FUNCTIONAL IDENTIFICATION OF RASGEF1 FAMILY OF EXCHANGE FACTORS AS ACTIVATORS OF RAP2, AND AS INTERACTING PARTNERS OF CCDC124

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BY
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I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

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Ph.D. in Molecular Biology and Genetics
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Coiled coil domain-124 gene is highly conserved among eukaryotes and the human counterpart encodes a protein with no domain similarities with any previously characterized eukaryotic proteins. In this study, we aimed to identify biological functions and interaction partners of human Ccdc-124. A yeast-two-hybrid analysis carried in this study has revealed that Ccdc-124 interacts with RasGEF1B which was predicted to be a member of Ras guanine exchange factors. The highly conserved RasGEF1 family of proteins contain C-terminal CDC25-homology domain (CDC25-HD) and an N-terminal RasGEF-N domain (Ras Exchange Motif, REM), and is of unknown function and specificity. In this thesis, the interaction of Ccdc-124 and RasGEF1 family of proteins was also established with co-immunoprecipitation and GST pull down assays. On the other hand, by using purified RasGEF1A and RasGEF1B proteins, as well as a large number of Ras family of G-proteins, we established that RasGEF1A and RasGEF1B function as very specific exchange factors for Rap2, a member of the Rap subfamily of Ras-like G-proteins. They do not act on Rap1 or other members of the Ras subfamily. On the other hand, Ccdc-124 protein did not change the stimulatory effect of RasGEF1 family of proteins on any of the tested G proteins *in vitro*. Furthermore, using reciprocal site-directed mutagenesis, we analyzed residues that allow RasGEF1 proteins to discriminate between Rap1 and Rap2, and we were able to identify Phe39 in the switch I region of Rap2 as a specificity residue. Mutation of the corresponding Ser39 in Rap1 changed the specificity and allowed the nucleotide exchange of Rap1(S39F) to be stimulated by RasGEF1B. This study describes for the first time GEFs that are uniquely specific for Rap2 among Rap family of G-proteins.

ÖZET

RAS GUANİN DEĞİŞİM FAKTÖRÜ-1 (RASGEF1) AİLESİ PROTEİNLERİN RAP2 AKTİVATÖRLERİ OLARAK TANIMLANMASI VE CCDC-124 İLE ETKİLEŞİMİNLERİNİN BELİRLENMESİ

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Ccdc-124 geni bütün ökaryot canlılarda korunmuştur. Ccdc-124 proteininde bilinen protein motiflerine gözlemlenmemiştir. Bu tez kapsamında yapılan çalışmalarda, maya ikili hibrit analizleri sonucunda, guanin nükleotid değişim faktörü olduğu düşünülen RasGEF1B'nin Ccdc-124 ile etkileştiği bulunmuştur. Fonksiyonu ve özelliği bilinmeyen, ancak yüksek oranda korunmuş olan RasGEF1 ailesi proteinlerinin, C-ucunda CDC25-Homoloji Bölgesi, N-ucunda ise RasGEF-N, Ras Değişim Motifi, bölgesi bulunmaktadır. Ccdc-124 ve RasGEF1 ailesi proteinlerinin birbirlerine bağlanması immünopresipitasyon ve GST çöktürme yöntemleri ile de doğrulanmıştır. Saflaştırılmış RasGEF1A, RasGEF1B ve birçok Ras ailesi proteinleri kullanılarak yapılan deneylerde, RasGEF1A ve RasGEF1B proteinlerinin Ras benzeri G poteinlerinden sadece Rap2'ye özgün değiştirici faktör olarak ekileştiği bulunmuştur. Bu proteinler Rap1 ve diğer Ras ailesi proteinlerine etki etmemişlerdir. Diğer yandan, Ccdc-124 proteini, *in vitro* deneylerde, RasGEF1 ailesinin hiçbir G proteini stimülasyonunu da etkilememiştir. Karşılıklı bölge-yönelimli mutagenez ile RasGEF1 proteinlerinin Rap1 ve Rap2 proteinlerini ayırt edici bölgesi analiz edilmiş ve Rap2'nin switch I bölgesinde yeralan fenilalanin39'un özgüllüğü sağlayan amino asit olduğu bulunmuştur. Rap1 üzerinde bu bölgeye karşılık gelen serin39'un mutasyonu ise RasGEF1B ile stimülasyonunu sağlamaktadır. Bu çalışma sonucunda insan hücrelerinde sadece Rap2'ye özgün bir nükleotid değiştirici faktörün varlığı ilk defa belirlenmiştir.

DEDICATION PAGE

To My Family

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1 INTRODUCTION

1.1 RAS family of small G proteins

Small GTP binding proteins together with their effectors and regulators have an important role in cell signaling pathways which affects almost all parts of the cell system. Most of these small GTP binding proteins belong to a superfamily called RAS (Colicelli 2004). Ras genes are found mutated in 20% of all human tumors. The highest incidences are found in adenocarcinomas of the pancreas (90%), colon (50%), and lung cancers (30%), thyroid tumors (50%), and myeloid leukemia (30%). The human Ras superfamily consists of at least 154 members divided into five principal families: the Ras, Rho, Rab, Arf, and Ran families (Wennerberg *et al.* 2005). This family of proteins has roles in the regulation of cell growth, proliferation, differentiation, apoptosis, adhesion and gene expression (Ehrhardt *et al.* 2002). For example, Ran G proteins are responsible for nuclear import and export, the regulation of nuclear envelope formation, and the control of spindle formation. Members of the Rab and Arf families play important roles in vesicle-associated processes, ranging from vesicle formation and transport to exocytosis. The Rho family is mainly involved in the regulation of cell shape, the cytoskeleton, and cell migration, whereas Ras family members regulate a variety of signaling pathways, resulting in transcription and cellular differentiation and proliferation (Bos *et al.* 2007).

1.2 GTPase Activating Proteins (GAPs)

Small G-Proteins are molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. Only in the GTP-bound form they interact with effector proteins and activate target pathways. For activation, GDP has to be released and a new GTP molecule has to be bound. To become inactivated, the bound GTP molecule has to be hydrolysed. Although G proteins are also called GTPases, the actual GTP hydrolysis reaction is in fact very slow for most of the G proteins, and efficient hydrolysis requires the interaction with a GAP, which

accelerates the cleavage step by several orders of magnitude. Several structural and biophysical studies have unraveled the reaction mechanism. The GTP-bound state is stabilized by backbone interactions with their switch I and switch II regions, and the γ-phosphate of GTP. Efficient GTP hydrolysis requires the presence of a trans element (typically an Arg residue) that interacts with the nucleotide to promote hydrolysis (Fig.1.1). The main contribution of different GAPs to catalysis is the stabilization of the intrinsically mobile catalytic machinery of the G protein and, in most cases, the insertion of a catalytic residue in *trans* (Weirich *et al.* 2008).

1.3 Guanine Nucleotide Exchange Factors (GEFs)

When G proteins are GTP bound, they create a binding site for their downstream effector proteins. Activation of RAS proteins is regulated by the catalytic action of GEFs. This diverse family includes GEFs acting on p21 Ras proteins (mSos, RasGRF, RasGRP) on Rap proteins (RasGRP, Epac, C3G, MR-GEF and RA-GEF), and on Ral proteins (RalGDS, Rlf, Rgl) (Quilliam *et al.* 2002); notably, most GEFs can act on more than one GTPase. The GDP/GTP cycle is highly regulated by GEFs that induce the release of the bound GDP to be replaced by the more abundant GTP. The large number of G proteins requires a multitude of GEFs to ensure signaling specificity. GEFs active on the Ras family of proteins share a catalytic domain of about 250 amino acids that is homologous with the catalytic domain of CDC25 in *Saccharomyces cerevisiae* and a Ras exchange motif (REM) domain. GEFs are usually multidomain proteins, many of which are protein or lipid interaction domains, indicating that they serve as localization signals and/or as scaffolds for the formation of protein complexes.

1.3.1 General Mechanism of GEF Function

The affinity of most small G proteins for GDP/GTP is in the lower nanomolar to picomolar range. The direct consequence of this high affinity is a slow dissociation rate of nucleotides with a half-life on the order of one or more hours. Because

exchange of GDP for GTP and, thus, activation of G proteins in biological processes occur within minutes or even less, exchange of GDP for GTP requires the activity of GEFs. Indeed, GEFs accelerate the exchange reaction by several orders of magnitude (Vetter and Wittinghofer 2001). The GEFs are often the targets of biological signals, which induce, inhibit, or modulate their catalytic activity. GEFs catalyze the dissociation of the nucleotide from the G protein by modifying the nucleotide-binding site such that the nucleotide affinity is decreased and, thus, the nucleotide is released and subsequently replaced. In general the affinity of the G protein for GTP and GDP is similar, and the GEF does not favor rebinding of GDP or GTP. Thus the resulting increase in GTP-bound over GDP-bound is due to the approximately ten times higher cellular concentration of GTP compared to GDP.

In contrast, the affinities of the exchange factor for the nucleotide-bound G protein and of the nucleotide for the exchange-factor-bound G protein (the ternary complexes) are much lower (Vetter and Wittinghofer 2001). Thus, the interaction of a GEF weakens the affinity for the nucleotide, and vice versa, the nucleotide weakens the affinity for the GEF. In the course of the exchange reaction the GEF displaces the bound nucleotide, and subsequently a new nucleotide displaces the GEF. The G-protein-bound nucleotide is sandwiched between two loops called switch 1 and switch 2. The switch regions together with the phosphate-binding loop (P loop) interact with the phosphates and a coordinating magnesium ion. Both phosphates and the magnesium ion are essential for the high-affinity binding of the nucleotide to the G protein (Vetter and Wittinghofer 2001). GEF binding induces conformational changes in the switch regions and the P loop, while leaving the remainder of the structure largely unperturbed.

1.3.2 Regulation of GEF Activity

Almost all GEFs are multidomain proteins regulated in a highly complex fashion. This regulation includes protein-protein or protein-lipid interactions, binding of second messengers, and posttranslational modifications. These interactions and modifications induce either one or more of three major changes: a translocation to a

specific compartment of the cell where the small G protein is located, the release from autoinhibition by a flanking domain or region, which covers the binding site for the small G protein, or the induction of allosteric changes in the catalytic domain (Bos *et al.* 2007).

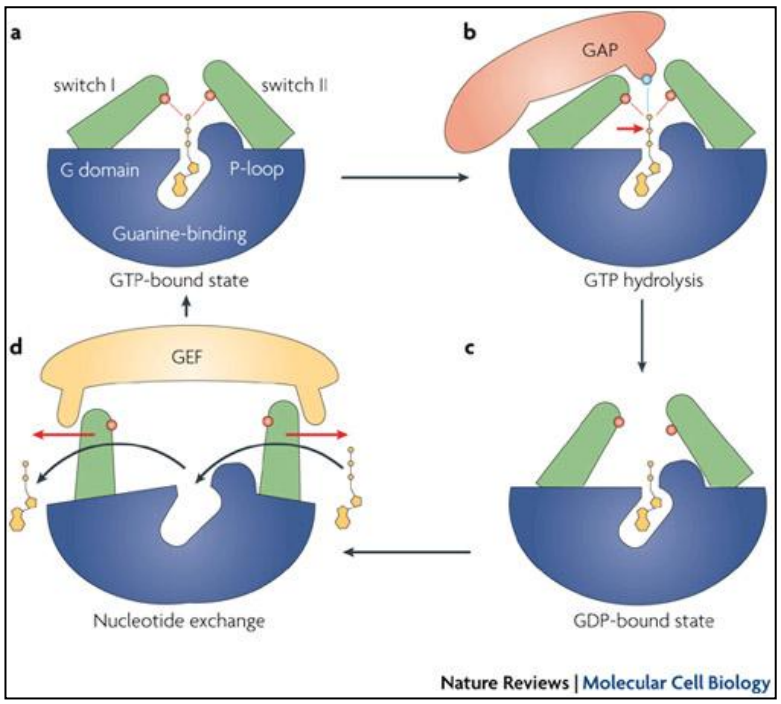


Figure 1.1: The switch mechanism of Nucleotide Exchange. Mechanism relies on the inherently low nucleotide-hydrolysis and nucleotide-exchange rates of these proteins. The GTP-bound state (panel a) is stabilized by backbone interactions (red circles) in two sequence motifs, known as switch I and switch II, and the γ-phosphate of GTP. Efficient GTP hydrolysis requires the presence of a *trans* element (blue circle; typically an Arg residue) that interacts with the nucleotide to promote hydrolysis (red arrow, panel b). The GDP-bound state is also stable, but the interactions between the switch motifs and the nucleotide are lost (panel c), in some cases leading to significant changers in the structure of the switch regions. Nucleotide exchange requires the contribution of an exchange factor (panel d), which stabilizes a relaxed conformation of the GTP-binding (G) domain (red arrows), causing GDP to be released and a new GTP molecule to enter the active site (Weirich *et al.* 2008).

1.4 RAP (Ras-Proximal) family of small G proteins

Rap proteins are Ras-like small G-proteins, which functions as molecular switches by cycling between a GDP-bound inactive and a GTP-bound active state. They control a wide variety of cellular process, most notably cell adhesion and cell junction formation, which play a crucial role in cell migration and tumor formation. On the basis of the sequence homology, the Rap family is divided into two subgroups, Rap1 and Rap2, with a total of five members: Rap1A, Rap1B, Rap2A, Rap2B, and Rap2C (Paganini *et al.* 2006). No functional differences have been reported between the members of each group. There is about 95% sequence identity between Rap1A and Rap1B, and about 90% identity between Rap2A, Rap2B, and Rap2C. Effector-binding regions, including switch I, show the highest conservation, and C-terminal regions the lowest. Similarly, Rap1 proteins are closely related (about 70% identical) to Rap2 subfamily members. Although identical or overlapping functions have been described for Rap1 and Rap2 proteins (Christian *et al.* 2003; McLeod *et al.* 2002; McLeod *et al.* 2004), a number of studies showing functional distinctions and different effector–protein interactions have been reported for Rap1 and Rap2 proteins (Fu *et al.* 2007; Huang *et al.* 2004; Imamura *et al.* 2003; Zhu *et al.* 2005).

1.4.1 RAP1

Rap-GEF/Rap signaling regulates the formation of adherens junctions. RAP1 was first identified as a gene that could reverse the loss of adhesion observed in NIH3T3 cells transformed by K-Ras (Kitayama *et al.* 1989). Subsequently, activation of Rap GTPases has been shown to increase cell adhesion and spreading and also to play a role in migration (Arthur *et al.* 2004; Enserink *et al.* 2004; Price and Bos 2004; Price *et al.* 2004). Although the mechanism of Rap activity is not completely understood, there is convincing evidence that activated Rap promotes adhesion through integrin activation (Bos *et al.* 2003; McLeod *et al.* 2004; Reedquist *et al.* 2000). RAP proteins are activated by mitogenic stimuli and function as regulators of integrin mediated cell adhesion and cell spreading. In cultured cells, RAP proteins do not show transforming activity. Rather, overexpression of RAP1A inhibits RAS

mediated transformation. However, RAP1A has been reported to bind and activate BRAF, suggesting that it has the capacity to promote mitogenesis and perhaps transformation in some cases. Two observations suggest contributions of RAP proteins in tumorigenesis, but with possible tissue type specificity. Activation of a RAP directed GEF or inactivation of a RAP directed GAP promotes hematopoietic tumor formation. Conversely the loss of an activator of RAP1 proteins has been found in a mouse osteosarcoma and in several nonhematopoietic human cancer cell lines (Colicelli 2004).

Recent studies have suggested that Rap1 may actually regulate adherens junctions. Cell-cell junctions are formed evenly around the lateral circumference of cells by homophilic interactions between the extracellular domains of E-cadherin, linked by their intracellular tail to catenins and to the actin cytoskeleton (Jamora and Fuchs 2002). Two recent studies in mammals have shed new lights on the connection between Rap1 and adherens junctions. In the first study, a Rap1 GTPase activator, DOCK4, was identified as a tumor suppressor (Yajnik *et al.* 2003). DOCK4 specifically activates Rap1 and regulates the formation of adherens junctions. In the second study, the authors demonstrated that ligation of the extracellular domain of E-cadherin enhances Rap1 activity, and that active Rap1 regulates the subsequent accumulation of at newly formed cellcell contact sites (Hogan *et al.* 2004). Data from the second study suggests that formation of adherens junctions is a two-step process. When cells first contact one another, small clusters of E-cadherin ligate through the homophilic interaction, which may, in turn, induce the activation of Rap1; activation of Rap1 may then activate inside-to-outside signaling through stimulating actin polymerization, which mediates the further recruitment of E-cadherin from the cytoplasmic or plasma membrane pool and facilitates the formation of mature E-cadherin-based adherens junctions. In Drosophila, it is reported that, Rap1 regulates the even distribution of adherens junctions of epithelial cells in wing imaginal disc (Knox and Brown 2002); Rap1 null cells have uneven adherens junctions and are dispersed into neighboring cells.

1.4.2 RAP2

Knowledge about Rap2 is limited. Rap2 is a member of the Ras family of small GTPases whose effector domain is almost identical to that of Ras, and can therefore bind most Ras effectors. Rap2 inhibits many Ras pathways including Ras-induced Raf activation at the plasma membrane (Ohba *et al.* 2000). Rap2 also binds to the Ral GEFs, Ral GDS, RGL and RLF (Nancy *et al.* 1999). These proteins are also Ras effectors and induce nucleotide exchange leading to the formation of active RalA. Rap2 is reported to localize mainly in the endoplasmic reticulum (ER), whereas Rap1 localizes at the Golgi apparatus (Beranger *et al.* 1991; Beranger *et al.* 1991). Unlike Rap1, Rap2 cannot reverse Ras-induced transformation of NIH 3T3 cells (Jimenez *et al.* 1991), and no biological phenotype has been linked to Rap2 in the literature. The regulation of Rap2 also remains unknown. Rap1GAP stimulates Rap2 GTPase activity in vitro, albeit significantly more weakly than Rap1 (Janoueix-Lerosey *et al.* 1992). PDZ-GEF1, also activates Rap2 and that GTP-bound Rap2 makes up more than 50% of the Rap2 in A14 and COS1 cells (Ohba *et al.* 2000).

In *Xenopus* embryos, Rap2 was shown to regulate activin/nodal signaling by modulating receptor trafficking (Choi *et al.* 2008). In the absence of ligand, Rap2 directs activin/nodal receptors into a Rab11-dependent recycling compartment, thereby avoiding degradation and maintaining cell-surface levels of receptors. Upon ligand addition, Rap2 no longer directs the receptors for recycling, but rather competes with Smad7 and delays receptor degradation, thus enhancing signaling (Colicelli 2004). Recently, specific Rap2-binding proteins such as RPIP8, TNIK and MAP4K4 were reported as candidate effectors; however, their cellular functions as Rap2 effectors are not fully established (Kardassis *et al.* 2009).

Further research in another eukaryote model, *C. elegans*, have revealed that there are three *Rap*-like genes. *rap-1* and *rap-2* are very similar to vertebrate *Rap-1b* and *Rap-2a*. *rap-3* shows less similarity to vertebrate *Rap* genes but is most similar to *Rap-1b*. Rap function in *C. elegans* involves the function and morphogenesis of hypodermal cells but not their generation or specification (Pellis-van Berkel *et al.* 2005). *rap-1* mutants display defects in hypodermal morphogenesis and function, including

formation of adherens junctions, secretion of cuticle and basement membrane, and hypodermal cell integrity and viability. While *rap-2* mutations on their own are wild-type, *rap-2* mutations enhance the effects of *rap-1*, suggesting that *rap-1* and *rap-2* act redundantly and can partially compensate for each other's loss (Pellis-van Berkel *et al.* 2005).

1.5 RAP Guanine Exchange Factors

Particularly, GEFs enhance the formation of the GTP-bound active conformation in response to upstream signals mediated by various cell surface receptors. To date, a number of GEFs for Rap proteins have been identified like, C3G, Epac (or cyclic AMP [cAMP]-GEF), CalDAG-GEFI, PDZ-GEF1, and GFR (Repac). These multi-domain proteins are highly regulated and responsible for the temporal and spatial activation of Rap. The Rap-GEFs Epac1 and Epac2 are directly regulated by cAMP and particular Epac2 modulates insulin secretion from pancreatic cells (Rehmann 2006). C3G binds to the adaptor protein Crk, being involved in tyrosine kinase-dependent activation of Rap1 (Gotoh *et al.* 1995). Epac/CAMPGEF is activated through direct association with cAMP, thereby stimulating Rap-dependent signaling (de Rooij *et al.* 1998; Kawasaki *et al.* 1998). Another Rap GEF, CalDAGGEF1, which contains calcium and diacylglycerol binding motifs, has a role in Rap activation in response to these second messengers (Kawasaki *et al.* 1998). Another Rap GEF is RAGEF-1 (also termed PDZ-GEF1, nRapGEP, or CNrasGEF), which exhibits GEF activity toward Rap1 and Rap2 (Liao *et al.* 1999; Liao *et al.* 2001). RA-GEF-1 contains putative cNMP-binding, REM, PDZ, and RA domains as well as the GEF catalytic domain. RA-GEF-2, whose structural features are intimately related to RA-GEF-1, exhibits GEF activity toward Rap1 and Rap2. GFR (Repac), which lacks the cAMP dependent regulatory sequences, is a constitutive activator of both Rap1 and Rap2 (Gao *et al.* 2001).

1.6 RasGEF1 Family

Proteins that act as Guanine exchange factors of Ras can be classified into at least two families, on the basis of sequence similarities, the CDC24 family and the CDC25

family. The size of the proteins of the CDC25 family range from 309 residues (LTE1) to 1596 residues (Sos). The sequence similarity shared by all these proteins is limited to a region of about 250 amino acids generally located in their C-terminal section (currently the only exceptions are sos and ralGDS where this domain makes up the central part of the protein). This domain has been shown, in CDC25 an SCD25, to be essential for the activity of these proteins.

The crystal structure of the GEF region of human Sos1 complexes with Ras has been solved. The structure consists of two distinct alpha helical structural domains: the N-terminal domain which seems to have a purely structural role and the C-terminal domain which is sufficient for catalytic activity and contains all residues that interact with Ras. A main feature of the catalytic domain is the protrusion of a helical hairpin important for the nucleotide-exchange mechanism. The N-terminal domain is likely to be important for the stability and correct placement of the hairpin structure. The signature pattern for this entry spans the helical hairpin.

The *rasgef* genes encode a subgroup of highly conserved Ras guanine nucleotide exchange factors. While EST projects revealed the presence of *rasgef* genes in organisms that range from nematodes to humans, their functions remain to be elucidated. In zebrafish two *rasgef* genes, *rasgef* and *rasgef1b*, have been identified and high throughput analysis revealed tissue specific embryonic expression for *rasgef1b*. The combined data generated from EST projects and genome-scanning gene-prediction programs suggest that vertebrates have three distinct *rasgef* genes and that only two of these genes are present in zebrafish. Phylogenetic analysis of the predicted RasGEF proteins show that the fish and mammalian proteins have slightly diverged during evolution. Furthermore, the cDNA of this zebrafish gene has a slightly higher homology to mammalian *rasgef1b* (73%) than to *rasgef1c* (66%) and *rasgef1a* (64%). In common, the RasGEF proteins possess two domains, a highly conserved carboxy-terminal RasGEF domain and a slightly less conserved amino-terminal RasGEFN domain (Figxx) with a yet unknown function. At the level of the protein sequence, vertebrate RasGEF1B proteins display 80–95% identity in the RasGEFN domain and 91–98% identity in the RasGEF domain. RasGEF domains

normally activate small GTPases of the Ras/Rho family by catalyzing the exchange of the inactive GDP-bound form to the activated GTP-bound form (Quilliam et al., 2002). It is shown that three *rasgef1b*-transcripts are generated from two transcriptional start sites and by alternative splicing. Detailed expression analyses show that *rasgef1b* is expressed in a subset of adaxial cells, in the anterior part of somites, in the rostral part of the mid-hindbrain boundary and in the rhombomere boundaries. In the larva, *rasgef1b* is further expressed in the pallium and the inner nuclear layer of the retina. It is also found that *rasgef1b* is expressed maternally and that the ubiquitous distribution of maternal transcripts disappears shortly after mid-blastula transition. At early epiboly stages, *rasgef1b* expression is restricted to the margin with low levels of expression on the ventral and high levels of expression on the dorsal side. It is also showed that early zygotic expression is regulated by Nodal and FGF signals and that these signals have different activities in regulating the level and distribution of early zygotic *rasgef1b* mRNA expression (Epting *et al.* 2007).

Whether or not RasGEF1B proteins have a similar function remains to be investigated, particularly because their GEF domains display characteristic differences when compared to the GEF domains of the related Ras activating proteins CDC25 and SOS (Jones *et al.* 1991). Till now, the only evidence for a biological function of RasGEF1B proteins comes from studies in invertebrates. *Drosophila* and *Caenorhabditis elegans* both contain a single *rasgef1b*-related gene, named *rasgef*, which shows 35% and 36–37% identity to the genes encoding the vertebrate RasGEF1B proteins, respectively (Kiger *et al.* 2003). In large scale RNAi experiments, knock down of *Drosophila* RasGEF in Schneider-cells was found to result in a slight change in cell morphology. However, no defects were found when *Drosophila* or *C. elegans rasgef* were knocked down in the entire organism (Rual *et al.* 2004).

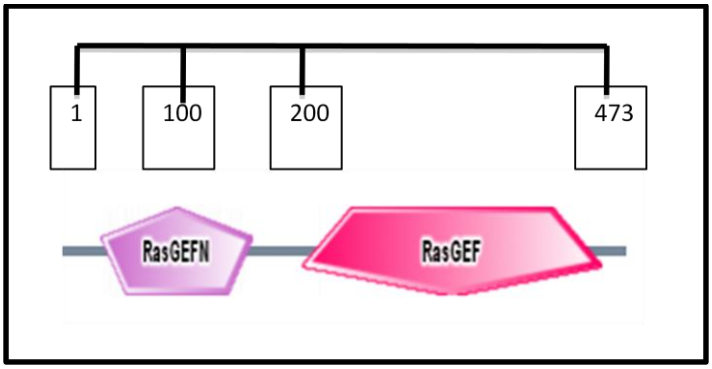


Figure 1.2: The RasGEF proteins possess two domains, a highly conserved carboxy-terminal RasGEF domain and a slightly less conserved amino-terminal RasGEFN domain. RasGEFN domain spans 33th to 161st residues, RasGEF domain through 201th-454th residues.

1.7 Objectives and Rationale

Previously, our laboratory has established the expression of a novel gene of unknown function, Ccdc-124, in a number of cell lines tested. Intrigued by a very high conservation of this gene among eukaryotes, we decided to establish the biological function of Ccdc-124. For this, we have first used the well known yeast-two-hybrid technique, and screened a liver cDNA library in order to identify interaction partners of Ccdc-124. These analysis have revaled RasGEF1B as a potential interaction partner. We have then validated these results by a number of *in vitro* and *in vivo* protein-protein interaction assays. Even though proteins belonging to RasGEF1 family were predicted to act as guanine exchange factors for Ras G-proteins, their precise biological functions were ambigious, and G-proteins stimulated by RasGEF1 family members were unknown. Therefore, we decided to test the guanine exchange factor activities of proteins belonging to this family, and we settled to identify Ras family of G-proteins stimulated by these GEFs in *in vitro* guanine nucleotide exchange assays. We have identified Rap2, a member of Ras family of G-proteins as substrate of RasGEF1A and RasGEF1B, and we then aimed to study residues having discriminatory roles in the interactions between RasGEF1s and Ras Family of G-proteins. In parallel to these studies, we have also studied the effect of Ccdc-124 in RasGEF1 stimulated guanine nucleotide exchange activities *in vitro*.

2 MATERIAL & METHODS

2.1 Yeast Two Hybrid

Bait cloning and Y2H screening were performed by Hybrigenics, S.A., Paris, France (http://www.hybrigenics.com).

Human CCDC124 (aa1-aa223) cDNA was PCR-amplified and cloned in a LexA, C-terminal fusion vector optimized by Hybrigenics. The bait construct was checked by sequencing the entire insert, and was subsequently transformed in the L40ΔGAL4 yeast strain (Fromont-Racine *et al.* 1997).A Human Liver random-primed cDNA library, transformed into the Y187 yeast strain and containing ten million independent fragments, was used for mating. High mating efficiency was obtained by using specific mating method (Legrain *et al*., 1998, 2000, 2002). The screen was first performed on a small scale to adapt the selective pressure to the intrinsic property of the bait. No autoactivation of the bait was observed. Then, the full-scale screen was performed in conditions ensuring a minimum of 50 million interactions tested, in order to cover five times the primary complexity of the yeast-transformed cDNA library (Rain *et al.* 2001). 98 millions of interactions were actually tested with Human CCDC124. After selection on medium lacking leucine, tryptophane, and histidine, 16 positive clones were picked, and the corresponding prey fragments were amplified by PCR and sequenced at their 5' and 3' junctions.

Sequences were then filtered and contiged (Formstecher *et al.* 2005) and compared to the latest release of the GenBank database using BLASTN (Altschul *et al.* 1997). A Predicted Biological Score (PBS) was attributed to assess the reliability of each interaction, as described previously (Formstecher *et al.* 2005). Briefly, the PBS relies on two different levels of analysis: firstly a local score takes into account the redundancy and independency of prey fragments, as well as the distributions of reading frames and stop codons in overlapping fragments. Secondly, a global score

takes into account the interactions found in all the screens performed at Hybrigenics using the same library. In addition, potential false-positives are flagged by a specific "E" PBS score. This is done by discriminating prey proteins containing "highly connected" domains, previously found several times in screens performed on libraries derived from the same organism. The PBS scores have been shown to positively correlate with the biological significance of interactions (Rain *et al.* 2001; Wojcik *et al.* 2002).

2.2 Northern Blotting

Northern blotting was performed on FirstChoice Northern Human Blot1 membrane (each lane containing 2 μg poly (A) RNA) (Ambion). DNA templates for probe preparation were formed via restriction enzyme digestion from PCR product cloned plasmids for Ccdc-124 and RasGEF1B or directly from PCR products for Gapdh and β-actin. PCR products for Gapdh and β-actin formed via primers which were kindly provided by Ayşe Elif Erson and Mehmet Öztürk's group, respectively. Then, DNA templates were labeled by north2south biotin random prime labeling kit (Pierce). Probes were synthesized as described in manufacturer's protocol. Probes and their sizes are presented in Table 2.6.

Gene	Starting material	Restriction Enzyme	Probe Size
Ccdc-124	p3XFlagCcdc-124	Apa I and Xba I	381 bp
RasGEF1B	p3XFlagRasGEF1B	Bgl II and BamHI	284 bp
Gapdh	Gapdh PCR product	-	408 bp
β-actin	β-actin PCR product	-	539 bp

Table 2.6: Northern blotting probes. Starting DNA material, used restriction enzymes for probe synthesis and synthesized probe sizes are indicated.

In order to hybridize the nucleic acids and perform the detection by synthesized probes, north2south chemiluminescent hybridization and detection kit (Pierce) was

utilized. Kit's protocol was exactly followed and resulting blots were exposed to film for approximately 1 min.

2.3 Cell Culture

In this study, Huh-7 (Human hepatocellular carcinoma), HeLA (Human Cervical carcinoma) and MCF-7 (Caucasian female breast adenocarcinoma) cell lines were used for immunoprecipitation, immunostaining and western analysis.

Cell lines were grown in high glucose Dulbecco's modified Eagle's medium (Gibco) with the addition of 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (Biochrom) at 37˚C in a 5% CO2 incubator.

2.3.1 Transfection

Transfection was performed via using FuGene-6 reagent (Roche). Optimized transfection reagent (µl) /plasmid DNA (µg) was 3:1. Firstly, transfection reagent was diluted in serum and antibiotics free medium. After 10 minutes of incubation, plasmid DNA was added and FuGene-plasmid DNA mixture was incubated for 30 minutes. During this time interval, medium of the cells was changed. Finally, FuGene-DNA complex was transfected to cells in a drop-wise manner.

