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A pull-down procedure for the identification of unknown GEFs for small GTPases

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ABSTRACT

Members of the family of small GTPases regulate a variety of important cellular functions. In order to accomplish this, tight temporal and spatial regulation is absolutely necessary. The two most important factors for this regulation are GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), the latter being responsible for the activation of the GTPase downstream pathways at the correct location and time. Although a large number of exchange factors have been identified, it is likely that a similarly large number remains unidentified. We have therefore developed a procedure to specifically enrich GEF proteins from biological samples making use of the high affinity binding of GEFs to nucleotide-free GTPases. In order to verify the results of these pull-down experiments, we have additionally developed two simple validation procedures: An *in vitro* transcription/translation system coupled with a GEF activity assay and a yeast two-hybrid screen for detection of GEFs. Although the procedures were established and tested using the Rab protein Sec4, the similar basic principle of action of all nucleotide exchange factors will allow the method to be used for identification of unknown GEFs of small GTPases in general.

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Introduction

Small GTPases of the Ras superfamily act as regulators of a variety of important functions such as cell proliferation (Ras),[1,2] remodeling of the cytoskeleton (Rho, Rac and Cdc42),[3] nuclear im- and export (Ran)[4] or vesicular transport (Rab, Sar and Arf).[5,6] They do so by switching between an inactive GDP-bound (guanosine-5′-diphosphate) and an active GTP-bound (guanosine-5′-triphosphate) state, only interacting with specific downstream effector proteins in the active conformation.[7] The interconversion between these states is mediated by GTPase activating proteins (GAPs) that catalyze hydrolysis of GTP and guanine nucleotide exchange factors (GEFs) that catalyze GDP/GTP-exchange.[8]

A recent review giving a comprehensive overview of known GEFs and GAPs for small GTPases and their mechanisms of action can be found in Cherfils *et al.* (2013).[9] The review illustrates the great diversity between the different families of structurally unrelated GEFs. For most small GTPase subfamilies, relative to the number of known members of the subfamily a similar or even higher number of GEFs have been identified to date (see Table 1). However, this is not true for the Rab-family, indicating that further GEFs still need to be identified for this family, but possibly also for other small GTPases. However, their great structural diversity hinders an easy identification of novel GEFs *in silico* based on primary sequences.

In the early days of small GTPase research, the first GEF proteins were found by phenotypic observations related to their function (e.g. SOS,[10] Dbl-GEFs[11]) and/or identified via standard fractionation approaches (e.g., RCC1[12]) of cell extracts or overexpression of the target gene and subsequent analysis of its GEF activity.[13-16] Similarly, Vps9-domain containing[17] and DENN-domain[18] GEFs were found via phenotypic defects associated with mutations or deletions and subsequent analysis of their interacting proteins and their function.[19,20]

In many cases, interactions of a protein with small GTPases containing the mutation corresponding to S17N in Ras (often termed the dominant inactive form of a small GTPase) served as an indication for GEF

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Table 1. Small GTPase families and their known GEFs.

GTPase family	Function	known GEFs	references
Ras (36 members)	Cell proliferation and differentiation	3 families (SOS, RasGRP and RasGRF), all carrying a catalytic Cdc25-homology domain (27 members in humans)	9,65-67
Rho (20 members in human)	Regulation of the cytoskeleton	Dbl-homology (DH) domain GEFs (70 members in human)	9,67,68
		Dock Homology Region (DHR) GEFs, 11 members	
		PRONE GEFs in plants	69
Rab (61 members in humans)	Vesicular trafficking	Vps9 domain GEFs (10 members)	9,67,70,71
		DENN domain GEFs (18 members)	72
		TRAPP complex	73
		Mon1-Ccz1	74
		Rabin8/Sec2	47,60
		Ric1-Rgp1	75,76
		BLOC-3	77
		Mss4	43
		REI-1	22
Arf (5 members + Arls), Sar (127 in total)	Vesicular trafficking	ArfGEFs (Sec7 catalytic domain, 16 members)	9,67
		Sec12	
Ran	Nuclear im- and export	RCC1	9,67
		Importin β	78
		RanBP1	79

activity. Consequently, yeast two-hybrid approaches using small GTPase mutants with lowered nucleotide binding affinity (such as the S17N mutation in Ras, but also others as discussed later) have been used to identify GEFs.[21-25] However, additional and efficient high throughput methodologies are still missing.

We have therefore established a procedure for specific enrichment of GEFs from biological samples making use of the known mechanistic principles of these exchange factors. In order to exchange one bound nucleotide for another, small GTPases have to pass through a nucleotide-free intermediate state. The basic mechanism of GEF action is the stabilization of this nucleotide-free intermediate. The mechanism catalyzed by GEFs can be described as a two-step process, with a first low-affinity encounter complex (Rab:GXP:GEF; GXP can be both GDP or GTP) in which the nucleotide is still bound, and a second high affinity nucleotide-free Rab:GEF complex (see Figure. 1A).[26-28] In the reverse reaction, another nucleotide can bind, thereby releasing the Rab protein from the GEF. Any directionality of this reaction (i.e. from a GDP-bound toward a GTP-bound small GTPase) is achieved by the excess of GTP over GDP present in cells under physiological conditions[29] and the relative affinities of the corresponding GTPase toward GDP and GTP. Because of the high concentrations of guanine nucleotides (0.5 mM GTP, 0.15 mM GDP) inside cells,[29] the nucleotide-free Rab:GEF complex is only short-lived *in vivo*. However, the complex can be stabilized by artificially depleting the environment of guanine nucleotides as has been done in many cases to obtain stable complexes for X-ray crystallography (see for example references[30-34]).

Because of the high-affinity binding of nucleotide-free GTPases and GEFs, the nucleotide-free GTPases should be a suitable bait for pull-down experiments in order to identify unknown GEFs for a given small GTPase from natural sources. We have established this procedure using the known Rab:GEF pair Sec4:Sec2, showing that the endogenous GEF protein can be specifically enriched from yeast cell lysate. Furthermore, we have established a straightforward and rapid procedure to validate putative targets from such pull-down experiments.

