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FROM UNSPECIFIC QUENCHING TO SPECIFIC SIGNALING:

Functional GTPase Assays Utilizing Quenching
Resonance Energy Transfer (QRET) Technology

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It is not certain that everything is uncertain.

- Blaise Pascal (1623-1662)

Kari Kopra

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ABSTRACT

The aim of the work presented in this study was to demonstrate the wide applicability of a single-label quenching resonance energy transfer (QRET) assay based on time-resolved lanthanide luminescence. QRET technology is proximity dependent method utilizing weak and unspecific interaction between soluble quencher molecule and lanthanide chelate. The interaction between quencher and chelate is lost when the ligand binds to its target molecule. The properties of QRET technology are especially useful in high throughput screening (HTS) assays.

At the beginning of this study, only end-point type QRET technology was available. To enable efficient study of enzymatic reactions, the QRET technology was further developed to enable measurement of reaction kinetics. This was performed using protein-deoxyribonucleic acid (DNA) interaction as a first tool to monitor reaction kinetics. Later, the QRET was used to study nucleotide exchange reaction kinetics and mutation induced effects to the small GTPase activity. Small GTPases act as a molecular switch shifting between active GTP bound and inactive GDP bound conformation.

The possibility of monitoring reaction kinetics using the QRET technology was evaluated using two homogeneous assays: a direct growth factor detection assay and a nucleotide exchange monitoring assay with small GTPases. To complete the list, a heterogeneous assay for monitoring GTP hydrolysis using small GTPases, was developed. All these small GTPase assays could be performed using nanomolar protein concentrations without GTPase pretreatment. The results from these studies demonstrated that QRET technology can be used to monitor reaction kinetics and further enable the possibility to use the same method for screening.

Keywords: guanosine triphosphatase (GTPase), high throughput screening (HTS), kinetic measurement, time-resolved luminescence, quenching resonance energy transfer (QRET)

Kari Kopra

Epäspesifisestä sammutuksesta spesifiseen signalointiin: Toiminnalliset sammutus-resonanssi-energiansiirtoon perustuvat GTPaasi määritykset

Solubiologia ja anatomia, Biolääketiteen laitos, Lääketieteellinen tiedekunta, Turun Yliopisto, Turku, Suomi
Molekyylilääketieteen tohtoriohjelma (TuDMM) ja Kansallinen bioinformatiikan ja rakennebiologian tutkijakoulu (ISB)
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TIIVISTELMÄ

Tämän tutkimuksen tavoitteena oli osoittaa sammutus-resonanssi-energiansiirtoon (QRET) pohjautuvan teknologian toimivuus aika-eroitteisissa lantanidien luminesenssiin perustuvissa määrityksissä. Molekyylien etäisyysriippuvuuteen perustuvassa QRET teknologiassa, reportterimolekyylin sitoutuminen kohdemolekyyliin voidaan havaita luminesenssi-signaalin muutoksena. Sammuttajamolekyylin ja lantanidikelaatin sisältävän reportterimolekyylin välinen heikko vuorovaikutus katkeaa kun reportterimolekyyli sitoutuu kohdemolekyyliinsä. QRET teknologian ominaisuudet ovat erityisen käyttökelposia tehoseulonta (HTS) menetelmissä.

QRET teknologian soveltuvuus määrityskäyttöön on osoitettu jo ennen tämän tutkimuksen aloittamista, mutta määritykset ovat perustuneet reaktion lopputuloksen mittaamiseen. Jotta pieniä entsyymireaktioita voitaisiin tutkia tehokkaasti, on reaktion kinetiikkaa voitava mitata. Ensimmäinen määritys, jossa kinetiikan määritys oli mahdollista, suoritettiin tutkimalla DNA:n ja proteiinin välistä vuorovaikutusta. Myöhemmin tätä kykyä ominaisuutta käytettiin guanosiinitrifosfataasien (GTPaasi) tutkimiseen, selvittämällä eri GTPaasien nukleotidin vaihdon tehokkuutta ja eri pistemutaatioiden vaikutusta GTPaasin toimintaan. GTPaasit ovat kytkinmolekyylejä, jotka ovat aktiivisia GTP:hen ja passiivisia GDP:hen sitoutuneena.

QRET teknologian soveltuvuutta reaktiokinetiikan mittaamiseen arvioitiin kahdessa erotusvapaassa määrityksessä: suora kasvutekijämääritys ja GTPaasien nukleotidin vaihdon kinetiikan määritys. GTPaasien tutkimusta täydennettiin vielä erotteluun perustuvalla GTP:n hydrolysoitumismäärityksellä. Aikaerotteiseen luminesenssiin perustuvat määritykset voitiin toteuttaa esikäsittelemättömillä GTPaaseilla. Tämän GTPaaseilla suoritetun tutkimuksen perusteella, QRET teknologia soveltuu sekä reaktiokinetiikan määrittämiseen että tehoseulontaan.

Avainsanat: aikaerotteinen luminesenssi, guanosiinitrifosfataasi (GTPaasi), kinetiikka määritys, sammutusenergiansiirto (QRET), tehoseulonta

TABLE OF CONTENTS

ABBREVIATIONS

Arf	ADP ribosylation factor (GTPase)
Arl	Arf-like (GTPase)
Arp	Arf-related (GTPase)
ATP	adenosine-5'-triphosphate
bFGF	basic fibroblast growth factor
CFP	cyan fluorescent protein
CTP	cytidine-5'-triphosphate
DH	Dbl homology
DHR	Dock homology region domain
DNA	deoxyribonucleic acid
EC_{50}	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
EFI	europium luminescence intensifier
Fab	fragment antigen-binding
FBA	bFGF binding aptamer
FLAIR	fluorescence activation indicator for Rho protein
FP	fluorescence polarization
FRET	Förster resonance energy transfer
FTI	farnesyltransferase inhibitor
GAP	GTPase-activating protein
GDF	GDI-displacement factors
GDI	GDP dissociation inhibitors
GDP	guanine-5'-diphosphate
GEF	guanine nucleotide exchange factor
GF	growth factor
GFP	green fluorescent protein
GMP	guanine-5'-monophosphate
GPCR	G-protein-coupled receptor
GSH	glutathione
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
GTPase	guanosine-tri-phosphatase
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
HTS	high throughput screening
Hvr	hypervariable region
IDCC	N-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide
kDa	kilodalton
Ln^{3+}	lanthanide (rare earth element group)
LOD	limit-of-detection
Mant	methylanthraniloyl (luminescent label)
Mant-GppNH	p2'(3')-O-(N-methylanthraniloyl)-guanosine 5'-[β,γ-imido]triphosphate
Mant-GTPγS	2'(3')-O-(N-methylanthraniloyl)-guanosine 5'-[γ-thio]triphosphate
MDCC	N-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamide

MG	malachite green
NDPK	nucleoside diphosphate kinase
NF1	neurofibromatosis type 1
Nflx	norfloxacin
nFRET	nonoverlapping FRET
NMR	nucleic magnetic resonance
ParM	bacterial actin homologue
PBD	p21-binding domain
PBP	phosphate binding protein
PCA	protein-fragment complementation
PH	pleckstrin homology
P_i	inorganic phosphate
PPI	protein-protein interaction
QRET	quenching resonance energy transfer
Rab	Ras-related proteins in brain (GTPase)
Raichu	Ras and interacting protein chimeric unit
Ral	Ras-like (GTPase)
Ran	Ras-like nuclear protein (GTPase)
RanBP	Ran-binding protein
Rap	Ras-proximal (GTPase)
Ras	rat sarcoma virus gene (GTPase)
RBD	Ras binding domain
RCC1	regulator of chromosomecondensation
REM	Ras exchange motif
RFP	red fluorescent protein
Rho	Ras homologous (GTPase)
SA	streptavidin
S/B	signal-to-background
ScFv	single-chain variable fragment
Sar	secretion-associated and Ras-related (GTPase)
split-FP	split-fluorescent protein
sNBD	succinimidyl 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoate
SOS	son of sevenless
Tamra	tetramethylrhodamine (luminescent label)
TEAA	triethylammonium acetate
TIRF	total internal reflection fluorescence
TNP	trinitrocyclohexadienylidene (luminescent label)
TLC	thin layer chromatography
TR-FTIR	time-resolved Fourier transform infrared
TRL	time-resolved luminescence
VBA	VEGF binding aptamer
VEGF	vascular endothelial growth factor
YFP	yellow fluorescent protein
2'/3'-AHC-GTP	2'-/3'-O-(6-aminohexylcarbamoyl)guanosine-5'-O-triphosphate
8-AHT-GTP	8-(6-aminohexylthio)guanosine-5'-O-triphosphate

LIST OF ORIGINAL PUBLICATIONS

I   Kari Kopra, Markku Syrjänpää, Pekka Hänninen and Harri Härmä (2014). Non-competitive aptamer-based quenching resonance energy transfer assay for homogeneous growth factor quantification. *Analyst* **139:**2016-2023.