2.4 Western blot analysis

Proteins from cell lines were isolated for western blotting analysis. Cell pellets were lysed in 50 µl lysis buffer consisting of 50mM Tris Base, 250mM NaCl, 1X proteinase inhibitor cocktail and 0.1% NP40. Protein concentrations were determined via using Bradford assay. Twenty micrograms of whole cell extracts were denatured in gel loading buffer [50 mM Tris-HCl pH 6.8, 1% SDS, 0.02% bromophenol blue, 10% Glycerol and 5% 2-mercaptoethanol] at 95°C for 5 min, resolved by SDS-PAGE using a 10% gel, and electrotransferred onto PVDF membranes (Millipore). The membranes were blocked in Blotto (Tris-buffered saline containing 0.5% Tween 20 and 5% nonfat milk powder) for 1 hour at room temperature. The membranes were incubated with first antibody (Table 2.7) for 1 hour, washed 3 times with Blotto and incubated with secondary antibody (see below) for 1 hour, immunocomplexes

were then detected via Super Signal West Dura (Pierce), and exposed to X-Ray films (AGFA) for 1 minute. The films were then developed using a hyper-processor developer (Amersham).

Primary antibody	Description	Concentration in blotto
Anti-Ccdc-124	Rabbit, polyclonal	2 µg/ml
Anti-Flag (Sigma)	Mouse, monoclonal	1 µg/ml
Anti-Calnexin	Rabbit, polyclonal	0.1 µg/ml
Anti-His-Probe(Santa Cruz)	Rabbit, polyclonal	1 µg/ml

Table 2.7: First antibodies used in Western Blot experiments.

2.5 Immunostaining

Cells that cultured on cover slips in 6-weel plates were washed 3 times with cold 1XPBS (137 mM NaCl, 2,7mM KCl, 1,4 mM KH_2PO_4, 4,3 mM Na_2HPO_4) then fixed with 4% Para-formaldehyde at room temperature for 15 min. After fixation cells were permeabilized with 1:1000 Triton X100 in 1XPBS for 6 min at room temperature. Cells then blocked with 5% BSA in1XPBS for 1 hour at room temperature, then incubated with first antibody which is diluted in blocking solution for 1 hour. After first antibody incubation cells were washed 3 times with 1XPBS and incubated with TRITC or FITC conjugated secondary antibody diluted in blocking solution for 1 hour at room temperature. Then cells were washed for 3 times with 1XPBS and counter stained with DAPI for 30 sec, washed with dH_2O and mounted with glycerol.

2.6 GST-Pull Down Assay

100µl 50% slurry (~50µl packed) GSH beads were washed 3 times with wash buffer (50mM Tris HCl pH 7.5, 100mM NaCl, 3mM β-Mercaptoethanol). First two vials

incubated (immobilized) with 200µg purified GST-RasGEF1 protein, second vial incubated (immobilized) with 200µg GST protein, fourth vial just incubated with wash buffer and rotated at 4°C for 1 hour. After immobilization, beads were washed 3 times. First and third vials incubated with eash buffer only, second and fourth vials incubated with 500µg Ccdc-124 protein and incubated at 4°C for 1 hour by rotating. Then beads were washed 9-10 times with wash buffer, and samples were eluted with 40µl 4x SDS loading buffer, boiled for 5 min and loaded on an SDS gel. For the control of of protein sizes 10mg/ml from each protein were loaded to the same gel. The gel then stained with coomasie for 15 min and destained with water.

2.7 Polarization Assay

2.7.1 IAEDANS Labelling

Cystein mutants of purified Ccdc-124 proteins were washed with 50mM Tris HCl (pH 7.5), 100mM NaCl, 5mM Ascorbic Acid in eppendorf concentrators and 0.5mg of them incubated with 10 times molar concentration of IAEDANS overnight at 4°C.

2.7.2 Fluorescence Polarization

Monitoring protein-protein interactions by using fluorescence polarization. After excitation with polarized light, the photons emitted by fluorescent probes are also polarized; however, rotational diffusion of a fluorophore over its excitedstate lifetime causes depolarization of the emitted photons. Fluorescence polarization measures the average angular displacement of a fluorophore over its excited-state lifetime, which is affected by the molecule's rate of rotational diffusion. Because the rate of rotational diffusion of a molecule is inversely related to its size, polarization provides a sensitive measure of changes in molecular volume that occur when the fluorescent protein is bound by a nonfluorescent protein (Harrington *et al.* 2003). Fluorescence polarization was used to monitor the association of IAEDANS-labeled Ccdd-124 with nonfluorescent RasGEF1B. Measurements were performed at 25°C in 20 mM Tris HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 3 mM β-mercaptoethanol in quartz cuvettes. Fluorescence data were recorded with a Fluoromax-2 spectrophotometer, with excitation and emission wavelengths of m-nucleotides at 336 nm and 450 nm,

respectively. Firstly the polarizations of 2.5μM IAEDANS labeled Ccdc-124 proteins alone were measured and then 20nM RasGEF1B protein was added each time with time laps.

2.8 Protein Imunoprecipitation Assay

70-90% confluent (100mm dish) cells were rinsed twice with PBS (10mM phosphate, 2.7mM potassium chloride, 137mM sodium chloride, pH 7.4) and 1 ml of lysis buffer (50mM Tris HCl, pH 7.4, with 150mM NaCl,1mM EDTA, and 1% TRITON X-100) was added and incubated for 15−30 minutes on a shaker at 4°C. Then cells were scraped, collected and centrifuged at 12,000Xg for 10 min. Supernatant transferred onto 40μl 50% slurry ANTI-FLAG M2 affinity gel (Sigma) and incubated overnight on shaker at 4°C. Subsequently the resin was washed three times with 1ml TBS. 20 μl of 2Xsample buffer (125mM Tris HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.004% bromphenol blue) was added to each sample. After denaturation by boiling for 3 min, samples were loaded on SDS-PAGE and immunoblotted with specific antibody against the Ccdc-124 protein and anti-Flag antibody against the fusion RasGEF1B protein.

2.9 Plasmid Constructs

cDNAs corresponding to the RasGEF1A and RasGEF1B genes were cloned into the pQE80L system by Hani Alotaibi and Pelin Telkoparan. Then these cDNAs were transferred into pGEX-4T-1 expression vector: cDNAs corresponding to RasGEF1A and RasGEF1B were amplified by PCR (Table 2.1) with the primers (Table 2.2) suitable for pGEX cloning, and then fragments were purified from agarose gel. RasGEF1A and RasGEF1B were digested at 37°C overnight with their suitable enzymes. pGEX-TEVn was also digested first with EcoRI at 37°C overnight and then EcoRI was inactivated at 65°C for 20 min. and divided into two vials. One was digested with BamHI and the other half was digested with NcoI at 37°C for 2 hours. Then digested samples were purified from agarose gel and ligated with RasGEF1A and RasGEF1B fragments (Table 2.3). After ligation they were transformed into Top10 competent cells and spread on 50μg/ml Amp included LB agar plates. After

incubating the plates overnight at 37°C colonies were picked and a colony PCR was made in order to select the positive colonies. Then the plasmids from positive colonies were isolated and sequenced.

DiRas2, M-Ras, R-Ras, RalB, TC21, RERG and Rheb were cloned into pGex4T1–TEV and expressed in E. coli BL21 DE3 cells. C-terminally truncated forms of H-Ras, Rap1A, Rap1B and Rap2A were cloned into ptac vectors and expressed in CK600K cells by Raphael Gasper.

Plasmid DNA	1 µl
Primer1 (100pmol)	0.5 µl
Primer2 (100pmol)	0.5 µl
dNTP	5 µM
DMSO	5 µl
5x HiFi Buffer	10 µl
H_2O	27.5 µl
HiFi Polymerase Qiagen	0.5 µl

Tablo 2.1: PCR Reaction of RasGEF1A and RasGEF1B by using pQE80L-RasGEF1A and pQE80L-RasGEF1B as template. Conditions: 94°C 4min, (94°C 30sec, 53°C 60sec, 72°C 60sec)x30, 72°C 4 min.

RASGEF 1A Forward (BamHI) 5’-CGT AGG ATC CAT GCC CCA GAC GTC CGT TGT C-3’
RASGEF 1A Reverse (EcoRI) 5’-GGT AGA ATT C TC AGG CTC TGT TCA GAA GGG TG-3’
RASGEF 1B Forward (Nco I) 5’-CTG CCC ATG GGC ATG CCT CAG ACT CCT CCC TTT TC-3’
RASGEF 1B Reverse (BamHI) 5’-CCT AGG ATC CAT GCC ACA GAC GCT GAG TGC C-3’

Table 2.2: RasGEF1A and RasGEF1B primers for cloning into pGEX system.

RasGEF1A Digestion	
DNA (~100ng)	28 µl
EcoRI Buffer	4 µl
EcoRI	2 µl
BamHI	2 µl
H_2O	4 µl
Total	40 µl

RasGEF1B Digestion	
DNA (~100ng)	28 µl
EcoRI Buffer	4 µl
EcoRI	2 µl
NCoI	2 µl
H_2O	4 µl
Total	40 µl

PGEX TEVn Digestion	
DNA	20 µl
EcoRI Buffer	3 µl
EcoRI	3 µl
H_2O	4 µl
Total	30 µl

PGEX TEVn-EcoRI Digestion with NcoI	
DNA	15 µl
EcoRI Buffer	0.5 µl
NcoI	1 µl
H_2O	3.5 µl
Total	20 µl

Ligation of PGEX TEVn with RasGEF1A and B	
5x ligase Buffer	4 µl
Vector (12.5ng/µl)	4 µl
Insert (~10.5ng/µl)	1 µl
Rapid Ligase	1 µl
H_2O	10 µl
Total	20 µl

Table 2.3: Digestion and ligation reactions of RasGEF1A, RasGEF1B and pGEX-Tevn

2.10 Protein Purification

2.10.1 Small Scale Expressions of pQe80L-RasGEF1 and pQe81L-Ccdc-124

BL21 codon+ RIL competent cells transformed with pQE80L-RasGEF1 and pQE81L-Ccdc-124 for test expression.

200 µl competent cell + 1 µl plasmid DNA on ice for 30 min, heat shock at 42°C for 1 min then add 500 µl LB and shake for 1 hour at 37°C. After 1 hour of shaking, 100 µl was spread over an agarose plate which has 50µg/ml ampisilin (Amp) + 25µg/ml chloramphenicol (Cam) in it and incubated overnight at 37°C. The next day half of the colonies grew on the plates scaped into 400 ml LB flasks which have 50µg/ml Amp and 25µg/ml Cam. Flasks were shaked at 23°C till they reached the A_{600} nm of 0.4. When they reached the A_{600} nm of 0.4, 1 ml of "uninduced" control was taken from each of them and the rest is induced with 100 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and divided into two flasks, one was grown at 16 °C the other was grown at 23 °C. Next day 1 ml of induced control was taken from each flask then they divided into three 50 ml falcons so that there were 3 samples for 3 different lysis buffers, lysis buffer1, 2 and 3 (see below).

Falcons were centrifuged and pellets were lysed with different lysis buffers. After dissolving pellets with lysis buffers, 100 µM phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride (PMSF) was added and each was sonicated with microtipped sonicator at the maximum level with 50% amplitude, 50 seconds for 2 times. 10 µl control samples were taken from them, then 1 ml from each sample was centrifuged at 4 °C and 14000 rpm for 30 min and 10 µl sample was taken as a Raw Extract (RE) control, the rest is incubated with 50 µl 50% Ni-beads for 30 min at 4 °C. 10 µl Flow Though (FT) control was also taken before the beads were washed. After 5 times washing step with their corresponding washing buffers, beads were eluted with 4x sample buffer. All the samples heated up to 95 °C for 5 min then loaded in 15% SDS gels and ran at 70mA/gel for 30 min and gels were stained with coomassie for 15 min then destained with water.

2.10.2 Large Scale Expression of Ccdc-124

5L culture of pQE81L Ccdc-124 in BL21 codon + RIL *E.coli* cells was grown at 23°C till the A_{600} nm reached to 0.5. 1ml of "uninduced" control sample was taken and the rest was induced with 100mM IPTG overnight and pelletted by centrifuging at 4400 rpm at 16 °C for 15 min. Pellets were resuspended with lysis buffer 1 (see below) and 100mM PMSF was added. Then pellets were lysed by using midi-tipped sonicator at the maximum level with 50% amplitude, 50 seconds for 2 times on ice. Cell lysate supernatants were applied to Ni-NTA-agarose column, pre-equilibrated with lysis buffer 1 at 4°C and washed first with wash buffer 1 then with high salt buffer. Then eluted with a linear gradient up to 1M imidazole in elution buffer (pH 8.0) and 4ml fractions. Fractions were loaded on a 15% SDS gel, and then all fractions pooled together and concentrated (with Millipore Amicon Ultra Centrifugal Filter 10 NMWL). Concentrated samples were loaded on Superdex S75 26 ⁄ 60 columns (Amersham Biosciences) (gel filtration). Fractions after gel filtration were loaded to a 15% SDS gel and the proper fractions were pooled together and concentrated.

Lysis Buffers:

Lysis Buffer 1: 50 mM Tris pH 7.5, 300 mM NaCl, 3 mM β-Mercaptoethanol, 0.5% NP40, 0.5% Triton X100

Lysis Buffer 2: 50 mM Tris pH 8, 300 mM NaCl, 3 mM β-Mercaptoethanol, 0.5% NP40, 0.5% Triton X100

Lysis Buffer 3: 50 mM Tris pH 7.5, 300 mM NaCl, 3 mM β-Mercaptoethanol

Lysis Buffer with Detergent: 50 mM Tris pH 7.5, 300 mM NaCl, 3 mM β-Mercaptoethanol, 0.5% NP40, 0.5% Triton X100

Lysis Buffer without Detergent: 50 mM Tris pH 7.5, 300 mM NaCl, 3 mM β-Mercaptoethanol.

Wash Buffers:

Wash Buffer 1: 50mM Tris HCl pH 7.5, 100mM NaCl, 3mM β-Mercaptoethanol

Wash Buffer 2: 50mM Tris HCl pH 8, 100mM NaCl, 3mM β-Mercaptoethanol

High Salt Buffer: 50mM Tris HCl pH 7.5, 300mM NaCl, 3mM β-Mercaptoethanol, 1mM ATP

Column Elution Buffer 1 pH 8.0: 50mM Tris HCl pH 7.5, 100mM NaCl, 3mM β-Mercaptoethanol, 20mM Glutathion

Column Elution Buffer 2 pH 8.0: 50mM Tris HCl pH 7.5, 100mM NaCl, 3mM β-Mercaptoethanol, 1M immidazole

2.10.3 Protein expression and purification of wild type G proteins

DiRas2, M-Ras, R-Ras, TC21, RalB, RERG, Rheb, ptac–H-Ras, ptac–Rap1A, ptac–Rap1B and ptac– Rap2A were expressed at 25°C after induction with 100 Mm IPTG at a A_{600} nm of 0.6. Cell lysate supernatants of GST-fused RasGEF1A and RasGEF1B were applied to a glutathione (GSH) column (Amersham Biosciences, Freiburg, Germany) pre equilibrated with buffer A [50 mm Tris (pH 7.5), 100 mM NaCl, 3 mM β-mercaptoethanol] at 4 °C and eluted with buffer A containing 20 mM GSH, and GST fusion proteins were purified by gel filtration on Superdex S75 16/60 columns (Amersham Biosciences). Cell lysate supernatants of the GST-fused G-proteins were applied to GSH Sepharose 4B (GE Healthcare, Chalfont St Giles) pre-equilibrated with buffer B [25 Mm Tris (pH 7.5), 500 Mm NaCl, 5 Mmdithioerythritol] at 4 °C, and either eluted with 20 Mm GSH and subsequently digested by Tobacco Etch Virus (TEV) protease (1mg/ml) in batches, or cleaved by thrombin (300U) on the column. Cleaved proteins were further purified by gel filtration and another GSH column to separate G-proteins from GST; ptac-cloned proteins were purified by Q-Sepharose and subsequent gel filtration.

2.10.4 Site Directed Mutagenesis

PGEX-4T-3–Rap1B, pGEX-4T-3–Rap2A and pQE81L-Ccdc-124 mutants were generated using a Stratagene QuickChange site-directed mutagenesis kit by using primers below (Table 2.4 and Table 2.5). For templates smaller than 5kb, 50ng DNA; longer than 5kb, 100ng DNA was used. After mutagenesis PCR reaction, samples were subjected to Dpn I digestion and transformed into Top10 competent cells. The plasmids were isolated and their sequences were verified by sequencing.

Ser12 to Cys (TCG→TGC) 5'GGTGAGAACACCAAGTGCGCAGCGGCCCGGG 3′
Ser92 to Cys (TCC→TGC) 5'CCGCGGGTGGCCACGTGCAGCAAGGTCACCC3′
Ser155 to Cys (AGC→TGC) 5′GCCATTGCAGTGCTCTGCGTGGCGGAGGAGGCG3′

Table 2.4: Ccdc-124 Mutagenesis Oligos.

Rap2A T27I ACC →ATC 5′GCAGTTCGTGACCGGCATCTTCATCGAGAAATACG-3′
Rap2A F39S TTC→TCC 5′CCCCACCATCGAGGACTCCTACCGCAAGGAGATCG-3′
Rap2A S66A TCC→GCC 5′CCGAGCAGTTCGCGGCCATGCGGGACCTGTAC-3′
Rap1B S39F TCT→TTT 5′CCTACGATAGAAGATTTTTATAGAAAGCAAGTTG-3′

Table 2.5: Mutagenesis Oligos of Rap2A and Rap1B.

2.10.5 Mant-GppNHp Exchange Reaction

Two milligrams from each protein was loaded with N-methylanthranil acid-labeled guanine nucleotide (mant-GNP) by incubating with 400µM mGppNHp and 1 U.mg-1 alkaline phosphatase (Roche Diagnostics, Indianapolis, IN, USA) in an exchange buffer [200mM (NH4)2SO4, 1mM ZnCl2] for 16 h at 4°C overnight at dark. Exchange of nucleotides was monitored by HPLC. After exchange, proteins were separated from free nucleosides and alkaline phosphatase by gel filtration (S75 10／30). Purified proteins were concentrated, flash-frozen in liquid nitrogen, and stored at 80 °C.

2.11 Nucleotide exchange reaction (GEF Assay)

The dissociation of a protein-bound nucleotide was determined in real time by fluorescence spectroscopy using a fluorescent N-methylanthraniloyl (mant) derivative of guanosine nucleotide mant-GppNHp, coupled at the 2’ (3’) hydroxyl group of the ribose.

In principle, each nucleotide-binding protein has a defined intrinsic rate of nucleotide release, which is often too low to be physiologically relevant. Specificity and activity of GEFs can be analyzed qualitatively by comparison of intrinsic and GEF-stimulated fluorescence measurements. This is performed in a fluorescence spectrometer (FluoroMax-4, Horiba) since these reactions are slow (>1000 sec). GEF and also GAP assays do not need post-translationally modified GTPases. Thus, all proteins and protein domains produced in *Escherichia coli* can be used.

Mant-GppNHp labeled G proteins are diluted with 50mM Tris HCl (pH 7.5), 100mM NaCl, 5mM $MgCl_2$ and 3mM β-Mercaptoethanol in quartz cuvettes. The intrinsic mant-fluorescence signal from 150nM G proteins then the signal after incubation with either 4µm or 200nm RasGEF1s with 10µm GDP in the fluorescence spectrometer was recorded real time using an excitation wavelength of 366 nm and an emission wavelength of 450 nm, an integration time of 2 sec at 25 °C. Data were fittet to a single exponential equation using grafit 5.0 (http://www.erithacus .com/grafit/).

2.12 Crystallization

Crystallization trials were conducted in 96-well sitting-drop Qiagen 96-well crystallization screens (see below). These 96-well plates have room for three samples per well, therefore we could screen three different protein concentrations per plate, allowing us to test 288 conditions per trial over 70 µl well solution. A nanoliter liquid handling system (TTP Labtech Mosquito) was used to dispense the protein samples to the rooms of the wells. Crystallization trials were screened for 30 days.

Qiagen Reservoir Solutions used for Crystallization

1. JCSG Core I
2. The Classics Suite
3. JCSG + Suite
4. The PEGS Suite
5. $AmSO_4$ Suite

3 RESULTS

3.1 Identification of Ccdc 124 as a Ubiquitously Expressed Gene Conserved in Lower and Higher Eukaryotes

3.1.1 History of Identification

Ccdc124 was firstly identified in a study carried out by Hani Alotaibi during his Ph.D. studies in our group where *cis*-acting transcriptional regulatory elements of the Na^+/I^- Symporter (NIS) gene were being analyzed. The aim was to determine the conserved non-coding regions of 3' downstream of the NIS gene by computational analysis, then assess these regions by luciferase system in order to find highly conserved and active regions as candidates for regulatory regions. 90 kb genomic DNA fragment including the NIS gene was analyzed in order to identify at least 50% conserved putative regulatory elements of NIS gene in human, mouse and rat. In that study, 10 conserved putative regions were identified via using VISTA program (Bary, *et al.*, 2003 and Couronne, *et al.*, 2003). One of these conserved regions (region 10) showed a high activity in luciferase system but it came out that it was not a 3' regulatory element of NIS but it was the 5' regulatory region of a putative gene called Loc115098 (Ccdc-124).

This gene is described as coiled-coiled domain containing 124 (Ccdc-124) in Genome Browser database at the University of California Santa Cruz. Ccdc-124 is conserved in all eukaryotes from the lower eukaryote fungus *Aspergillus nidulans* to *Homo sapiens*. This gene has 75% identity scores in mammals, 50% identity in nematodes and insects and 30-40% identity in plants and ascomycetes. Its protein has no domain similarities with any known protein. The gene contains 4 coding exons and it is predicted to encode a 223 amino acid protein in human. Molecular weight and isoelectric point of the protein were calculated as 25835.2 Da and 9.54, respectively via using ProtParam tool. It consists of 43 negatively charged, 51

positively charged residues, which means, 42% of the protein consists of charged residues (Fig.3.1).

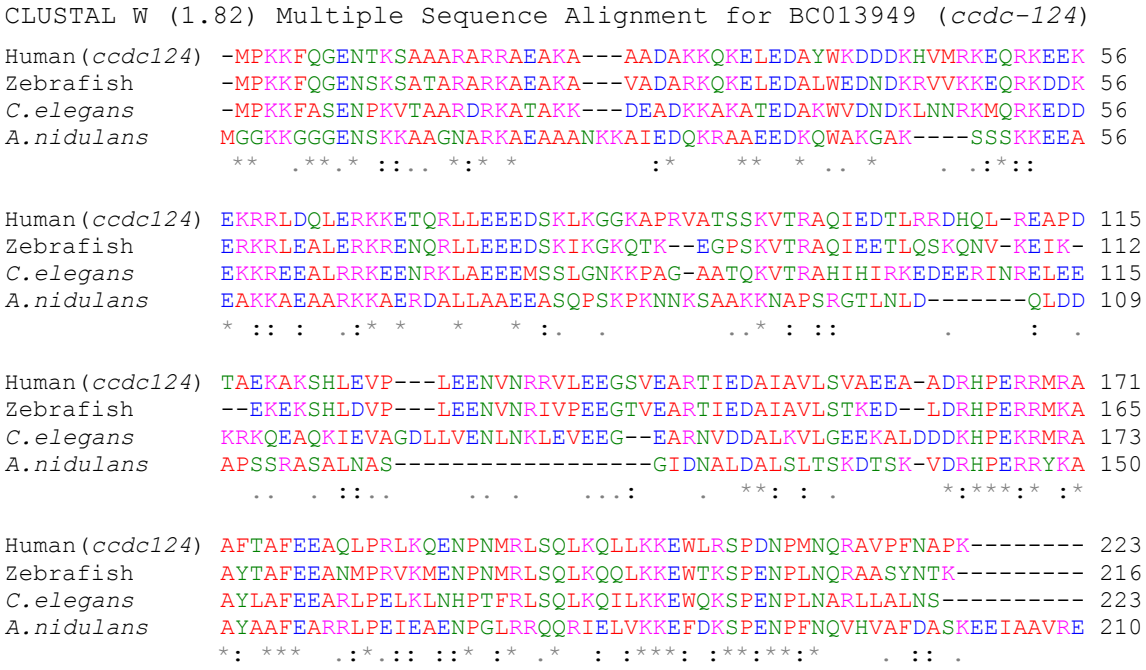


Figure 3.1: High conservation of ccdc-124 gene protein sequence in eukaryotes. Protein sequences of ccdc124 from different eukaryotes (human, zebrafish, *C. Elegans* and *A. nidulans*) were aligned by using Clustal-W multiple sequence analysis program. In the figure, green shows hydroxyl and amino groups containing amino acids; red shows, little hydrophobic amino acids; blue shows acidic amino acids; pink shows alkaline amino acids. 42% of the human CCDC-124 consists of positively charged (K, R, H, D, E) amino acids.

3.1.2 Expressional Analysis of Ccdc 124

Northern blot analysis of Ccdc-124 was carried on a human tissue RNA transferred membrane (Human Blot 1, Ambion) and biotin labeled probes (Fig.3.2 and Fig.3.3).

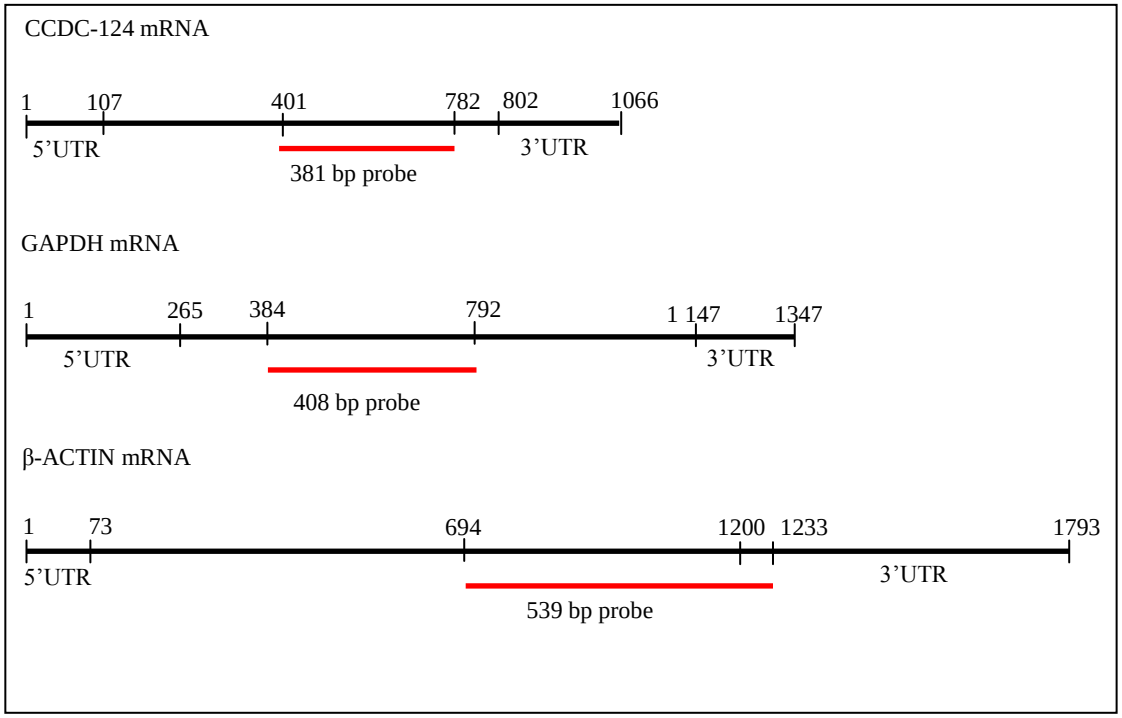


Figure 3.2: Northern blotting probes for Ccdc-124, Gapdh and β-actin. mRNA size, 5'UTR, 3'UTR and probe size & location are indicated for each gene.

According to the results of Northern Blot analysis, Ccdc-124 is ubiquitously expressed in all human tissues tested.

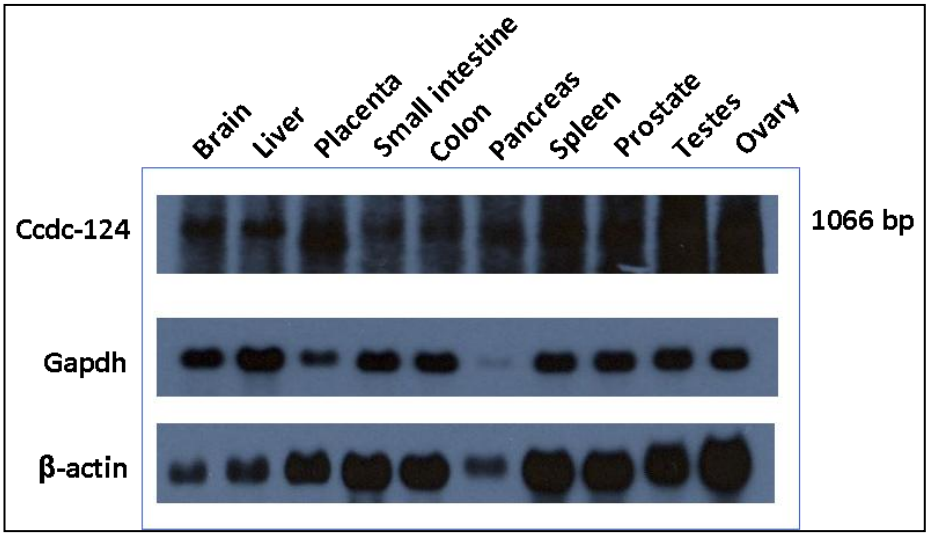


Figure 3.3: Human tissue expression analysis of CCDC-124 by Northern blotting. Probes were labeled via biotin and hybridized to Human Blot 1 membrane (see M.M.). Blots were exposed to X-ray films. β-actin and Gapdh were used as internal controls.

3.2 Characterization of the protein encoded by Ccdc124

3.2.1 Generation of polyclonal Antibody

The 24-mer Ccdc-124 N-terminus peptide (MPKKFQGENTKSAAARARRAEAK-[C]-amide) was synthesized by a private company (Cambridge Research Biochemicals) and two rabbits were been immunized by using this peptide in a KLH (keyhole limpet haemocyanine) conjugated form. After the 6th immunization, reactivity towards the N-terminus peptide has been analyzed by the Company by Elisa analysis, and these polyclonal anti-Ccdc-124 antibodies were purified using affinity purification method. Then antibodies were shipped to our laboratory together with sufficient amounts (10 mg) of reactive peptides.

Subsequently, we have carried out an immunoblot analysis using the antibodies generated aginst the N-terminus peptide of Ccdc 124. We have transfected Huh-7 human hepatocellular carcinoma cell lines with both N- and C- terminus Flag-tagged versions of Ccdc 124 gene in pCMV-14 expression vectors, and then carried out a Western blot analysis using total proteins from these cells (see M.M.). In this Western blot analysis (Fig.3.4 right panel) above mentioned polyclonal Ccdc-124 antibody have led to the detection of a 33 kD protein. This result is in agreement with Ccdc-124 bands obtained using Anti-flag antibodies (Fig.3.4 left panel).