Results

Pull-down procedure and analysis by mass spectrometry

In order to specifically enrich GEF proteins from cell lysates, Sec4 was first biotinylated using the commercially available EZ-Link™ Maleimide-PEG2-Biotin and immobilized on streptavidin magnetic beads (SMBs). Subsequently, the immobilized Sec4 was incubated with yeast cell lysate spiked with exogenously added alkaline phosphatase to degrade all nucleoside tri-, di- and monophosphates present in the samples and to allow stable complex formation between the GTPase and GEFs. The idea behind this was that complex formation between the immobilized GTPase and the corresponding GEF would lead to release of the bound nucleotide and degradation by the alkaline phosphatase, thus yielding a high-affinity nucleotide-free GEF:GTPase complex. After several washing steps using standard buffer, bound GEFs were specifically eluted in 2 subsequent steps with buffer containing 500 μM GTP and 10 mM GTP, respectively. As negative controls, the same experiments were

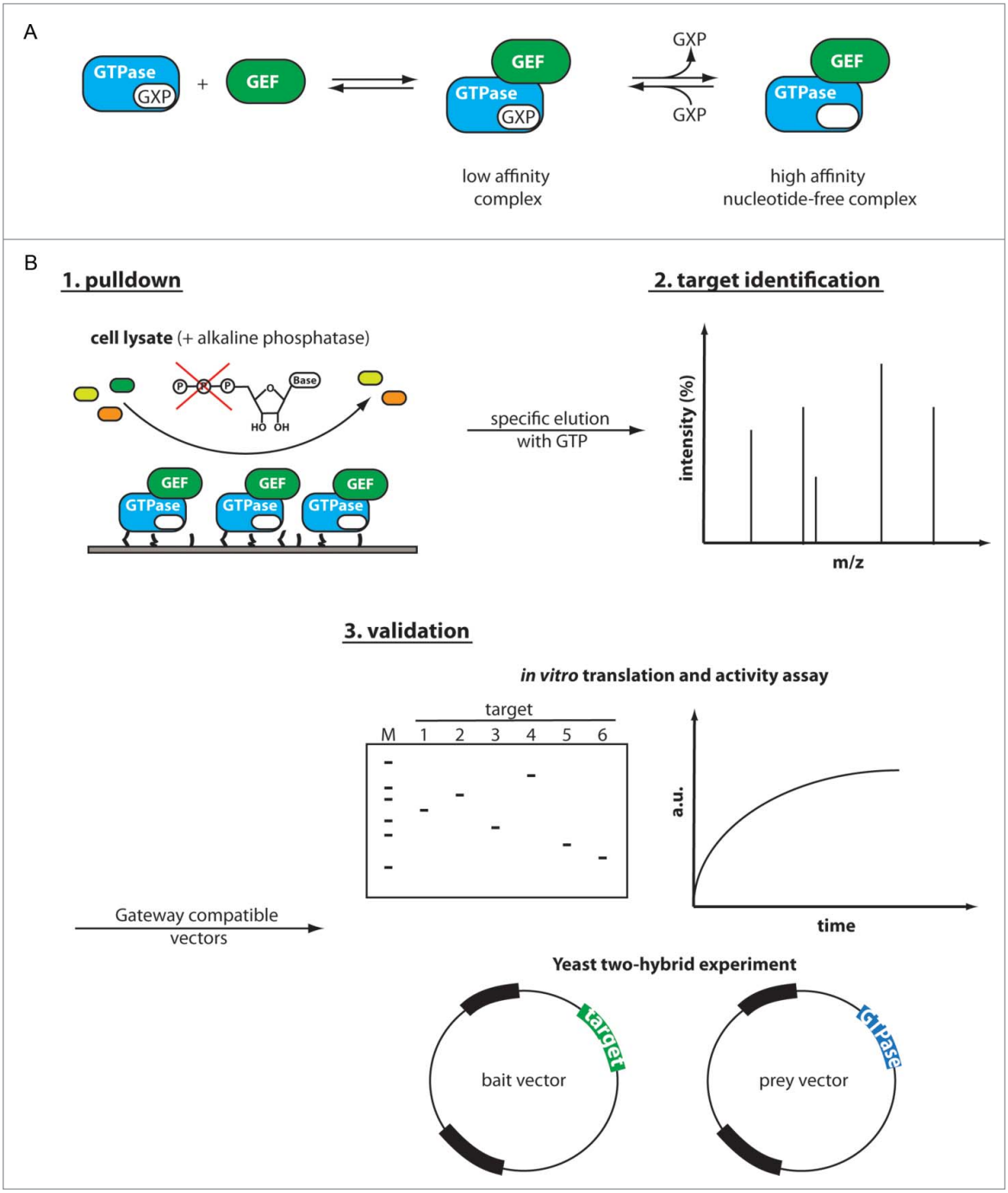

Figure 1. Scheme for the specific enrichment and identification of unknown GEFs of small GTPases. (A) General reaction scheme of guanine nucleotide exchange factors for small GTPases. GEFs operate by transiting from a low affinity ternary GTPase:GXP:GEF intermediate to a high-affinity binary GTPase:GEF complex and back. In a first step, the GEF binds the GTPase:GXP complex with low affinity. GEF-mediated release of the nucleotide in the second step leads to a high affinity GEF:GTPase complex. In the reverse reaction, a different guanine nucleotide can bind, thereby completing the exchange reaction. (B) We envision to enrich the specific GEFs from cell lysates by exploiting the high-affinity of the intermediary nucleotide-free GTPase:GEF complex. The immobilized GTPase of interest is incubated with cell lysate containing its cognate GEF (green) among several other proteins (yellow and orange). Upon formation of a binary GTPase:GEF complex, the released nucleotide is degraded by exogenously added alkaline phosphatase to further stabilize the GTPase:GEF complex. After washing and removal of unbound proteins, the GEF can be specifically eluted by addition of GDP or GTP followed by subsequent identification via mass spectrometry. Since complex mixtures such as cell lysates may give rise to false positive targets, we designed the following procedures for GEF validation: I) An *in vitro* translation system coupled with an activity based GEF assay and II) a Y2H experiments with specifically designed GTPase mutants favoring GEF-interaction. The use of Gateway compatible vectors for both validation procedures greatly simplifies the cloning of different target proteins into expression vectors or vectors for Y2H experiments.

performed using SMBs without immobilized Sec4. The eluted samples from all experiments were digested with trypsin and the peptide pattern was subsequently analyzed via mass spectrometry (MS) in a nano-HPLC coupled Quadrupole-Orbitrap Mass Spectrometer (Fig. 1B, see materials and methods for the exact procedure). In order to allow statistical evaluation of the experiments, every experiment was repeated thrice with two experimental replicates (6 experiments in total) and only proteins that were specifically enriched in the Sec4 pull-down experiments, but not in the control experiments, were regarded as significant hits.

A total of 10 different proteins were significantly enriched in at least one of the triplicate experiments compared to the control experiments (Fig. 2 and Table S1). Besides several other proteins, both known GEFs of Sec4 (Sec2 and Dss4[35,36]) were among these 10 proteins, indicating that the method will be suitable for identification of unknown GEFs for other GTPases as well. Most notably, Sec2 showed the highest label free quantification (LFQ) intensities in most experiments and was significantly enriched in all triplicate experiments (Fig. 2). In contrast, Dss4 was only significantly enriched in one triplicate. In addition to the known GEFs, two proteins known to interact with Rab proteins (Ivy1[37] and Mrs6[38]) were enriched as well as several proteins not known to have a function in Rab-mediated vesicular trafficking.