II   Kari Kopra, Alessio Ligabue, Qi Wang, Markku Syrjänpää, Olga Blaževitš, Stefan Veltel, Arjan van Adrichhem, Pekka Hänninen, Daniel Abankwa and Harri Härmä (2014). A homogeneous quenching resonance energy transfer assay for the kinetic analysis of the GTPase nucleotide exchange reaction. *Anal Bioanal Chem* **406**:4147-4156.

III Maja Solman, Alessio Ligabue, Olga Blazevits, Alok Jaiswal, Yong Zhou, Hong Liang, Kari Kopra, Camilo Guzman, Harri Härmä, John Hancock, Tero Aittokallio and Daniel Abankwa (2015). Cancer associated mutations in the switch III-region of Ras increase tumorigenicity by nanocluster augmentation. *Submitted manuscript.*

IV   Kari Kopra, Anita Rozwandowicz-Jansen, Markku Syrjänpää, Olga Blaževitš, Alessio Ligabue, Stefan Veltel, Urpo Lamminmäki, Daniel Abankwa and Harri Härmä (2015). GTP-specific Fab fragment-based GTPase activity assay. *Anal Chem* **87**:3527-3534.

In addition, some unpublished data is presented.

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1 INTRODUCTION

The complexity of biological systems and the processes involved in medical diagnostics, food safety assurance and environmental monitoring have for long challenged researchers in these fields. Biochemical assays have been developed with the aim to provide answers in such dilemmas. Traditionally, these methods are classified as heterogeneous and homogeneous assays, based on whether there are separation steps or not (Davies, 2005). The method chosen to resolve the problem in hand is selected based on the need. Homogeneous methods are fast and could provide real-time data. These assays are also easy to automate and more cost-effective compared to heterogeneous assays. Homogeneous assay is the method of choice when a fast and simple method is needed, like in point-of-care or high throughput screening (HTS). On the other hand, heterogeneous assays provide more sensitive approaches, because of the additional separation step. However, the inclusion of this additional step requires extra equipment and effort, and the real-time monitoring capability is lost.

Small guanosine-tri-phosphatases (GTPases) are a large group of hydrolase enzymes that are divided into several families based on their sequence homology. These cytosolic proteins regulate several important cellular functions; cell growth, differentiation, and movement. Due to their central role, GTPases have inherent oncogenic signaling properties, making them potential as anti-cancer drug targets. GTPases are normally in guanine-5'-diphosphate (GDP) bound inactive conformation, but after stimulus, guanine-5'-triphosphate (GTP) is bound and GTPase is activated. These processes are controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GTPases are crucial part of many kinase cascades, and understanding the functions and the structural aspects of the GTPases is crucial in finding novel drug molecules to control this complex protein-protein interaction (PPI) network.

The enzymatic nature of the GTPases sets a special requirement for the development of an efficient assay method, i.e. the method should be sufficiently sensitive and provide kinetic data with low amounts of proteins. To fulfill this, a homogeneous separation free assay technique is the method of choice. Recently, a large variety of homogeneous luminescence based methods, which try to meet these requirements, have been developed. With luminescence, real-time observation of binding event becomes possible. However, in many of the currently known methods, the reporter group may alter the biochemical properties of the GTPase reaction. Thus the trends in method development have proceeded towards label-free techniques. These methods, however suffer from large protein consumption and low or medium throughput.

2 REVIEW OF THE LITERATURE

Small GTPases, hereinafter called just GTPases, work as intracellular molecular switches and are involved in regulating key cellular processes (Vetter and Wittinghofer, 2001). Mutations in GTPases are frequently found in human cancers and in several developmental disorders, demonstrating their significance as signaling molecules (Fernandez-Medarde and Santos, 2011; Prior et al., 2012). For many years, heterogeneous radiolabel techniques were the method of choice for GTPase nucleotide exchange research, but the trend has been towards homogeneous methods using luminescent GTP analogs or label-free techniques (Hemsath and Ahmadian, 2005). The review begins by introducing the human GTPases, their most important regulator molecules, GEFs, GAPs, and GDP dissociation inhibitors (GDIs), and their relevance in human diseases. Subsequently, the different bioanalytical assay methods used to study GTPases are described. The main focus is on *in vitro* assays, but the most important *in vivo* methods will also be introduced.

2.1 Human GTPases and GTPase controlling molecules

The GTPase superfamily comprises more than 150 members in the human genome and is divided into five subfamilies (Ras, Rho, Arf, Rab, and Ran) based on their sequence and functional similarities (Vetter and Wittinghofer, 2001; Wennerberg et al., 2005). Ras is the founding member of a family containing a multitude of 21-25 kilodalton (kDa) peripheral membrane GTPases that control signal transduction cascades. GTPases work as intracellular molecular switches and are involved in regulating key cellular processes including cell division, signal transduction, motility, vesicle transport, senescence, and apoptosis (Vetter and Wittinghofer, 2001; Colicelli, 2004; Wennerberg et al., 2005). In this section, GTPases and GTPase regulators are described. Furthermore, known associations between GTPases and diseases are described.

2.1.1 Human GTPase superfamily

All GTPases are formed by a single polypeptide chain, and act as "molecular switches" that are active in the GTP- and inactive in the GDP-state (Figure 1). GTPases are composed of six β-sheet surrounded by five α-helices. This conserved domain includes motifs that recognize guanine base, β-phosphate, and Mg^{2+} (Vetter and Wittinghofer, 2001). These conserved motifs are a part of p-loop and switch I-II, which are involved in nucleotide exchange and hydrolysis. However, the actual molecular switch includes GTPase, GEF, and GAP proteins together. GTPases are classified into five families, but the boundaries between families are not strict and not all proteins can be assigned to any of these families. Also there are a large number of structurally and functionally diverse proteins that possess GTPase like function but fall outside the GTPase superfamily.
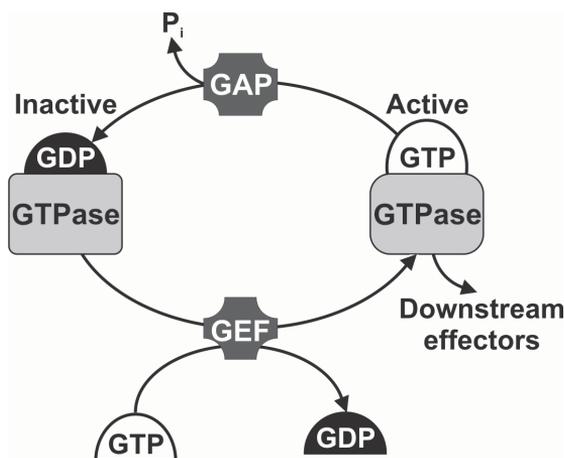
Figure 1. The GTPase activation/de-activation cycle. GTPases are intracellular molecular switches cycling between inactive (GDP-bound) and active (GTP-bound) state. The GTPase activation is controlled by guanine nucleotide exchange factors (GEF), which catalyze the GDP dissociation enabling GTP association. Active GTP-GTPase can activate downstream effectors. The GTPase activating protein (GAP), catalyze the GTP γ-phosphate hydrolysis, cycling the GTPase back to inactive GDP bound state.

RasGTPases

The first GTPase superfamily member found was rat sarcoma virus gene (Ras) (Der et al., 1982). Today, there are more than 30 known members in the Ras family (Reuther and Der, 2000; Csépányi-Kömi et al., 2012). The Ras subfamily is divided in three major groups; 1. Ras (e.g. gene expression control, cell growth, cell differentiation, and cell survival), 2. Ral (e.g. vesicle trafficking, cell polarization, and apoptosis), 3. Rap (adhesion-related functions e.g. integrin augmentation). Minor groups include, e.g. Rheb, R-Ras, M-Ras, Rin, and Rit (Reuther and Der, 2000; Csépányi-Kömi et al., 2012).

