426**, 243–53.

Pfeffer, S.R. (1996) Transport vesicle docking: SNAREs and associates. *Annual review of cell and developmental biology* **12**, 441–61.

Popovic, D. & Dikic, I. (2014) TBC1D5 and the AP2 complex regulate ATG9 trafficking and initiation of autophagy. *EMBO reports* **15**, 392–401.

Prag, G. et al. (2003) Mechanism of ubiquitin recognition by the CUE domain of Vps9p. *Cell* **113**, 609–20.

Preuss, M.L. et al. (2006) A role for the RabA4b effector protein PI-4Kbeta1 in polarized expansion of root hair cells in Arabidopsis thaliana. *The Journal of Cell Biology* **172**, 991–8.

Preuss, M.L. et al. (2004) The Arabidopsis Rab GTPase RabA4b localizes to the tips of growing root hair cells. *The Plant cell* **16**, 1589–603.

Rak, a et al. (2000) Crystal structure of the GAP domain of Gyp1p: first insights into interaction with Ypt/Rab proteins. *The EMBO Journal* **19**, 5105–13.

Rittinger, K. et al. (1997) Structure at 1.65 A of RhoA and its GTPase-activating protein in complex with a transition-state analogue. *Nature* **389**, 758–62.

Rivera-Molina, F.E. & Novick, P.J. (2009) A Rab GAP cascade defines the boundary between two Rab GTPases on the secretory pathway. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 14408–13.

Rojas, A.M. et al. (2012) The Ras protein superfamily: Evolutionary tree and role of conserved amino acids. *Journal of Cell Biology* **196**, 189–201.

Roos, J. et al. (2014) RabGAP22 Is Required for Defense to the Vascular Pathogen Verticillium longisporum and Contributes to Stomata Immunity M. Gijzen, ed. *PLoS ONE* **9**, e88187.

Rudack, T. et al. (2012) Ras and GTPase-activating protein (GAP) drive GTP into a precatalytic state as revealed by combining FTIR and biomolecular simulations. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 15295–300.

Rueckert, C. & Haucke, V. (2012) The oncogenic TBC domain protein USP6/TRE17 regulates cell migration and cytokinesis. *Biology of the Cell* **104**, 22–33.

Rutherford, S. & Moore, I. (2002) The Arabidopsis Rab GTPase family: another enigma variation. *Current opinion in plant biology* **5**, 518–28.

Saito, C. & Ueda, T. (2009) Chapter 4: functions of RAB and SNARE proteins in plant life. *International review of cell and molecular biology* **274**, 183–233.

Salminen, A. & Novick, P.J. (1987) A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell* **49**, 527–38.

Seewald, M.J. et al. (2002) RanGAP mediates GTP hydrolysis without an arginine finger. *Nature* **415**, 662–6.

Segev, N., Mulholland, J. & Botstein, D. (1988) The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell* **52**, 915–24.

Scheffzek, K. (1997) The Ras-RasGAP Complex: Structural Basis for GTPase Activation and Its Loss in Oncogenic Ras Mutants. *Science* **277**, 333–38.

Sivars, U., Aivazian, D. & Pfeffer, S.R. (2003) Yip3 catalyses the dissociation of endosomal Rab-GDI complexes. *Nature* **425**, 856–9.

Sklan, E.H. et al. (2007) TBC1D20 Is a Rab1 GTPase-activating Protein That Mediates Hepatitis C Virus Replication. *The Journal of Biological Chemistry* **282**, 36354–61.

Sorokina, E.M. & Chernoff, J. (2005) Rho-GTPases: New members, new pathways. *Journal of Cellular Biochemistry* **94**, 225–31.

Speth, E.B. et al. (2009) Subcellular localization and functional analysis of the Arabidopsis GTPase RabE. *Plant physiology* **149**, 1824–37.

Stewart, M. (2007) Molecular mechanism of the nuclear protein import cycle. *Nature reviews. Molecular cell biology* **8**, 195–208.

Suda, Y. et al. (2013) Rab GAP cascade regulates dynamics of Ypt6 in the Golgi traffic. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 18976–81.

Sunada, M. et al. (2016) Functional analyses of the plant-specific C-terminal region of VPS9a: the activating factor for RAB5 in Arabidopsis thaliana. *Journal of plant research* **129**, 93–102.