We wondered whether the established method might also be suitable to identify proteins that directly interact with the active (i.e., GTP-bound) GTPases, but not with GEF proteins. Therefore, in an additional experiment, biotinylated Sec4:GppNHp (GppNHp – Guanosine-5′-[β,γ-imido]triphosphate) was immobilized on the SMBs for similar pull-down experiments, but without the addition of alkaline phosphatase. Interestingly, both Ivy1 and Mrs6 were again specifically enriched in these pull-down experiments as well as the known Sec4 effector protein Sro7[39] (besides several other proteins; see Table S2 and Fig. S1) and were identified via MS. In contrast, neither Sec2 nor Dss4 were detected via MS, thus indicating that nucleotide-free GTPases need to be used to specifically enrich their corresponding GEFs.

Validation of putative targets

Since 10 different proteins were specifically enriched in the previous Sec4 pull-down experiments compared to the control experiments and pull-down experiments from complex samples generally contain many false positive results, we set up a strategy to validate these putative binding partners. The methods used aimed at detecting the activity of potential GEFs, but also to verify other potential interaction partners that were co-enriched in the pull-down experiments. Because commonly used recombinant expression and purification strategies are laborious and time-consuming when multiple different putative targets need to be tested, we used a commercially available *in vitro* transcription/translation (IVTT)

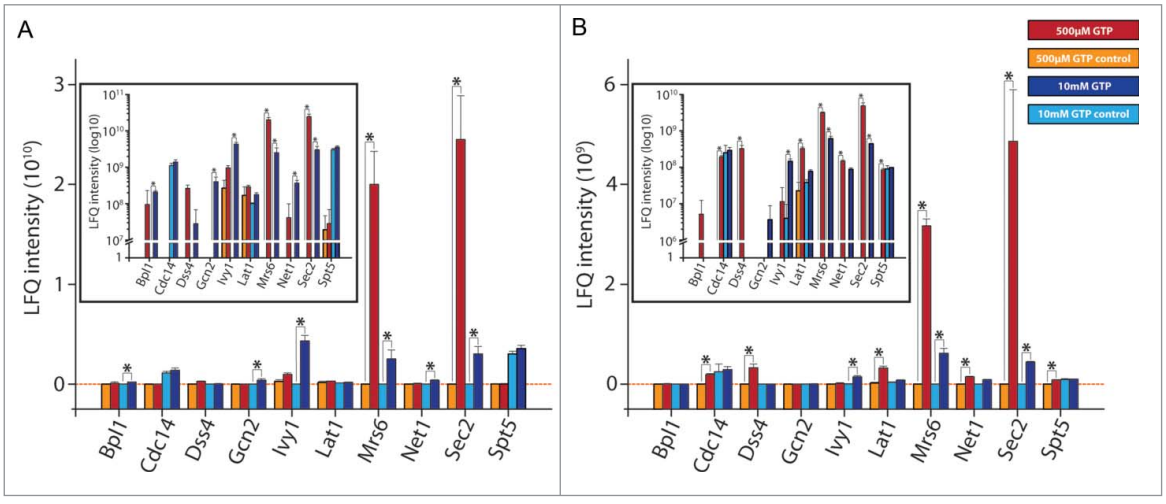

Figure 2. Proteins that were specifically enriched in the pull-down experiments. (A) and (B) show the two independent experimental replicates of the pull-down experiments with each single experiment performed in triplicate. The average values of the label free quantification (LFQ) with their standard deviations are indicated in the graphs for all proteins that were significantly enriched in at least one triplicate (red: elution with 500 μM GTP; orange: control experiment not containing immobilized Sec4 and elution with 500 μM GTP; blue: elution with 10 mM GTP; cyan: control experiment not containing immobilized Sec4 and elution with 10 mM GTP; * indicates statistically relevant enrichment comparing the pull-down and the control experiment (t-test)). The insets show the same graphs with logarithmic scale of the LFQ intensities for better visualization of the differences between the experiments and the corresponding controls.

system[40,41] coupled with a fluorescence based exchange assay to validate the GEF(s) among the other identified targets. Independently, all putative targets were used in yeast two-hybrid (Y2H) experiment to allow validation of GEFs and other binding partners of the GTPase (Fig. 1B). To simplify the procedure, Gateway compatible vectors were used that allow easy shuttling of the genes coding the putative targets for the different validation steps into expression vectors as well as vectors for the Y2H screen.

Validation of putative targets via IVTT and GEF assay

For the IVTT system, all constructs were cloned into expression vectors containing N-terminal His_6-tags via the Gateway cloning system.[41] Subsequently, all constructs were produced *in vitro* in small scale IVTT-expressions (typically ~40 μl) and expression of the different proteins was confirmed via western-blotting against the His_6-tag with an α-His_6-tag-antibody (Fig. S2a). Out of 10 proteins, 7 could be readily produced in detectable amounts and these were directly used for a fluorescence based GEF activity assay without further purification. For the activity assay, Sec4 was preparatively loaded with the fluorescent nucleotide mantGDP and displacement of mantGDP with GDP was monitored. In these experiments, only the known GEF Sec2 induced a significant increase in the rate of nucleotide exchange (Fig. 3 and Fig. S2). Dss4 did not increase the rate of nucleotide exchange in these experiments. However, keeping in mind the 23-fold lower activity of Mss4 toward Rab8 (Mss4 is the human homolog of Dss4; $k_{cat}/K_M = 8.5{\cdot}10^3\ M^{-1}\ s^{-1}$) and the 285-fold lower activity of Dss4 toward Ypt1 ($k_{cat}/K_M = 7.2{\cdot}10^2\ M^{-1}s^{-1}$) compared to Sec2:Sec4 ($k_{cat}/K_M = 2.0{\cdot}10^5\ M^1\ s^{-1}$) as well as the proposed function of Mss4 as a chaperone rather than a GEF,[42,43] this is not surprising. Thus, a 23- to 285-fold higher expression level would be necessary for Dss4 compared to Sec2 to obtain a similar exchange activity.