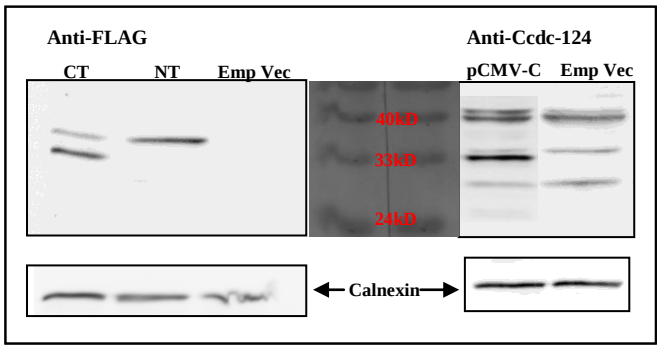


Figure 3.4: Western Blot Analysis of Ccdc-124. Left panel shows the C-terminally and N-terminally FLAG tagged Ccdc-124, cells were transfected either with pCMV-3XFLAG-ccdc124 (NT), pCMV-ccdc124-3XFLAG (CT) or pCMV14 (Emp Vec) plasmids and blotting was performed with anti-FLAG antibody; right panel shows the endogenous and over-expressed Ccdc-124 protein, cells were transfected with pCMV-ccdc124 (pCMV-C) plasmid and blotting was performed with above mentioned specific polyclonal antibody.

3.2.2 Subcellular localization of Ccdc-124

Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells (cultured cells, cell suspensions) by use of a specific antibody, which binds to it, thereby allowing visualization and examination under a microscope. It is a valuable tool for the determination of cellular contents from individual cells. Cells to be stained can be attached to a solid support like cover slips to allow easy handling in subsequent procedures. Cells are fixed and cell membrane is permeabilized with certain reagents, then proteins are first labeled with their specific antibody and stained with a specific secondary antibody which is conjugated with a fluorescent dye.

We have assessed the presence and subcellular localizations of Ccdc-124 protein in a number of human cell lines. These included the ER(+) mammary tumor cell line model MCF-7 and a cervical cancer cell model HeLa. Cells were transfected either with N-Terminally FLAG tagged, C-Terminally FLAG tagged, or untagged pCMV-Ccdc-124 plasmid. Ccdc-124 proteins were detected with either anti-FLAG antibody or polyclonal anti-Ccdc-124 antibody and stained with TRITC or FITC conjugated secondary antibody and nuclear stain was made with DAPI (Fig.3.5, Fig.3.6, Fig.3.8, Fig.3.9 and Fig.3.10) (see M.M.). Beside these, samples without DAPI staining were made in order to detect any interference of DAPI with the antibodies or the filters of the microscope (Fig.3.7 for HeLa cells and Fig.3.11 for MCF-7 cells). As a result of these experiments, it was observed that Ccdc-124 protein was mainly localized in the cytoplasm of the cell and ubiquitously expressed.

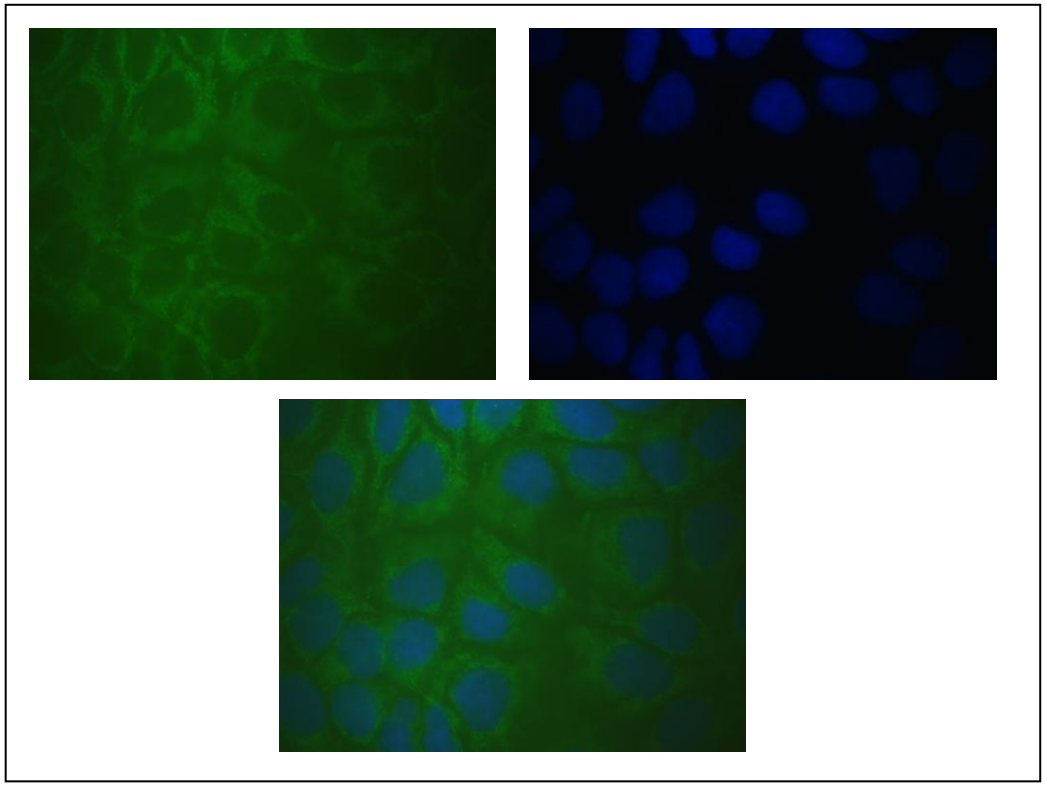


Figure 3.5: Immunocytochemistry of Ccdc-124. HeLa cells were fixed and stained with polyclonal anti-Ccdc-124 antibody and FITC conjugated anti-rabbit antibody. **A**, Endogenously expressed Ccdc-124; **B**, Nuclear stain with DAPI; **C,** Merged image. (40X)

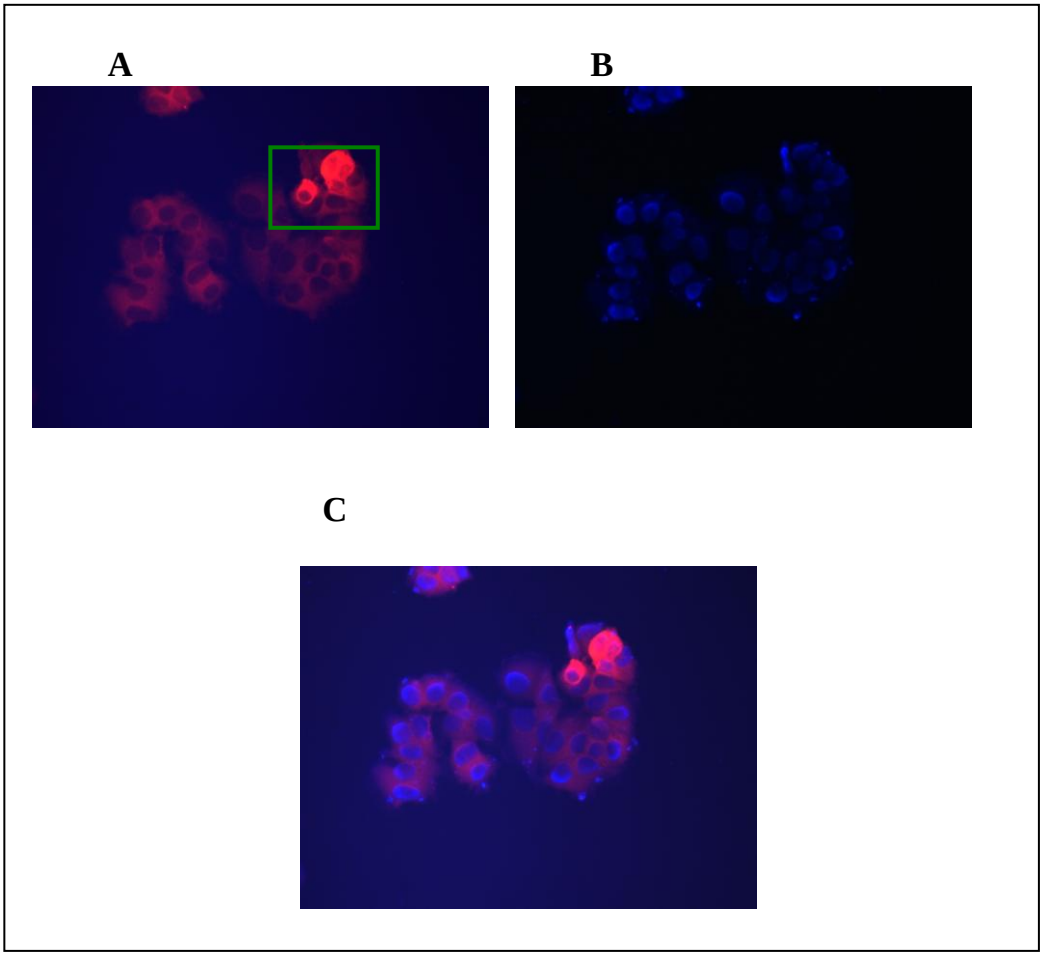


Figure 3.6: Immunocytochemistry of Ccdc-124. pCMV-Ccdc-124 transfected HeLa cells were fixed and stained with polyclonal anti-Ccdc-124 antibody and TRITC conjugated anti-rabbit antibody. **A**, Endogenously expressed (faint red) and transfected (bright red marked by the green rectangular line) Ccdc-124; **B**, Nuclear stain with DAPI; **C,** Merged image. (40X)

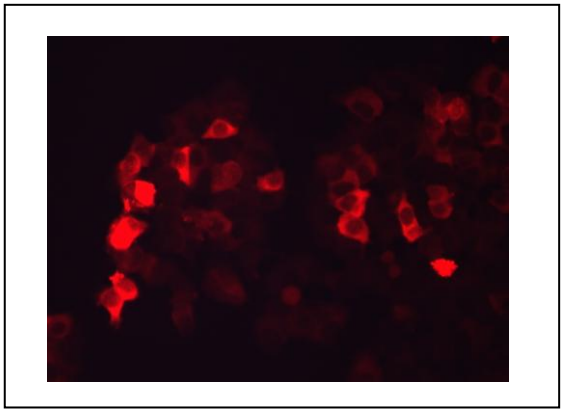


Figure 3.7: Immunocytochemistry of Ccdc-124 without DAPI stain. pCMV-Ccdc-124 transfected HeLa cells were fixed and stained with polyclonal anti-Ccdc-124 antibody and TRITC conjugated anti-rabbit antibody without nuclear (DAPI) staining. (40X)

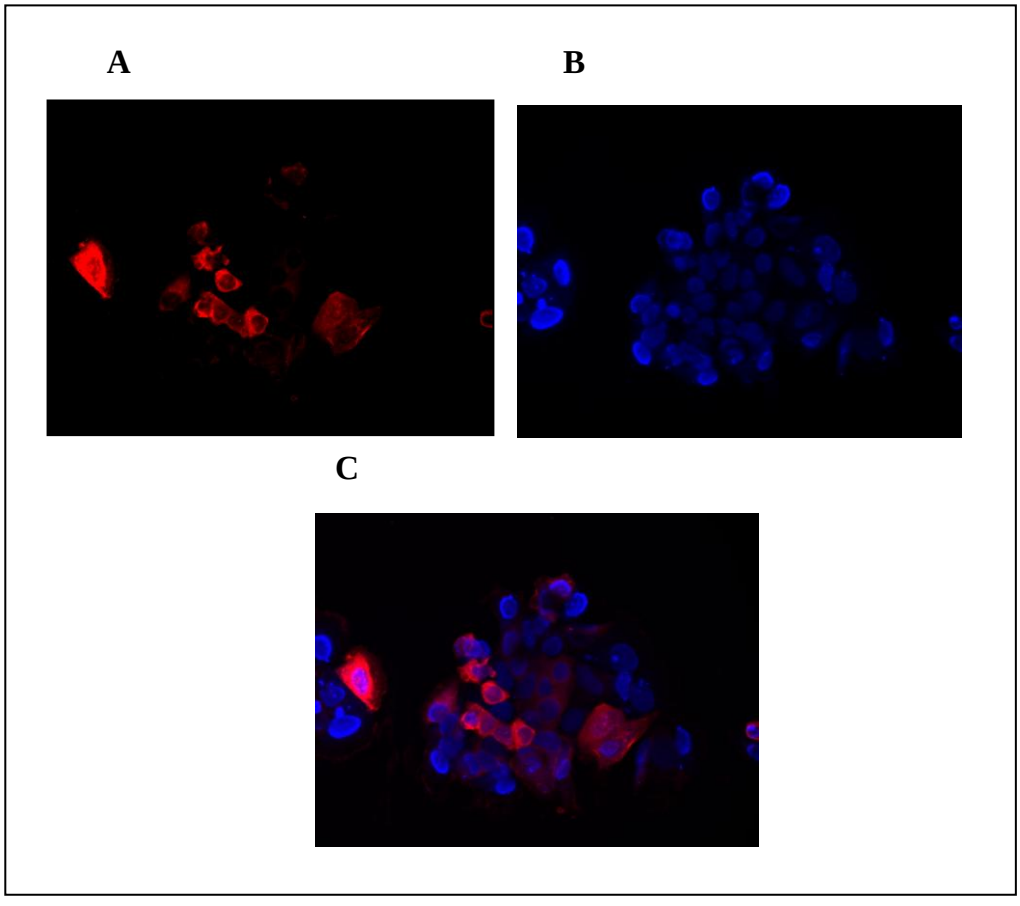


Figure 3.8: Immunocytochemistry of Ccdc-124. pCMV-Ccdc-124-3XFLAG transfected HeLa cells were fixed and C terminally FLAG tagged Ccdc-124 were stained with anti-FLAG antibody and TRITC conjugated anti-mouse antibody. **A**, Transfected Ccdc-124 stain; **B**, Nuclear stain with DAPI; **C,** Merged image. (40X)

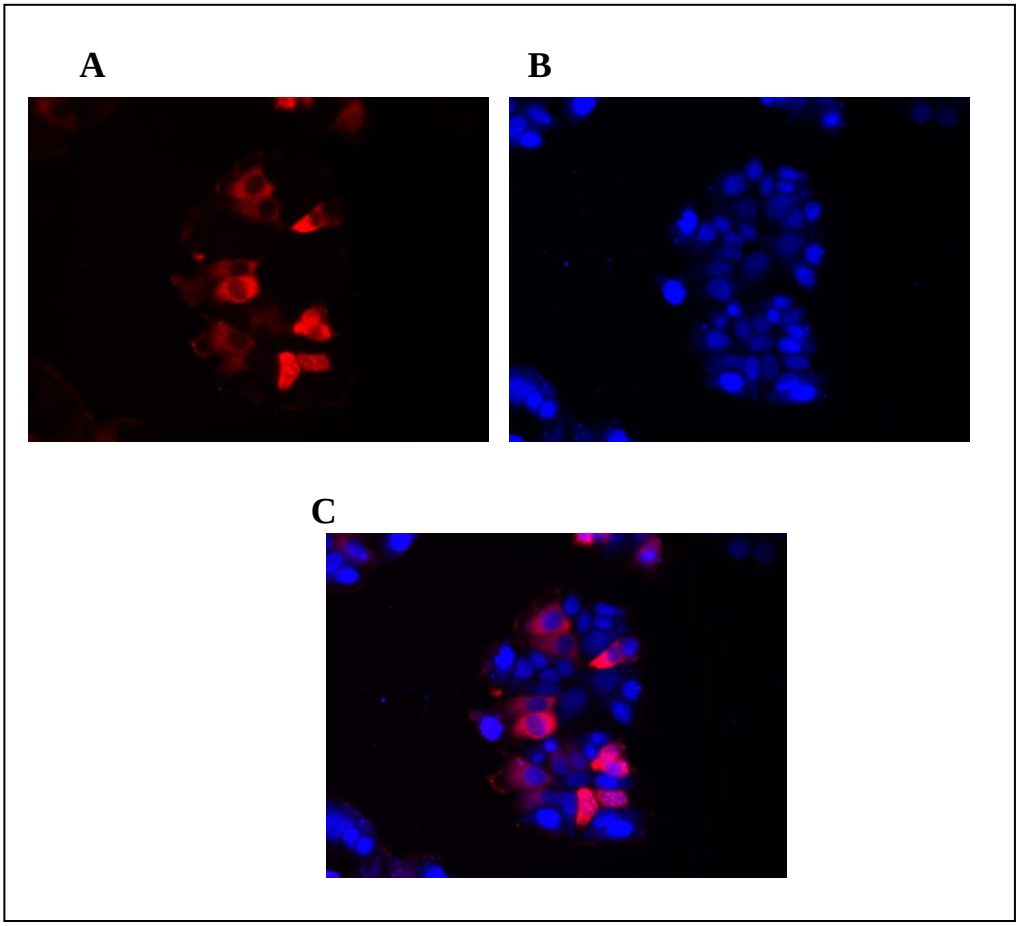


Figure 3.9: Immunocytochemistry of Ccdc-124. pCMV-3XFLAG-Ccdc-124 transfected HeLa cells were fixed and N terminally FLAG tagged Ccdc-124 were stained with anti-FLAG antibody and TRITC conjugated anti-mouse antibody. **A**, Transfected Ccdc-124 stain; **B**, Nuclear stain with DAPI; **C,** Merged image. (40X)

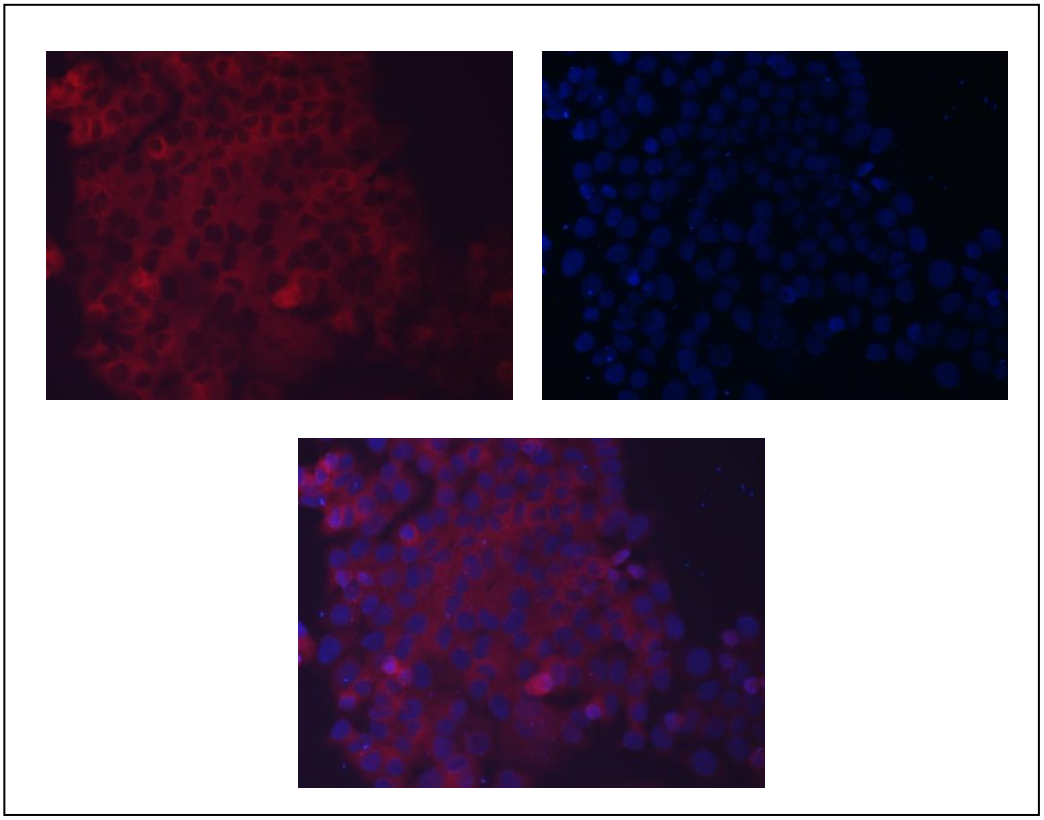


Figure 3.10: Immunocytochemistry of Ccdc-124. pCMV-Ccdc-124 transfected MCF-7 cells were fixed and stained with polyclonal anti-Ccdc-124 antibody and TRITC conjugated anti-rabbit antibody. **A**, Endogenously expressed and transfected Ccdc-124; **B**, Nuclear stain with DAPI; **C,** Merged image. (40X)

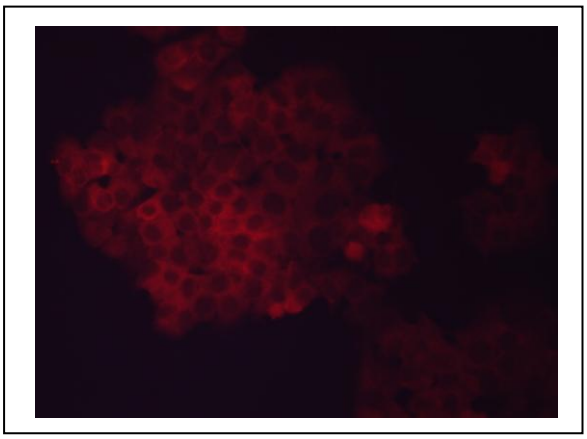


Figure 3.11: Immunocytochemistry of Ccdc-124 without DAPI stain. pCMV-Ccdc-124 transfected MCF-7 cells were fixed and stained with polyclonal anti-Ccdc-124 antibody and TRITC conjugated anti-rabbit antibody without nuclear (DAPI) staining. (40X)

3.3 Identification of RasGEF1 Family Members as Proteins Interacting with Ccdc124 *in vitro* and *in vivo*

3.3.1 Identification of proteins that interact with human CCDC124 by yeast two-hybrid (Y2H) screening.

Bait cloning and Y2H screening were performed by Hybrigenics, S.A., Paris, France (http://www.hybrigenics.com). A Human Liver random-primed cDNA library, transformed into the Y187 yeast strain and containing ten million independent fragments, was used for mating. High mating efficiency was obtained by using specific mating method (Legrain et al., 1998, 2000, 2002). And all the fragments obtained from the assay gave the same result which was RasGEF1B (Fig.3.12).

Clone Name	Type Seq	Gene Name (Best Match)	Start..Stop	Frame	Sens	%Id 5p	%Id 3p	PBS
pB29_A-6	5p/3p	Human RASGEF1B	489..1515	IF		99.5	99.7	A
pB29_A-8	5p/3p	Human RASGEF1B	489..1515	IF		97.7	99.1	A
pB29_A-12	5p/3p	Human RASGEF1B	489..1515	IF		98.1	99.9	A
pB29_A-7	5p/3p	Human RASGEF1B	489..1515	IF		96.9	99.3	A
pB29_A-15	5p/3p	Human RASGEF1B	489..1515	IF		97.6	99.6	A
pB29_A-13	5p/3p	Human RASGEF1B	489..1515	IF		96.3	99.4	A
pB29_A-14	5p/3p	Human RASGEF1B	489..1515	IF		99.6	99.7	A
pB29_A-5	5p/3p	Human RASGEF1B	489..1515	IF		98.0	99.5	A
pB29_A-3	5p/3p	Human RASGEF1B	489..1515	IF		97.0	99.6	A
pB29_A-9	5p/3p	Human RASGEF1B	495..1503	IF		97.2	99.6	A
pB29_A-1	5p/3p	Human RASGEF1B	495..1503	IF		97.8	100.0	A
pB29_A-4	5p/3p	Human RASGEF1B	495..1503	IF		98.2	99.9	A
pB29_A-2	5p/3p	Human RASGEF1B	495..1503	IF		98.7	99.9	A
pB29_A-16	5p/3p	Human RASGEF1B	495..1503	IF		99.1	99.6	A
pB29_A-11	5p/3p	Human RASGEF1B	555..1660	IF		98.7	98.5	A

Figure 3.12: Yeast-two-hybrid analysis revealed RasGEF1B as a potential interaction partner of Ccdc-124. A human liver random primed cDNA library (in pB29 plasmid) transformed into the yeast Y187 strain was used for mating with the L40ΔGAl4 strain containing human Ccdc124 as a bait. 16 positive clones have appeared on selective medium (see M.M.). All positive clones were picked, the corresponding prey fragments were amplified by PCR and sequenced at their 5' and 3' junctions. Names of positive clones were indicated (pB29_A-xx). All fragments were in frame with Gal4 Activation Domain (IF). A in boxes indicate 99.9% confidence rate in the interaction. Start and stop codons of fragments that are in frame are indicated.

3.3.2 RasGEF1 family was validated as interacting partners of Ccdc124.

3.3.2.1 Small Scale Protein Expressions

Following the results obtained from yeast-two-hybrid analysis, in order to assess in vitro interaction capacities of Ccdc-124 and RasGEF1 family members, and validate the interactions between these proteins, we first expressed these proteins in bacteria and purified them. Ccdc-124 and RasGEF1 family proteins were cloned in an IPTG (isopropyl-beta-D-thiogalactopyranoside) inducible pQE81 and pQE80 *E. coli* expression system, respectively. In order to assess the best expression and purification conditions for pQE81L-Ccdc124, pQE80L-RasGEF1A and pQE80L-RasGEF1B, a small scale test expression was designed. Proteins were expressed in bacteria growing in TB medium at two different temperatures, 16°C and 23°C, and lysed with three different lysis buffers and their corresponding wash buffers (see M.M.). After purification, proteins were loaded on a 15% SDS gel and stained with coomassie blue dye (Fig.3.13).

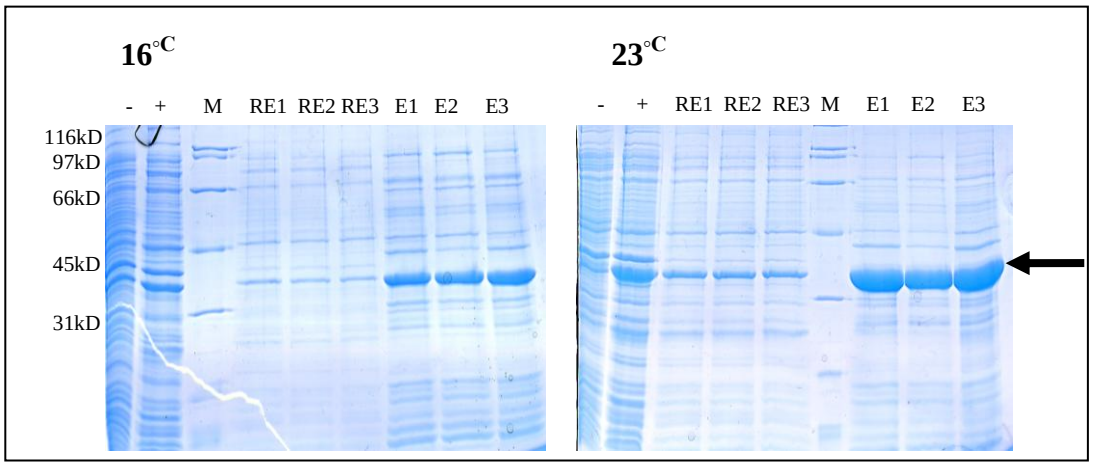


Figure 3.13: Small scale expressions of pQE81L Ccdc-124 in Bl21DE3 cells at 16°C and 23**°C.** "-" and "+" stand for not induced and IPTG induced controls, "RE" stands for Raw Extract lysate obtained after centrifugation not yet incubated with beads, "E" stands for Elution after incubation with Ni-Beads. Numbers "1,2,3" stand for three different lysis buffers (see M.M.).

In order to be sure that the major band (see arrow in Fig.3.13) on the coomassie stain of Ccdc-124 is the expected band, samples were loaded to two gels, one for

coomassie and the other one for blotting with the anti-His antibody against the His tag of induced Ccdc-124 (Fig.3.14).

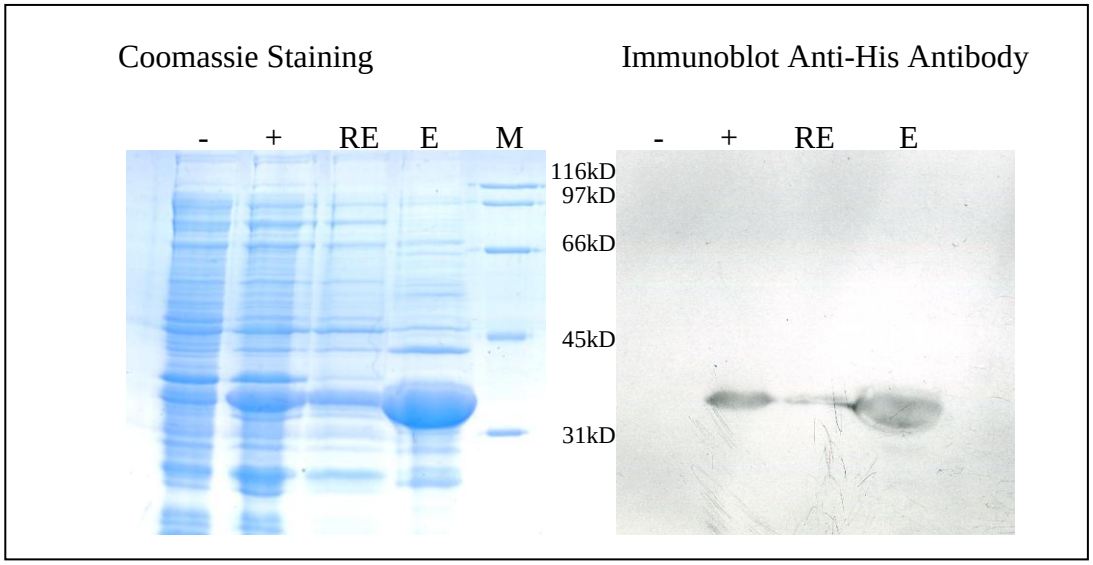


Figure 3.14: Coomassie and Western Blot analysis of small scale pQE81L Ccdc-124 expression. "-" and "+" stand for not induced and IPTG induced controls, "RE" stands for Raw Extract lysate after centrifugation but before incubation with beads, "E" stands for Elution after incubation with Ni-Beads. Western blot assay was performed with polyclonal anti-His-probe antibody (1:1000, Santa Cruz) and anti-Rabbit-Peroxidase antibody (1:2000, Sigma). Samples were obtained from cells incubated at 23**°C** (see above).

Because the small scale expression and purification of pQE81L-Ccdc124 was successful, the large scale expression was again performed using the pQE system (see below).

We then performed small scale expression and purification of RasGEF1 family members. According to the coomassie stainings of overnight induced RasGEF1B, the protein were not detected at the expected size which was approximately 57 kD (see arrow for the expected position of the band in Fig.3.15). For this reason in addition to overnight inductions, 2 hours and 3 hours inductions at 16ºC and 23ºC were also performed (Fig.3.16), but still no band was detected. Apart from this, a western blot was performed with the overnight induced samples in order to monitor once again the right size of RasGEF1B with anti-His antibody (Fig.3.17). According to the blot

results, RasGEF1B was observed at the expected size (57 kD) in the 23°C sample, even if it is not clearly visible in the coomassie-gel.

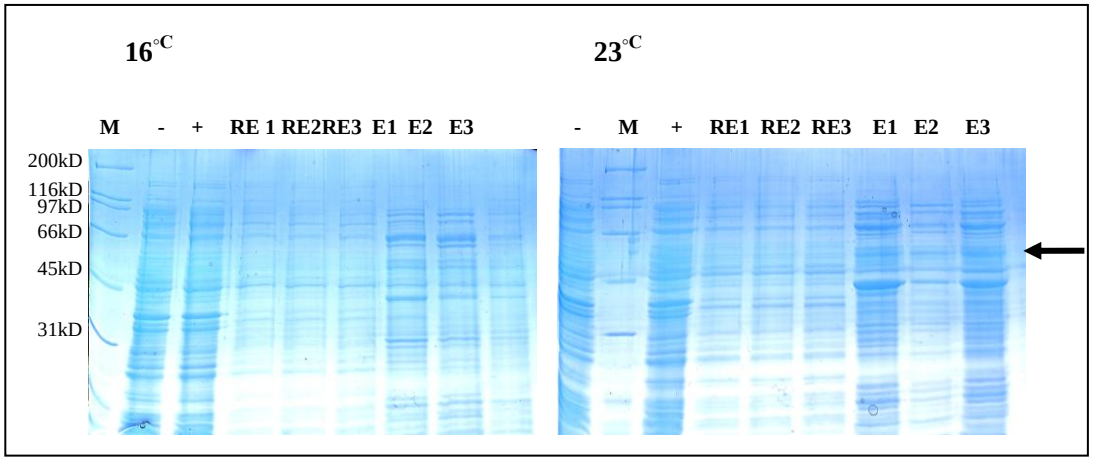


Figure 3.15: Overnight small scale expression of pQE80L RasGEF1B in Bl21DE3 cells at 16°C and 23°C, respectively. "-" and "+" stand for uninduced and IPTG induced controls, "RE" stands for Raw Extract lysate after centrifugation but before incubation with beads, "E" stands for Elution after incubation with Ni-Beads. "1,2,3" numbers stand for three different lysis buffers (see M.M). The arrow indicate expected position of RasGEF1B band.