The most notable members in Ras subfamily are H-Ras, N-Ras, and K-Ras (A and B), which share up to 85% of sequence homology (Reuter and Der, 2000). Rap (Ras-proximal) proteins are involved in integrin-mediated cell adhesion and spreading (Collicelli, 2004). Ral (Ras-like) proteins have role in cell proliferation and polarization (Feig, 2003). Ras proteins are attached to the membrane through CAAX-motif signaling sequences in the carboxyl terminus. This motif enables Ras farnesylation and also N-Ras and K-Ras geranylgeranylation. Another Ras modification is palmitate fatty acid, attached to other Ras proteins except in K-Ras4B which possesses lysine-rich polybasic sequence (Reuter and Der, 2000). These Ras modifications determine the Ras location and transport to the plasma membrane. Ras proteins mediate their effects mainly through a complex kinase cascades and the knowledge of the related molecules and their links to diseases are constantly increasing (Takai et al., 2001; McCubrey et al., 2006; Siegfried et al., 2013). A simplified representation of the Ras downstream signaling network is depicted in Figure 2.
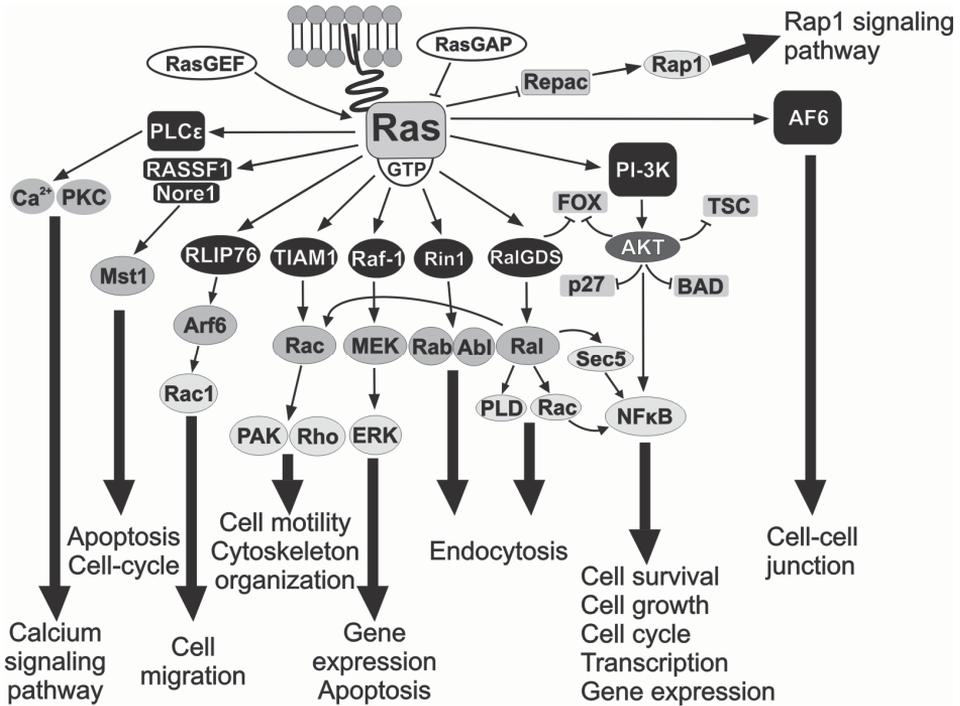
Figure 2. A simplified overview of the Ras downstream signaling network. Ras regulates several important cellular functions as cell growth, differentiation, and movement through complex signaling network.

RhoGTPases

The Ras homologous (Rho) family is one of the major subfamilies, comprising 22 known protein members mostly regulating actin related functions (Csépányi-Kömi et al., 2012). There are three major member groups of the Rho family, Cdc42, Rac, and Rho, and many minor groups (Takai et al., 2001). The three main Rho groups are interconnected and the activation signal is transmitted from one RhoGTPase to another (Takai et al., 2001). Rho proteins are lipid modified at their carboxyl terminus, containing farnesyl or geranylgeranyl groups. Rho plays an important role in actin cytoskeleton reorganization enabling cell shape change, motility, adhesion, and cytokinesis. Furthermore, cellular events, like the formation of stress fibers, focal adhesion in fibroblast, smooth muscle contraction, and cell growth have been linked to Rho (Takai et al., 2001).

ArfGTPases

The ADP ribosylation factor (Arf) subfamily contains Arf 1-6, but also secretion-associated and Ras-related (Sar), Arf-like (Arl), and Arf-related (Arp) proteins. Arf group's main function is to control intracellular vesicular traffic (D'Souza-Schorey and Chavrier, 2006). Unlike other GTPases, Arf proteins are N-terminally lipid modified (myristoyl fatty acid), which is essential for membrane association and biological activity (Donaldson and

Honda, 2005). SarGTPases identity to Arf is low (<30%), but they are functionally related initiating vesicle budding (Kahn et al., 2006). Non-coherent ArlGTPases are a divergent group from the other GTPases, displaying large conformational changes (Kahn et al., 2006). Arp proteins share 30-40% sequence similarity to other Arf family members, but exhibits unusual features like high intrinsic GTPase activity (Schürmann et al., 1999).

RabGTPases

The largest GTPase subfamily is the Ras-related proteins in brain (Rab) family, comprising over 60 members in humans (Schwartz et al., 2007; Hutagalung and Novik, 2011). Like Arfs, Rab proteins are involved in membrane trafficking and intracellular signaling. Rabs are C-terminally modified, containing geranylgeranyl groups controlling Rab interactions with membrane and GDIs (Schwartz et al., 2007). Also most Rabs include a C-terminal prenylation signal and N-terminal myristylation site like ArfGTPases (Collicelli, 2004).

RanGTPase

The Ras-like nuclear protein (Ran) has been defined as a separate family or as a part of Rab family (Colicelli, 2004; Yudin and Fainzilber, 2009). The single RanGTPase regulates nuclear import and export, the mitotic spindle, and reconstruction of the nuclear envelope (Weis, 2003). Ran also has a role in cytoskeletal dynamics and importin-dependent transport (Clarke and Zhang, 2008; Yudin and Fainzilber, 2009).

2.1.2 Guanine nucleotide exchange factors (GEFs)

GTPases are rarely in nucleotide free conformation, but normally they are in inactive GDP-bound conformation. In the cell there is an excess of GTP over GDP, but the intrinsic nucleotide exchange is very low in case of most of the GTPases. GEFs are needed to enable GTPase activation by increasing the GDP release (Lenzen et al., 1998). The ability to increase the nucleotide exchange is partly due to the GEF proteins higher affinity to the GDP-bound GTPase, and partly due to ability to stabilize the nucleotide free conformation enabling GTP binding (Lenzen et al., 1998).

For over 150 GTPases there is an even higher number of equal molecular size GEFs (Wennerberg et al., 2005; Bos et al., 2007). The number of GEFs and GTPases corresponds in most of the subfamilies, except in Rho where GEFs outnumbers GTPases nearly 4-fold (Csépányi-Kömi et al., 2012). This complexity guarantees the specificity of the signaling network. GEFs are multidomain proteins comprising of protein and lipid interaction domains (Bos et al., 2007). The exact nucleotide exchange mechanism varies depending on the GTPase and GEF. However, the basic principle is similar; GEF activates the GTPase by either helping in the GDP removal and/or stabilizing the intermediate conformation. The principle of GEF dependent nucleotide exchange is depicted in Figure 3, using RhoGTPase-GEF interaction as an example.
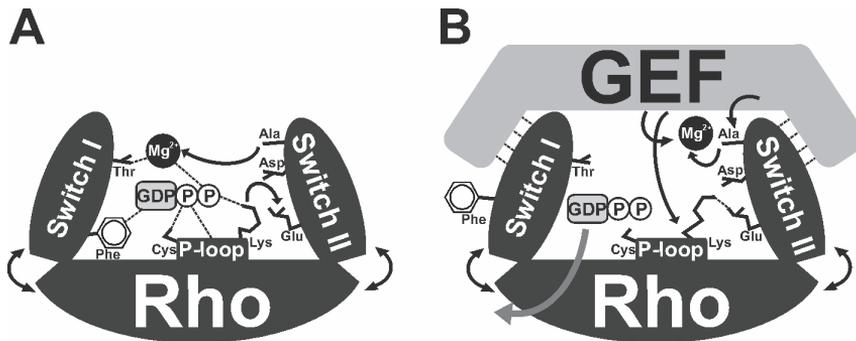
Figure 3. Guanine nucleotide exchange factor (GEF) catalyzed GDP dissociation. (A) Rho-GTPase is normally in GDP-bound inactive form. P-loop together with switch I and II are responsible for nucleotide binding in magnesium (Mg^{2+}) dependent manner. (B) GEF interacts with RhoGTPase switches and with Mg^{2+} by changing the GTPase conformation and enabling GDP dissociation.

GEF proteins are regulated at four levels; 1. localization, 2. intramolecular inhibitory sequence, 3. PPI, and 4. GEF activity down-regulation (Schmidt and Hall, 2002). The localization is controlled by activation of cell surface receptors, through regulating domains (e.g. pleckstrin homology (PH)), and with nuclear import and export signals (Prokopenko et al., 1999; Tatsumoto et al., 1999; Bustelo 2000; Cherfils and Zeghouf, 2013). The intramolecular inhibition usually occurs through GEF autoinhibition (Schmidt and Hall, 2002). In the third state the PPI can control GEF activity independently by oligomerization of the GEFs or through interaction of different domains in the GEF and the GTPase (Hart et al., 1998; Zhu et al., 2001). Finally, the GEF activity is down-regulated through GEF inhibitors or ubiquitination/degradation (Bustelo et al. 1997; Nielsen et al., 1997).