Validation of putative targets via yeast-two hybrid experiments

In a second validation step, all putative targets were cloned into a Gateway-compatible pGBKT7 bait-vector for Y2H-screening. In order to impede GTPase-prenylation that could interfere with targeting of Sec4 to the nucleus required for monitoring protein-protein interactions in Y2H, $Sec4_{WT}$ (WT – wild type) without the two C-terminal Cys-residues ($Sec4_{WT}\Delta C$) was used as a prey. Additionally, we constructed different mutants of Sec4

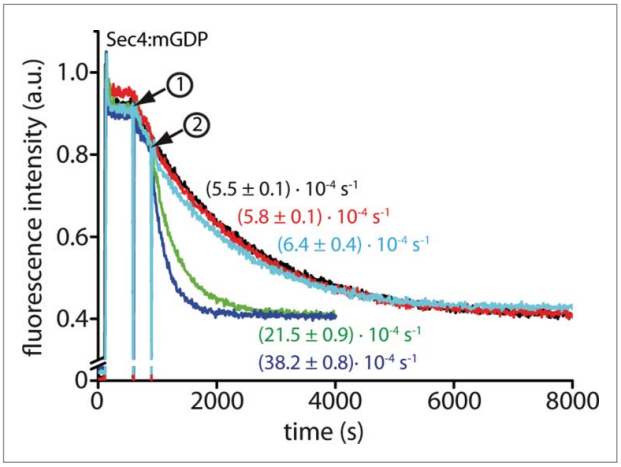

Figure 3. Nucleotide exchange assay using proteins from cell free expression. Sec4:mantGDP (1 μM) was incubated with 200 μM GDP (step 1) without addition of cell-free expressed protein showing the intrinsic rate of nucleotide exchange (black curve) and after addition (step 2) of 20 μl cell-free expression mixture (red curve) as control experiments. These experiments show that the cell-free expression mixture does not contain factors that accelerate nucleotide exchange. Additionally, similar experiments were repeated after expression of the putative targets (green and blue curves: 10 μl and 20 μl cell free expression mixture after expression of Sec2, respectively; cyan: 20 μl cell free expression mixture after expression of Dss4). Observed rate constants are indicated for each curve.

mimicking the GDP-bound inactive state ($Sec4_{S34N}\Delta C$), the GTP-bound active state ($Sec4_{Q79L}\Delta C$) and a xanthosine 5′-triphosphate- (XTP-) binding mutant ($Sec4_{D136N}\Delta C$).[44] The D136N-substitution in $Sec4_{D136N}\Delta C$ changes the preference of the GTPase from guanine to xanthosine nucleotides. The latter mutant was designed since the environment of the small GTPase cannot be depleted of nucleotides *in vivo* (e.g. in a Y2H screen), hence not allowing the stable GEF:$GTPase_{WT}$ complex formation that is necessary for the Y2H experiment. Since XTP occurs at extremely low abundance in cells[45] we reasoned that this mutant might be effectively uncomplexed with any nucleotide and therefore faithfully represent a nucleotide-free Sec4 mimic. Expression of all bait and prey constructs in yeast were tested via western-blotting using Gal4 DNA-binding domain (Gal4 DBD) or Gal4 transcriptional activation domain (Gal4 TA) specific antibodies (Fig. S4) indicating that all except Net1 and Spt5 were well expressed.

All 10 putative targets were first tested against an empty prey-vector (Fig. 4A), and this showed that only Mrs6 caused auto-activation in the Y2H screen. In a second step, the experiments were repeated against the prey-vectors containing the different Sec4 mutants. Growth on selective media lacking histidine indicated that both Dss4 and Sec2 interact with the dominant