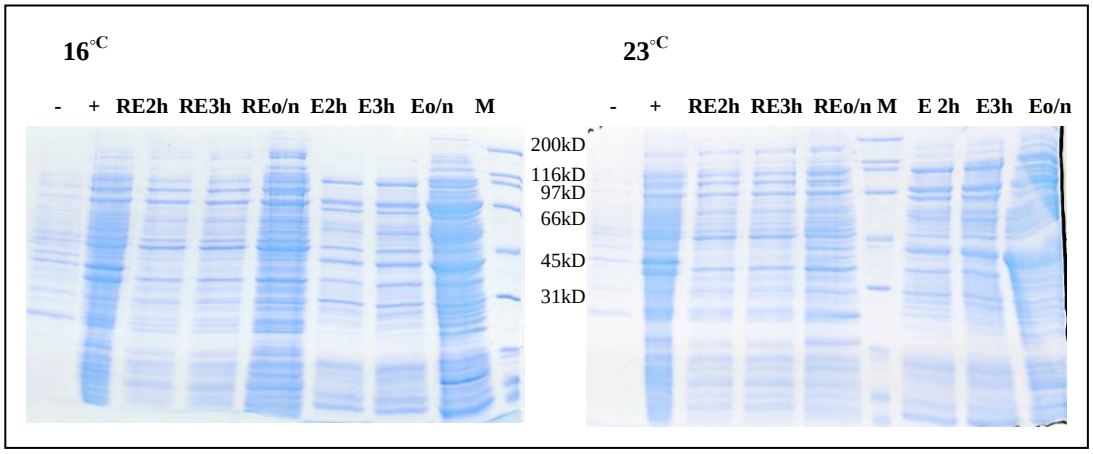


Figure 3.16: 2 hour and 3 hour small scale expression of pQE80L RasGEF1B in Bl21DE3 cells at 16°C and 23°C, respectively. "-" and "+" stand for uninduced and IPTG induced controls, "RE" stands for Raw Extract lysate after centrifugation but before incubation with beads, "E" stands for Elution after incubation with Ni-Beads. "2h","3h" and "o/n" stand for 2 hours, 3 hours and overnight, respectively. RasGEF1B band is again not observable.

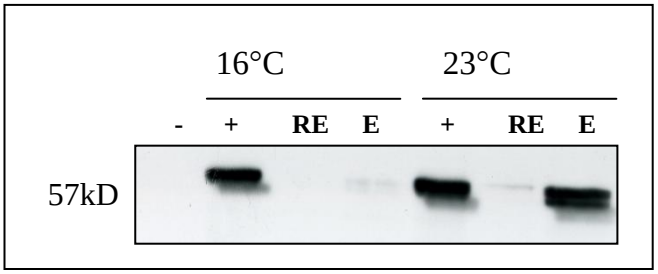


Figure 3.17: Western blot analysis of pQE80L RasGEF1B with anti-Histidine antibody. "-" and "+" stand for not induced and induced controls, "RE" stands for Raw Extract lysate after centrifugation but before incubation with beads, "E" stands for Elution after incubation with Ni-Beads. His-tagged RasGEF1B was detected with anti-His antibody (1:1000, Santa Cruz) and anti-Rabbit antibody (1:2000, Sigma)

It is well known that protein tags could affect the solubility of the proteins. For that reason, in order to assess whether His-tags could affect purification of RasGEF1 family members, RasGEF1A and RasGEF1B inserts were taken out of the pQE vector system which had His tags, and they were sub-cloned into pGEX system which has GST tags (see M.M). In fact, pGEX system could also be a better choice for purification of these proteins because they are relatively more efficient in terms of protein expression as compared to pQE vector system. Also, GST/GSH-bead system based purification has advantages in terms of purification/binding efficiency as compared to His/Ni-Bead based systems. After cloning RasGEF1A and RasGEF1B into pGEX-TEV-N expression system (see M.M.), again small scale test expressions were performed (Fig.3.18).

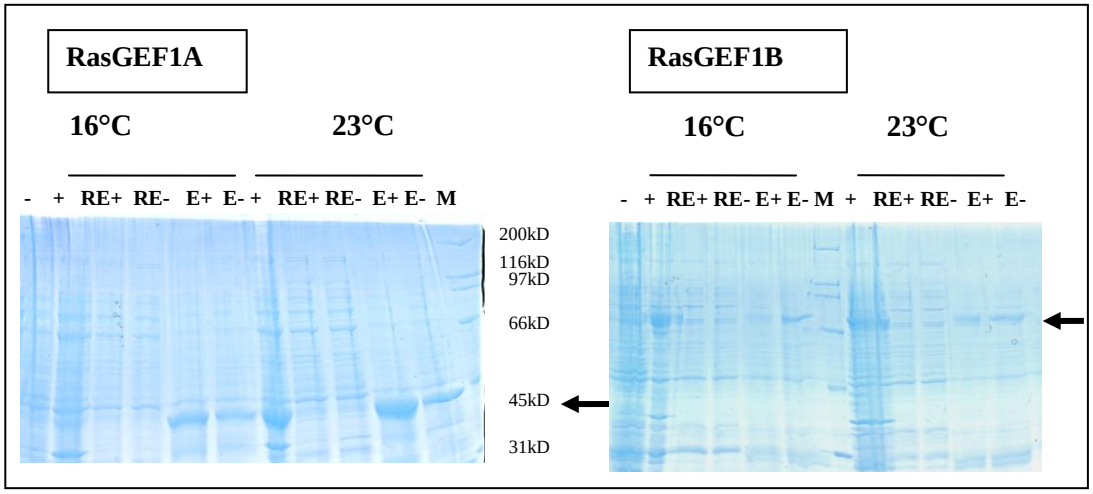


Figure 3.18: Overnight small scale expression of pGEXTEVn-RasGEF1A in Bl21DE3 cells at 16°C and 23°C, respectively. "-" and "+" stand for not induced and IPTG induced controls, "RE" stands for Raw Extract lysate after centrifugation but before incubation with beads, "E" stands for Elution after incubation with GSH-Beads. – and + signs after RE and B stand for lysis buffer with or without detergent (see M.M).

Small scale expressions were performed for pGEX-RasGEF1A and 1B at 16°C and 23°C with lysis buffers with or without detergent, in order to establish best buffer and temperature conditions for purification. The major band that was detected on the coomassie staining of the test expression of pGEX-RasGEF1A was ~45 kD (see arrow in the left panel of Fig.3.18), which normally should be ~75kD (~55kD RasGEF1A+~20kD GST Tag). In order to understand whether the aberrant size was due to an error in the sequence of the sub-cloned RasGEF1A insert, the DNA sequence of the insert was analyzed. Unfortunately, according to the sequencing results, RasGEF1A had 5 PCR generated mutations and one of them was created a stop codon. That result explained why the dominant band at the small scale expression of RasGEF1A was located at ~45 kD and not at the expected size, approx. 75 kD. As a result, we decided to convert this mutated RasGEF1A insert to wild-type by site directed mutagenesis (see M.M.). After an extensive mutagenesis effort (5 different *in vitro* mutagenesis PCR reactions) we were able to successfully get the wild-type RasGEF1A (sequencing results not shown) and continued our experiments with that plasmid (see below). On the other hand, pGEX-TEV-n-RasGEF1B was detected at right size (see arrow in right panel of Fig.3.18). As a result of these test

expressions, we got the best expression and purification results from 23°C with the lysis buffer without detergent, so it was decided to proceed with that conditions.

3.3.2.2 Large Scale Culture of CCDC-124

5L culture of pQE81L-Ccdc-124 in BL21 *E.coli* cells were grown at 23°C. Supernatant of the lysed cells were loaded to Ni-NTA-Agarose column and eluted with increasing concentrations of imidazole. Half of the fractions were loaded on an SDS-gel, and existence of Ccdc-124 protein was assessed in all fractions (Fig.3.19, panel A). Then, fractions containing Ccdc-124 were pooled together and concentrated for the gel filtration step (Fig.3.19 panel B). After gel filtration, fractions were loaded to an SDS gel and fractions between 27-38 and 39-51 pooled separately and named as pool I and pool II respectively. Pool I was used in all experiments, since it has a higher purity. (Fig.3.19 panel C) (see M.M.). Concentrations of Pool I and II are 28.8mg/ml and 30mg/ml, respectively.

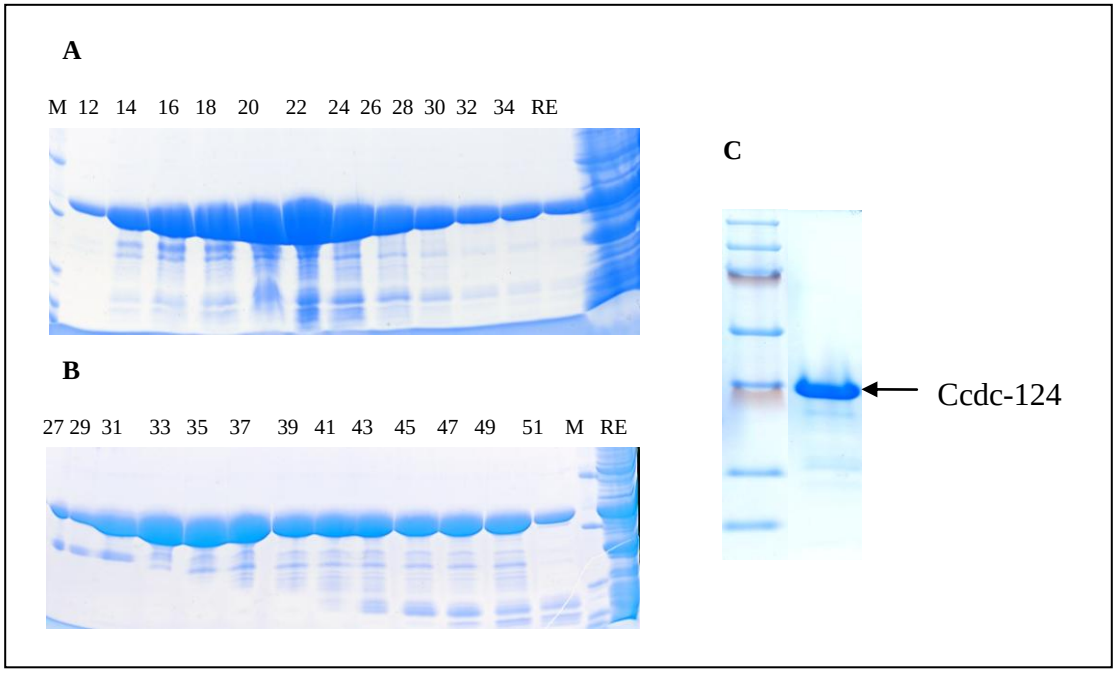


Figure 3.19: Purification of pQE81L-Ccdc-124. A, Fractions of ccdc-124 protein after purification on Ni-NTA-column were loaded on a gel; **B,** then fractions were collected and concentrated and loaded on Superdex S75 26 / 60 column and fractions coming from gel filtration were loaded on another gel; **C,** fractions between 27 and 51 were collected in two pools and concentrated. Purified Ccdc-124 protein from the purer pool loaded on a gel.

3.3.2.3 Large Scale Culture of pGEX-RasGEF1B

pGEX-RasGEF1B was expressed in 10L culture of *E.coli*, induced and collected as before. Supernatants were loaded on to GSH columns and fractions were loaded on a SDS-gel (see Fig.3.20, below). 1mg/ml TEV was used for the cleavage of the GST tag of pGEX-TEV-n-RasGEF1B.

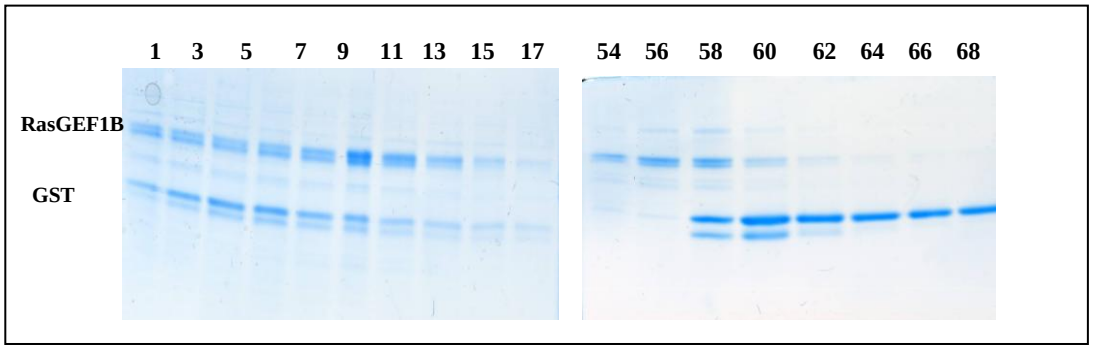


Figure 3.20: Purification of RasGEF1B protein. Fractions of RasGEF1B protein after purification on GSH-column were loaded on a gel, and then selected fractions were collected for TEV cleavage**.**

According to the purities of fractions observed in gels in Fig.3.20 (above), fractions between 1 to 19 and 53 to 57 were pooled into two tubes. Then, these two pools of RasGEF1B were subjected to TEV cleavage. After TEV cleavage, intensive protein precipitation was observed: both supernatant and the precipitated parts were loaded to another SDS gel and stained with coomassie blue dye (Fig.3.21). According to the results obtained in that gel, nearly all of the cleaved RasGEF1B proteins were precipitated. As a conclusion, it was decided to purify RasGEF1 proteins without separating them from their GST tags.

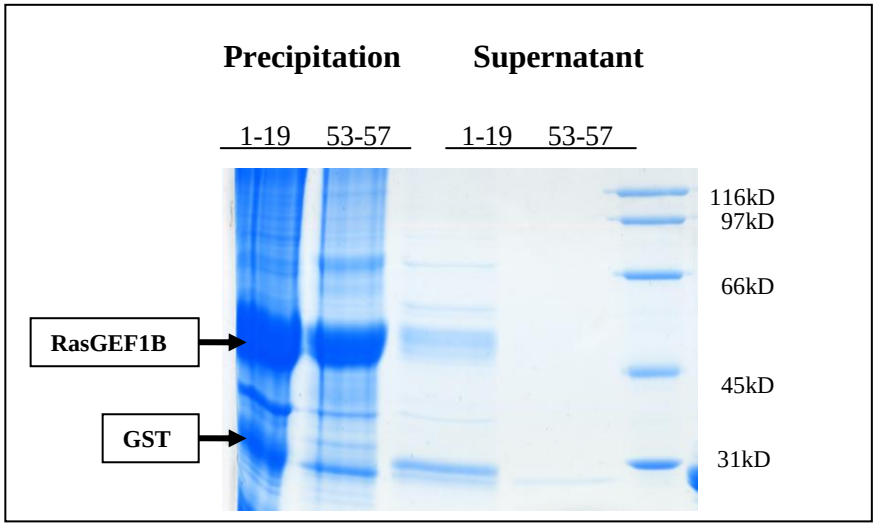


Figure 3.21: TEV Cleavage of GST-RasGEF1B protein. When the GST tag is removed, RasGEF1B protein (see arrow) precipitated immediately. Both precipitation and the supernatant after TEV cleavage loaded on a gel.

Then, the purifications were repeated by using RasGEF1B having GST tags on them (without TEV treatments, see Fig.3.22). Again they were pooled to two goups (Pool I; fractions 6-9, pool II; fractions 10-14, see Fig.3.23), and purities were monitored on a novel coomassie gel. Concentrated proteins were gel-filtrated and kept for the following experiments (see below).

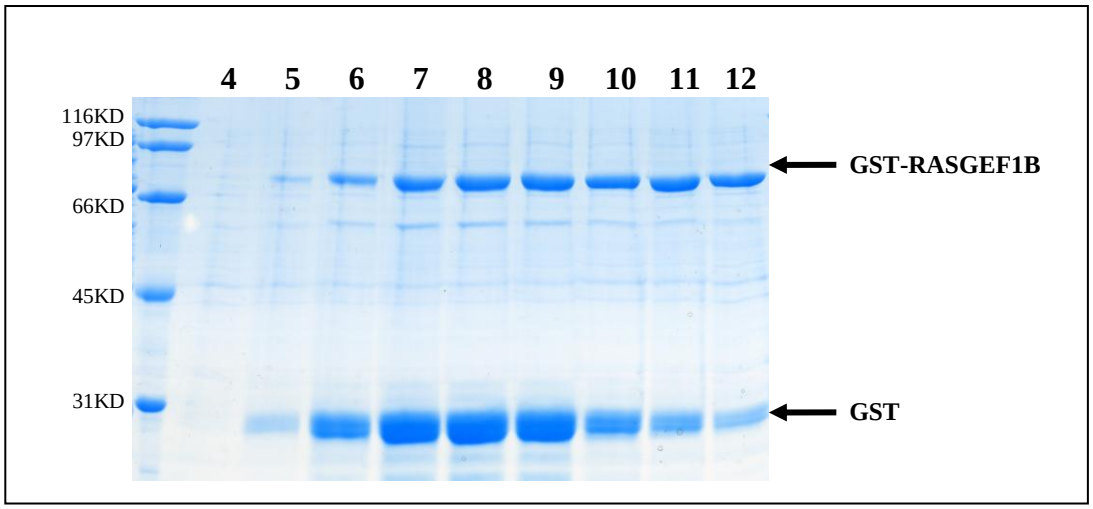


Figure 3.22: Purification of RasGEF1B protein. Fractions of RasGEF1B protein after purification on GSH-column were loaded on a gel, and then selected fractions were collected for gel filtration**.**

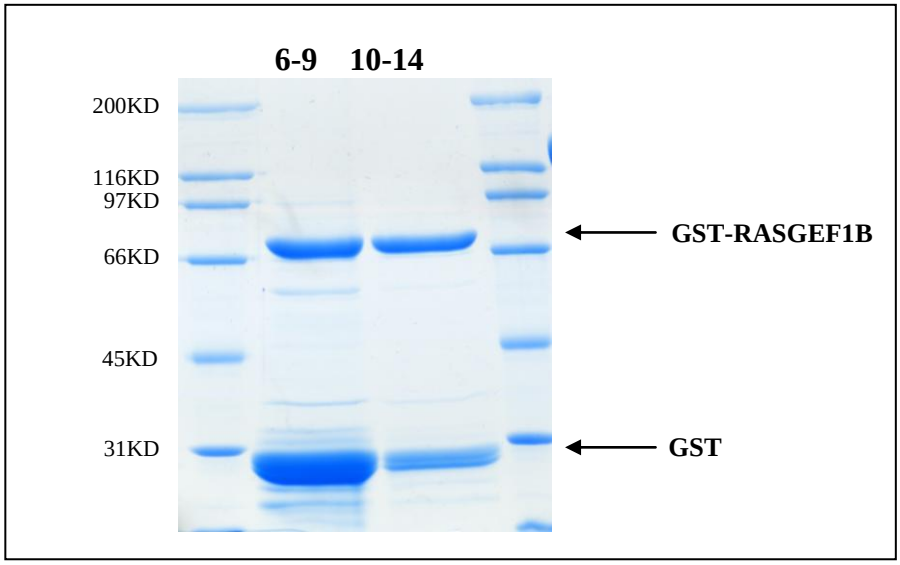


Figure 3.23: Purified GST tagged RasGEF1B protein pool. After gel filtration, pools were concentrated and loaded on a gel, the concentration of the first pool was 14.25mg/ml (178µM) and the second pool was 10mg/ml (125µM).

3.3.2.4 Large Scale Culture of RasGEF1A

RasGEF1A was expressed in *E. coli* as a 10L culture and purified with GSH-Beads in two 5 ml columns (Fig.3.24 panel A). Then the elution was gel filtrated with Superdex S75 16/60 columns and fractions were loaded on a 15% SDS gel (Fig.3.24 panel B). Fractions between 22-34 were collected, concentrated and concentrations were measured (Fig.3.24 panel C). The concentration of the protein was established as 8.5mg/ml (113µM).

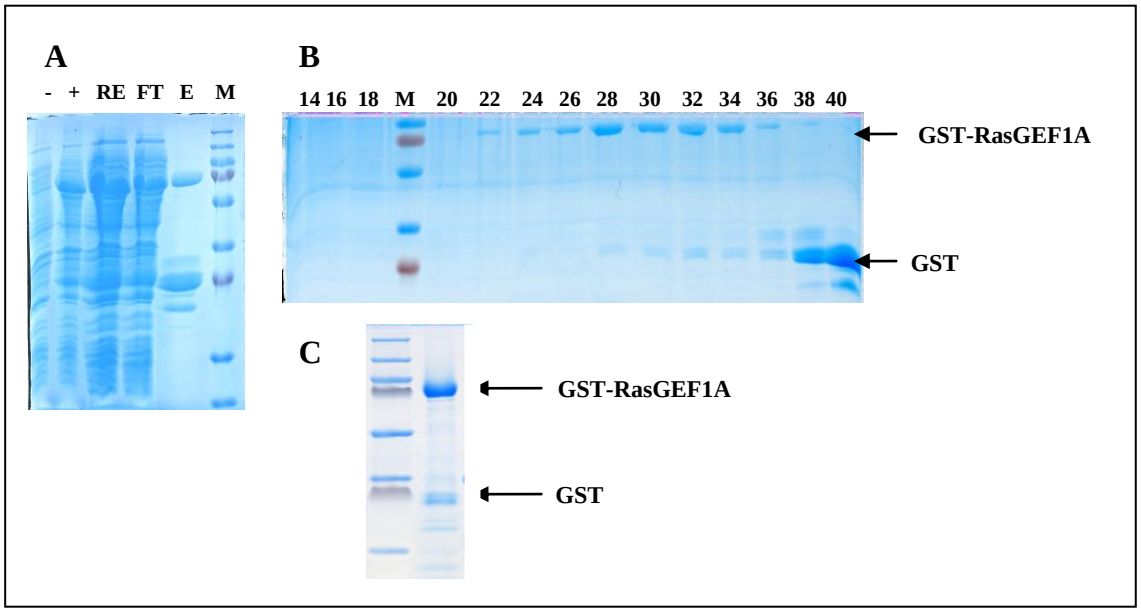


Figure 3.24: RasGEF1A protein purification. A, GST-RasGEF1A was purified with GSH beads. – and +: uninduced and IPTG induced controls, respectively; RE: Raw Extract after lysis but before incubation with beads; FT: Flow Through after incubation with beads; E: Elution; M: Marker. **B,** Elution was subjected to gel filtration**.** Factions between 22 and 34 were collected and pooled together. **C,** Collected fractions were concentrated and loaded on a gel.

3.3.3 GST Pull Down Assay

The pull-down assay is an *in vitro* method used to determine physical interaction between proteins. A tagged protein or the bait (GST, His6, biotin etc.) purified in an appropriate expression system (e.g., *E. coli*) is immobilized on a glutathione affinity gel. The bait serves as the secondary affinity support for identifying new protein partners or for confirming a previously suspected protein partner to the bait.

In our case, GST tagged RasGEF1A and RasGEF1B proteins were used as bait and Ccdc124 was used as prey. RasGEFs were alone incubated with the GSH beads in order to detect the level of RasGEF1 binding to the beads, or alternatively RasGEF1s and Ccdc124 were incubated together in order to visualize the interaction between RasGEF1s and Ccdc124 (Fig.3.25). GSH beads were also incubated with Ccdc124 alone in order to detect the background interaction of Ccdc124 to the GSH beads in

the absence of GST-RasGEF1s, or GSH beads first incubated with GST protein then with Ccdc124 in order to detect the binding capacity of Ccdc124 to GST tag. Apart from these samples, RasGEF1A or 1B, Ccdc124 and GST proteins were loaded to the gel to determine the sizes of the proteins (Fig.3.25). As a result we have clearly establied that both RasGEF1A and RasGEF1B interact with Ccdc124 *in vitro.* This result validated our yeast-two-hybrid results that had initially indicated a possible interaction between RasGEF1s and the novel conserved eukaryotic protein of unknown function, Ccdc-124.

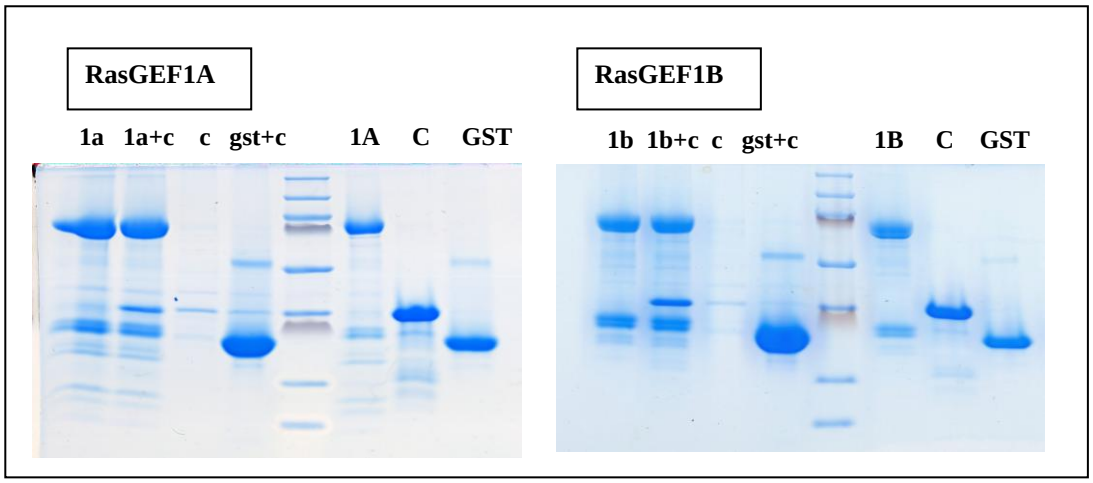


Figure 3.25: GST-Pull Down Assay of GST-RasGEF1A and GST-RasGEF1B. 1a and 1b stand for GST-RasGEF1A and GST-RasGEF1B proteins, respectively; c stands for Ccdc-124 protein, gst stands for GST protein. First Lane: GST-RasGEF proteins were immobilized on GSH-Beads (which shows the immobilized RasGEF proteins on GSH-Beads); Second Lane: Ccdc-124 proteins were incubated with GSH-Beads immobilized with GST-RasGEF proteins(which shows the interaction between RasGEFs and Ccdc-124); Third Lane: Ccdc-124 proteins were incubated with GSH-Beads only (which shows the interaction capacity of Ccdc-124 with the GSH-Beads in the absence of an interacting partner); Fourth Lane: Ccdc-124 proteins were incubated with GSH-Beads immobilized with GST protein (which shows the interaction capacity of Ccdc-124 with the GST Tag); Fifth Lane: Marker; Sixth Lane: GST-RasGEF proteins; Seventh Lane: Ccdc-124 protein; Eighth Lane: GST protein.

3.3.4 In vitro Interaction of RasGEF1B and Ccdc-124: Polarization

The fluorescence polarization immunoassay is another way to show *in vitro* interaction of two proteins. This method depends upon the fact that a conjugate of a fluorescing substance and a small molecule, moves very quickly in solution so that polarized light which is passed through the solution and excites the fluorescing substance is depolarized in the case of the emission due to the rotation of the conjugate taking place between impingement and emission. The degree of depolarization is thereby inversely proportional to the rate of rotation of the molecule. In the case of binding the fluorescing substance to a large molecule, for example in the case of binding of a fluorescent-labelled Ccdc-124(33kD) to RasGEF1B (75 kD), the movement of the whole conjugate is so very considerably limited that the polarized light is now no longer depolarized from the point of time when it impinges up to the point of time when it is emitted but rather again emerges polarized from the solution.

We used IAEDANS as a fluorescent dye. IAEDANS is an organic fluorophore and stands for 5-(2-[(iodoacetyl)amino]ethylamino)naphthalene-1-sulfonic acid. It has a peak excitation wavelength of 336 nm and a peak emission wavelength of 490 nm. The extinction coefficient of the dye is 5700. It usually reacts with thiols like cystein. Since Ccdc-124 protein doesn't have any Cystein residues, it was decided to transform some amino-acids of Ccdc-124 into Cystein. For this purpose, I decided to change 3 Serins into Cysteins since their size and structures are similar. These Serins were selected from the coiled coil domain of the protein, because it is necessary to label relatively stable (inflexible) parts of the protein in order to obtain reliable results.

Ser12, Ser92 and Ser156 were transformed into Cysteins by site directed mutagenesis. These 3 cystein mutants of Ccdc-124 proteins were purified from a large scale culture as before (Fig.3.26, Fig.3.27, Fig.3.28 and Fig.3.29).

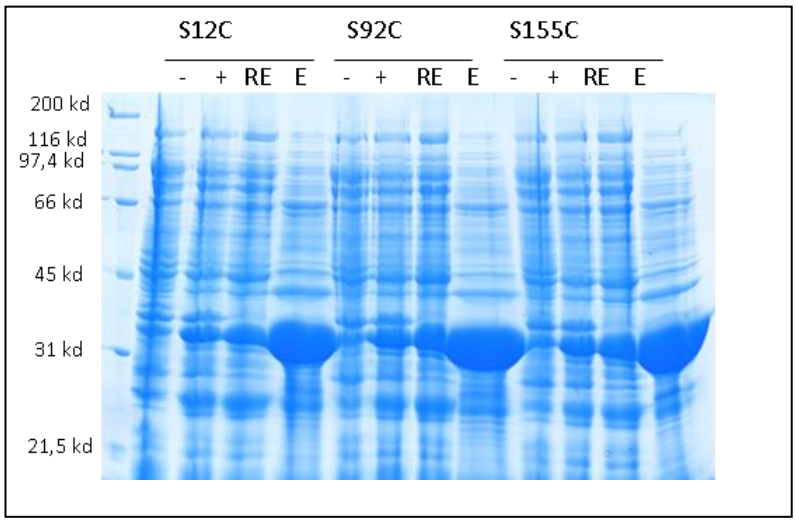


Figure 3.26: Purification of Ser12, Ser92 and Ser156 Ccdc-124 proteins. A, Proteins were purified with Ni-beads. – and +: uninduced and IPTG induced controls, respectively; RE: Raw Extract after lysis but before incubation with beads; E: Elution.

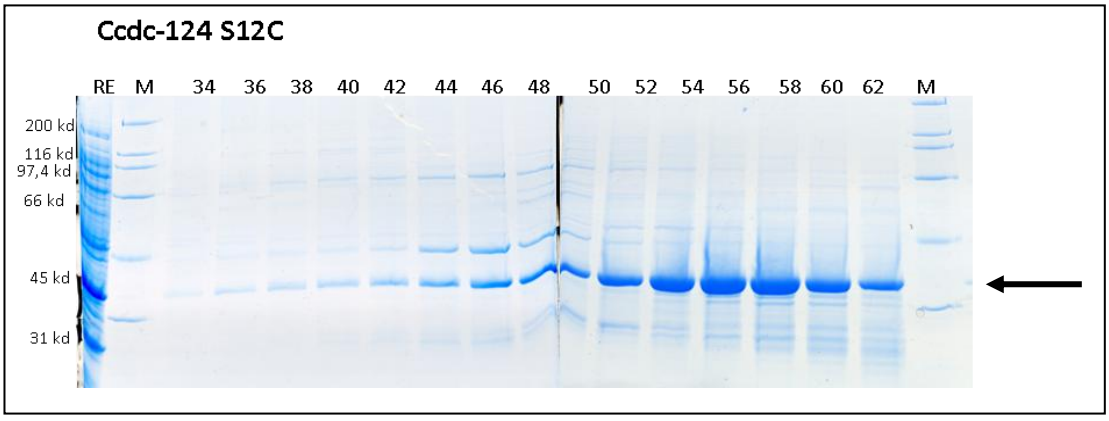


Figure 3.27: Elution of Ccdc-124 S12C was subjected to gel filtration. Factions between 50 and 63 were collected and pooled together and concentrated up to 46.4 mg/ml.