RasGEFs

There are around 30 known RasGEFs containing the Cdc25 homology domain. Usually the catalytic Cdc25 domain is in combination with a stabilizing Ras exchange motif (REM). Cdc25 is by far the most widely studied GEF domain and it can activate Ras, Rap, and Ral (Cherfils and Zeghouf, 2013). Other domains, e.g. Ras/Rap1-associating domain and pleckstrin homology (PH) domain, allow the protein-protein and protein-lipid interactions (Liao et al., 1999). RasGEFs are mostly localization controlled through lipid products (Caloca et al., 2003). Autoinhibition, membrane targeting, and lipid sensing regulatory segments also play important roles in RasGEF control (Jun et al. 2013).

RhoGEFs

The GTPase subfamily with the largest number of identified GEFs is the RhoGTPases (around 80). There are three main catalytic domain in RhoGEFs, Dbl homology (DH), DOCK, and PRONE. In most RhoGEFs, a tandem domain defines the catalytic and the targeting domains (PH), and forms a minimal structural unit to promote nucleotide

exchange (Schmidt and Hall, 2002; Rossman et al., 2005). The DH includes three homology regions that form the interaction pocket. The PH domain mostly modulates membrane binding and is located adjacent to the C-terminus of the DH domain, but also can participate in the GTPase binding (Schmidt and Hall, 2002; Rossman et al., 2002). The RhoGEF family containing Dock Homology Region domain (DHR) is comprised of 11 members. DHR and DH domains are unrelated but both controls Rho activation (Cote and Vuori, 2007; Rossman et al., 2005). DOCKs mostly control Rac and Cdc42. In plants, PRONE domain-containing RhoGEFs are dominant and works similarly to DH containing GEFs, even though the domains are unrelated (Thomas et al., 2007).

ArfGEFs

The Sec7 domain is the characteristic catalytic domain in ArfGEFs. The number of known ArfGEFs is approximately 20, but their function is less defined than the functions of Rho- or RasGEFs (Casanova, 2007; Donaldson and Jackson, 2011). ArfGEFs are divided into five groups, from which four families are related, but the fifth family has no sequence homology (Richardson et al., 2012; Zhai et al. 2012). ArfGTPases undergoes exceptionally large structural rearrangement during membrane-location and activation. In the nucleotide exchange, the membrane acts as cofactor simultaneously ensuring the correct membrane attachment. Even less is known about ArfGEF other than the Sec7 domain containing proteins. Also no GEFs for Arl and Arp have been described (Cherfils and Zeghouf, 2013).

RabGEFs

RabGEFs are a structurally diverse group of proteins and their size varies from small proteins to multiprotein complexes. Also their mechanisms behind nucleotide exchange occur through unrelated structural changes, and their activities vary significantly (Lee et al., 2009; Stein et al., 2012). The group of over 50 RabGEFs is divided into four types of unrelated GEF groups (Hutagalung and Novick, 2011). The most common catalytic domain for RabGEFs is Vsp9. Another conserved catalytic domain is DENN, and together with Vps9 these are mostly the activators for endocytic Rabs and their specificities are partly overlapping (Vázquez-Martínez and Malagón, 2011; Cherfils and Zeghouf, 2013). The nucleotide exchange mechanism for the Vps9 domain-containing GEFs utilizes the catalytic glutamine like ArfGEFs. The DENN domain protects the switch I but does not modify the nucleotide binding site.

RanGEFs

The single RanGTPase has one extremely effective GEF protein called RCC1 (regulator of chromosome condensation). This RanGEF performs a pivotal role in catalyzing the GDP dissociation by a mechanism in which switch I is not displaced. The difference is due to a 10-fold higher affinity of the Ran protein to GDP than GTP. The RanGEF binds both Ran-GDP and Ran-GTP forms (Moore, 1998; Seki et al., 1996; Moore, 1998).

2.1.3 GTPase activating proteins (GAPs)

The intrinsically slow GTP hydrolysis can be accelerated by large multidomain GAP proteins. The correct function of GAP is necessary to enable GTPase signaling by increasing the hydrolysis reaction in biologically relevant level. The catalytic GAP domain or domains (20-50 kDa) operate the catalytic function in GTP hydrolysis, which is a key reaction in many biological processes, e.g. protein synthesis, visual perception, vesicular transport, protein targeting, growth control, and differentiation (Bourne et al., 1990).
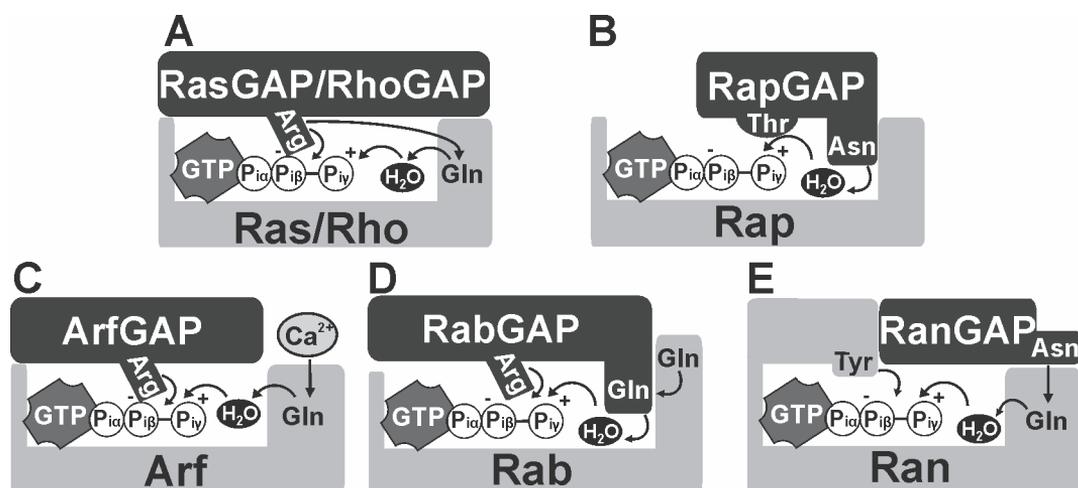
Figure 4. The GTPase activating protein (GAP) assisted GTP hydrolysis. The intrinsic GTP hydrolysis in GTPases is very slow, and GAP proteins accelerate the reaction in H_2O and Mg^{2+} dependent manner. The GTP hydrolysis mechanism with Ras/Rho (A), Rap (B), Arf (C), Rab (D), and Ran (E) GTPases varies but one or more important amino acids, arginine, glutamine, and/or asparagine, is involved in GTP hydrolysis.

The overall number of GAPs matches the number of GTPases. However, not all proteins with a GAP domain have been proved to exhibit hydrolysis activity (Brandt and Grosse, 2007). The arginine finger is the most important region in the GAP structure (Scheffzek et al., 1997). In the trimeric membrane G proteins, the arginine finger is located in Gα subunit. The catalytic mechanism in GAP-assisted GTP hydrolysis is not universal for all G-domain containing proteins, and some also work independent of arginine or glutamine in active site of the GAP domain (Figure 4). The hydrolysis mechanisms inside the GTPase families are roughly similar, exhibiting the bond cleavage between the γ- and β-phosphate. This occurs after GAP binding, when in-line nucleophilic attack of the γ-phosphate of GTP can take place in the presence of a water molecule (Cherfils and Zeghouf, 2013). The specificity profile of GAP proteins and the control of GAPs and GEFs are quite similar (Bernards and Settleman, 2004).

RasGAPs

There are approximately 15 proteins belonging RasGAP family, which can be divided as RasGAPs and RapGAPs. RasGAPs can be further divided into four and RapGAPs into three subfamilies (Ligeti et al., 2012). RasGAP mainly downregulates Ras function but it also works as intrinsic effector and has a role in Rho activation (Tocqué et al., 1997; Leblanc et al., 1998). In the basic form, the catalytic domain is located near the C terminus while the N terminus is essential for downstream signaling. Regulative domains mediate lipid interactions, Ras-induced transformation, and interactions with proteins (Musaccihio et al., 1992; Clark et al., 1993; Lemmon, 2005; Sot et al., 2013). A characteristic arginine-finger can be found in RasGAPs (Figure 4A). The arginine-finger stabilizes the negative charge development at the GTP phosphate group and serves as a helper in Ras switch II glutamine positioning (Glennon et al., 2000). RapGAPs do not use an arginine-finger, as this is replaced by threonine (Figure 4B). The reaction is mediated through catalytic asparagine working similarly as the glutamine-finger in the RabGAPs (Scrima et al. 2008).

RhoGAPs

The large RhoGAP family comprises over 70 known proteins containing RhoGAP domain (Xu et al. 2013). RhoGAPs have a number of functional domains enabling interactions with Rho and effector molecules. The RhoGAP domain shares over 20% sequence homology among family members, especially in catalytic arginine-finger region (Moon and Zheng, 2003; Rittinger et al., 1997). RhoGAPs shares the same basic catalytic GTP hydrolysis mechanism as RasGAPs (Figure 4A). Most of the RhoGAPs displays activity towards more than one RhoGTPase members (Tcherkezian and Lamarche-Vane, 2007).