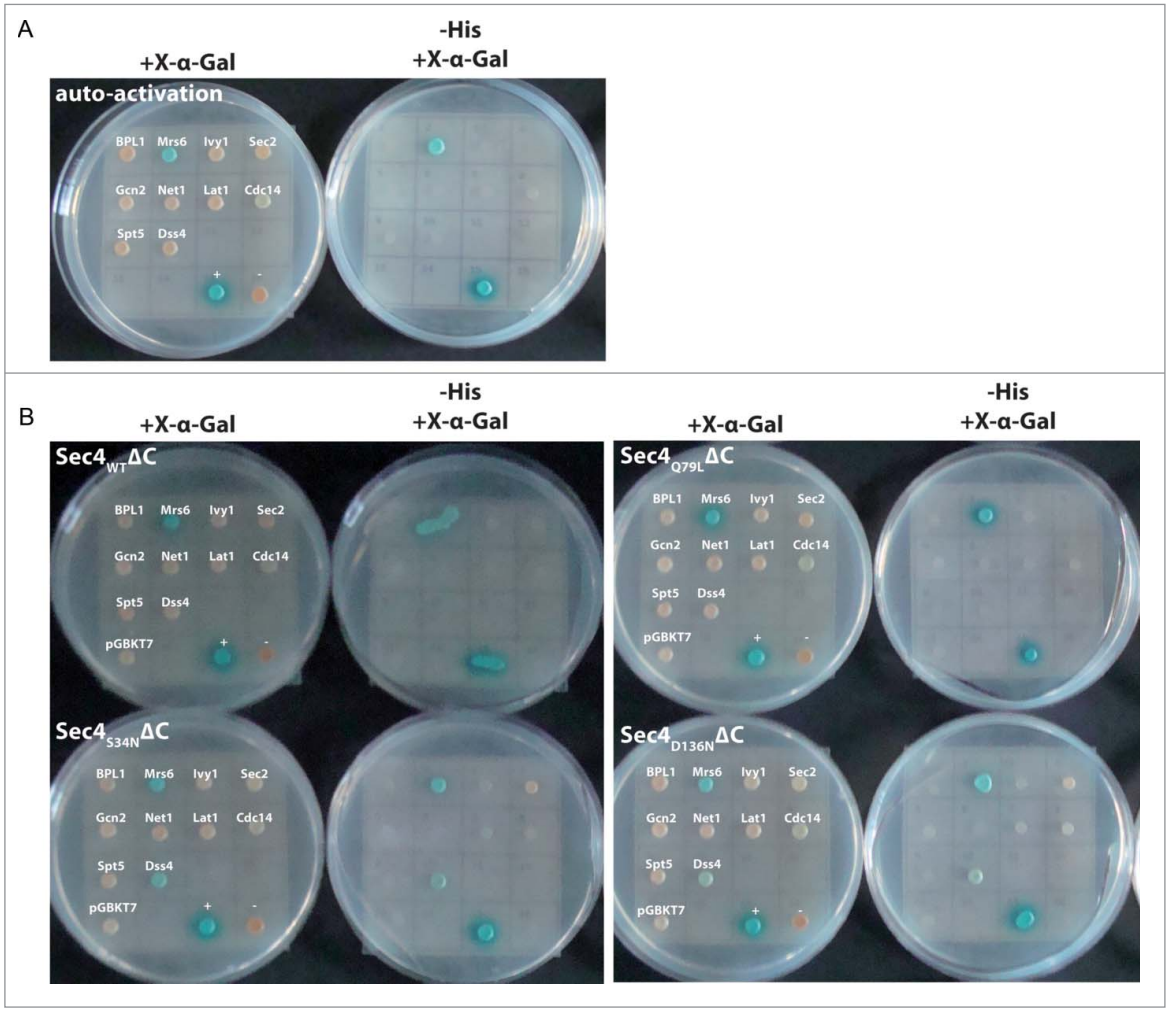

Figure 4. Results of the yeast two-hybrid screens with Sec4. (A) All 10 putative targets from the pull-down experiments were cloned into bait-vectors and tested for auto-activation against empty prey-vectors. In these experiments, only Mrs6 displayed auto-activation. (B) The Y2H experiments were repeated using the prey-vectors containing $Sec4_{WT}\Delta C$, $Sec4_{S34N}\Delta C$, $Sec4_{Q79L}\Delta C$ or $Sec4_{D136N}\Delta C$ to test for interaction with putative binding partners. Growth on the selective medium lacking histidine (-His) indicates an interaction of both known GEFs (Sec2 and Dss4) with $Sec4_{S34N}\Delta C$ and $Sec4_{D136N}\Delta C$, but not with $Sec4_{WT}\Delta C$ and $Sec4_{Q79L}\Delta C$. However the interaction is probably weak (as expected for an enzymatic interaction) and turnover of the chromogenic substrate X-α-Gal could only be observed for Dss4, but not Sec2 ((+) and (−): positive (pGBKT7–53/pGADT7-T) and negative (pGBKT7-Lam/pGADT7-T) controls).

negative mutant $Sec4_{S34N}$ as well as the supposedly nucleotide-free (and xanthosine nucleotide specific) $Sec4_{D136N}$ variant, but not with $Sec4_{WT}$ or the constitutively active $Sec4_{Q79L}$ (Fig. 4B and Table 2). We also observed a very weak positive signal for the non-GEF protein Cdc14 in the Y2H experiments using $Sec4_{D136N}$. In addition to growth on the selective medium we also observed turnover of the chromogenic substrate X-α-Gal in the case of Dss4, but not Sec2. For the latter this is presumably a consequence of the low-affinity interaction between GEFs and small GTPases in the presence of high concentrations of nucleotides present in cells. Furthermore these results suggest that $Sec4_{D136N}\Delta C$ still binds endogenous nucleotides under these conditions *in vivo*, and this is in keeping with the observation that for the corresponding Ras mutant ($H\text{-}Ras_{D119N}$) the affinity of GDP (and presumably GTP), while much weaker than that of the wild-type protein, is still in the submicromolar range.[46] We consequently also tested a double mutant ($Sec4_{N133I,\ D136N}$) since both mutations have been reported to strongly reduce guanine nucleotide binding affinity.[44] However $Sec4_{N133I,\ D136N}$ was not expressed in yeast (Fig. S4) probably because it was toxic toward yeast arising from sequestering the complete cellular pool of

Table 2. Results of the Y2H experiments shown in Figure 4.

	Bpl1	Mrs6	Ivy1	Sec2	Gcn2	Net1	Lat1	Cdc14	Spt5	Dss4
autoactivation	–	+++	–	–	–	–	–	–	–	–
$Sec4_{WT}\Delta C$	–	+++	–	–	–	–	–	–	–	–
$Sec4_{S34N}\Delta C$	–	+++	–	+	–	–	–	–	–	++
$Sec4_{Q79L}\Delta C$	–	+++	–	–	–	–	–	–	–	–
$Sec4_{D136N}\Delta C$	–	+++	–	+	–	–	–	(+)	–	++

endogenous cognate nucleotide exchange factors (as already suggested in the original publication on these mutations[44]). Despite this, for human GTPases the corresponding double mutant might be a better mimic of a nucleotide-free protein without having the toxic side effects seen in yeast.

We subsequently used other known Rab:GEF pairs with different mutations of the Rab proteins in a similar experiment to verify the general applicability of this approach. The pairs tested included Rab8:Rabin8 and Rab8:GRAB (Rabin8 and GRAB are Sec2 homologues from human)[47] as well as Rab1b:DrrA[30] (Fig. 5). All