Szumlanski, A.L. & Nielsen, E. (2009) The Rab GTPase RabA4d regulates pollen tube tip growth in Arabidopsis thaliana. *The Plant cell* **21**, 526–44.

Takai, Y., Sasaki, T. & Matozaki, T. (2001) Small GTP-binding proteins. *Physiological reviews* **81**, 153–208.

Talarek, N. et al. (2005) A novel link between a rab GTPase and Rvs proteins: the yeast amphiphysin homologues. *Cell biochemistry and function* **23**, 253–66.

Tcheperegine, S.E., Gao, X.-D. & Bi, E. (2005) Regulation of Cell Polarity by Interactions of Msb3 and Msb4 with Cdc42 and Polarisome Components. *Molecular and Cellular Biology* **25**, 8567–80.

Trehin, C. et al. (2013) QUIRKY interacts with STRUBBELIG and PAL OF QUIRKY to regulate cell growth anisotropy during Arabidopsis gynoecium development. *Development* **140**, 4807–17.

Ueda, T. et al. (2001) Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of Arabidopsis thaliana. *The EMBO Journal* **20**, 4730–41.

UniProt Consortium (2015) UniProt: a hub for protein information. *Nucleic acids research* **43**, D204-12.

Vaibhava, V. et al. (2012) Optineurin mediates a negative regulation of Rab8 by the GTPase-activating protein TBC1D17. *Journal of Cell Science* **125**, 5026–39.

Veltel, S. et al. (2008) The retinitis pigmentosa 2 gene product is a GTPase-activating protein for Arf-like 3. *Nature structural & molecular biology* **15**, 373–80.

Vernoud, V. et al. (2003) Analysis of the small GTPase gene superfamily of Arabidopsis. *Plant physiology* **131**, 1191–208.

Vetter, I.R. (2014) The Structure of the G Domain of the Ras Superfamily. In A. Wittinghofer, ed. *Ras Superfamily Small G Proteins: Biology and Mechanisms 1*. Vienna: Springer Vienna,, 25–50.

Vollmer, P. et al. (1999) Primary structure and biochemical characterization of yeast GTPase-activating proteins with substrate preference for the transport GTPase Ypt7p. *European Journal of Biochemistry* **260**, 284–90.

Wang, W. & Ferro-Novick, S. (2002) A Ypt32p exchange factor is a putative effector of Ypt1p. *Molecular Biology of the Cell* **13**, 3336–43.

Wennerberg, K. (2005) The Ras superfamily at a glance. *Journal of Cell Science* **118**, 843–46.

Winge, P. et al. (2000) Genetic structure and evolution of RAC-GTPases in Arabidopsis thaliana. *Genetics* **156**, 1959–71.

Winter, D. et al. (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PloS one* **2**, e718.

Wittinghofer, A. & Vetter, I.R. (2011) Structure-Function Relationships of the G Domain, a Canonical Switch Motif. *Annual Review of Biochemistry* **80**, 943–71.

Wuichet, K. & Søgaard-Andersen, L. (2015) Evolution and diversity of the Ras superfamily of small GTPases in prokaryotes. *Genome biology and evolution* **7**, 57–70.

Yalovsky, S. (2015) Protein lipid modifications and the regulation of ROP GTPase function. *Journal of experimental botany* **66**, 1617–24.

Yamano, K. et al. (2014) Mitochondrial Rab GAPs govern autophagosome biogenesis during mitophagy. *eLife* **3**, e01612.

Yorimitsu, T., Sato, K. & Takeuchi, M. (2014) Molecular mechanisms of Sar/Arf GTPases in vesicular trafficking in yeast and plants. *Frontiers in plant science* **5**, 411.

Zheng, H. et al. (2005) A Rab-E GTPase mutant acts downstream of the Rab-D subclass in biosynthetic membrane traffic to the plasma membrane in tobacco leaf epidermis. *The Plant cell* **17**, 2020–36.

Zhu, H., Liang, Z. & Li, G. (2009) Rabex-5 is a Rab22 effector and mediates a Rab22-Rab5 signaling cascade in endocytosis. *Molecular Biology of the Cell* **20**, 4720–9.