ArfGAPs

There are at least ten ArfGAP subfamilies in the human genome (Cukierman et al., 1995). Like ArfGEF, ArfGAPs are also a structurally diverse group, and their size varies from small proteins to large complexes. The ArfGAP domain contains an architectural zinc-finger motif and catalytic arginine (Figure 4C) (Goldberg, 1999; Scheffzek et al., 1998). The role of ArfGAPs is not only catalytic, but it is also important in membrane trafficking (Nie and Randazzo, 2006). The localization and regulation of ArfGAP is not well understood, but the function of ArfGAP seems to be specific to Arf over Arl or Sar (Inoue and Randazzo, 2007; Kahn et al., 2008).

RabGAPs

RabGTPases are less dependent on GAP function than the other GTPases due to their prominent intrinsic GTP hydrolysis (Barr and Lambright, 2010). A multitude of RabGAPs have been identified, of which most contain a catalytic TBC domain working in parallel to those of Ras- and RhoGAPs (Pan et al., 2006). However, a single non-TBC GAP has also

been identified (Clabecq et al., 2000). In TBC domain-containing RabGAPs, there is arginine-finger and additional glutamine-finger dual structure in the catalytic site (Figure 4D) (Pan et al., 2006). The additional finger functions similarly to the asparagine moiety in RapGAPs (Scrima et al. 2008). However, it should be pointed that the dual-finger structure is not always found in TBC domain-containing GAPs.

RanGAPs

In RanGTPases, GTP hydrolysis mechanism differs from other GTPases involving the RanGAP and Ran-binding proteins (RanBPs) (Bischoff et al., 1995). The RanBP itself cannot mediate the hydrolysis but works as a coactivator of RanGAP by relieving the inhibition caused by the C-terminus of Ran (Bischoff et al., 1995; Seewald et al. 2002). Because RanGEF (RCC1) is located in the nucleus and RanGAP is in the cytosol, gradient is created across the nuclear pore (Görlich and Kutay, 1999). The RanGAP function does not utilize the arginine-finger, but Ran itself provides the machinery for hydrolysis (Figure 4E). The glutamine residue in Ran has a crucial importance in GTP hydrolysis and the RanGAP function by stabilizing the switch II region and positioning the catalytic glutamine (Seewald et al. 2002). The mechanism is related to what has been found with Gα proteins (Tesmer et al., 1997).

2.1.4 GDP dissociation inhibitors (GDIs)

The guanine nucleotide dissociation inhibitors (GDIs) are the third major class of GTPase regulatory proteins. GDIs work as down-regulators for Rho- and RabGTPases, typically preventing the nucleotide exchange and membrane association (Dovas and Couchman, 2005). The GDI possess three functions; 1. inhibition of nucleotide exchange, 2. inhibition of GTP hydrolysis, and 3. inhibition of GTPase binding to membrane (Figure 5).

Mammalian RhoGDIs are divided in three groups; RhoGDIα, RhoGDIβ, and RhoGDIγ (DerMardirossian and Bokoch, 2005). GDP dissociation inhibition preventing the GEF binding to Rho is the main GDI function (Ueda et al., 1990). The hydrolysis inhibition and the affinity to Rho-GTP is condition dependent and less understood (Sasaki et al., 1993; Chuang et al., 1993). However, it is known that the GDIs can also interact with Rho-GTP and prevent both GAP induced and intrinsic hydrolysis. GDI can also maintain the Rho in the cytosol by harboring the membrane-targeting moiety within the hydrophobic pocket (Keep et al., 1997). In all cases, the GDI function is regulated by phosphorylation (DerMardirossian and Bokoch, 2005; DerMardirossian et al., 2006). Large RabGDIs perform the same function as RhoGDIs without any structural similarity (Boguski and McCormick, 1993; Keep et al., 1997). In the case of Rab, the inhibition of the membrane association is the primary GDI function. RabGDIs are controlled by phosphorylation, but also with GDI-displacement factors (GDF). GDFs function by releasing Rab from GDI in endosomal Rabs (Dirac-Svejstrup et al., 1997; DerMardirossian and Bokoch, 2005).
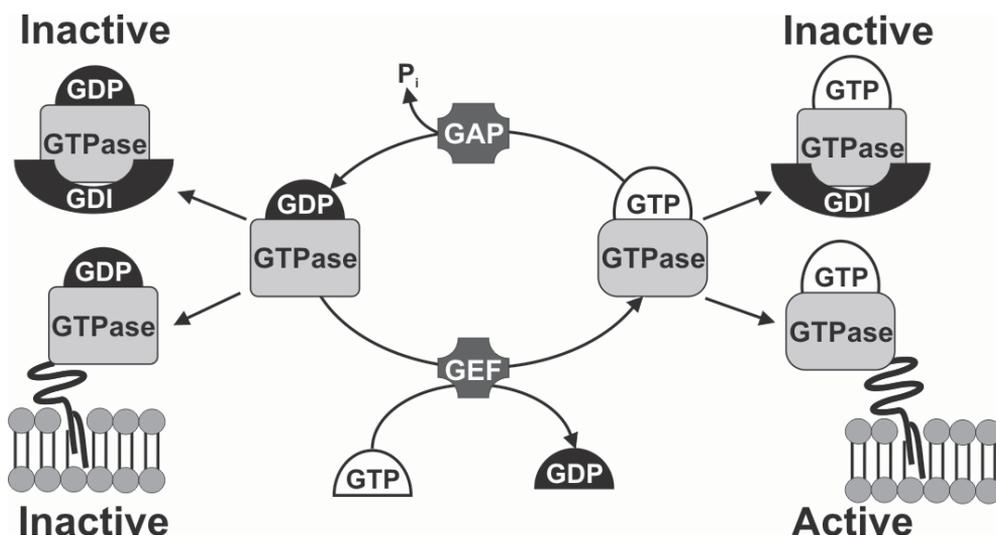
Figure 5. GDP dissociation inhibitor (GDI) function in the GTPase cycle. GDI proteins are GTPase down-regulators, which prevents mostly the GTPase membrane anchorage and/or nucleotide exchange. GDIs prefer binding to GDP-bound GTPase but it can interact also with active GTP-GTPase. GDI has been described to Rho and Rab family GTPases.

δ subunit of retinal rod phosphodiesterase (PDEδ)

The δ subunit of retinal rod phosphodiesterase (PDEδ) display GDI-like function solubilizing farnesylated Ras isoforms and enhancing the diffusion in the cytoplasm (Chandra et al., 2012). PDEδs are not normally counted as a GDI but in the group of GDI-like solubilizing factor. PDEδ possess GDI-like pocket which can trap farnasylated Ras into cytoplasm but also concentrate depalmitoylated Ras at the Golgi (Hanzal-Bayer et al., 2002; Chandra et al., 2012). The PDEδ interact normally with the prenyl-moiety which is often found in Ras and Rap. However, prenylation is not essential for GTPase binding and PDEδ can interact also with non-farnasylated ArlGTPases (Nancy et al., 2002).

2.1.5 GTPases in cancer and other diseases

Disturbances in GTPase signaling can result severe disease states. The most common disease is mutational activation of Ras, which is found in over 30% of human cancers (Karnoub and Weinberg, 2008). This high diseases relevance makes GTPases attractive pharmacological targets, but so far no successful Ras inhibition strategies are in clinical use. GTPases are strictly regulated by GEF and GAP proteins which can also be mutated, but are often classified as undruggable targets (Vigil et al., 2010). However, a useful inhibition strategy will probably require combinatory pathway blocking and tailored Ras isoform and mutation specific therapeutic approaches (Vigil et al., 2010; Mattingly, 2013; Cox et al., 2014).

Cancer

Overactivity of GTPases is the most common cause of disease. Cancers are frequently linked to mutations of RasGTPases at three loci, Gly12, Gly13, and Gln61 (Prior et al., 2012). K-Ras mutations are the predominant form in cancer and occur usually in early states of tumor progression. N-Ras and H-Ras are found in less than 10% of all cancers and there is clear link between the cancer type and the mutated Ras subfamily (Fernández-Medarde and Santos, 2011). Other mutated GTPases than Ras are rarely found in cancer (Vigil et al., 2010). Ras mutations usually occur early in the tumor progression and the continued mutant Ras expression is cruzial for tumor maintenance and linked to poor prognosis. Mutations cause constantly active GTP-Ras population due to impared GAP stimulation (Bos et al., 2007; Cox et al., 2014). K-Ras mutations are most often found in biliary tract, intestine, and pancreas cancers. N-Ras linked cancers are located in hematopoietic and lymphoid tissue, skin, and intestine. H-Ras mutations are found in urinary track and salivary gland cancers (Karnoub and Weinberg, 2008; Fernández-Medarde and Santos, 2011). Ras mutation frequency in cancer and the isoform-specificity of the point mutations are listed in Table 1.