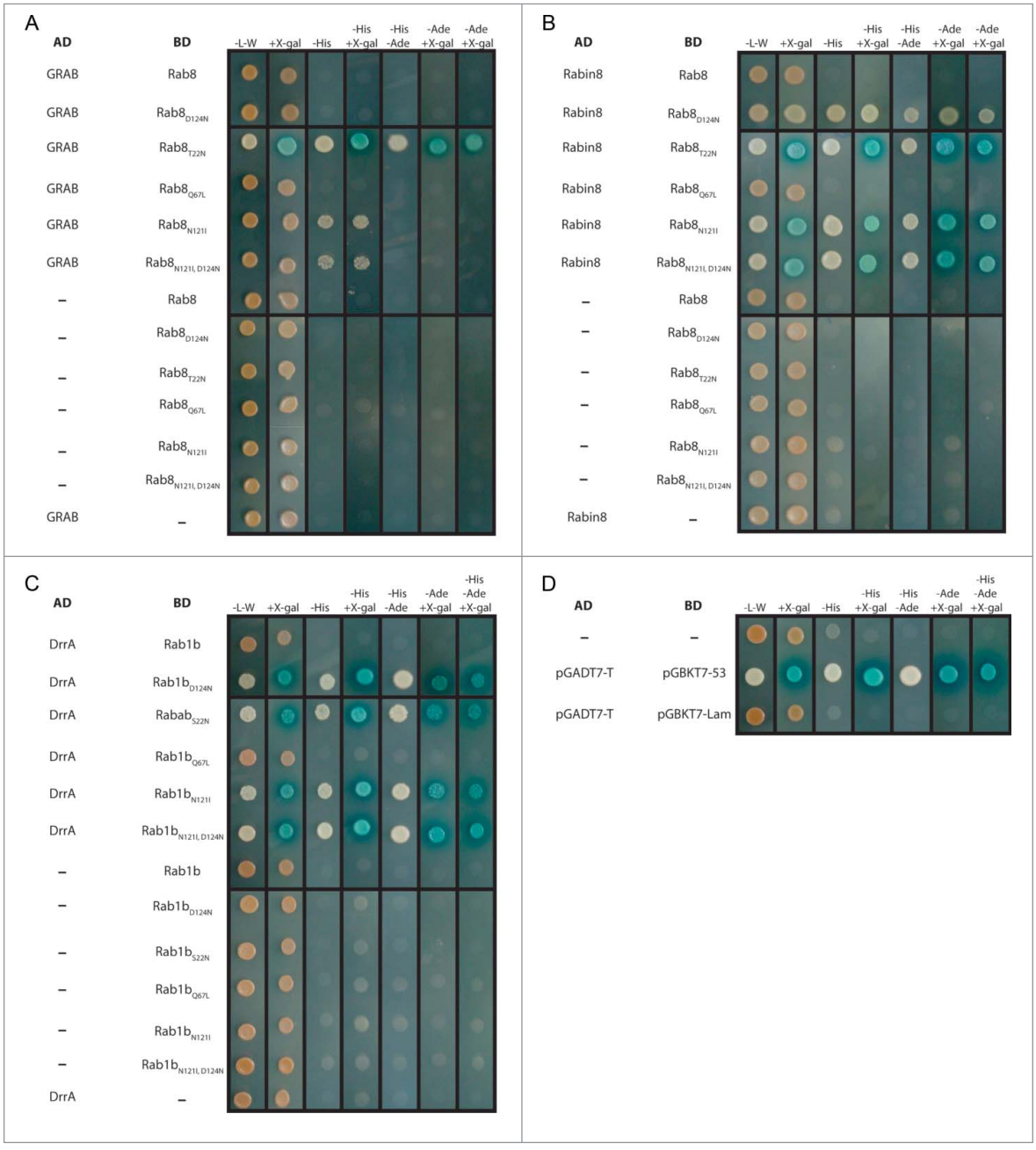

Figure 5. Results of the yeast two-hybrid screens with known Rab:GEF pairs. (A) The GEF domains of GRAB (aa 73–154 cloned into the vector pGADT7) and (B) Rabin8 (aa 153–237 in vector pGADT7) were tested for interaction with Rab8A (aa 1–203 in vector pGBKT7) and different mutants of Rab8 ($Rab8_{T22N}$, $Rab8_{Q67L}$, $Rab8_{N121I}$, $Rab8_{D124N}$, $Rab8_{N121I,\ D124N}$). (C) In a similar experiment, the GEF domain of the Legionella pneumophila GEF DrrA (aa 340–533) was tested for interaction with Rab1B (aa 1–199) and the corresponding mutants of Rab1B ($Rab1_{S22N}$, $Rab1_{Q67L}$, $Rab1_{N121I}$, $Rab1_{D124N}$, $Rab1_{N121I,\ D124N}$). All experiments were also performed with the corresponding empty vectors (—) to exclude auto-activation. (D) Shown are the positive (pGBKT7–53/pGADT7-T) and negative (pGBKT7-Lam/pGADT7-T) controls as well as the control with empty vectors (—/—).

experiments indicated an interaction between the GEFs and most of those Rab mutants impaired in nucleotide binding, but not with wildtype Rabs or the constitutively active (Q67L in Rab1b and Rab8) mutant. However we observed notable differences in the strength of the observed signals and the possible stringency of selection in the Y2H experiments. Notably, the Rab proteins containing the mutations N121I and D124N only showed interactions at low stringency of selection for $Rab8_{N121I}$:GRAB (Fig. 5A) and $Rab8_{D124N}$:Rabin8 (Fig. 5B) and completely missing interaction for $Rab8_{D124N}$:GRAB (Fig. 5A). In contrast, the mutation corresponding to S22N in Rab1b and T22N in Rab8 showed strong interaction with all GEFs tested and under maximum possible stringency of selection (Fig. 5A-C), thus making this mutation the preferable one for identification of interacting GEFs.

Discussion

GEFs play a crucial role for correct spatial and temporal activation of small GTPases. For Rab-proteins, it has emerged over the last few years that GEFs have an important function in localization of the Rab-proteins to the correct intracellular membranes.[48] Furthermore, many intracellularly surviving pathogens provide GEFs to manipulate small GTPase signaling and regulate events in infected host cells.[49] However, despite the apparent importance of GEF molecules, for many small GTPases the corresponding natural or pathogenic GEF(s) have yet to be identified.

In contrast to Rab GAPs, where systematic analyses of their functional roles and their corresponding Rab substrates have been performed based on the strong sequence similarity among the GAPs,[50] a similar approach is not possible for GEFs due to their great structural diversity.[9] We have therefore shown in this publication that a pull-down procedure can be used to specifically enrich GEFs for small GTPases by depleting their environment of nucleotides and thus making use of the high affinity of GEFs for nucleotide-free GTPases. A similar procedure has previously been applied to specifically enrich GEFs for Rho proteins[51] making use of a mutation corresponding to G15A in Ras, a mutation that causes a dramatic decrease of nucleotide binding affinity.[52] To the best of our knowledge, this is the first time that a pull-down procedure for small GTPases has been set up for specific enrichment of nucleotide exchange factors among the different GTPase interacting proteins by making use of the basic mechanism of GEFs and using the wildtype form of a GTPase. Since nucleotide-free GTPases (and GTPases carrying mutations that render them effectively nucleotide-free) are often inherently unstable and difficult to handle, this greatly simplifies the pull-down procedure and makes it more generally applicable. Testing the principle for the Rab protein Sec4 showed that the procedure can be applied successfully and both known exchange factors (Sec2 and Dss4) were specifically enriched and identified by this approach. However, even though the method worked very well for these Rab:GEF pairs, one has to keep in mind that missing factors in positive or negative feedback loops as observed in Rab cascades,[53] the absence of post-translational modifications[8] or putative GEFs containing transmembrane regions might interfere with an easy identification via this method.

Since pull-down experiments from complex samples always give rise to many false positive targets, we furthermore established validation procedures to identify the GEFs among the putative targets. One further development of this procedure could be the use of stable-isotope labeling of amino acids in cell culture (SILAC) to further reduce the number of false positives detected via mass spectrometry and consequently minimize the effort needed to validate the putative targets.[54]

The validation procedures reported in this publication include detection of the activity of potential GEFs as well as Y2H screens to allow identification and verification of GEFs and other general binding partners of small GTPases. The results using activity-based detection of GEF proteins presented above indicate that only potent GEFs can be easily identified by this approach (as shown for Sec2), while for less potent GEFs, more elaborate experiments will have to be performed. However the catalytic efficiencies (k_{cat}/K_M) of yet identified GEFs for small GTPases cover the whole range from 10^2 M^{-1} s^{-1} to 10^7 M^{-1} s^{-1} ($k_{cat}/K_M = 2.0 \cdot 10^5$ M^{-1} s^{-1} for Sec2),[55] thus making the procedure generally applicable for many of them.