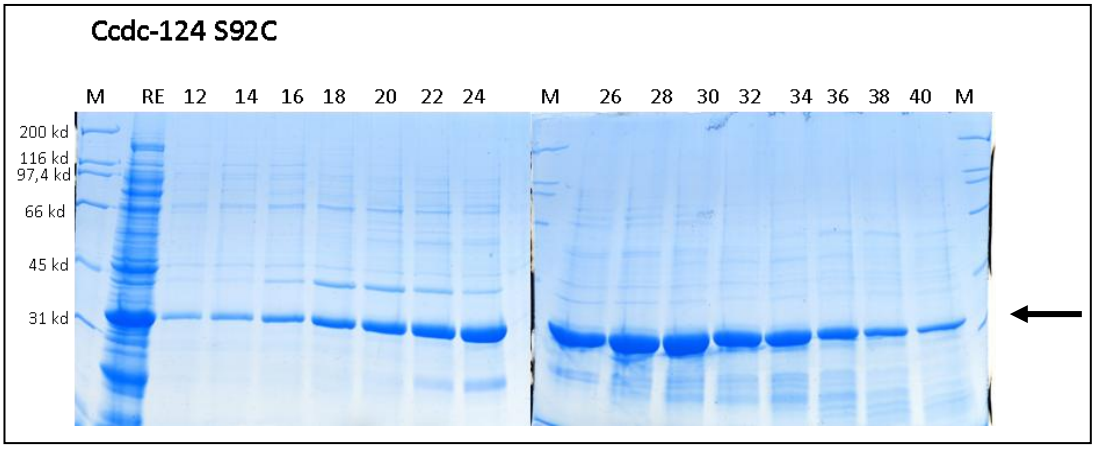


Figure 3.28: Elution of Ccdc-124 S92C was subjected to gel filtration. Fractions between 16-25 and 26-41 were pooled and concentrated up to 56.3 mg/ml and 67 mg/ml respectively.

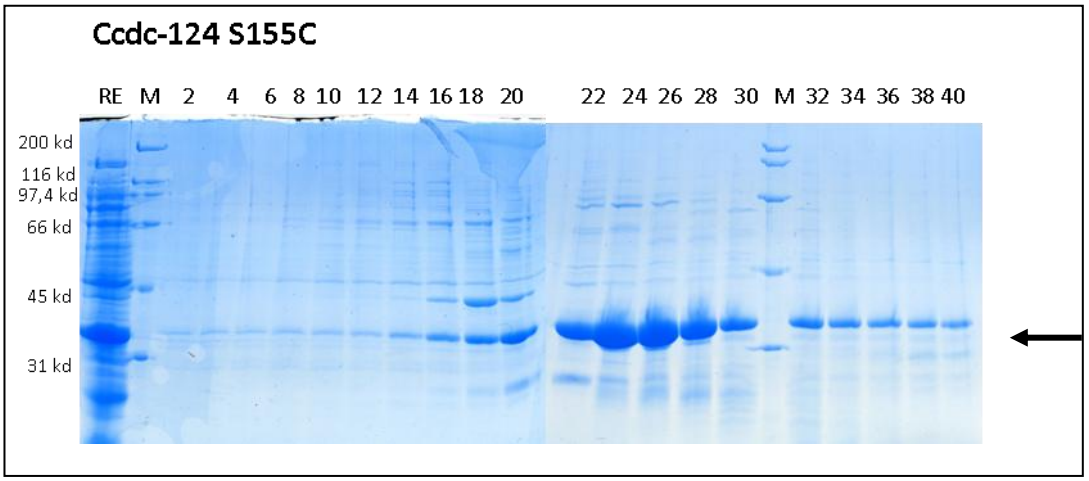


Figure 3.29: Elution of Ccdc-124 S155C was subjected to gel filtration. Fractions between 16-30 and 31-41 were pooled and concentrated up to 58 mg/ml and 17.3 mg/ml respectively.

2mg of these proteins were labeled with IAEDANS overnight and subjected to gel filtration in order to get rid of unlabeled proteins and free IAEDANS (Fig.3.30). Possible protein fractions (fractions that gave paek for 336nm for Iaedans and 280nm for protein at the same time) were given to gel. For every mutant, fractions around 6th fraction were pooled and concentrated.

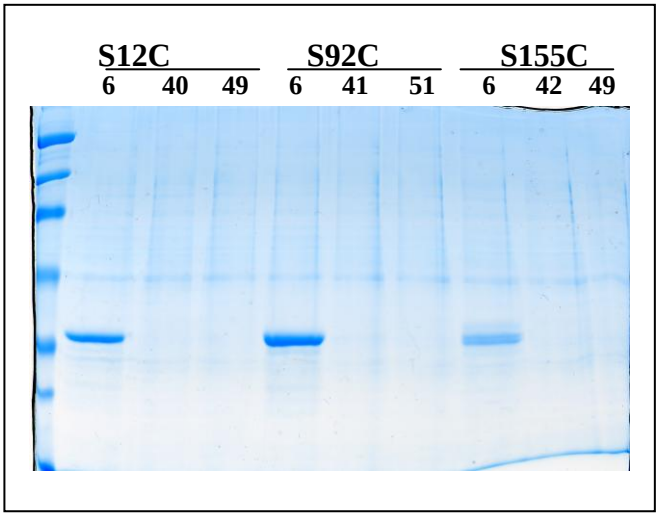


Figure 3.30: Selected fractions after gel filtration for Iaedans labeling. Fractions from 2-12 from S12C, 3-12 for S92C, and 4-11 for S155C were collected and concentrated to 78.9µM, 89.47µM and 87.7µM, respectively.

These IAEDANS labeled Ccdc-124 proteins were then used for polarization assay with RasGEF1B protein. In this assay, the polarizations of labeled Ccdc-124 proteins were measured first and subsequently, RasGEF1B proteins were added with certain molar concentrations and with certain time laps. In normal conditions, if these two proteins bind together: polarization of the labeled Ccdc-124 protein changes every time RasGEF1B protein is added to the solution and polarization should reach to a saturation level when they reach to equilibration. In our case, it was obvious that these two proteins interact with each other because every time RasGEF1B was added, the polarizations were changed but since RasGEF1B was not a "happy protein" and precipitates easily we could not sustain that experiment long enough to reach saturation (Fig.3.31).

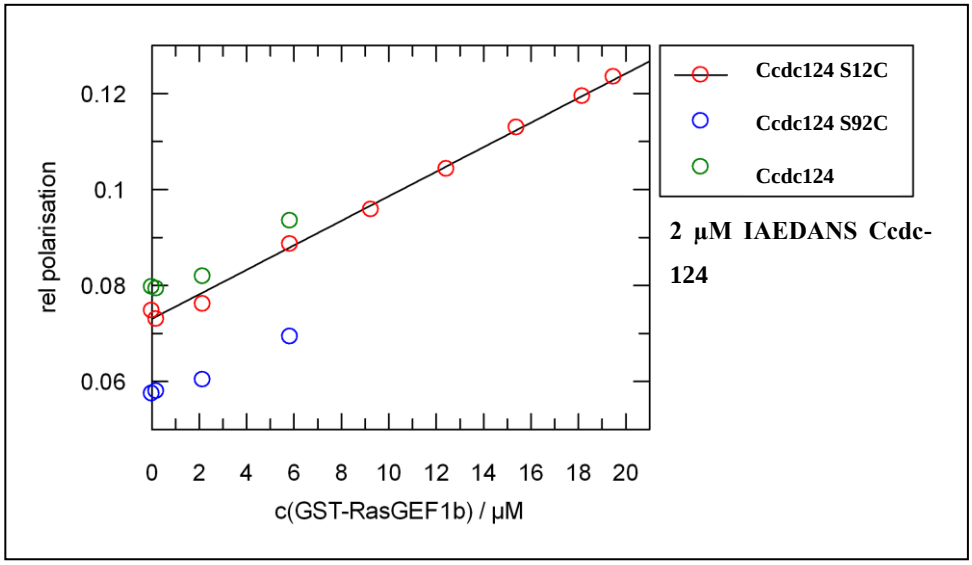


Figure 3.31: Polarization assay of IAEDANS labeled Ccdc-124 protein with RasGEF1B protein. A representative graph showing the polarization of RasGEF1B and Ccdc-124. Because of the limited concentration of RasGEF1B protein, assay was continued with Ccdc-124 S12C after 6µM of RasGEF1B.

3.3.5 In Vivo Detection of Interaction between Ccdc-124 and RasGEF1B with Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) is considered to be the gold standard assay for protein-protein interactions. The protein of interest is isolated with a specific antibody. The antibody-protein complex is then precipitated usually using protein-G or protein-A sepharose which binds most antibodies. If there are any protein/molecules that bind to the first protein, they will also be precipitated. Co-precipitated protein can then be identified by Western blot analysis or by sequencing a purified protein band.

Since there was no commercially available antibody of RasGEF1B, we used a C terminally FLAG tagged (Fig.3.32) and N terminally Myc tagged RasGEF1B plasmid (p3XFLAG-RasGEF1B) and anti-FLAG antibody to detect RasGEF1B protein.

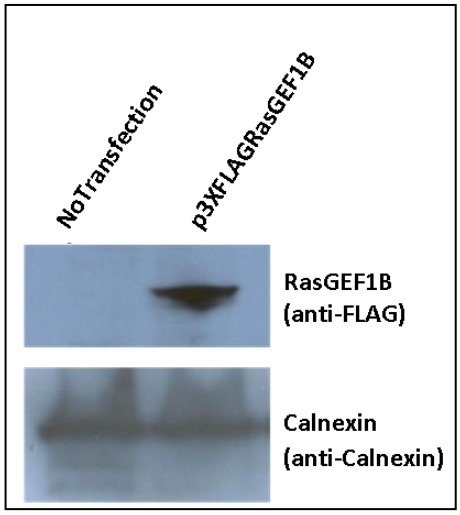


Figure 3.32: Western Blot analysis of RasGEF1B. p3XFLAG-RasGEF1B transfected HeLa cells were blotted with anti-FLAG antibody, equal protein loadings were assessed by blotting the same membrane with anti-calnexin antibody.

In the first round of Co-IPs we used ANTI-FLAG® M2 Affinity Gel (Sigma). P3XFLAG-RasGEF1B and pCMV-Ccdc-124 transfected Hela and MCF-7 cells were lysed and RasGEF1B protein was precipitated with ANTI-FLAG® M2 Affinity Gel and co-precipitated Ccdc-124 protein was analyzed with its specific antibody (Fig.3.33). But unfortunately, the secondary antibody that was used for western blot also detects the dissociated heavy and light chains of the immobilized M2 antibody which gives around 25 and 50kDa bands (which is a common inconvenience of IP) and these bands partially overlap with the Ccdc-124 and RasGEF1B bands and made it hard to detect the bands (results not shown).

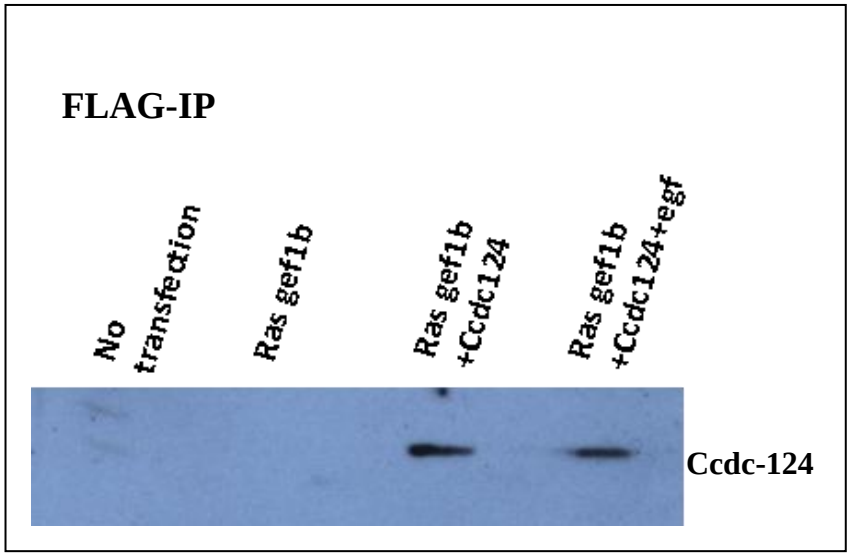


Figure 3.33: FLAG-IP of Hela cells. HeLa cells were co-transfected with P3XFLAG-RasGEF1B and pCMV-Ccdc-124, immunoprecipitated with anti-FLAG and blotted with anti-Ccdc-124.

Because of the inconveniences of using secondary antibody in our system, we changed to c-Myc immunoprecipitation and anti-FLAG-HRP blot system. In this system p3XFLAG-RasGEF1B and p3XFLAG-Ccdc-124 plasmids were co-transfected to Hela cells and RasGEF1B was precipitated with anti-c-Myc antibody, since it has also a Myc tag at the N terminus. And western blot was performed with HRP conjugated anti-FLAG antibody in order to eliminate the use of secondary antibody. Performing the blot with anti-FLAG-HRP also gave us the chance to demonstrate RasGEF1B and Ccdc-124 proteins on the same membrane at the same time (Fig.3.34).

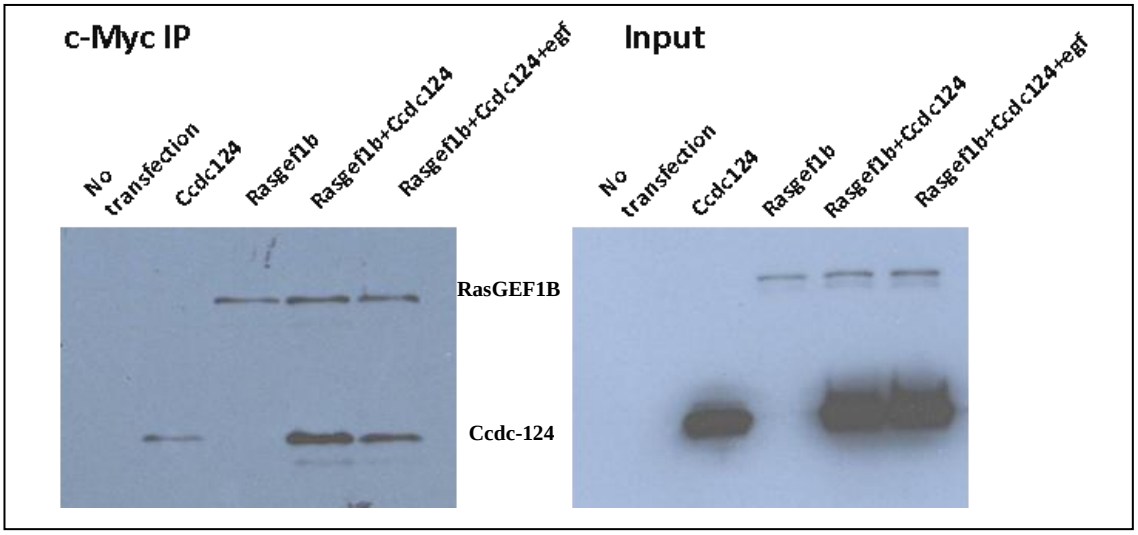


Figure 3.34: Myc IP of HeLa cells with FLAG-HRP antibody. HeLa cells were co-transfected with P3XFLAG-RasGEF1B and p3XFLAG-Ccdc-124, immunoprecipitated with anti-MYC and blotted with anti-FLAG.

3.3.6 Studies towards Crystalization of Ccdc-124

Protein crystallization occurs when the concentration of protein in solution is greater than its limit of solubility and so the protein is in a super-saturated state. In order to crystallize a protein, the purified protein undergoes slow precipitation from an aqueous solution. As a result, individual protein molecules align themselves in a repeating series of "unit cells" by adopting a consistent orientation. The importance of protein crystallization is that it serves as the basis for X-ray crystallography, wherein a crystallized protein is used to determine the protein's three-dimensional structure via X-ray diffraction. Protein crystallization is inherently difficult because of the fragile nature of protein crystals. Proteins have irregularly shaped surfaces, which results in the formation of large channels within any protein crystal. Therefore, the non-covalent bonds that hold together the lattice must often be formed through several layers of solvent molecules. In addition to overcoming the inherent fragility of protein crystals, the successful production of x-ray worthy crystals is dependent upon a number of environmental factors because so much variation exists among proteins, with each individual requiring unique conditions for successful

crystallization. Therefore, attempting to crystallize a protein without a proven protocol can be very tedious. Some factors that require consideration are protein purity, pH, concentration of protein, temperature, and precipitants. In order for sufficient homogeneity, the protein should usually be at least 97% pure. pH conditions are also very important, as different pH's can result in different packing orientations. Buffers, such as Tris-HCl, are often necessary for the maintenance of a particular pH. Precipitants, such as ammonium sulfate or polyethylene glycol, are compounds that cause the protein to precipitate out of solution.

Two of the most commonly used methods for protein crystallization fall under the category of vapor diffusion. These are known as the *hanging drop* and *sitting drop* methods (Fig.3.35). Both entail a droplet containing purified protein, buffer, and precipitant being allowed to equilibrate with a larger reservoir containing similar buffers and precipitants in higher concentrations. Initially, the droplet of protein solution contains an insufficient concentration of precipitant for crystallization, but as water vaporizes from the drop and transfers to the reservoir, the precipitant concentration increases to a level optimal for crystallization. Since the system is in equilibrium, these optimum conditions are maintained until the crystallization is complete.

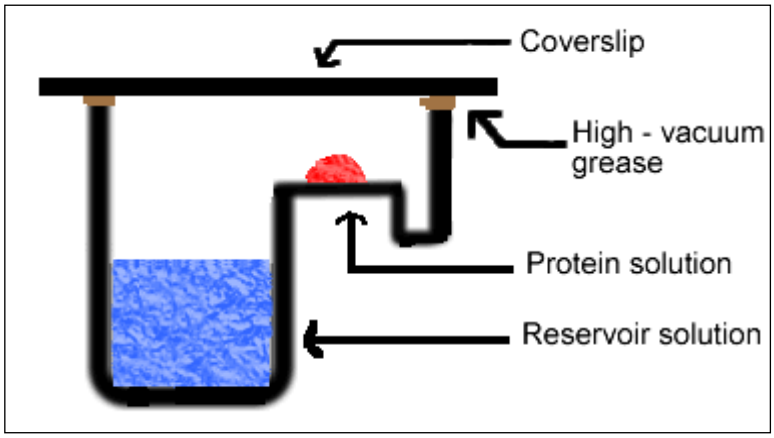


Figure 3.35: Diagram of sitting drop method. In this method, the protein drop sits on a pedestal above the reservoir solution, as opposed to hanging. This is our method of choise for obtaining crystals of Ccdc-124.

We used sitting drop method for the crystallization of Ccdc-124 protein. 5 different, 96 well plate reservoir solutions (Qiagen) (see M.M.) were used at 4°C and 16°C. With these solutions, three different protein concentrations can be subjected to crystallization at the same time. We used 10mg/ml, 20mg/ml, 30mg/ml, 40mg/ml, 50mg/ml and 60mg/ml of protein concentrations. Reservoirs were screened for 30 days. We use 5 different 96 well plate, which means 480 different solutions and each well combined with 6 different Ccdc-124 concentrations, which means 2880 samples and we used 2 different temperatures, which means 5760 protein crystallization conditions (Fig.3.36).

Unfortunately we couldn't get any crystals from these experiments. It is most probably due to the extremely soluble structure of the protein. In order to crystallize, protein should precipitate first, but since Ccdc-124 protein had a high solubility we couldn't get enough precipitations even at the high concentrations such as 60mg/ml.

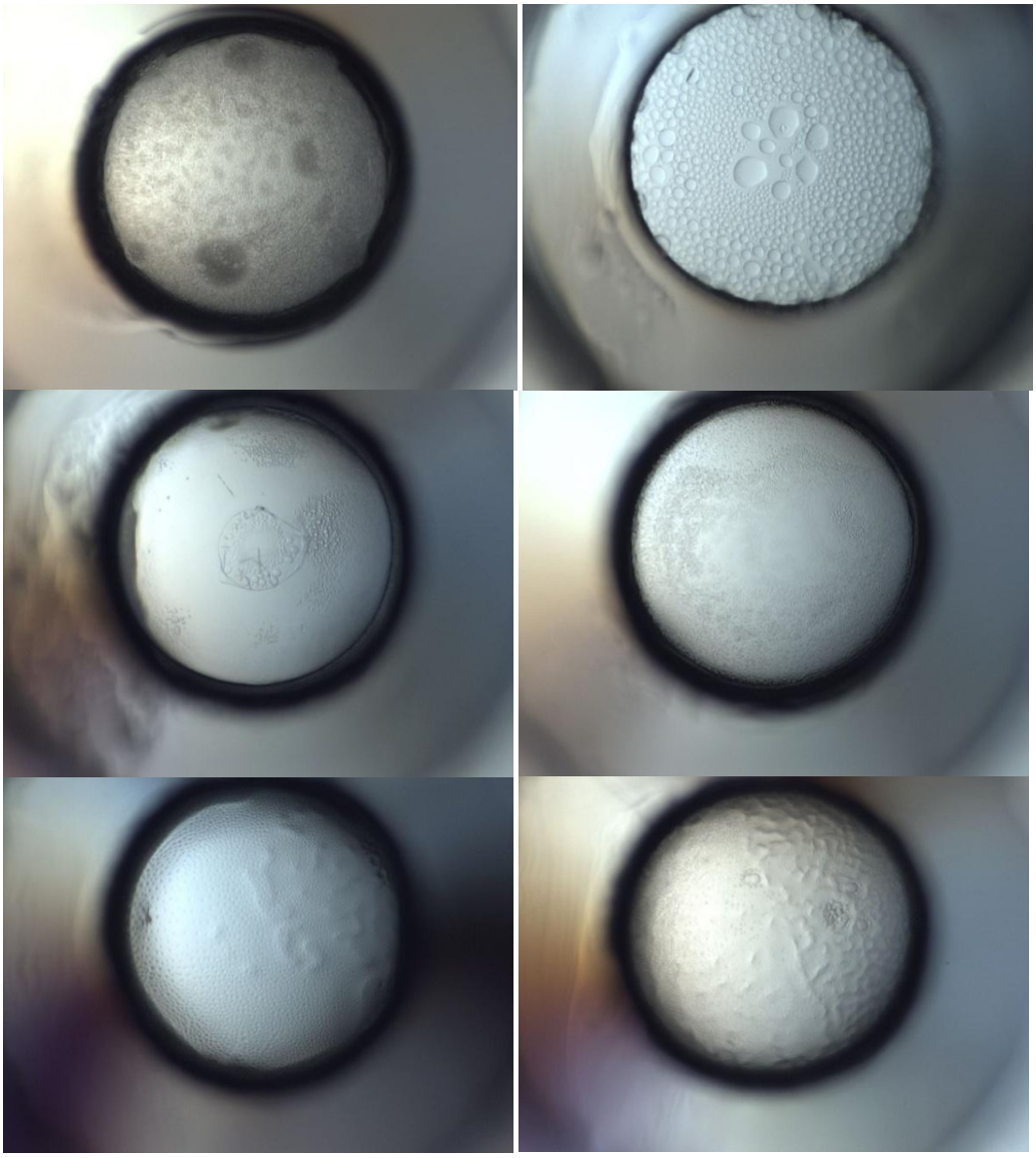


Figure 3.36: Some examples from crystallization results of Ccdc-124 out of 5760 results from different temperature and solution combinations.

3.4 Establishment of Specificities of RasGEF1A and RasGEF1B

N-Methyl-3‘-O-anthranoyl (MANT) guanine nucleotide analogs are environmentally sensitive fluorescent probes, very useful to study proteins which bind guanine nucleotides. The ribose modification interferes minimally with the nucleotide’s ability to serve as an enzyme substrate (van den Berghe *et al.* 1997). MANT-

nucleotides have been used to monitor a variety of nucleotide-dependent enzyme functions. It has been shown that on binding to Ras the fluorescence emission of mant-GDP increases approximately two-fold when it binds to the protein. This fluorescence analogue is used to study the interaction of small G proteins with their guanine exchange factors. The slow time-dependent fluorescence change at 450 nm, when mant-GDP labeled G Protein is incubated in the presence of 100-fold excess of unlabeled GDP, representing the intrinsic GDP dissociation rate (Remmers *et al.* 1994).

Nucleotide exchange activities of two RasGEF1 proteins were assessed for using the fluorescence assay. G-proteins were loaded with fluorescent mant-nucleotide and incubated with RasGEF1A and RasGEF1B in the presence of a large excess of unlabeled nucleotide. As it has previously been shown that the stimulation of nucleotide dissociation by GEFs is independent of the nature of the nucleotide (Esters *et al.* 2001; Klebe *et al.* 1995; Lenzen *et al.* 1995), for reasons of stability and convenience mGppNHp was used in the following assays, which can be incorporated in the presence of alkaline phosphatase. The release of fluorescent nucleotide was followed as a decrease in fluorescence in real time and was fitted to a single exponential. To identify the specificity of RasGEF proteins, various G-proteins from the Ras subfamily, such as H-Ras, R-Ras, M-Ras, TC21, Di-Ras2, Rheb, RERG, RalB, Rap1A, Rap1B, and Rap2A were used (Colicelli 2004; Karnoub and Weinberg 2008; Takai *et al.* 2001). In order to asses the role of Ccdc-124 on the GEF activity of RasGEF1B, Ccdc-124 protein was included to the GEF assays performed with RasGEF1B.

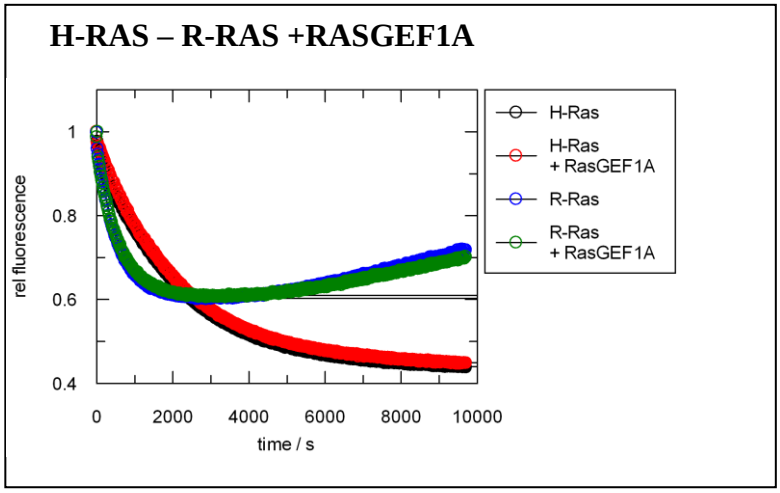


Figure 3.37: Guanine Nucleotide Exchange Assay of RasGEF1A on H-Ras and R-Ras. Black circles show the intrinsic fluorescence dissociation rate of H-Ras (150nM) by itself; Red circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of H-Ras (150nM); Blue circles show the intrinsic fluorescence dissociation rate of R-Ras (150nM) by itself and the Green circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of R-Ras(150nM).

G proteins have an intrinsic nucleotide dissociation rate and these rates are different for each G protein. Guanine exchnge factors increase the nucleotide dissociation rates of their G protein substrates. In figure 3.37, the intrinsic nucleotide dissociation rates of H-Ras and R-Ras is 1.77 x 10^{-4}.s^{-1} and 1.08 x 10^{-3}.s^{-1} respectively. The dissociation rates of H-Ras and R-Ras with RasGEF1A is 1.82 x 10^{-4}.s^{-1} and 1.14 x 10^{-3}.s^{-1} respectively (Table 3.2). These rates clearly shows that RasGEF1A did not further stimulate the dissociation rates of these G proteins.

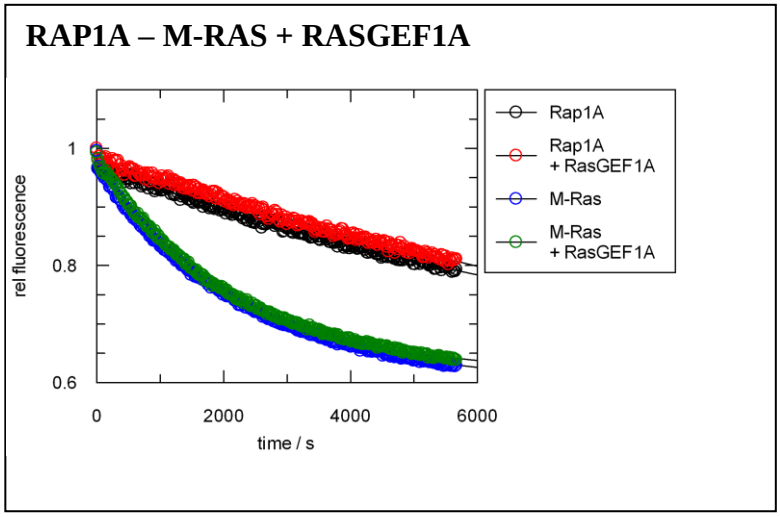


Figure 3.38: Guanine Nucleotide Exchange Assay of RasGEF1A on Rap1A and M-Ras. Black circles show the intrinsic fluorescence dissociation rate of Rap1A (150nM) by itself; Red circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of Rap1A (150nM); Blue circles show the intrinsic fluorescence dissociation rate of M-Ras (150nM) by itself and the Green circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of M-Ras (150nM).

The intrinsic nucleotide dissociation rates of Rap1A and M-Ras is 1.53 x 10^{-4}.s^{-1} and 3.74 x 10^{-4}.s^{-1} respectively. The dissociation rates of Rap1A and M-Ras with RasGEF1A is 1.81 x 10^{-4}.s^{-1} and 3.98 x 10^{-4}.s^{-1} respectively (Table 3.1 and 3.2). These rates clearly shows that RasGEF1A did not further stimulate the dissociation rates of these G proteins (Fig 3.38).

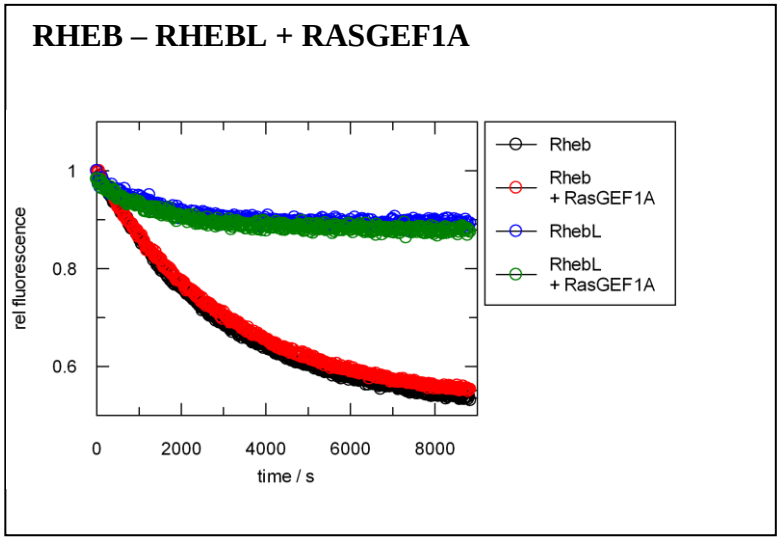


Figure 3.39: Guanine Nucleotide Exchange Assay of RasGEF1A on Rheb and RhebL. Black circles show the intrinsic fluorescence dissociation rate of Rheb (150nM) by itself; Red circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of Rheb (150nM); Blue circles show the intrinsic fluorescence dissociation rate of RhebL (150nM) by itself and the Green circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of RhebL. (150nM)

The intrinsic nucleotide dissociation rates of Rheb and RhebL is 3.59 x 10^{-4}.s^{-1} and 6.61 x 10^{-4}.s^{-1} respectively. The dissociation rates of Rheb and RhebL with RasGEF1A is 3.87 x 10^{-4}.s^{-1} and 6.69 x 10^{-4}.s^{-1} respectively (Table 3.2). These rates clearly shows that RasGEF1A did not further stimulate the dissociation rates of these G proteins (Fig 3.39).