Table 1. Ras mutations and isoform-specific point mutations in human cancer. Data represent overall and Ras isoform-specific mutation frequency in selected cancers, and most frequent point mutations based on COSMIC database (Prior et al., 2012; Cox et al., 2014).

Cancer	% K-Ras mutation frequency	Point mutation	% N-Ras mutation frequency	Point mutation	% H-Ras mutation frequency	Point mutation	% Ras mutation frequency	Most mutated gene
PDCA	100	12D, 12V	0	-	0	-	97.7	KRas
CRC	86	12D, 12V, 13D	14	-	0	-	52.2	APC
MM	55	12A, 12D, 61H	45	61K, 61R	0	-	42.6	NRas
LAC	96	12C, 12V, 12D	3	-	1	-	32.2	EGFR
SCM	3	-	94	61R, 61K	3	-	29.1	BRaf
UCEC	84	12D, 12V	14	-	2	-	24.6	PTEN
TC	8	12D, 12C, 13D	65	61K, 61R	27	61R, 61K, G12V	12.5	BRaf
AML	27	12D, 13D, 12V	59	12D, 13D	14	-	11.4	NPM1
BUC	33	12D, 12V	13	-	54	12V, Q61R	10.6	TERT
HNSCC	9	12D, 12V, 12C	5	-	86	12V, 61L	5.5	NOTCH1

Abbreviations: AML, Acute myeloid leukaemia; APC, Adenomatous polyposis coli; BUC, Bladder urothelial carcinoma; CRC, Colorectal adenocarcinoma; EGFR, epidermal growth factor receptor; HNSCC, Head and neck squamous cell carcinoma; LAC, Lung adenocarcinoma; MM, Multiple myeloma; NPM, Nucleophosmin; PDCA, Pancreatic ductal adenocarcinoma; PTEN, Phosphatase and tensin homolog; SCM, Skin cutaneous melanoma; TC, Thyroid carcinoma; TERT, Telomerase reverse transcriptase; UCEC, Uterine corpus endometrioid carcinoma.

In the Rho family, Rac1 mutations have been found in melanomas and RhoGEF mutations in various cancers (Wertheimer et al., 2012; Davis et al., 2013). Both Rac and RacGEF overexpression and mutations play a more central role in cancer progression than cancer development (Parri and Chiarugi, 2010; Vigil et al., 2010; Wertheimer et al., 2012). Deleted, silenced or methylated RhoGAP (DLC1) has also been linked to cancer (Vigil et al., 2010). ArfGTPases are regulators of the proliferative and invasive properties of cancer cells (Boulay et al., 2008; Muralidharan-Chari et al., 2009). Arf plays a role in modulating Rho dependent signaling, which can also lead to cancer. Overexpression of ArfGEFs and ArfGAPs has also been implicated in oncogenesis (Ha et al., 2008; Tsai et al., 2012). ArfGAP overexpression in cancer makes cells apoptosis resistant (Vigil et al., 2010). Elevated expression of Rab gene has been found in several cancers and Rans plays a role in cancer development, aggressiveness, progression, and prognostic (Cheng et al., 2005; Abe et al., 2008; Yuen et al., 2012). Ran silencing-induced apoptosis could be potential way to treat cancer even the mutation is in some other GTPase family (Yuen et al., 2012).

RASopaties

In addition to cancer, defective GTPase signaling has been linked to immunological and inflammatory disorders. These defects are termed as RASopathies (Fernández-Medarde and Santos, 2011; Smith et al., 2013). Neurofibromatosis type 1 (NF-1), Noonan syndrome, Costello syndrome, leopard syndrome, cardiofaciocutaneous syndrome, legius syndrome, and other developmental disorders belong to this group of diseases (Karnoub and Weinberg, 2008; Fernández-Medarde and Santos, 2011).

NF-1 is an autosomal dominant disease caused by mutation of the *NF1* gene (Fernández-Medarde and Santos, 2011). NF-1 is Ras activity controlling GAP protein and its mutation causes the loss of NF-1 function (Cichowski and Jacks, 2001). Capillary malformation-arteriovenous malformation is also caused by overactive Ras due to another mutated GAP protein (p120GAP), and predisposes the patient for cancer (Aoki et al., 2008). Legius syndrome is related to NF-1 but also another disorder, Noonan syndrome. Legius syndrome is caused by a mutation of Raf (Fernández-Medarde and Santos, 2011). In hereditary gingival fibromatosis type 1 the son-of-sevenless (SOS) GEF protein is overactivated due to a frame-shift mutation (Hart et al., 2002). Leopard syndrome and Noonan syndrome are related to mutations in the gene producing non-receptor tyrosine kinase phosphatase. There are also *Raf* gene mutations found in leopard syndrome and *Ras*, *SOS*, *Raf*, *MEK* and *SHOC* gene mutations in Noonan syndrome. Both diseases are linked to cardiac defects and developmental abnormalities (Karnoub and Weinberg, 2008; Fernández-Medarde and Santos, 2011). Leopard syndrome and Noonan syndrome share many phenotypical characteristics with cardiofaciocutaneous syndrome (Tidyman and Rauen, 2008). In Costello syndrome, two glycine's in positions 12 and 13 in H-Ras are mutated, leading to skin changes and susceptibility to cancer (Aoki et al., 2008).

Ras inhibition

There are no Ras inhibitors in the clinical use and thus Ras has been termed as "undruggable". However, improved understanding of Ras and advances in drug discovery has energized efforts to defeat Ras-driven cancers. Recently, multiple research programs have been established (Cox et al., 2014; Stephen et al., 2014). Ras signaling can be inhibited using direct inhibitors, interfering membrane association, targeting Ras downstream effectors, or using synthetic lethal interactors. Collection of promising inhibitors is listed in Table 2.

Table 2. Selected collection of potential Ras or Raf targeting inhibitors. Ras activation can be targeted directly or by affecting the membrane association or downstream effector activation (Maurer et al., 2012; Sun et al., 2012; Baker and Der, 2013; Hocker et al., 2013; Cox et al., 2014).

Strategy	Function	Target in screening	Drug	Disease
Nucleotide exchange inhibition	SOS1-mediated nucleotide exchange	K-Ras 4B	DCAI	
		H-Ras	HBS3 peptide	
		K-Ras	BZIM	
		K-Ras	0QY and 0QX	
		K-Ras	SRJ23	
	Mutation specific nucleotide exchange	K-Ras G12C	SML-8-73-1	
		K-Ras G12C	Shokat compound	
Membrane association blocking	Farnesyltransferase inhibitor	Ras	Lonafarnib	Progeria
		Ras	Tipifarnib	AML
	Ras farnesylcysteine mimetic		Salirasib	PC
	PDEδ inhibition	K-Ras	Deltarasin	
Raf effector inhibition	Ras-Raf interaction inhibition	H-Ras	IND12	
		C-Raf	Kobe 0065 and Kobe 2602	
		H-Ras and C-Raf	MCP1	
		B-Raf	Vemurafenib	Melanoma
		Raf[1]	Sorafenib	RCC, HCC, TC

Abbreviations: AML, Acute myelogenous leukemia; HCC, Hepatocellular carcinoma; PC, Pancreatic cancer; PDE δ, δ subunit of retinal rod phosphodiesterase; RCC, Renal cell carcinoma; TC, Thyroid cancer.
[1] Multikinase inhibitor

The picomolar guanosine nucleotide binding and the lack of sufficient binding pocket in the Ras surface complicates the discovery of effective direct Ras inhibitors. Ras isoform specific post-translational modifications further complicate the Ras inhibition, and thus effector inhibition has so far showed the greatest promice. The use of farnesyltransferase inhibitors (FTIs) were the first highly studied strategy to block Ras activity. FTIs block the

H-Ras membrane association, but unfortunately cannot work with N-Ras or K-Ras which are modified also by geranylgeranyltransferases (Liu et al., 2010). Other strategies to interfere Ras membrane association are based on mimiting Ras C-terminal modification; lipid modifications (palmitoylation/depalmitoylation), CAAX-sequence processing enzymes, or post-translational modifications affecting Ras localization (Marom et al., 1995; Barcelo et al., 2013; Cox et al., 2014). In last few years, low affinity Ras binding inhibitors blocking the nucleotide exchange or Raf binding have been introduced (Maurer et al., 2012; Sun et al., 2012; Hocker et al., 2013; Cox et al., 2014). However, affinities for these inhibitors are too low to be sufficient for clinical testing. Maybe the most promising strategy to affect Ras signaling is Ras effector inhibition. Approximately thirty inhibitor affecting Raf-MEK-ERK-pathway are currently in clinical studies, but the complexity of the signaling network has challenged researchers (Morris et al., 2013). According to present knowledge, blocking of multible kinase cascade nodes is needed to provide effective inhibition. Other major pathway which inhibition is in clinical evaluation is PI3K-AKT-mTOR (Cox et al., 2014). Combined strategies to block Raf-MEK-ERK- and PI3K-AKT-mTOR-pathways have already reached clinical trials (Britten, 2013). Also Ral and Rac1 inhibitors have shown some potential (Cox et al., 2014). Genes with synthetic lethal interactions with mutant Ras have also been identified, but they usually are tissue-specific and univeral interactors might be impossible to find (Cox et al., 2014).