A second approach for validation and identification of GEFs is provided by the Y2H experiments used in this publication. The possibility to identify GEFs via this approach has previously been shown for other exchange factors in several publications (see e.g., refs. 21-25) by using mutants of small GTPases with a lowered nucleotide affinity. Also, a modified yeast two-hybrid approach making use of the fact that small GTPases interact with their effectors only after activation by a GEF has been used in the past to characterize GEFs.[56]

Binding of both Dss4 and Sec2 to $Sec4_{S34N}$ and $Sec4_{D136N}$ observed in the Y2H experiments can be easily explained by the preferential binding of GEFs to nucleotide-free GTPases and more generally by the preferential binding of GEFs to the disordered open conformation of the switch regions of small GTPases.[9] Several effects contribute to this. First, the S34N mutation in GTPases leads

to a strongly reduced nucleotide affinity (which is more strongly pronounced for GTP than for GDP),[57] while the D136N mutation leads to a dramatic reduction of nucleotide affinity because of the loss of an important specific interaction with the nucleobase. The S34N mutant should be regarded as a hybrid nucleotide-free and GDP-bound mimic. Consistently, a specific interaction has previously been observed in Y2H experiments for Rabin8 (the human homolog of Sec2) and the constitutively inactive mutant $Rab8_{T22N}$, but not with $Rab8_{WT}$ or the constitutively active $Rab8_{Q67L}$ mutant.[23] In addition to the aforementioned effects, reported relative affinities of small GTPases toward GTP and GDP usually show a slightly (approximately 2- to 4-fold) higher nucleotide-affinity of the GTPases toward GTP compared to GDP,[57,58] thus making the GDP-bound state a thermodynamically slightly better binding partner of GEFs than the GTP-bound state.

The general applicability of the Y2H approach was shown in this publication using different GEFs together with their known Rab substrates and different mutants thereof. These experiments indicate that comparative Y2H experiments using mutants of small GTPases mimicking the different activity states (i.e. active, inactive or nucleotide-free) can give valuable insights regarding the function of interacting proteins. However, it has to be borne in mind that many protein-protein interactions are of transient nature with K_D or K_M-values generally in the μM range and the mutations used are only mimics of the different activity states. In some cases they might even hinder GEF binding.[59] Therefore only the combination of specific enrichment of potential GEFs combined with different validation steps will give conclusive answers as to whether a certain protein is a GEF or not.

Materials and methods

Pull-down procedure

Purified full length Sec4 (purification as described[60]) was biotinylated using EZ-Link™ Maleimide-PEG2-Biotin (Life Technologies) as described by the manufacturer and 1.5 nmole biotinylated Sec4 was immobilized on streptavidin magnetic beads (SMBs, New England Biolabs, 250 μl of the bead solution was thoroughly washed with phosphate buffered saline (PBS; 137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4, 1.8 mmol/l KH_2PO_4) pH 7.0 prior to usage).

Yeast cell lysate was prepared by growing yeast cells in YPDA medium to OD_{600nm}=1.7–3.0, harvesting the cells, and resuspending them in PBS pH 7.0 supplemented with protease inhibitors (1 mM phenylmethanesulfonylfluoride and each 5 μg/ml chymostatin, leupeptin, antipain and pepstatinA). Subsequently the cells were lysed using a French press and the supernatant was cleared by centrifugation (75600 g, 10 °C, 1 h).

The yeast lysate (total protein concentration 1 mg/ml) was spiked with 25 units of alkaline phosphatase per ml cell lysate and stored on ice 1 h before usage. A volume of 1 ml lysate was added to the immobilized Sec4 and incubated for 45 min on ice before removing the supernatant and washing three times with 500 μl PBS pH 7.0. Addition of cell lysate and washing with PBS was repeated twice before eluting the bound proteins with 100 μl PBS pH 7.0 containing 500 μM GTP and subsequently 100 μl PBS containing 10 mM GTP. All experiments were performed in triplicate with two biological replicates using a different batch of Sec4 and yeast cell lysate. Additionally similar experiments without immobilized Sec4 were performed as negative controls.

Mass spectrometry

For mass spectrometry, samples from the pull-down were digested in solution using proteomics grade porcine trypsin protease (Sigma Aldrich). A volume of 100 μl of digestion buffer (50 mM Tris pH 7.5, 1 mM DTT, 13.3 % w/v carbamide, 5 μg/ml trypsin) were added to the sample and the mixture was incubated for 1 h at room temperature in a tube rotator at 40 rpm. A volume of 100 μl of alkylation buffer (50 mM Tris pH 7.5, 13.3 % w/v carbamide, 55 mM iodoacetamide) was added and the sample was further incubated overnight at 37°C and 350 rpm in a thermomixer. The reaction was stopped by addition of 2 μl Trifluoroacetic acid.

Digested samples were purified using StageTips made of dual layer blank-outs from Empore™ 2215-C18 extraction discs (3M) as described previously.[61] Shortly, StageTips were activated with 100μl methanol and equilibrated by two further centrifugation cycles with 100 μl 0.1 % formic acid in H_2O. Samples were applied to the tips twice; after a first cycle of centrifugation, the flow-through was loaded and centrifuged again. StageTips were washed with 100 μl 0.1 % formic acid and subsequently bound peptides were eluted into a clean tube with 2 × 20 μl elution buffer (80 % acetonitrile, 0.1 % formic acid). Eluted samples were dried using a centrifugal evaporator and stored at −20 °C until measurement.

For analysis, these tryptic peptides were dissolved in 20 μl 0.1 % TFA in water and analyzed by nano-HPLC/MS/MS. Briefly, the tryptic digests were separated and analyzed on a UltiMate™ 3000 RSLCnano system coupled on-line to a Q-Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer equipped with a nano-spray source (Nanospray Flex Ion Source, all Thermo Scientific, Germany). All solvents were LC-MS grade. 3 μl of

the peptide solution were injected onto a pre-column cartridge (5 μm, 100 Å, 300 μm ID × 5 mm, Thermo Scientific, Germany) using 0.1 % TFA in water as eluent with a flow rate of 30 μl/min. Desalting was performed for 5 min with eluent flow to waste followed by back-flushing of the sample during the whole analysis from the pre-column to the PepMap100 RSLC C18 nano-HPLC column (2 μm, 100 Å, 75 μm ID × 25 cm, nano-Viper, Thermo Scientific, Germany) using a linear gradient starting with 95 % solvent A (0.1 % formic acid in water) / 5 % solvent B (0.1 % formic acid in acetonitrile) and increasing to 30 % solvent B after 95 min using a flow rate of 300 nl/min.