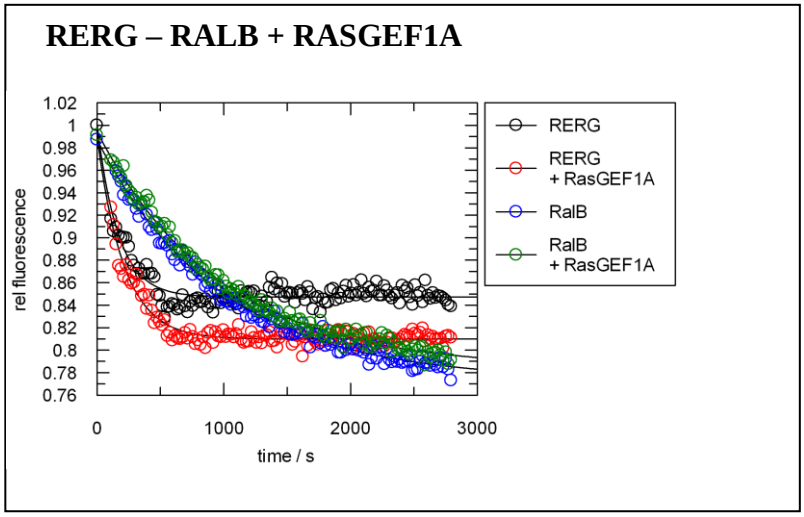


Figure 3.40: Guanine Nucleotide Exchange Assay of RasGEF1A on Rerg and RalB. Black circles show the intrinsic fluorescence dissociation rate of Rerg (150nM) by itself; Red circles show the effect of RasGEF1A (4μM) on the fluorescence dissociation rate of Rerg (150nM); Blue circles show the intrinsic fluorescence dissociation rate of RalB (150nM) by itself and the Green circles show the effect of RasGEF1A (4μM) on the fluorescence dissociation rate of RalB (150nM).

The intrinsic nucleotide dissociation rates of RERG and RalB is 5.49 x 10^{-3}.s^{-1} and 9.76 x 10^{-4}.s^{-1} respectively. The dissociation rates of RERG and RalB with RasGEF1A is 6.05 x 10^{-3}.s^{-1} and 9.53 x 10^{-4}.s^{-1} respectively (Table 3.2). These rates clearly shows that RasGEF1A did not further stimulate the dissociation rates of these G proteins (Fig 3.40).

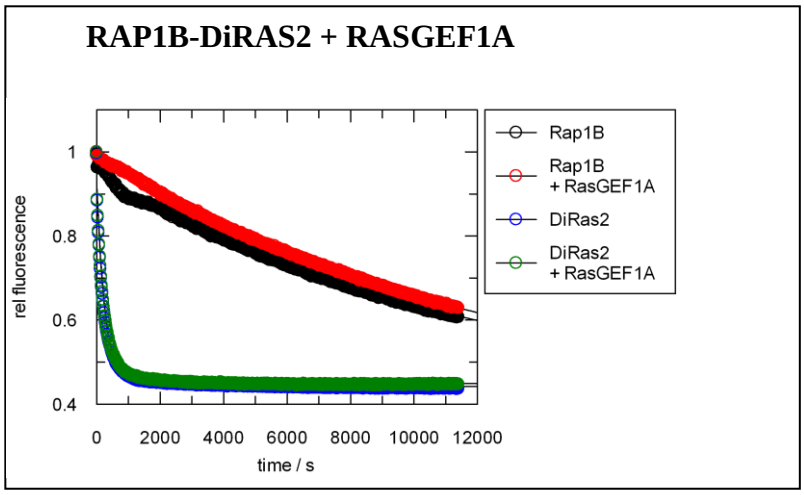


Figure 3.41: Guanine Nucleotide Exchange Assay of RasGEF1A on Rap1B and DiRas2. Black circles show the intrinsic fluorescence dissociation rate of Rap1B (150nM) by itself; Red circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of Rap1B (150nM); Blue circles show the intrinsic fluorescence dissociation rate of DiRas2 (150nM) by itself and the Green circles show the effect of RasGEF1A (4µM) on the fluorescence dissociation rate of DiRas2 (150nM).

The intrinsic nucleotide dissociation rates of Rap1B and DiRas2 is 5.64 x 10^{-5}.s^{-1} and 1.92 x 10^{-3}.s^{-1} respectively. The dissociation rates of Rap1B and DiRas2 with RasGEF1A is 4.69 x 10^{-5}.s^{-1} and 1.99 x 10^{-3}.s^{-1} respectively (Table 3.1 and 3.2). These rates clearly shows that RasGEF1A did not further stimulate the dissociation rates of these G proteins (Fig 3.41).

The rate of nucleotide release from Rap2A in the presence of 4 µM RasGEF1A was too fast to detect, for this reason different concentrations of RasGEF1A were tested on Rap2A (Fig.3.42) and according to these results 200 nm of RasGEF1A was chosen to use in the experiments.

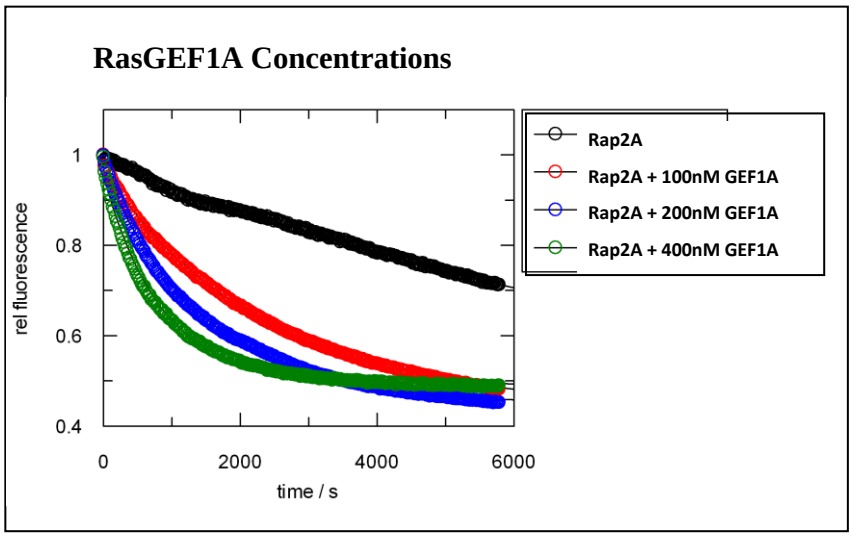


Figure 3.42: RasGEF1A concentrations on Rap2A. Because of the effect of 4µM RasGEF1A was too fast to detect its effect on Rap2A, different concentrations of RasGEF1A were tested on Rasp2A in order to asses the best concentration for RasGEF1A. Black circles show the intrinsic fluorescence dissociation rate of Rap2A (150nM) by itself; Red circles show the effect of 100nM RasGEF1A, blue circles show the effect of 200nM RasGEF1A and green circles show the effect of 400nM RasGEF1A on the fluorescence dissociation rate of Rap2A (150nM). The Concentration of 200nM was chosen.

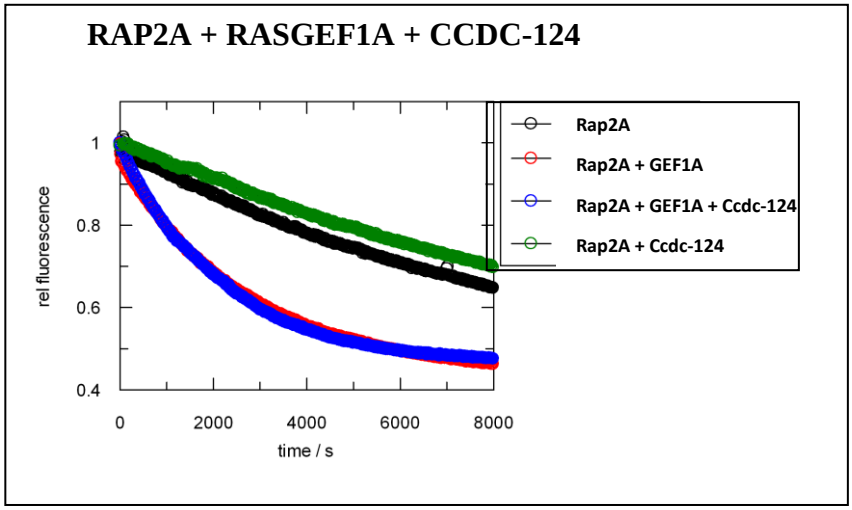


Figure 3.43: Guanine Nucleotide Exchange Assay of RasGEF1A with Ccdc-124 on Rap2A. Black circles show the intrinsic fluorescence dissociation rate of Rap2A (150nM) by itself; the Green circles show the effect of Ccdc-124 (40µM) (10 times the concentration of GEF) on the fluorescence dissociation rate of Rap2A (150nM); Red circles show the effect of RasGEF1A (200nM) on the fluorescence dissociation rate of Rap2A (150nM); Blue circles show the effect of RasGEF1A (200nM) together with Ccdc-124 (40µM) on the fluorescence dissociation rate of Rap2A (150nM).

Figure 3.43 shows the GEF activity of RasGEF1A on Rap2A and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of Rap2A is $2.02 \times 10^{-5}.s^{-1}$ and this rate was not significantly changed by addition of Ccdc-124 which is $3.78 \times 10^{-5}.s^{-1}$ The dissociation rate of Rap2A with RasGEF1A is $1.82 \times 10^{-4}.s^{-1}$ and once more the rate was not significantly changed with Ccdc-124 which is $2.12 \times 10^{-4}.s^{-1}$ (Table 3.1). These results indicate that RasGEF1A stimulate the nucleotide dissociation of Rap2A but Ccdc-124 does not further stimulate or inhibit this effect.

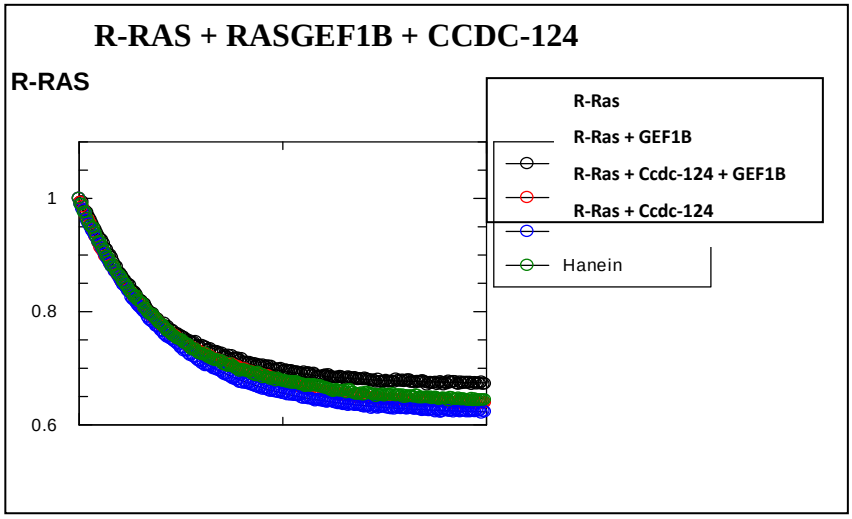


Figure 3.44: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on R-Ras. Black circles show the intrinsic fluorescence dissociation rate of R-Ras (150nM) by itself; the Green circles show the effect of Ccdc-124 (40µM) on the fluorescence dissociation rate of R-Ras (150nM); Red circles show the effect of RasGEF1B (4µM) on the fluorescence dissociation rate of R-Ras (200nM); Blue circles show the effect of RasGEF1B (4µM) together with Ccdc-124 (40µM) on the fluorescence dissociation rate of R-Ras (150nM).

Figure 3.44 shows the GEF activity of RasGEF1B on R-Ras and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of R-Ras is $1.08 \times 10^{-3}.s^{-1}$ and this rate was not significantly changed by addition of Ccdc-124 which is $9.50 \times 10^{-4}.s^{-1}$ The dissociation rate of R-Ras with RasGEF1B is $9.03 \times 10^{-4}.s^{-1}$ and once more the rate was not significantly changed with Ccdc-124 which is $9.37 \times 10^{-4}.s^{-1}$ (Table 3.2). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of R-Ras and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of R-Ras.

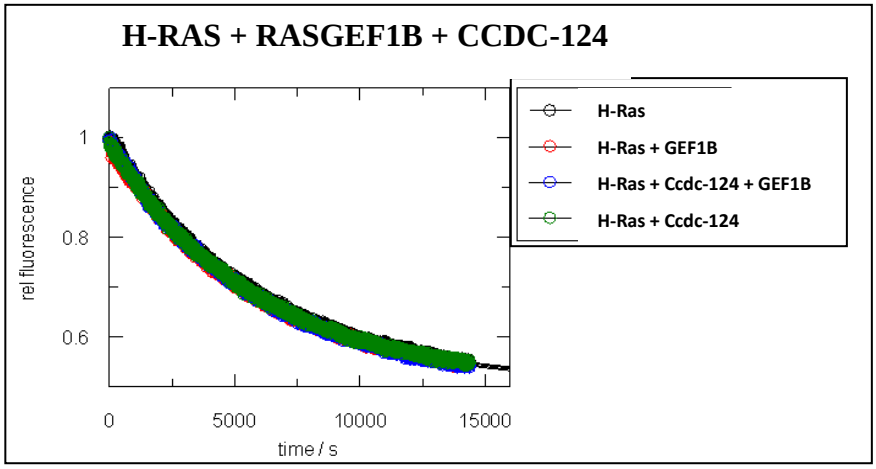


Figure 3.45: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on H-Ras. Black circles show the intrinsic fluorescence dissociation rate of H-Ras (150nM) by itself; the Green circles show the effect of Ccdc-124 (40µM) on the fluorescence dissociation rate of H-Ras (150nM); Red circles show the effect of RasGEF1B (4µM) on the fluorescence dissociation rate of R-Ras (150nM); Blue circles show the effect of RasGEF1B (4µM) together with Ccdc-124 (40µM) on the fluorescence dissociation rate of H-Ras (150nM).

Figure 3.45 shows the GEF activity of RasGEF1B on H-Ras and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of H-Ras is $1.77 \times 10^{-4}.s^{-1}$ and this rate was not significantly changed by addition of Ccdc-124 which is $1.73 \times 10^{-4}.s^{-1}$ The dissociation rate of H-Ras with RasGEF1B is $1.74 \times 10^{-4}.s^{-1}$ and once more the rate was not significantly changed with Ccdc-124 which is $1.73 \times 10^{-4}.s^{-1}$ (Table 3.2). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of H-Ras and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of H-Ras.

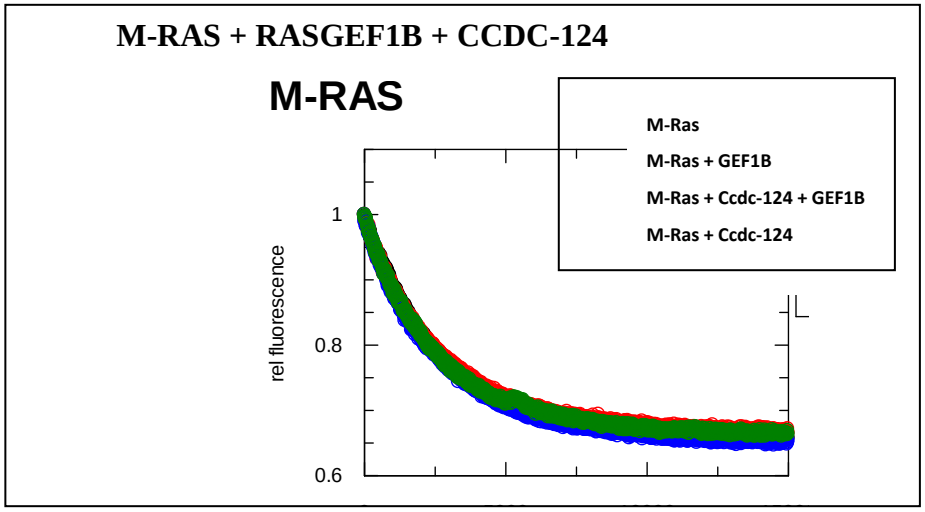


Figure 3.46: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on M-Ras. Black circles show the intrinsic fluorescence dissociation rate of M-Ras (150nM) by itself; the Green circles show the effect of Ccdc-124 (40μM) on the fluorescence dissociation rate of M-Ras (150nM); Red circles show the effect of RasGEF1B (4μM) on the fluorescence dissociation rate of M-Ras (150nM); Blue circles show the effect of RasGEF1B (4μM) together with Ccdc-124 (40μM) on the fluorescence dissociation rate of M-Ras (150nM).

Figure 3.46 shows the GEF activity of RasGEF1B on M-Ras and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of M-Ras is $3.74 \times 10^{-4}.s^{-1}$ and this rate was not significantly changed by addition of Ccdc-124 which is $3.83 \times 10^{-4}.s^{-1}$ The dissociation rate of M-Ras with RasGEF1B is $3.70 \times 10^{-4}.s^{-1}$ and once more the rate was not significantly changed with Ccdc-124 which is $3.66 \times 10^{-4}.s^{-1}$ (Table 3.2). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of M-Ras and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of M-Ras.

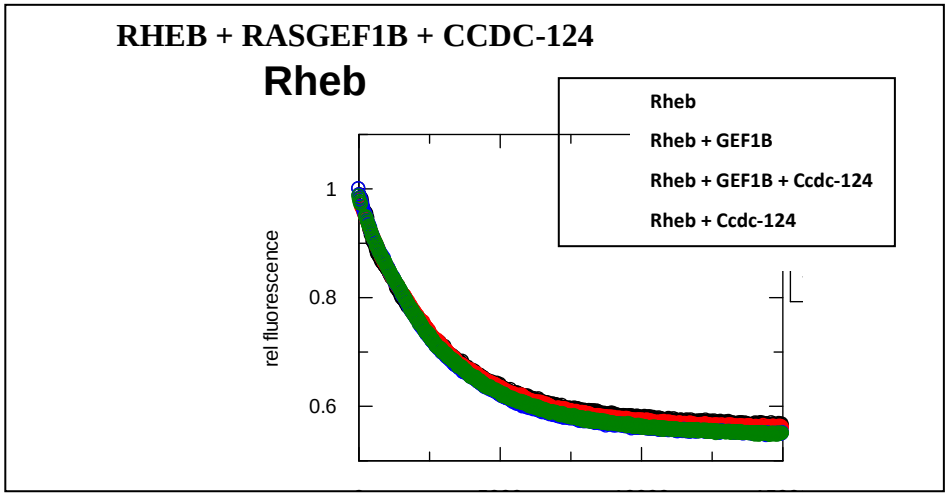


Figure 3.47: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on Rheb. Black circles show the intrinsic fluorescence dissociation rate of Rheb (150nM) by itself; the Green circles show the effect of Ccdc-124 (40µM) on the fluorescence dissociation rate of Rheb (150nM); Red circles show the effect of RasGEF1B (4µM) on the fluorescence dissociation rate of Rheb (150nM); Blue circles show the effect of RasGEF1B (4µM) together with Ccdc-124 (40µM) on the fluorescence dissociation rate of Rheb (150nM).

Figure 3.47 shows the GEF activity of RasGEF1B on Rheb and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of Rheb is $3.59 \times 10^{-4}.s^{-1}$ and this rate was not significantly changed by addition of Ccdc-124 which is $3.46 \times 10^{-4}.s^{-1}$ The dissociation rate of Rheb with RasGEF1B is $3.46 \times 10^{-4}.s^{-1}$ and once more the rate was not significantly changed with Ccdc-124 which is $3.58 \times 10^{-4}.s^{-1}$ (Table 3.2). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of Rheb and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of Rheb.

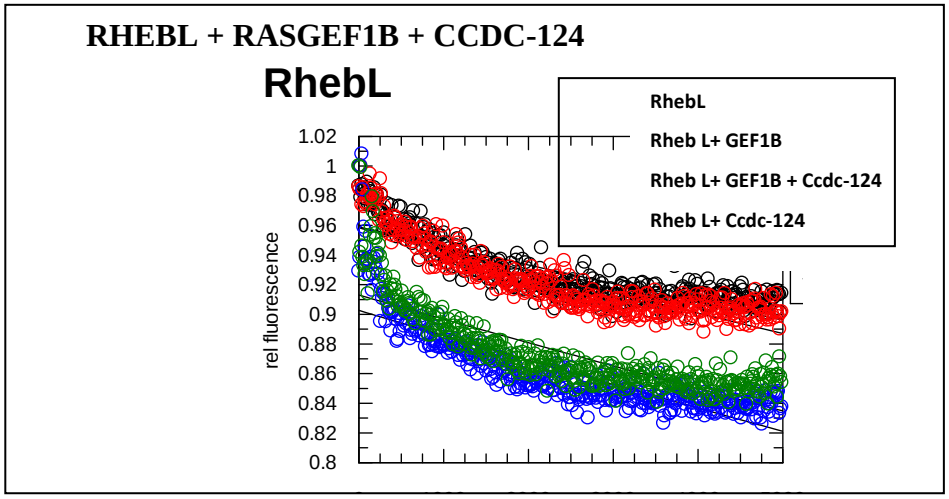


Figure 3.48: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on RhebL. Black circles show the intrinsic fluorescence dissociation rate of RhebL (150 nM) by itself; the Green circles show the effect of Ccdc-124 (40μM) on the fluorescence dissociation rate of Rheb (150 nM); Red circles show the effect of RasGEF1B (4μM) on the fluorescence dissociation rate of RhebL (150 nM); Blue circles show the effect of RasGEF1B (4μM) together with Ccdc-124 (40μM) on the fluorescence dissociation rate of RhebL (150 nM).

Figure 3.48 shows the GEF activity of RasGEF1B on RhebL and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of RhebL is $1.44 \times 10^{-5}.s^{-1}$ and this rate was not significantly changed by addition of Ccdc-124 which is $1.90 \times 10^{-5}.s^{-1}$ The dissociation rate of RhebL with RasGEF1B is $1.70 \times 10^{-5}.s^{-1}$ and once more the rate was not significantly changed with Ccdc-124 which is $2.00 \times 10^{-5}.s^{-1}$. These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of RhebL and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of RhebL.

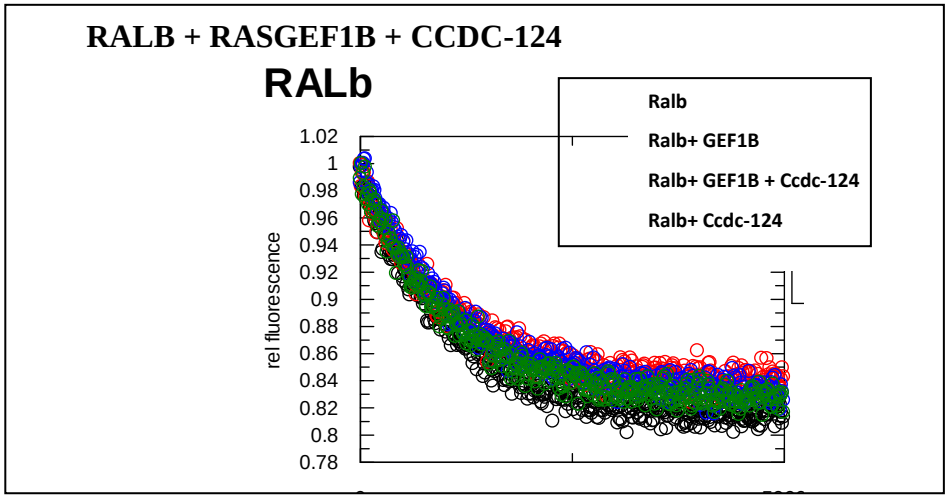


Figure 3.49: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on RalB. Black circles show the intrinsic fluorescence dissociation rate of RalB (150nM) by itself; the Green circles show the effect of Ccdc-124 (40µM) on the fluorescence dissociation rate of RalB (150nM); Red circles show the effect of RasGEF1B (4µM) on the fluorescence dissociation rate of RalB (150nM); Blue circles show the effect of RasGEF1B (4µM) together with Ccdc-124 (40µM) on the fluorescence dissociation rate of RalB (150nM).

Figure 3.49 shows the GEF activity of RasGEF1B on RalB and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of RalB is 9.76 x 10^{-4}.s^{-1} and this rate was not significantly changed by addition of Ccdc-124 which is 9.66 x 10^{-4}.s^{-1} The dissociation rate of RalB with RasGEF1B is 9.46 x 10^{-4}.s^{-1} and once more the rate was not significantly changed with Ccdc-124 which is 9.32 x 10^{-4}.s^{-1} (Table 3.2). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of RalB and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of RalB.

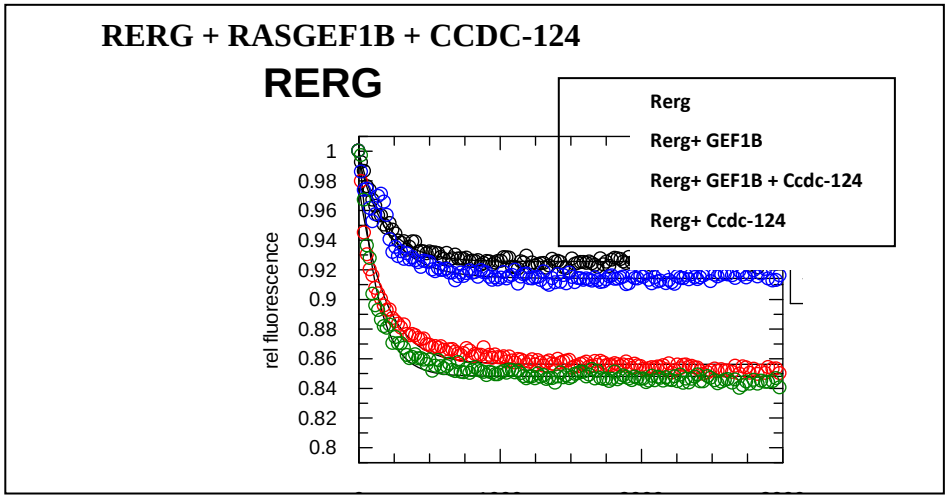


Figure 3.50: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on Rerg. Black circles show the intrinsic fluorescence dissociation rate of Rerg (150nM) by itself; the Green circles show the effect of Ccdc-124 (40μM) on the fluorescence dissociation rate of Rerg (150nM); Red circles show the effect of RasGEF1B (4μM) on the fluorescence dissociation rate of Rerg (150nM); Blue circles show the effect of RasGEF1B (4μM) together with Ccdc-124 (40μM) on the fluorescence dissociation rate of Rerg (150nM).

Figure 3.50 shows the GEF activity of RasGEF1B on Rerg and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of Rerg is 5.49 x 10^{-3}.s^{-1} and this rate was not significantly changed by addition of Ccdc-124 which is 7.69 x 10^{-3}.s^{-1} The dissociation rate of Rerg with RasGEF1B is 5.37 x 10^{-3}.s^{-1} and once more the rate was not significantly changed with Ccdc-124 which is 4.55 x 10^{-3}.s^{-1} (Table 3.2). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of Rerg and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of Rerg.

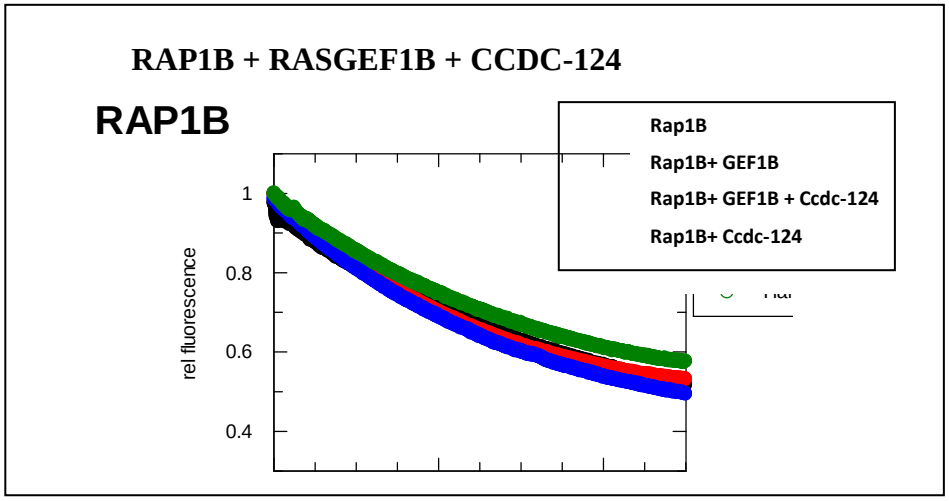


Figure 3.51: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on Rap1B. Black circles show the intrinsic fluorescence dissociation rate of Rap1B (150nM) by itself; the Green circles show the effect of Ccdc-124 (40μM) on the fluorescence dissociation rate of Rap1B (150nM); Red circles show the effect of RasGEF1B (4μM) on the fluorescence dissociation rate of Rap1B (150nM); Blue circles show the effect of RasGEF1B (4μM) together with Ccdc-124 (40μM) on the fluorescence dissociation rate of Rap1B (150nM).

Figure 3.51 shows the GEF activity of RasGEF1B on Rap1B and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of Rap1B is 5.64 x 10^{-5}.s^{-1} and this rate was not significantly changed by addition of Ccdc-124 which is 5.90 x 10^{-5}.s^{-1} The dissociation rate of Rap1B with RasGEF1B is 5.66 x 10^{-5}.s^{-1} and once more the rate was not significantly changed with Ccdc-124 which is 5.32 x 10^{-5}.s^{-1} (Table 3.1). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of Rap1B and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of Rap1B.

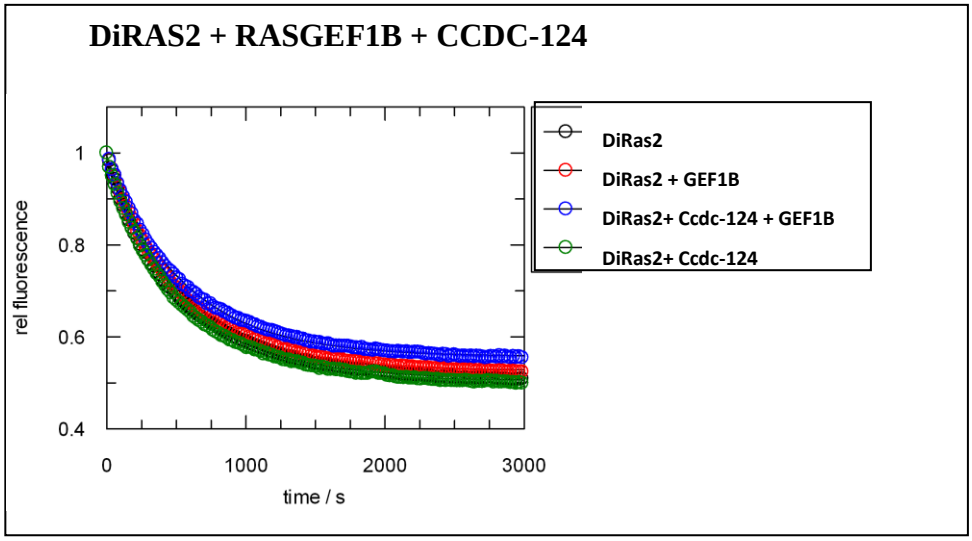


Figure 3.53: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on DiRas2. Black circles show the intrinsic fluorescence dissociation rate of DiRas2 (150nM) by itself; the Green circles show the effect of Ccdc-124 (40µM) on the fluorescence dissociation rate of DiRas2 (150nM); Red circles show the effect of RasGEF1B (4µM) on the fluorescence dissociation rate of DiRas2 (150nM); Blue circles show the effect of RasGEF1B (4µM) together with Ccdc-124 (40µM) on the fluorescence dissociation rate of DiRas2 (150nM).