2.2 GTPase preparation for *in vitro* GTPase assays

In the natural environment, GTPases are always bound to a nucleotide. However, most of the *in vitro* assays developed to study GTPases cannot be performed directly with GTPase in its natural state. Thus the preparation of nucleotide free GTPase or GTPase preloaded with a nucleotide analog is needed (Tucker et al., 1986; John et al., 1989; Smith and Rittinger, 2002).

2.2.1 GTPase expression and purification

Nowadays, GTPases are usually expressed from plasmids in bacterial production systems, usually the *E. coli* BL21 strain. Because large amounts of high purity proteins are required, the production and purification needs to be optimized. Although the optimal conditions vary between proteins, the basic protocols are similar (Eberth and Ahmadian, 2009). Molecular tags, such as glutathione S-transferase (GST) and hexahistidine, enable the efficient purification (Smith and Rittinger, 2002). The GST-fusion tag works with most of the GTPases and their regulatory proteins and it can be enzymatically cleaved after purification. Cleavage is often needed to decrease the size. The GST-tag can, however, be utilized for pull-down assays using glutathione (GSH) beads (Surviladze et al., 2010). The hexahistidine-tag is smaller than the GST-tag, and it does not require removal. The purification of GTPases and their regulators is normally executed in a multistepped process in which *E. coli* cells are lysed and the protein of interest is isolated. The final purification

is usually performed using gel filtration, and proteins are concentrated (10-20 mg/ml) with ultrafiltration (Eberth and Ahmadian, 2009). Suitable storage conditions are critical to preserve protein activity and to avoid rapid loss of activity at room temperature or due to freeze-thaw cycle (Gibbs et al., 1984). Thus, snap-freezing of these proteins is a mandatory step (Eberth and Ahmadian, 2009). In addition, GTPases undergo intrinsic hydrolysis and thus GTP loaded GTPases cannot be stored for long periods (Schlicting et al., 1989; French et al., 1994).

2.2.2 Preparation of nucleotide-free and luminescent nucleotide-bound GTPases

Most of the methods used to study GTPases *in vitro* require nucleotide free GTPase, which can then be preloaded with sufficient nucleotide analog. Activity assays are usually made with purified proteins. In cell lysate, problems can arise due to insufficient protein concentrations, protein complexes, and multiple regulators (Eberth and Ahmadian, 2009).

The ethylenediaminetetraacetic acid (EDTA)/Mg^{2+}-system was the first method used to create GTPases loaded with a desired nucleotide (John et al., 1989). The method is based on the ability of GTPase to bind nucleotides in a magnesium dependent manner (Burstein and Macara, 1992; John et al., 1993; Zhang et al., 2000). Magnesium chelation using EDTA depletes the nucleotide from the GTPase, enabling the purification of nucleotide free GTPase or GTPase reloading. Unfortunately, nucleotide free GTPases lose their activity far more easily than nucleotide loaded GTPases (John et al., 1990). Nucleotide free "apoproteins" can however be stabilized using guanine-5'-monophosphate (GMP) and/or guanosine (John et al., 1990). With the early protocols it was only possible to generate small quantitates of nucleotide free GTPase (Purich and MacNeal, 1978; Feuerstein et al., 1987; John et al., 1990). Nowadays, the alkaline phosphatase based method can provide large quantities of nucleotide free GTPases. The system is based on GDP degradation to GMP, inorganic phosphate (P_i), and guanosine. GMP and guanosine have very low affinities to GTPase, and GDP or GTP can instantly replace it (Eberth and Ahmadian, 2009). To enable quantitative nucleotide exchange using the EDTA/Mg^{2+} system, a 50-100-fold excess of nucleotide is needed, whilst with alkaline phosphatase the need is only 10-fold (Smith and Rittinger, 2002).

2.3 Non-luminescence based methods for functional GTPase study

The traditional way to study GTPases is by the heterogeneous binding assay. These assays provide a robust and background-independent way to study nucleotide exchange and effector binding in different assay matrixes. However, the usefulness of these assays is limited due to a number of drawbacks, i.e. high material consumption and the laborious protocol. In this section non-luminescence or label-free GTPase assays techniques are introduced.

2.3.1 High-performance liquid chromatography (HPLC)

Heterogeneous methods like high-performance liquid chromatography (HPLC) rely on an efficient separation step. HPLC can be used to determine GTP hydrolysis, although, as in other heterogeneous assays, the hydrolysis kinetics cannot be monitored. Label-free GTP hydrolysis monitoring using HPLC is GTPase family independent low throughput method (Ahmadian et al., 1999; Hemsath and Ahmadian, 2005). The HPLC separation can be performed using ion-exchange HPLC or reverse-phase HPLC (Smith and Rittinger, 2002). With reverse-phase HPLC, a problem arises from hazardous acetonitrile. In ion-exchange HPLC, the high salt concentration can harm the equipment or change the reaction (Smith and Rittinger, 2002). HPLC assays can be performed without labels using absorbance monitoring or with radioactive labels (Rubio et al., 2004). HPLC methods rely on GDP and GTP separation and nucleotide absorbance detection (254 nm). The nucleotide separation in HPLC is due to phosphate group induced effect, and the retention time increases along with the number of phosphates. Unfortunately, the UV absorbance spectrum of nucleotides partially overlaps with that of proteins, and thus the presence of GTPases must be taken account (Smith and Rittinger, 2002). In GTP hydrolysis assays the most severe problem is free GTP. GTPases needs to be preloaded with a high excess of GTP, part of the GTP stays unbound and cannot be hydrolyzed. Bound and non-bound GTP cannot be separated in label-free HPLC assays, but in radioactivity based HPLC methods the problems from GTPase and free GTP can be avoided (Will et al., 2001; Rubio et al., 2004).

Similarly to HPLC, thin layer chromatography (TLC) enables separation with equal sensitivity (Rubio et al., 2004). All separation methods can be used together with radiolabels, but without radiolabels, efficient separation is needed and thus HPLC is usually the method of choice. High-performance capillary electrophoresis (HPCE) possesses certain advantages over HPLC in nucleotide separation (Ng et al., 1992). The resolving power of HPLC and HPCE are equal, but HPCE can be performed with lower sample volumes and shorter analysis times (25 min). Also the columns used in HPCE are superior to HPLC columns (Ng et al., 1992). The separation efficiency, detector sensitivity, and GTPase studied, determine the amount of GTPase, regulator, and nucleotide needed. When assays to study intrinsic and GAP induced GTP hydrolysis are compared, sample and reagent requirements are totally different. However, in all cases high micromolar concentrations of protein and nucleotide are required.

Absorbance

Direct absorbance from nucleotides is used in HPLC-based methods for GTP hydrolysis detection, but normally the direct nucleotide absorbance is not high enough. Other absorbance based methods are usually based on P_i detection using Malachite Green (MG) or enzyme coupled assays. Colorimetric P_i detection using MG is based on the reaction between molybdate and P_i at a low pH (Itaya and Ui, 1966). In acidic conditions the

reaction produces phosphomolybdate, which in complex with MG changes the MG color from brown to green (640-650 nm). Although colorimetric assays, have been fairly successfully used for screening, they have a rather low sensitivity (Quan and Robinson, 2005; Monroy et al., 2013). Enzyme coupled assays, on the other hand, are not suitable for screening due to their high costs. Using 2-amino-6-mercapto-7-methylpurine ribonucleoside and purine-nucleoside phosphorylase the GTP hydrolysis is detectable from the observation of an increased absorbance (360 nm) in the presence of P_i (Webb, 1992; Webb and Hunter, 1992).

2.3.2 Nucleic magnetic resonance (NMR)

Nucleic magnetic resonance (NMR) imaging provides an efficient tool for label-free GTPase research. NMR can be used with unmodified GTPases and thus disturbances from labels are avoided. Recently, the throughput attainable with NMR has increased and today GTPase assays can be performed in a routine fashion.

NMR has given a new perspective to GTPase research, providing a real-time reaction and structure change monitoring platform (Marshall et al., 2012). Most GAPs and GEFs are large multidomain proteins and thus most *in vitro* assays are performed with truncated and more soluble proteins. In assays with truncated proteins, inputs involving, e.g. autoinhibition, phosphorylation, and changes in subcellular localization are lost. Real-time NMR utilizes radioactively labeled GTPases by monitoring nucleotide-dependent structural changes over time, independently to GTPase or GTPase reaction (Marshall et al., 2009; Mazhab-Jafari et al., 2010; Marshall et al., 2012).