For coupling of the nano-HPLC to the Quadrupole-Orbitrap Mass Spectrometer, a standard coated SilicaTip (ID 20 μm, Tip-ID 10 μM, New Objective, Woburn, MA, USA) was used. Mass spectra were acquired using a so called TOP10 method, i.e., full scan spectra were acquired using a mass range of m/z 300 to 1650 with a resolution of 70000, followed by up to ten high energy collision dissociation (HCD) MS/MS scans at a resolution of 17500 of the most intense at least doubly charged ions. The dynamic exclusion was set to 20 s.

Data evaluation was performed using MaxQuant software[62] (v.1.4.1.2) including the Andromeda search algorithm and searching the yeast reference proteome of the uniprot database. Briefly, the search was performed for full enzymatic trypsin cleavages allowing two miscleavages. For protein modifications carbamidomethylation was chosen as fixed and oxidation of methionine and acetylation of the N-terminus as variable modifications. The mass accuracy for full mass spectra was set to 20 ppm for the first and 4.5 ppm for the second search and to 20 ppm for MS/MS spectra. The false discovery rates for peptide and protein identification were set to 1 %. Only proteins for which at least two peptides were quantified were chosen for further validation. Relative quantification of proteins was carried out using the label free quantification algorithm implemented in MaxQuant. Briefly, samples resulting from affinity enrichments with SEC4 to the solid support and those resulting from similar enrichment using SMBs without Sec4 were grouped. Label free quanitification intensities (LFQ) were logarithmized (log2) and proteins which were not quantified at least two times in at least one of the groups were filtered out. Missing values were imputed using small normal distributed values and a t-test (FDR 0.05) was performed. Proteins which were statistically significant outliers in the t-test were considered as hits if at least two unique peptides were identified in each technical replicate of the Sec4 group and non in the controls or if at least 4 unique peptides were identified in each technical replicate in the Sec4 group.

Cell-free expression and GEF activity assay

All putative targets were cloned into the pDONR201 entry vector (Invitrogen) for Gateway compatible cloning. For proteins bigger than 120 kDa, smaller fragments were designed using the predict protein website (https://www.predictprotein.org/) to avoid fragments containing only parts of a folded domain. *In vitro* translation was performed using Lexsy cell extract (Jena Bioscience) as described by the manufacturer after transferring the target genes into pCellFree_G01 expression plasmids.[41] To verify successful translation, *in vitro* translated crude protein lysates were used for immunoblotting. Equal amount of lysate was mixed with 2x SDS loading buffer and boiled for 5 min at 95 °C. Samples were run on either 10 % or 15 % SDS-PAGE and transferred to the Polyvinylidenfluorid (PVDF) membrane. Membranes were blocked with TBS/0.05 % Tween 20 containing 5 % powdered skim milk, followed by incubation with monoclonal mouse anti-His antibody (Sigma). After extensive washing with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.6) / 0.05 % Tween 20, a horseradish peroxidase-conjugated secondary antibody (Cayman chemical company) was used for detection. Images were taken with a GelDoc system (Bio Rad) using the SuperSignal West Dura Substrate (Thermo Scientific).

GEF activities of *in vitro* translated crude extracts were measured with Sec4 preparatively loaded with mantGDP as described previously[42,60] in a buffer containing 20 mM Hepes pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$ and 5 mM DTE on a Fluromax-3 fluorescence spectrometer (Horiba Jobin Yvon Inc.) at 25°C. The change in mant fluorescence (excitation λ_{ex} =360 nm; emission λ_{em} =440 nm) was fitted to a single exponential equation using Origin 9.0.

Yeast two-hybrid experiments

Gateway compatible yeast two-hybrid vectors were designed based on the commercially available pGBKT7 vector (Clontech Laboratories, Inc.). The Gateway cloning cassette was introduced into the multiple cloning site using the NdeI and NotI restriction sites. Additionally the kanamycine resistance gene was replaced with a gentamicin resistance gene to be compatible with the donor vector pDONR201 (Invitrogen) used in this study (a vector map of the resulting Y2H vector is shown in Fig. S3).

The coding sequences for all putative targets from the pull-down screens were transferred from the corresponding pDONR201 entry vectors (see above) into the Gateway compatible pGBKT7 bait vector, the coding sequences for $Sec4_{WT}\Delta C$ and the different mutants thereof were cloned into the pGADT7 prey vector (Clontech Laboratories, Inc.). Using the one-step transformation protocol for yeast in stationary phase,[63] the generated vectors containing the putative targets or the Sec4 mutants as well as the empty vectors (to test for auto-activation) were transformed into yeast strains AH109 (pGBKT7 bait vectors) or Y187 (pGADT7 prey vector) and transformants were selected on SD plates deficient of tryptophane (SD-Trp) or leucine (SD-Leu), respectively. Subsequently, all different combinations of the AH109 yeast strain containing the bait vectors and the Y187 yeast strain containing the prey vectors were mated and plated on SD plates deficient of tryptophane and leucine (SD-Trp-Leu) and grown for 3 days (30 °C). Protein-protein interactions were subsequently screened by resuspending a single colony from the mating plates in 50 μl 0.9 % NaCl solution and plating 3 μl thereof on SD-Trp-Leu plates containing X-α-Gal with and without histidine and growing the cells for 3–4 days at 30 °C. To further analyze the strength of interactions of known Rab:GEF pairs, the expression of two or three reporter genes have been tested, using varying stringent growth conditions. The strength of interaction was estimated from the comparative growth of yeast cells on minimal media lacking either adenine or histidine or both. Mating of yeast cells (Y2HGold or AH109 strain) containing either the pGBKT7–53 or pGBKT7-Lam vectors with yeast cells (Y187 strain) containing the pGADT7-T vector (all Clontech Laboratories, Inc.) served as positive and negative controls, respectively.

For expression control of the different proteins in yeast, proteins were extracted according to[64] and expression was confirmed via western-blot (primary antibodies rabbit Gal4 DBD antibody sc-577 for bait proteins and mouse anti GAL4-TA sc-1663 for prey proteins, Santa Cruz Biotechnology, Inc.). The BCIP-NBT solution from Santa Cruz Biotechnology Inc. was used for detection using an alkaline phosphatase coupled secondary antibody.

Abbreviations

DBD	DNA binding domain
GAP	GTPase activating protein
GEF	guanine nucleotide-exchange factor
GDP	Guanosine-5′-diphosphat
GppNHp	Guanosine-5′-[β,γ-imido]-triphosphat
GTP	Guanosine-5′-triphosphat
GXP	Guanine nucleotide
IVTT	*in vitro* transcription/translation
LFQ	label free quantification
MS	mass spectrometry
PBS	phosphate buffered saline
SMBs	streptavidin magnetic beads
TA	transcriptional activation domain
WT	wild type
XTP	Xanthosine-5′-triphosphat
Y2H	yeast two-hybrid

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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