Figure 3.45 shows the GEF activity of RasGEF1B on DiRas2 and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of DiRas2 is 1.92 x 10^{-3}.s^{-1} and this rate was not significantly changed by addition of Ccdc-124 which is 1.97 x 10^{-3}.s^{-1} The dissociation rate of DiRas2 with RasGEF1B is 1.86 x 10^{-3}.s^{-1} and once more the rate was not significantly changed with Ccdc-124 which is 1.77 x 10^{-3}.s^{-1} (Table 3.2). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of DiRas2 and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of DiRas2.

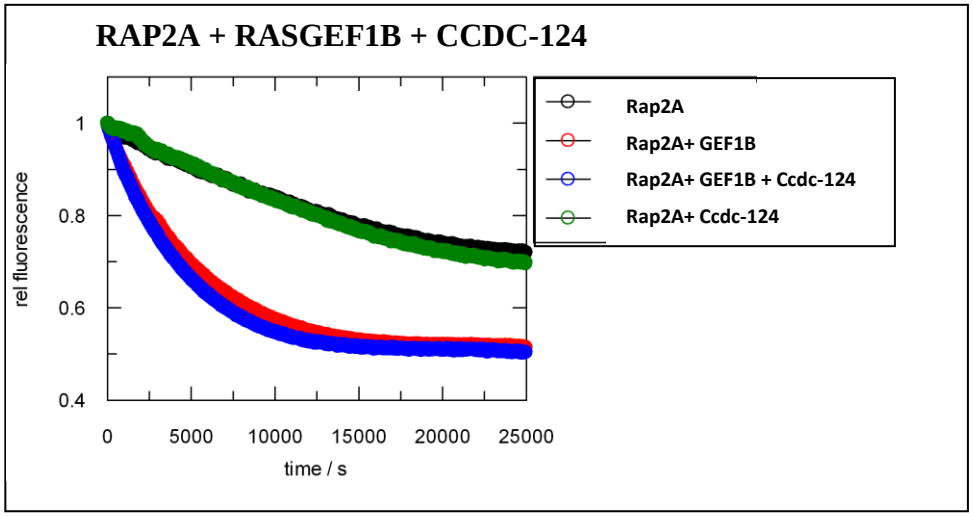


Figure 3.54: Guanine Nucleotide Exchange Assay of RasGEF1B with Ccdc-124 on Rap2A. Black circles show the intrinsic fluorescence dissociation rate of Rap2A (150nM) by itself; the Green circles show the effect of Ccdc-124 (40μM) on the fluorescence dissociation rate of Rap2A (150nM); Red circles show the effect of RasGEF1B (4μM) on the fluorescence dissociation rate of Rap2A (150nM); Blue circles show the effect of RasGEF1B (4μM) together with Ccdc-124 (40μM) on the fluorescence dissociation rate of Rap2A (150nM).

Figure 3.54 shows the GEF activity of RasGEF1B on Rap2A and the effect of Ccdc-124 protein on this stimulation. The intrinsic nucleotide dissociation rate of Rap2A is 2.02 x 10^{-5}.s^{-1} and this rate was not significantly changed by addition of Ccdc-124 which is 1.17 x 10^{-5}.s^{-1} The dissociation rate of Rap2A with RasGEF1B is 1.79 x 10^{-4}.s^{-1} and once more the rate was not significantly changed with Ccdc-124 which is 1.83 x 10^{-4}.s^{-1} (Table 3.1). These results clearly shows that RasGEF1B does not stimulate the nucleotide dissociation of Rap2A and Ccdc-124 does not have a further effect on the nucleotide dissociation rates of Rap2A.

As expected, the intrinsic dissociation rates of the G-proteins varied remarkably, with Di-Ras2 and RERG having rates as high as 1.92 x 10^{-3}.s^{-1} and 5.49 x 10^{-3}.s^{-1}, respectively. Regardless of the protein concentrations used in the reactions, RasGEF1A or RasGEF1B did not significantly stimulate nucleotide exchange on most tested Ras family members, such as H-Ras, R-Ras, M-Ras, TC21, Di-Ras2, Rheb, RERG, or RalB (Fig.3.37 to 3.56). However, both RasGEF1A and RasGEF1B

stimulated exchange on Rap2A (Fig.3.42, Fig.3.43 and Fig.3.54), whereas they had no GEF activities on the other members of the Rap subfamily, Rap1A or Rap1B, demonstrating that the RasGEF1 family members show a similar specificity. However, the presence of Ccdc-124 protein in the exchange assay did not affect exchange rate of any G-protein neither with RasGEF1A nor with RasGEF1B (Fig3.43 to Fig.3.54).

Although the concentration of RasGEF1A was reduced 20 times, the rate of nucleotide release from Rap2A ($1.82 \times 10^{-4}.s^{-1}$) was only comparable to the rate of release stimulated by RasGEF1B ($1.79 \times 10^{-4}.s^{-1}$), indicating a higher activity of RasGEF1A than of RasGEF1B. Altogether, these results clearly indicated that both RasGEF1A and RasGEF1B belong to a novel class of RapGEFs that are specific for Rap2.

Representative fluorescence traces for the RasGEF1A and RasGEF1B reactions are shown in Table.3.1 and Table 3.2 and the experiments are summarized in Fig.3.55. Figure 3.56 shows the effects of RasGEF1A and RasGEF1B on Rap family of proteins.

	Intrinsic	RasGEF1A (200 nM)	RasGEF1B (4 μM)
Rap1a wt	1.53e-004	1.81e-004	1.34e-004
Rap1b wt	5.64e-005	4.69e-005	5.66e-005
Rap1b S39F	5.29e-005	6.15e-005	1.80e-004
Rap2A wt	2.02e-005	1.82e-004	1.79e-004
Rap2A T27I	1.30e-005	1.03e-004	8.75e-005
Rap2A F39S	2.27e-005	2.71e-005	2.86e-005
Rap2A S66A	2.51e-005	2.59e-004	2.65e-004

Table 3.1: Representative nucleotide exchange rates of Rap1 and Rap2 proteins and their mutant forms with RasGEF1A and RasGEF1B.

	Intrinsic	RasGEF1A	RasGEF1B
H-Ras	1.77e-004	1.82e-004	1.74e-004
R-Ras	1.08e-003	1.14e-003	9.03e-004
M-Ras	3.74e-004	3.98e-004	3.70e-004
TC21	7.17e-004	7.17e-004	6.96e-004
RalB	9.76e-004	9.53e-004	9.G46e-004
Rheb	3.59e-004	3.87e-004	3.46e-004
Di-Ras2	1.92e-003	1.99e-003	1.86e-003
RERG	5.49e-003	6.05e-003	5.37e-003

Table 3.2: Representative nucleotide exchange rates of Ras family of G proteins with RasGEF1A and RasGEF1B.

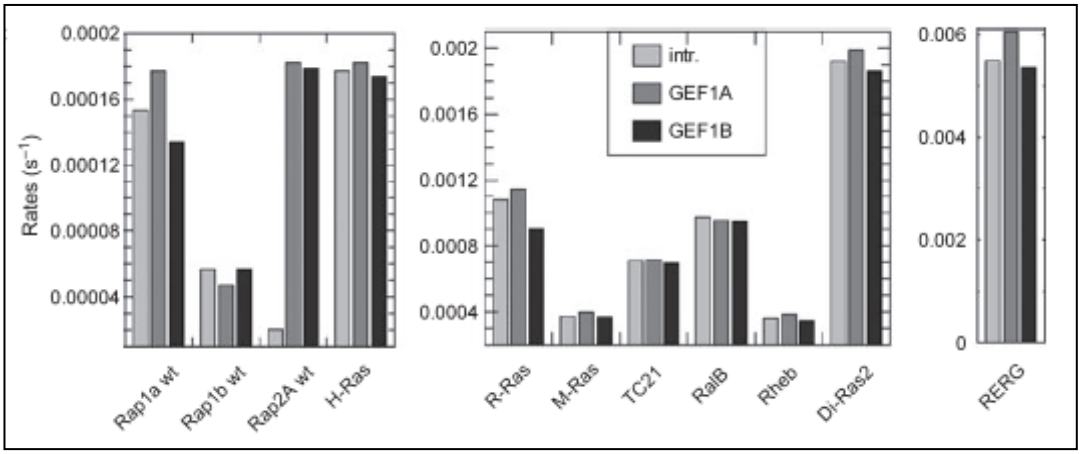


Figure 3.55: Guanine nucleotide exchange rates of Ras family of G-proteins in the presence of RasGEF1A and RasGEF1B. G-proteins indicated in each set of bar graphs were loaded with mant-GNP, and dissociation of the fluorescent nucleotide in the absence (intrinsic rate) or in the presence of 200nM RasGEF1A or 4µM RasGEF1B were followed. Values were obtained from at least three independent experiments, and representative graphs are displayed. GEF1A and GEF1B stand for RasGEF1A and RasGEF1B, respectively.

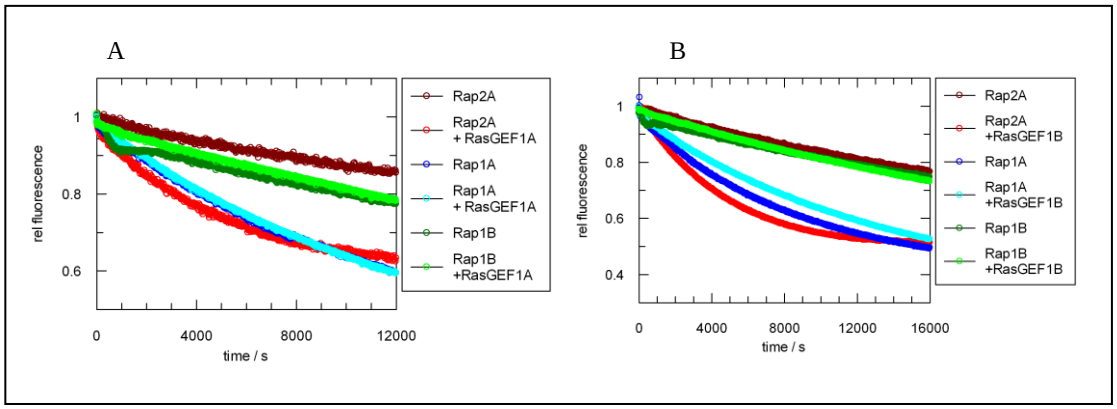


Figure 3.56: Guanine nucleotide exchange assays of Rap family of G-proteins in the presence of RasGEF1A and RasGEF1B. G-proteins were loaded with mant-GNP and guanine nucleotide exchange reaction of 150nM Rap2A, Rap1A and Rap1B with 200nM RasGEF1A (A) and with 4µM RasGEF1B (B). Dark Red circles show the intrinsic fluorescence dissociation rate of Rap2A by itself; Red circles show the effect of RasGEFs on the fluorescence dissociation rate of Rap2A; Dark Blue circles show the intrinsic fluorescence dissociation rate of Rap1A by itself; Light Blue circles show the effect of RasGEFs on the fluorescence dissociation rate of Rap1A; Dark Green circles show the intrinsic fluorescence dissociation rate of Rap1B by itself; Light Green circles show the effect of RasGEFs on the fluorescence dissociation rate of Rap1B;

3.5 Mutational Analysis of RasGEF1/Rap2 Interaction and Determination of Phe 39 as a specification residue.

Rap1A, Rap1B and Rap2A are closely related proteins. Their switch I region (amino acids 25–40) and switch II region (amino acids 57–75), both of which were previously shown to contain critical residues for G-protein–GEF interactions(Boriack-Sjodin *et al.* 1998; Rehmann *et al.* 2008; van den Berghe *et al.* 1999), are nearly identical. In an effort to identify residues that are critical for the specificity of RasGEF1A and RasGEF1B interactions with Rap2, the switch I and switch II regions of Rap proteins were compared and residues that are localized in these conserved functional domains, but differ between the two G-proteins were identified(Fig.3.57). In parallel, by taking advantage of a previously solved structure of Rap1B in complex with Epac2 (Rehmann *et al.* 2008), a structural model was prepared in order to detect residues in Rap2A that are distinct from their counterparts in Rap1A and Rap1B and are expected to interact with RasGEF1s (Fig. 3.58). The predominant part of the interface between Rap2A and RasGEF1B includes conserved regions of the GEF protein, with the exception of two regions that are in close contact with the G-protein but do not show sequence conservation. The first region includes the interface for binding to the switch I region of Rap2A, and the second is located around helix α 2 of Rap, C-terminal of the switch II region (Fig.3.58). The contact area around the switch II region of Rap is conserved between RasGEF1B and Epac2. The only switch I residue that is not conserved between Rap1 and Rap2 is Phe39 (Rap2A), which corresponds to Ser39 inRap1B (Fig.3.57). The side chain of Ser39 is encircled by the switch I region (Fig.3.58) suggesting that Phe39 of Rap2 will cause an entirely different conformation of this loop region. Interestingly, this region is not conserved between RasGEF1B and Epac2. Helix α2 of Rap is in direct contact with an α-helix in Epac2 that is remodeling the switch II region for the GEF reaction. This interaction mostly depends on the main chain of Epac2, and the only side chain interaction is established by Ser66 (Rap2A), which is an Ala (Ala66) in Rap1B (Fig.3.57 and Fig.3.58). Therefore, Phe39 and Ser66 in Rap2 were identified as possible discriminatory candidates for the G-protein–GEF interaction. A third

possible candidate was Thr27 in Rap2A, as this hydrophilic residue was replaced with a hydrophobic Ile in Rap1B, and structural data indicated this residue to be in contact with a conserved region of Epac2 (Fig.3.58).

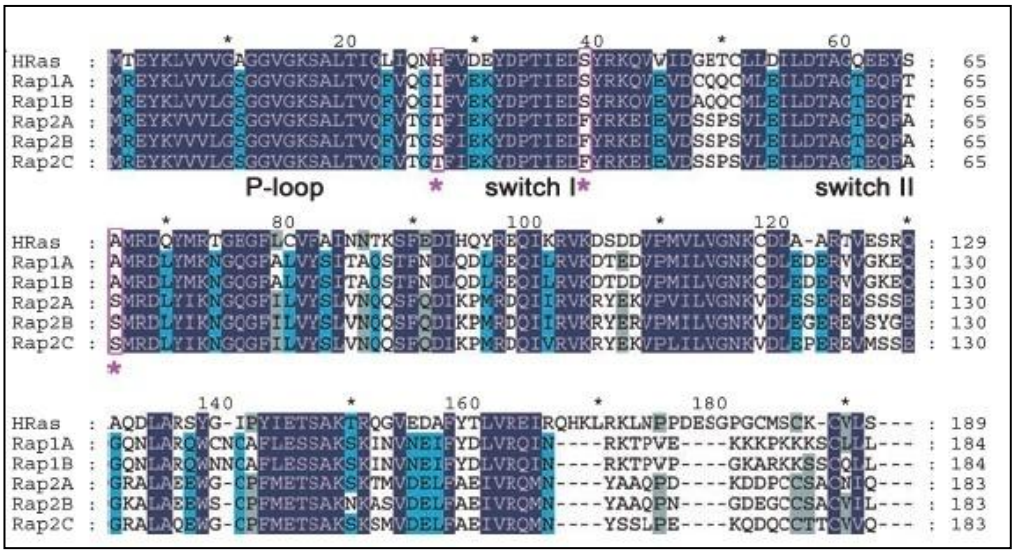


Figure 3.57: Positions of residues selected for mutational analysis. Sequence alignment of H-Ras, Rap1A, Rap1B, Rap2A, Rap2B and Rap2C. Residues that have 100%, 80% and 60% similarity are in dark blue, blue and gray, respectively. Thr27, Phe39, and Ser66, chosen for mutational analysis, are indicated by blue boxes and an asterisk below.

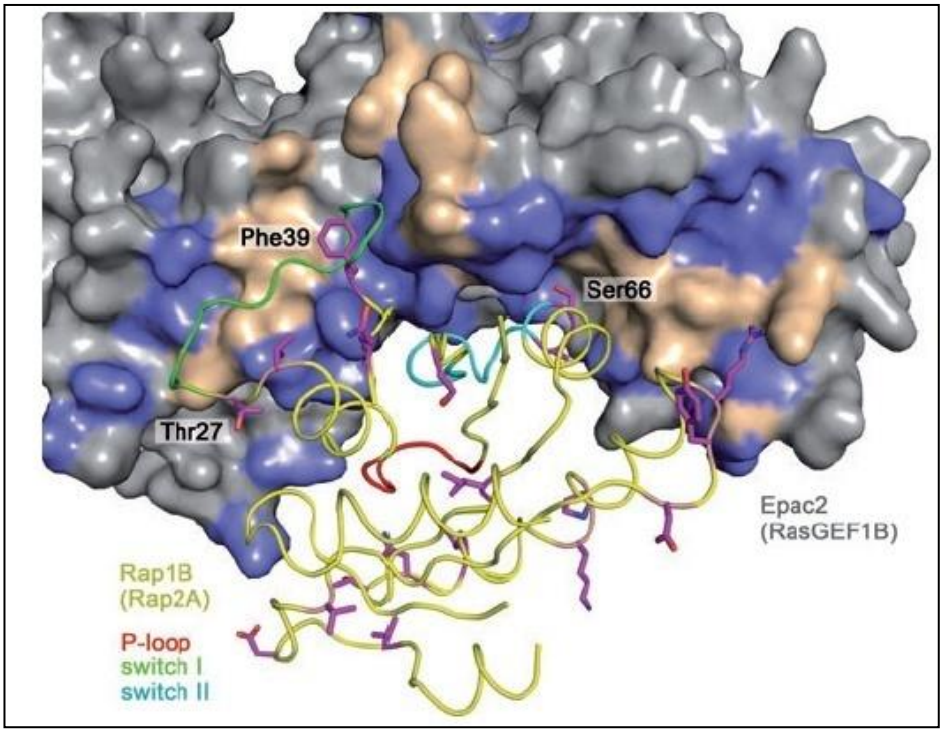


Figure 3.58: Predictive model of a Rap2A–RasGEF1B complex. The sequence of RasGEF1B was overlain with Epac2 from the Rap1B–Epac2–cAMP complex (Protein Data Bank code: 3CF6 (Rehmann *et al.* 2008), using MODELLER software (Marti-Renom *et al.* 2000), remodeling flexible loops and sequence differences caused by gaps in sequence alignment. Residues that were different between Rap1B and Rap2A were mutated in Rap1B–Epac2–cAMP by using COOT software (Emsley and Cowtan 2004). Sequence differences between Rap2A and Rap1B are highlighted in magenta; the secondary structure of Rap1B is in yellow. Switch I (green), switch II (cyan) and P-loop (red) regions are indicated. RasGEF1B is shown as a gray surface with conserved residues (as compared with Epac2) in blue, and interface residues that are not conserved in light brown.

We then carried out a comparative mutational analysis of the interaction between RasGEF1 and Rap proteins by converting Thr27, Phe39 and Ser66 in Rap2 to the residues found at the same positions in Rap1. Rap2 proteins carrying T27I, F39S and S66A mutations were expressed (Fig.3.59).

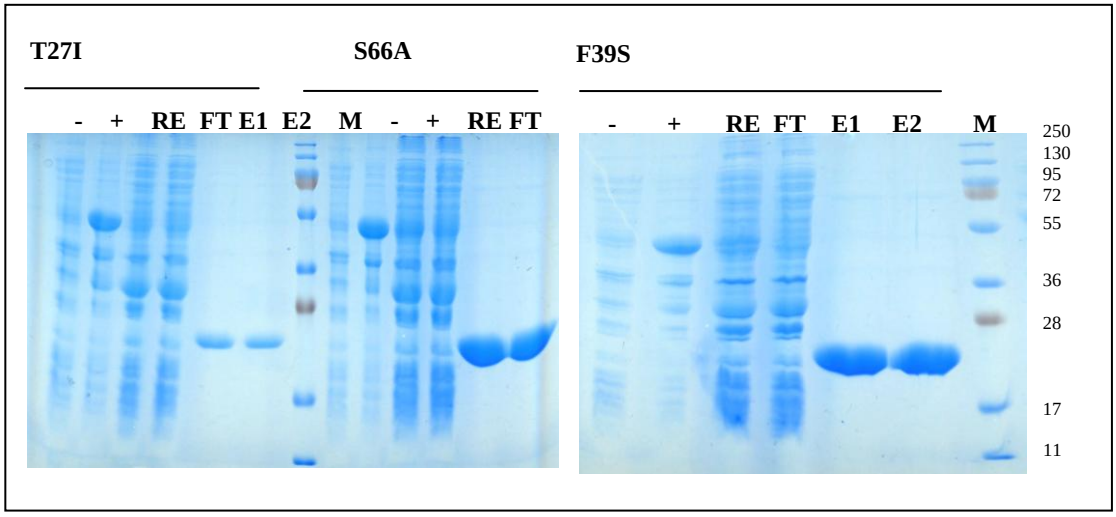


Figure 3.59: Purifications of Rap2A T27I, Rap2A S66A and Rap2A F39S proteins. Three Rap2A mutants were purified with GSH-Beads and GST tags were cleaved with Thrombin on the column and it is named as E1, then the residual proteins left on the column collected with elution buffer and named as E2 . – and +: uninduced and IPTG induced controls, respectively; RE: Raw Extract after lysis but before incubation with beads; FT: Flow Through after incubation with beads; E: Elution, M: Marker.

After GSH purifications of Rap2A mutants, eluates were controlled with HPLC to verify the presence of Rap proteins but not GST, since they have nearly the same size. The logic of distinguishing the Rap proteins from the GST proteins is: Rap proteins are naturally bound to nucleotides like GMP, GDP and GTP but mostly they are found as GDP bound. If the eluates contain Rap proteins instead of GST protein, they should preserve free nucleotides in the solution after proteins are precipitated with heat. For that purpose, 100µM of each protein eluate was prepared in 25µl H_2O and proteins were precipitated by heating the samples at 95 $^{\circ}$C, after the removal of the precipitated proteins by centrifugation, supernatants were subjected to HPLC after a control run with GDP. According to these HPLC results, all eluates had high concentrations of GDP which is the indicator of the presence of Rap proteins (Fig.3.60, Fig3.61 and Fig.3.62).

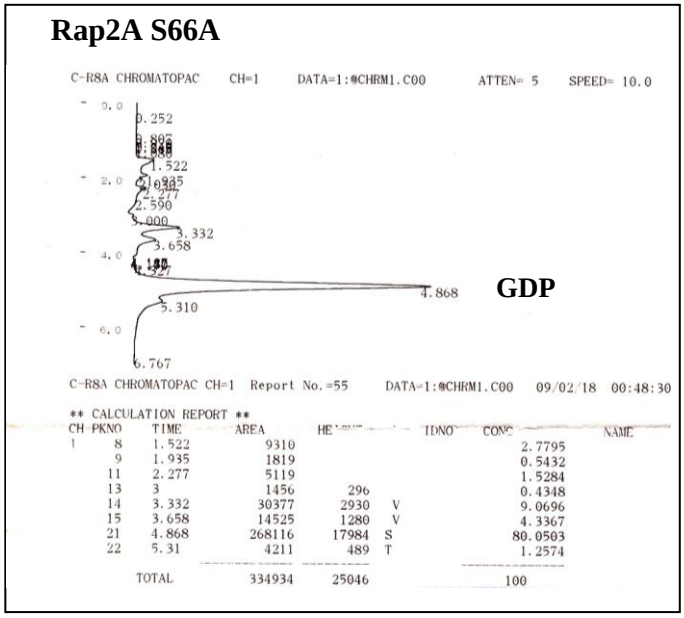


Figure 3.60: HLPC reaction of Rap2A S66A. 100µM purified Rap2A S66A was subjected to HPLC reaction to measure the concentrations of nucleotides in order to confirm the presence of the GTP binding protein Rap2A.

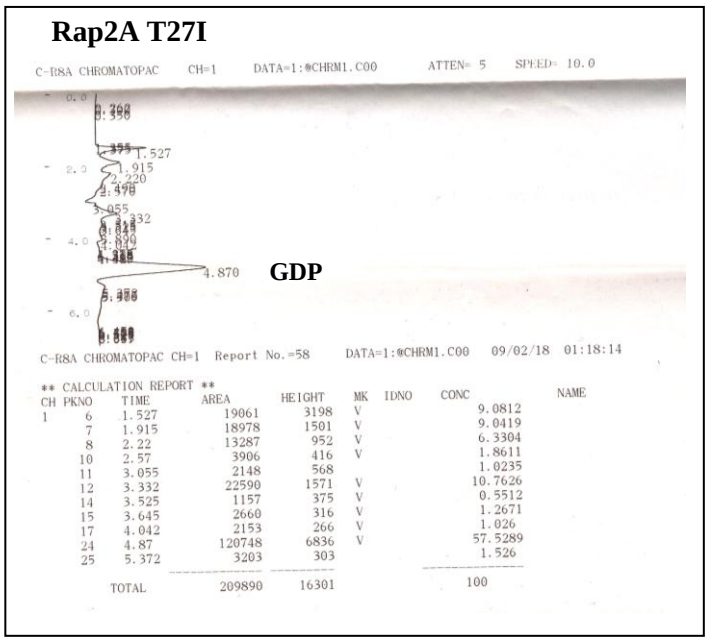


Figure 3.61: HLPC reaction of Rap2A T27I. 100µM purified Rap2A T27I was subjected to HPLC reaction to measure the concentrations of nucleotides in order to confirm the presence of the GTP binding protein Rap2A.

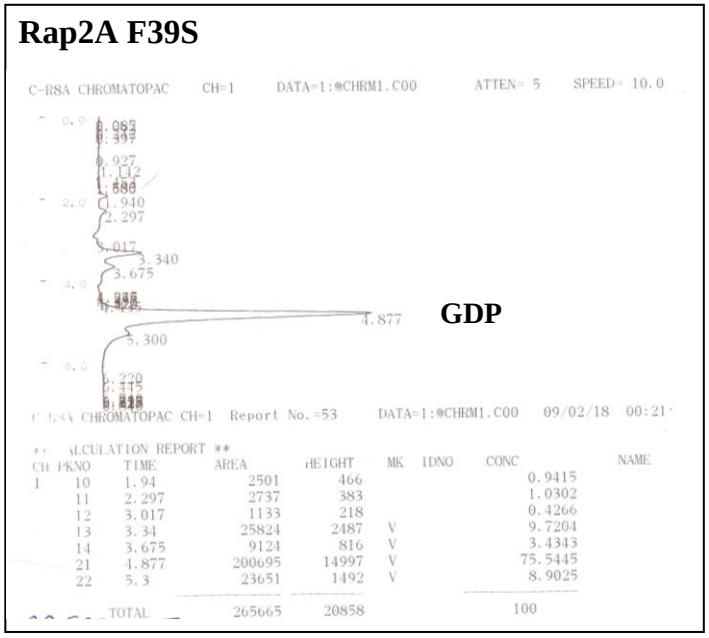


Figure 3.62: HLPC reaction of Rap2A F39S. 100µM purified Rap2A F39S was subjected to HPLC reaction to measure the concentrations of nucleotides in order to confirm the presence of the GTP binding protein Rap2A.

After these controls, 2mg from each Rap2A mutant protein was subjected to mant-GppNHp exchange with alkaline phosphatase and another round of HPLC analysis was performed to control the exchange reaction (data not shown). Subsequently, mant-GppNHp exchanged Rap2A mutant proteins were subjected to gel filtration in order to get rid of unlabelled proteins. Possible fractions were loaded on an SDS gel (Fig.3.63) and positive fractions from each sample were pooled together and concentrated. The presence of mant-GppNHp was tested again with HPLC (Fig.3.64, Fig3.65, Fig.3.66 and Fig3.67).

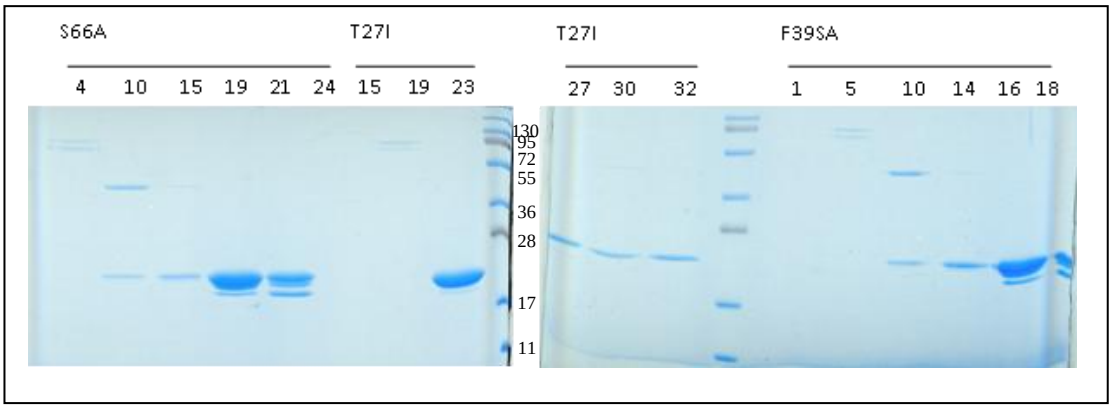


Figure 3.63: Gel filtrations of Rap2A T27I, S66A and F39S proteins after mGppNHp exchange raection. Fractions between 20-25 from S66A, 26-32 from T27I and 14-19 from F39S were collected and concentrated up to 2.81 mg/ml, 2.6mg/ and 3.2mg/ml respectively.

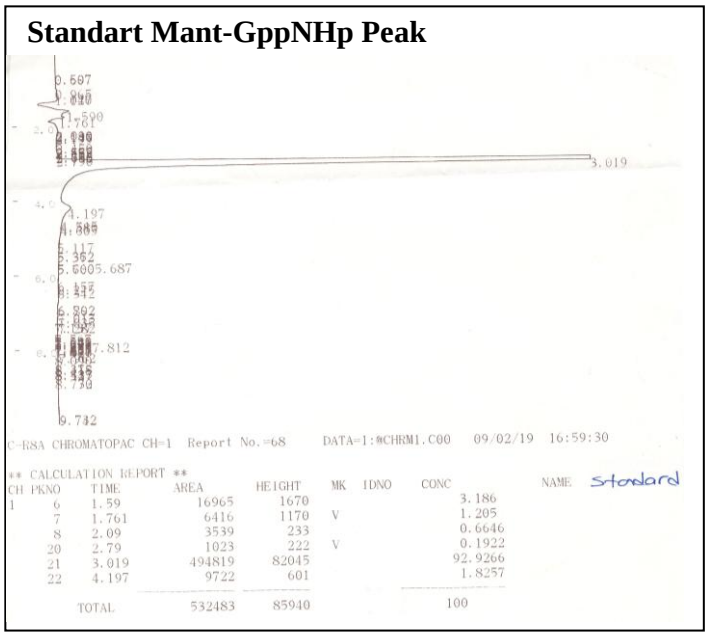


Figure 3.64: Mant-GppNHp retention time on HPLC. A standart m-GppNHp control sample was run on HLPC to determine the retention time of m-GppNHp which was around 3 min.

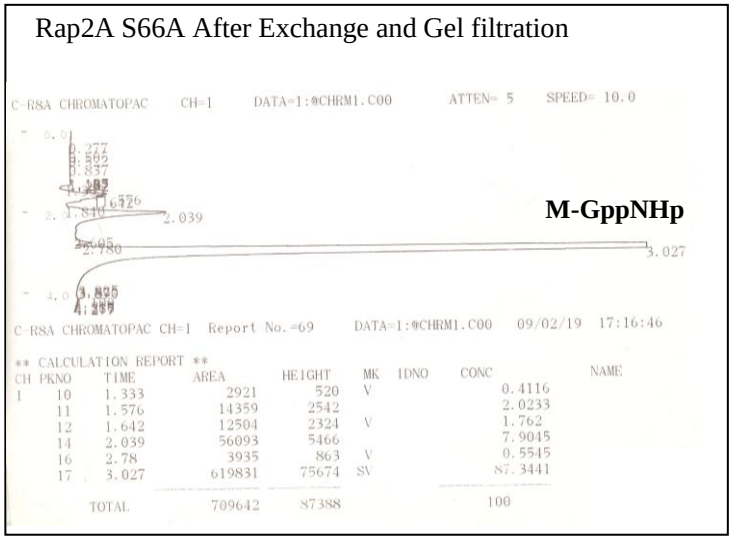


Figure 3.65: Mant-GppNHp retention time of Rap2A S66A on HPLC. 100µM Mant-GppNHp loaded Rap2A S66A protein was run on HPLC to verify the exchange reaction and to determine the concentration of loaded protein.

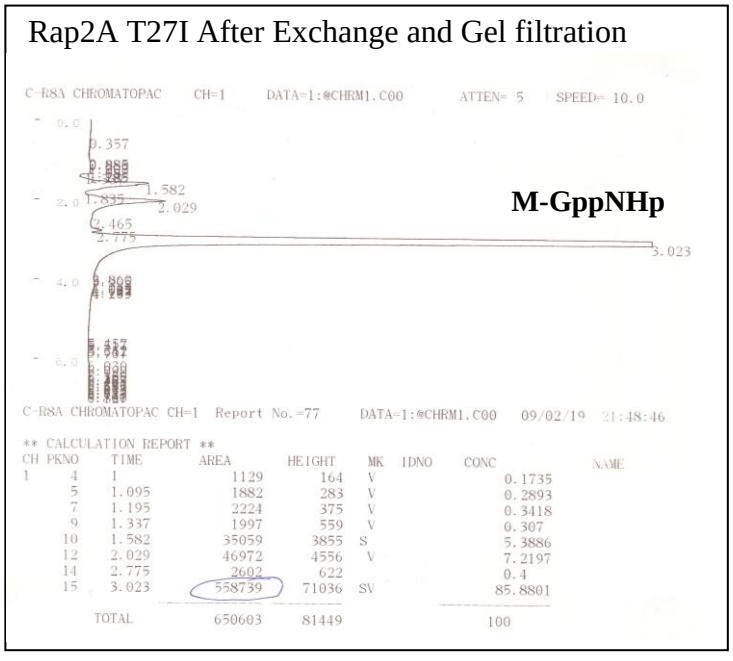


Figure 3.66: Mant-GppNHp retention time of Rap2A T27I on HPLC. 100µM Mant-GppNHp loaded Rap2A T27I protein was run on HPLC to verify the exchange reaction and to determine the concentration of loaded protein.

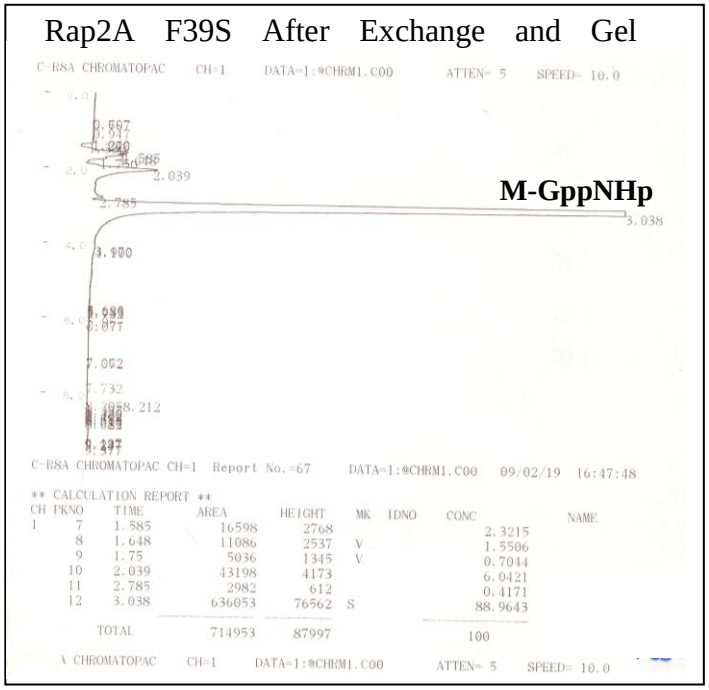


Figure 3.67: Mant-GppNHp retention time of Rap2A F39S on HPLC. 100µM Mant-GppNHp loaded Rap2A F39S protein was run on HPLC to verify the exchange reaction and to determine the concentration of loaded protein.

These mant-GppNHp labeled Rap2A mutants then subjected to GEF assay with RasGEF1 family of proteins in order to analyze the discriminatory amino acids. According to the GEF assay results, mutating Ser66 in the switch II region of Rap2 did not affect exchange rates by RasGEF1s (Fig.3.68), even though this region was previously shown to contain residues important for G-protein-GEF interactions (Mistou *et al.* 1992; Mosteller *et al.* 1995; Quilliam *et al.* 1996). Mutation T27I in the switch I region of Rap2 slowed down the exchange in the presence of RasGEF1s (Fig.3.68) but the effect was also small. Clearly, the most dramatic effect was obtained by mutating residue 39 of Rap2, which completely abolished nucleotide exchange of Rap2(F39S) catalyzed by RasGEF1A or RasGEF1B (Fig.3.68), indicating that Phe39 is essential for the catalytic interaction of Rap2 with RasGEF1 family members.

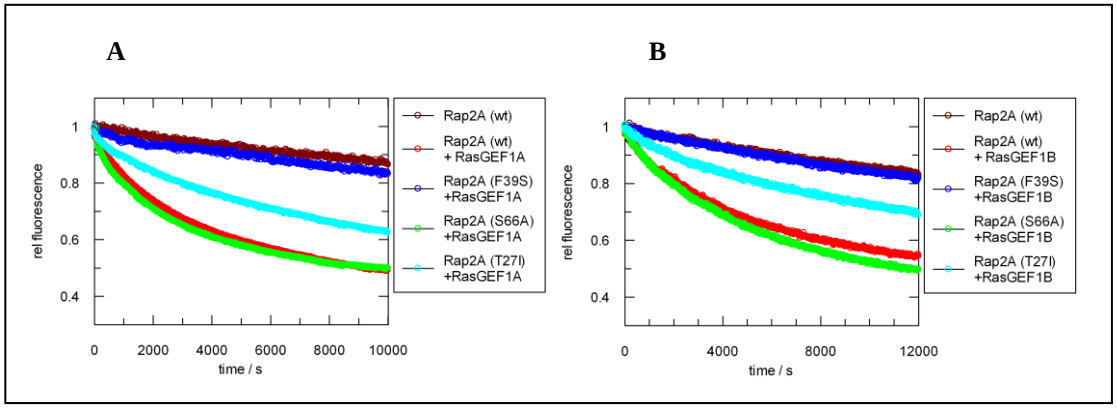


Figure 3.68: Guanine nucleotide exchange rates of wild-type and mutant Rap2A proteins. Wild-type or mutant Rap2A proteins were loaded with fluorescent mant-GNP, and the release of nucleotide was measured in real time in the absence or presence of GEFs. Values were obtained from at least three independent experiments, and representative graphs are displayed. (A) Guanine nucleotide exchange reaction of 150 nM wild-type Rap2A, Rap2A(F39S), Rap2A(S66A) and Rap2A(T27I) with 200 nM RasGEF1A. (B) Guanine nucleotide exchange reaction of 150 nM wild-type Rap2A, Rap2A(F39S), Rap2A(S66A) and Rap2A(T27I) with 4 µM RasGEF1B.

In order to assess whether simply converting the residue at the same position in Rap1 to Phe (as in Rap2A) could lead to the stimulation of exchange by RasGEF1 family members, Ser39 in Rap1 was mutated to Phenylalanine by site directed mutagenesis, expressed in *E.coli* and purified with GSH beads. In our first trial, we tried to remove the GST tag on the column but most of the protein precipitated, that is why in our second trial we made 3 different elutions; first elution was made with glutathione buffer without thrombin cleavage (in order to have a backup GST tagged protein in case of the other elutes precipitate), second elution was made with glutathione buffer and then elution was subjected to thrombin cleavage and in the third one, thrombin was added directly to the GSH column (Fig.3.69). Since the second elute had enough Rap1B S39F protein, we continued to mGppNHp exchange with that elute, and then exchange was controlled with HPLC as before (Fig.3.70).

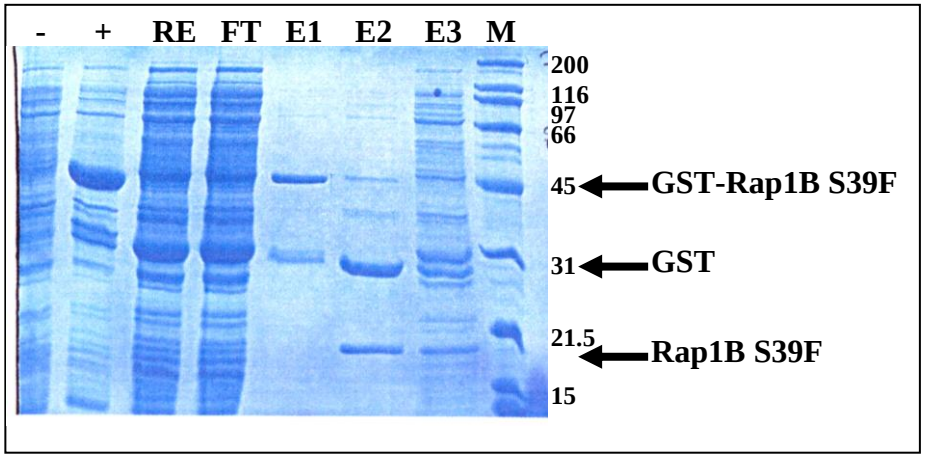
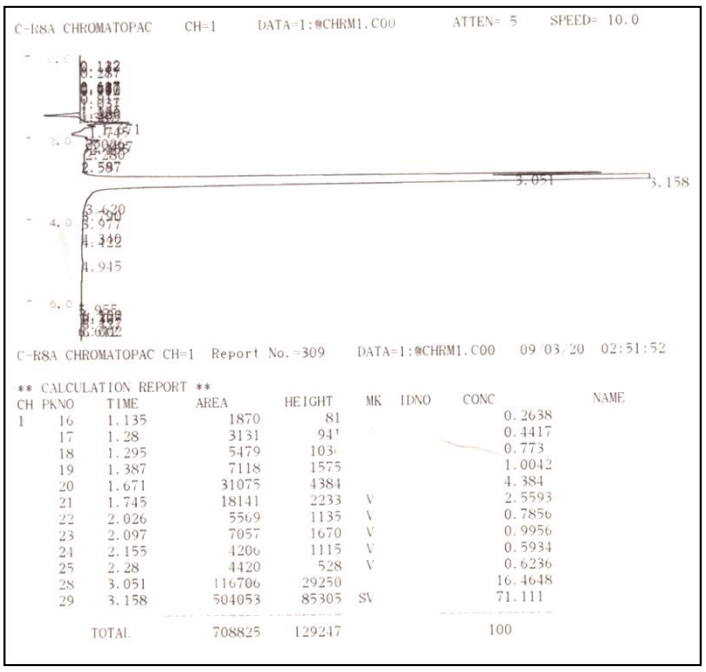


Figure 3.69: Purification of Rap1B S39F protein. Rap1B S39F mutant was purified with GSH-Beads and GST tags were cleaved with Thrombin. – and +: uninduced and IPTG induced controls, respectively; RE: Raw Extract after lysis but before incubation with beads; FT: Flow Through after incubation with beads; E1: Elution with glutathion buffer without thrombin cleavage; E2: Elution with glutathion buffer, then cleavage with thrombin; E3: Thrombin cleavage on the column; M: Marker.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C-R8A CHROMATOPAC CH=1 Report No.=309 DATA=1:@CHRM1.C00 09/03/20 02:51:52

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	16	1.135	1870	81			0.2638	
	17	1.28	3131	94			0.4417	
	18	1.295	5479	103			0.773	
	19	1.387	7118	1575			1.0042	
	20	1.671	31075	4384			4.384	
	21	1.745	18141	2233	V		2.5593	
	22	2.026	5569	1135	V		0.7856	
	23	2.097	7057	1670	V		0.9956	
	24	2.155	4206	1115	V		0.5934	
	25	2.28	4420	528	V		0.6236	
	28	3.051	116706	29250			16.4648	
	29	3.158	504053	85305	SV		71.111	
	TOTAL		708825	129247			100	

Figure 3.70: Mant-GppNHp retention time of Rap1B S39F on HPLC. 100µM Mant-GppNHp loaded Rap1B S39F protein was run on HPLC to verify the exchange reaction and to determine the concentration of loaded protein.

Subsequently, the guanine nucleotide exchange (GEF) activities of RasGEF1s on mutant Rap1(S39F) were tested (Fig.3.71). Interestingly, we observed that even though having Phe instead of Ser at position 39 of Rap1 was not sufficient for RasGEF1A to stimulate nucleotide exchange of Rap1(S39F), RasGEF1B could stimulate the exchange of mutant Rap1(S39F), albeit at a slower rate than that of Rap2 (Fig.3.71). However, RasGEF1A could not stimulate the exchange of mutant Rap1(S39F). In order to assure that RasGEF1A can not stimulate the exchange, increasing concentrations of RasGEF1A (up to 20 μM which is 200 times higher than the concentrations used for the other GEF assays) was used for the GEF assay of RasGEF1A with Rap1(S39F) (Fig.3.72). Even though the concentrations of RasGEF1A was increased to 20 μM, Rap1B(S39F) exchange did not affected (Fig.3.72). These results indicate that Phe39 is not only essential for stimulation of Rap2 by the RasGEF1 family of exchange factors, but is also sufficient, at least partially, for the specificity between Rap1 and Rap2 and for RasGEF1B to act as a cognate GEF on Rap1.

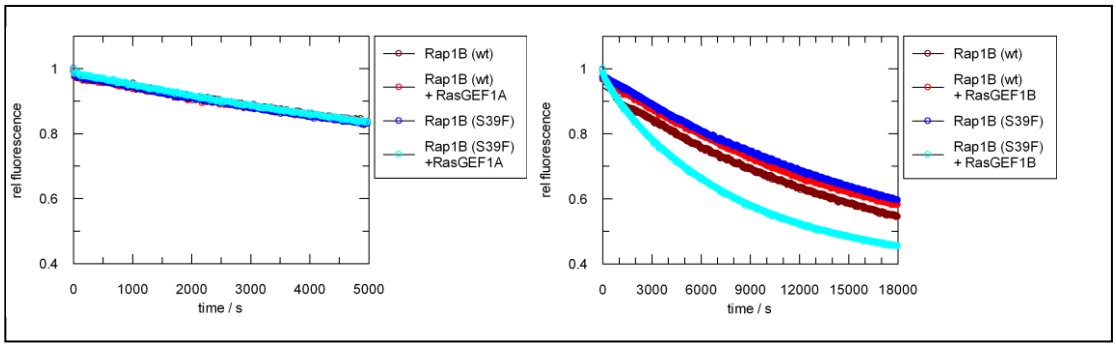
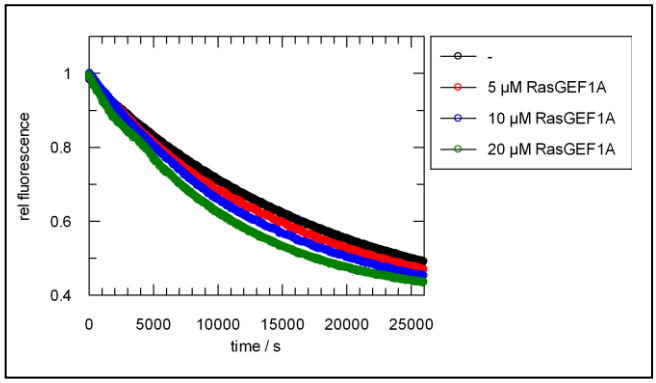


Figure 3.71: Guanine nucleotide exchange rates of wild-type and mutant Rap1B proteins. Wild-type or mutant Rap1B proteins were loaded with fluorescent mant-GNP, and the release of nucleotide was measured in real time in the absence or presence of GEFs. Values were obtained from at least three independent experiments, and representative graphs are displayed. (A) Guanine nucleotide exchange reaction of 150 nM wild-type Rap1B and Rap1B(S39F) with 200 nM RasGEF1A. (B), with 4 μM RasGEF1B.



Parameter	Value	Std. Error
Initial value	6.62e-001	4.55e-004
Rate constant	5.29e-005	7.88e-008
Offset	3.23e-001	5.24e-004

Parameter	Value	Std. Error
Initial value	6.36e-001	3.80e-004
Rate constant	6.56e-005	1.01e-007
Offset	3.56e-001	4.57e-004

Parameter	Value	Std. Error
Initial value	6.42e-001	2.55e-004
Rate constant	7.63e-005	8.81e-008
Offset	3.64e-001	3.11e-004

Parameter	Value	Std. Error
Initial value	6.18e-001	3.42e-004
Rate constant	8.86e-005	1.54e-007
Offset	3.71e-001	4.04e-004

Figure 3.72: Guanine nucleotide exchange (GEF) assay results of increasing concentrations of RasGEF1A on Rap1B(S39F). 150 nM Rap1B(S39F) loaded with mGppNHp were subjected to GEF assay in the absence (black circles) and in the presence of 5 µM (red circles), 10 µM (blue circles) and 20 µM (green circles) RasGEF1A.

4 DISCUSSION

The promoter of Ccdc-124 was identified in an unrelated study, and intrigued by high conservation rates of this gene in eukaryotes we decided to study cellular functions of this protein. The gene was found in fission yeast *Schizosaccharomyces pombe* and in a homothallic fungal mold *Aspergillus nidulans*, however it is absent in the genome of the budding yeast model *Saccharomyces cerevisiae* (Fig. 3.1, and results not shown). It could be speculated that Ccdc-124 could have a role in cytokinesis which is mechanistically different in *S. cerevisiae* (budding) as compared to other eukaryote models (fission/central division). As shown in Fig 3.1 and presented in corresponding section in "Results", Ccdc-124 is conserved with an impressive rate among all eukaryotes, and we assumed that these high conservation rates reflect its basic cellular functions. An initial screening of a cDNA library originated from mRNAs obtained from human liver cells by using Ccdc-124 as a bait in a yeast-two-hybrid platform (performed in collaboration with Hybrigenics S.A., Paris, France) have only revealed RasGEF1B as its potential interaction partner. We have then validated these results by two different experimental methods such as GST-pull down assays (Fig.3.25) and by co-immunoprecipitation experiments (Fig.3.33 and Fig.3.34). RasGEF1A, another member of RasGEF1 family was also included in these experiments (Fig.3.25). Both of these *in vitro* and *in vivo* (cell culture) analytic methods have further revealed an interaction of Ccdc-124 with RasGEF1 family members.

The cellular functions of RasGEF1 proteins were not sufficiently studied, and they were not appropriately established. There were only two studies that have addressed RasGEF1 family members: A multiple human tissue northern blot analysis carried out by Ura et al. (Ura *et al.* 2006) revealed that brain and spinal cord are two tissues with very strong RasGEF1A expression. These authors reported that RasGEF1A

could act as a GEF for H-Ras, K-Ras, and N-Ras, but sufficient experimental details were not given, and nor were other Ras proteins tested in comparison (see below). Regarding RasGEF1B, in fact the gene encoding murine RasGEF1B was first identified in a study reporting upregulation of its expression in macrophages in response to mucin-like glycoproteins of a protozoan parasite, Trypanosoma cruzi, and it was initially named GPIc4 (Ferreira *et al.* 2002). A study on tissue-specific expression of RasGEF1B homolog in developing zebrafish embryos indicated that this gene is strongly expressed in midbrain and hindbrain tissues (Epting *et al.* 2007). No other functional studies were carried out on this family of GEFs. In frame of this thesis work, we have expressed and purified Ccdc-124, RasGEF1A, RasGEF1B, as well as mutant forms of Rap2A and Rap1B in bacteria, and in collaboration with the group of Prof. Alfred Wittinghofer at Max-Planck Institute for Structural Biology we have, on one hand systematically tested stimulatory potentials of RasGEF1 family members on guanine nucleotide exchange in a large set of Ras superfamily of G proteins, and on the other hand assessed a possible effect of Ccdc-124 on the activities of RasGEF1 family members. As a result of these *in vitro* analysis using purified proteins we have unequivocally shown for the first time that RasGEF1A and RasGEF1B are specific for Rap2, and they do not activate nucleotide exchange of the other member of Rap family, Rap1 (see below for a longer discussion of these results). However, we could not identify any function of Ccdc-124 on GEF activities in this *in vitro* experimental set-up (Fig.3.43 and Fig.3.54). This result does not necessarily mean non-functionality of Ccdc-124 in RasGEF1 stimulated GDP/GTP exchange of Rap2. In fact, previous analysis carried out by Serap Erkek during her Master's project in our laboratory revealed that Ccdc-124 contains several potential phosphorylation sites (Thr, Ser, or Tyr), and it is possible that these modifications could modulate the functions of the protein. This might be reason why in an experimental set-up involving bacteria purified proteins, functionality of Ccdc-124 may remain unnoticed. It may however affect the function or kinetic parameters of RasGEF1 family of proteins in response to extracellular ligands, or the intracellular environment in a living cell system.

Purification of His-tagged Ccdc-124 turned out to be without major problems. We have purified ample amounts for GEF assays, as well as for crystallization purposes. Furthermore, aliquots of purified Ccdc-124 were sent to the laboratory at Bilkent, and mice were injected (50 μg/animal/immunization) with purified proteins in an effort to generate monoclonal antibodies against Ccdc-124 (Irem Gürbüz and Uygar Tazebay, personal comm.). However, purification of His-tagged RasGEF1B were rather unefficient. It is well known that expression systems and tags can affect the expression and purification efficiencies. For that reason, RasGEF1A and RasGEF1B were cloned into pGEX vector system which has GST-tag. That system indeed increased the expression and purification efficiencies of these proteins compared to His-tag system but still the amount of the proteins were much less than the amount of Ccdc-124 protein. On the other hand, when we tried to cleave the GST-tag, proteins were immediately precipitated. That is why, we purified RasGEF1 proteins with GST tag. In order to complete all the experiments we had to purify RasGEF1 proteins more than 5 times. On the other hand, since polarization assays take a lot of time and protein, we couldn't exactly completed the polarization assays because after some point RasGEF1B protein started to precipitate. But the increase in the polarization rates most probably indicate the binding of Ccdc-124 and RasGEF1B proteins.

Previous studies focusing on the cellular functions of Rap proteins have revealed that they could function independently of Ras. Rap1 has been studied extensively in many different cell types and organisms, and it has critical roles in cytoskeletal rearrangement, integrin-mediated cell adhesion, platelet activation, and cadherin-mediated cell junction formation (Bos 2006; Bos *et al.* 2001). Even though the expression patterns of Rap1 and Rap2 overlap, much less is known about a particular function of Rap2, indicating that the two subgroups (Rap1 and Rap2) could have different functions and be differentially regulated (Fu *et al.* 2007; Schmidt *et al.* 2001; Zhu *et al.* 2005). Supporting the notion of different functions, it has been shown that Rap2 is not involved in integrin activation but rather in Wnt–b-catenin signaling during dorsoventral patterning in Xenopus development (Choi and Han 2005), and that Rap2, but not Rap1, is expressed in red blood cells (Greco *et al.* 2006). We screened a large set of small GTP-binding proteins of the Ras subfamily

for stimulation of nucleotide exchange by two previously uncharacterized putative GEFs of the Cdc25 family, RasGEF1A and RasGEF1B. Both factors stimulated nucleotide exchange of only Rap2A, and did not have any effect at all on other distantly or closely related members of the Ras family. Comparison of exchange reaction rates of RasGEF1A and RasGEF1B suggested that RasGEF1A leads to about 20-fold faster release of nucleotide from Rap2A than that seen in the presence of RasGEF1B (Table 3.1). We noticed that the nucleotide exchange rate of RasGEF1B is also slower than that of other RapGEFs, such as Epac or C3G (Gasper *et al.* 2008). RasGEF1A and RasGEF1B are comparatively small GEFs, without any apparent additional domain that could potentially be autoinhibitory. However, weak affinities between GEFs and their cognate G-proteins have been noticed previously, and could indicate that, in vivo, RasGEF1 activity is substantially increased by translocation to the plasma or internal membranes, which increases the local concentration of substrate and enzyme. A multiple human tissue northern blot analysis carried out by Ura et al. (Ura *et al.* 2006) revealed that brain and spinal cord are two tissues with very strong RasGEF1A expression. In contrast to our data, these authors reported that RasGEF1A could act as a GEF for H-Ras, K-Ras, and N-Ras. In our opinion, this might be an artifactual result, as in their work both G proteins and GEFs were containing GST-tags, and dimerization of GSTs might have led to artifactual close interactions between GEFs and G proteins. In our hands, when unfused Ras proteins were used rather than GST-linked proteins, and phosphocellulose filter binding was avoided, RasGEF1A has no effect on the nucleotide exchange of classic Ras proteins in the solution assay.

The gene encoding murine RasGEF1B was first identified in a study reporting upregulation of its expression in macrophages in response to mucin-like glycoproteins of a protozoan parasite, Trypanosoma cruzi, and it was initially named GPIc4 (Ferreira *et al.* 2002). A study on tissue-specific expression of RasGEF1B homolog in developing zebrafish embryos indicated that this gene is also strongly expressed in midbrain and hindbrain tissues (Epting *et al.* 2007). Interestingly, specific functions of Rap2 were identified in different neuronal processes, such as synaptic depotentiation and modulation of neuronal morphology (Fu *et al.* 2007;

Schmidt *et al.* 2001; Spilker *et al.* 2008; Zhu *et al.* 2005), and it is possible that the two RasGEF1 family members play critical roles in the spatial and temporal regulation of Rap2 activity at different rates in hippocampal neurons during the control of excitatory synapses.

Although the structural model of the RasGEF1– Rap2 complex suggested Ser66 in the switch II region to be located in or close to the interface, the S66A mutation in Rap2A did not affect nucleotide exchange by RasGEF1 family members, suggesting that the mild Ser to Ala substitution can easily be accommodated in the interface. This is in line with the structure of the Epac–Rap1 complex, where Ala66 makes a main chain water-mediated contact with Asn803 of Epac (Rehmann *et al.* 2008). Whereas wild-type Rap2 is not stimulated by C3G, the double-mutated (T65A ⁄ S66A) Rap2 can be partially stimulated by C3G, just as a more extensive quadruple mutation of the switch II region made Ras partially responsive to C3G (van den Berghe *et al.* 1999). All of this supports the notion that the switch II region is involved in binding and specificity, and that the interface between Cdc25-GEFs and their cognate G-proteins shows great plasticity (Boriack-Sjodin *et al.* 1998; Rehmann *et al.* 2008). Conversion of Thr27 in switch I of Rap2A to Ile (as in Rap1) reduces the nucleotide exchange rate (Fig.3.68), indicating a functional role for this residue. The most dramatic effect is, however, due to the F39S mutation in Rap2A, which completely abolished nucleotide exchange activity by the two cognate GEFs, RasGEF1A and RasGEF1B (Fig.3.68). Residue 39 is located in the switch I region previously shown to be critical in Ras–Cdc25, Rap–Epac and Rap1–C3G interactions (Boriack-Sjodin *et al.* 1998; Rehmann *et al.* 2008; van den Berghe *et al.* 1999). Furthermore, it was previously shown that mutations at Thr35 and Glu37 positioned in the switch I region of Rap1 decreased its interaction with C3G by a factor of four (van den Berghe *et al.* 1999). These previous data are in agreement with our results regarding the critical role of the switch I region and, in particular, Phe39 in the interaction of Rap2A with RasGEF1A and Ras- GEF1B. Phe39 is also a specificity determinant, as the S39F mutation in Rap1B allows RasGEF1B to act on Rap1B. As RasGEF1A does not have the same stimulatory effect on Rap1(S39F), regardless of its concentration (from 200 nm up to 20 μm; Fig.3.72), this suggests that the two

GEFs establish different contacts even though they are 61% identical. These results indicate that even a single amino acid difference in the switch I region of Rap GTP-binding proteins may determine the specificities of interactions with cognate nucleotide exchange factors. In fact, in a previous study, residue 39 was also shown to determine the specificity of interaction of Rap2 with the downstream effector RAPL/NORE (Miertzschke *et al.* 2007). The Phe39 to Ser39 sequence difference in the switch I region of Rap1 and Rap2 is thus a major determinant of the activation of different Rap group members and their downstream biological effects.

5 FUTURE PERSPECTIVES

In this thesis, we have first identified the interaction of Ccdc-124 and RasGEF1 family of proteins. Then, we have shown that RasGEF1 family members, RasGEF1A and RasGEF1B stimulate guanine nucleotide exchange of Rap2, but not of Rap1, in *in vitro* assay conditions. Under same conditions, Ccdc-124 neither stimulates, nor inhibits RasGEF1 functions, even though the two proteins interact with each other. Immediate future studies will include assessments of Rap2 regulatory functions of RasGEF1s and the effect of Ccdc-124 on this regulation (if any) *in vivo*. For this purpose RasGEF1 family members and Ccdc-124 can be knocked-down by specific shRNA molecules, and the biological read-outs can be monitored. In fact, interestingly, both canonical and non-canonical Wnt pathways were shown to be under control of Rap2 proteins, and not of Rap1 (Choi *et al.,* 2005). Therefore, as one of the pathways that could be modulated by such knock-downs could be the Wnt pathway, we are planning to analyze Wnt pathway activities and the stability of β-catenin in RasGEF1/Ccdc-124 down-regulated cellular conditions.

Subcellular localizations of RasGEF1 family members and Ccdc-124 were not fully established. Even though we have observed a cytoplasmic localization pattern for Ccdc-124, we have not correlated it with the localizations of well established Golgi, ER, or vesicle specific markers. There are a number of molecular ways to modulate activities of GEFs such as 1) binding of small molecules (cAMP, DAG, Ca^{++}), 2) protein-protein interactions leading to stimulation/inhibition of GEFs, 3) acting as binding partners that bring them close to regulatory proteins, 4) or changing their subcellular localizations in order to bring them to proximities of their substrates. It is plausible to think that Ccdc-124 could stimulate RasGEF1 family members by changing their localizations, and if this is the case, then it would have been impossible to detect its stimulatory effect by our assay system. Ccdc-124 could

maintain this activity after going through post-translational modifications that have not been thoroughly analyzed yet. In fact, in bioinformatics data bases, Ccdc-124 is predicted to be a phospho-protein. Future work should adress if Ccdc-124 is modified *in vivo*, and how these *in vivo* modifications might affect the biological functions of the protein.

Ccdc-124 is a relatively small gene composed of 4 exons, and the region it covers is around 3 kb in total. Therefore, in theory deleting the gene in mice genome would be relatively easy, and detection of knock-outs will be less complicated as compared to genes of superior complexity. We have already obtained mouse Bac clones having the region with the locus encoding Ccdc-124 as well as all vector systems to create Cre-Lox constructs in our laboratory. Following resuts obtained from shRNA experiments where Ccdc-124 will be knocked-down in cells, and its biological effect on signaling pathways (Wnt/β-catenin) will be assessed, we are also planning to make Ccdc-124 knock-out mice. These mice might let us establish unequivocally the biological function of this novel conserved gene.

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