The most widely used two-dimensional heteronuclear NMR spectroscopy relies on monitoring nucleotide-induced changes from chemical shifts of each amino acid residue (Marshall et al., 2009). The overlay of 2D heteronuclear single quantum coherence spectra with ^{1}H/^{15}N is first created with active and inactive GTPase. The changes in active and inactive state reveal chemical-shift perturbations. These changes caused by chemical environment and protein structure can be used to determine kinetic information or mutation induced effects (Smith et al., 2013; Smith and Ikura, 2014). Defects involving expression, catalysis, allosteric regulation, and membrane targeting are distinguishable.

NMR is relative fast, and a single measurement takes from tens of seconds to tens of minutes. However, required millimolar protein concentration particularly limits the suitability of NMR in large scale assays (Marshall et al., 2009; Marshall et al., 2012). One advantage is that the real-time NMR is cell lysate compatible, enabling the study of full-length GTPases in their natural environment (Marshall et al., 2012). However, membrane-tethered GTPases are not sensitively detected by NMR, and assays with living cell are impossible (Marshall et al., 2012). NMR-based fragment screening is widely used due to

its easy automation and the ability to obtain high-resolution structures (Palmioli et al., 2009; Patrigi et al., 2011; Maurer et al., 2012; Sun et al., 2012; Gao et al., 2014).

Time-resolved Fourier transform infrared spectroscopy

Time-resolved Fourier transform infrared (TR-FTIR) difference spectroscopy is also applied to monitor GTPase reaction mechanisms (Gerwert, 1993; Cepus et al., 1998; Allin et al., 2001). TR-FTIR is advantageous for GTPase reaction studies because no crystallization is needed and the resolution is at the atomic level (Cepus et al., 1998; Allin et al., 2001). This is a major advantage compared to X-ray crystallography and NMR, which require solubilized proteins. TR-FTIR is used to reveal the GTP hydrolysis mechanisms, and the involvement of PPI in hydrolysis (Allin et al., 2001). High resolution is crucial to understand enzymatic reactions as well as detailed changes in GTPase geometry and charge distribution. The detailed understanding of the enzymatic catalysis helps towards understanding the changes in GTP hydrolysis induced by oncogenic mutations (Rudack et al., 2012).

2.3.3 Radionucleotides

Radionucleotides have provided a sensitive platform traditionally used to examine GTP binding proteins. For example, the filter-binding technique enables assays with nanomolar GTPase concentrations, and radiolabel-based assays are still more sensitive than recently developed methods (Tucker et al., 1986). Radionucleotides usually bind to GTPase without interferences (Rensland et al., 1995; Mazhab-Jafari et al. 2010). With GTPases, radioligands like [^{3}H]GDP and [γ-^{32}P]GTP have been used in heterogeneous assay platforms to study nucleotide exchange and GTP hydrolysis. Unfortunately, a separation step is always needed when radioligands are used. The scintillation proximity assay (SPA), however, is an exception and provides a homogeneous radionucleotide based platform. Nevertheless SPA is a popular method in drug screening and heterotrimeric G protein research, GTPases are rarely studied with it (Glickman et al. 2008).

The most frequently used radioligand based assay is the heterogeneous nitrocellulose filter binding assay performed with [γ-^{32}P]GTP (Goldberg, 1999). This assay enables GTP hydrolysis determination and allows multiple turnovers. When hydrolysis occurs, the γ-^{32}P is cleaved and it passes through the filter providing low radioactive signal. The correct behavior in the assay is also easy to confirm using [α-^{32}P]GTP, which always stays in the membrane (Tanaka et al., 1991). Beside filters, charcoal based centrifugal separation is also useful (Ligeti et al., 1993). The charcoal separation is based on γ-^{32}P liberation in an aliquot of the clear supernatant. The difference between these separation techniques is in the scintillation monitoring performed either in membrane or in solution. However, their disadvantages, such as separation, extensive manual manipulation, low intrinsic precision, radioactive waste, and limited data collection are much the same (Lenzen et al., 1995).

The use of radioligands enables multiple turnover measurements of GTP hydrolysis, which are not usually achievable with luminescence based assays. With multiple turnovers using [γ-^{32}P]GTP, the signal change can be increased compared to single turnover (Kunzelmann et al., 2006). With the rapid quench flow apparatus, partial reaction kinetics can also be monitored. 5'-O-(3-[^{35}S] thiotriphosphate) ([^{35}S]GTPγS) is the unhydrolyzable GTP analog enabling permanent GTP association to the GTPase. [^{35}S]GTPγS is still in use especially in membrane based assays (Yamashita et al., 1988; Milligan, 2003; Harrison and Traynor, 2003). G-protein-coupled receptors (GPCR) linked to G$_\alpha$-proteins are studied dominantly using [^{35}S]GTPγS (Milligan, 2003; Harrison and Traynor, 2003). Unfortunately, radiolabels causes unwanted effects in cells, e.g. cell-cycle arrest and apoptosis, and thus cell membrane is used (Dover et al., 1994; Yeargin and Haas, 1995). Radioactivity is also considered as a risk, requiring strict protocols of exposure and waste handling. For these reasons, new luminescence based assays are replacing the old radioactivity based methods.

2.4 Luminescence based methods for functional GTPase study

The most important properties of an ideal GTPase cycle monitoring method are sensitivity and ability to monitor reaction kinetics. With luminescent nucleotide analogs, GTPase reaction kinetics can be monitored with reasonable sensitivity. The sensitivity in the luminescence based methods comes from the reporter luminophore, which exhibits a change (positive or negative) in the luminescence properties due to nucleotide/GTPase interaction. The kinetic data is usually produced with environmental-sensitive reporters attached to the nucleotide and/or GTPase structure. Unfortunately, luminescent reporters often disturb the GTPase reaction and thus different approaches have been developed. These problems arise because the reporter or reporters interfere with either protein-protein or protein-nucleotide interactions. In this section, luminescence based methods to study GTPases are introduced.

2.4.1 Luminescently labeled nucleotides

Luminescent GTP or GDP analogs are useful for GTPase research enabling homogeneous assays and kinetic monitoring. Methylanthraniloyl- (mant), tetramethylrhodamine (tamra), and BODIPY-labels conjugated on the 2'- or 3'-oxygens of the ribose are the most common, but labels for the 8- or N2-position of the guanine or γ-phosphate are also available (Figure 6).

Environment-insensitive labels, like lanthanide (Ln^{3+}) chelates, are also usable. A luminophore that will only conjugate with a single isomer of the nucleotide is preferred to avoid mixed kinetics. This property is highlighted in stopped-flow measurements where 2'-conjugation is preferred (Rensland, 1991; Klebe, 1995a; Klebe, 1995b). In an ideal case, the nucleotide analog behaves like a natural nucleotide, modification is tolerated, and signal change is sufficient (Scheffzek, 1998; Ostermann et al., 1999; Mazhab-Jafari, 2010).
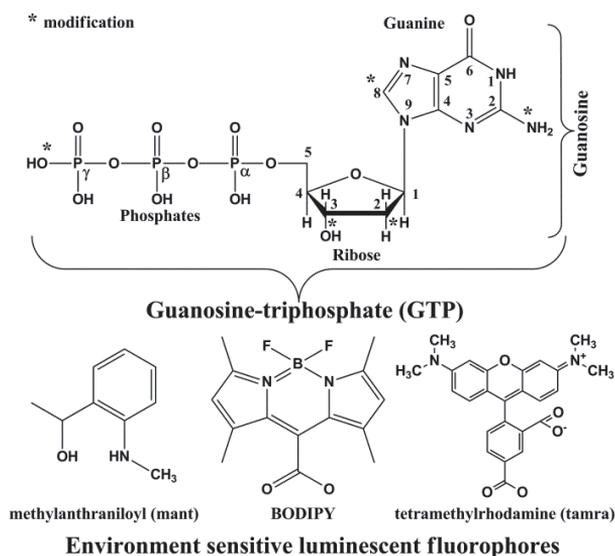
Figure 6. GTP and luminescent GTP analogs. GTP can be modified with different luminophores to create luminescent GTP analog. The most often used luminophores are environment-sensitive e.g. methylanthraniloyl (mant), BODIPY, and tetramethylrhodamine (tamra). Modifications (*) can be pointed either in 2'- or 3'-position in ribose, 8- or N2-point in guanine or in γ-phosphate.

Mant-conjugated nucleotides

Mant is an environmental-sensitive label that is by far the most widely used in GTPase studies (Neal et al., 1990). This is due the compact size of the luminophore (Figure 6). Usually the 2'-O- and/or 3'-O-isomers of a GDP or GTP are used for label conjugation (Hiratsuka, 1983; John at al., 1990). Mant-nucleotides are in less polar environment when bound to the GTPase leading enhancement in luminescence. This signal change enables nucleotide exchange reaction monitoring (Figure 7A) (Hiratsuka, 1983; John at al., 1990). To provide a strong relative increase in luminescence, the lower concentration limit for the mant is approximately ten nanomolar. Usually the selected mant as well as proteins concentrations are in micromolar range (Eberth and Ahmadian, 2009). The assay conditions also need to be optimized, e.g. by removing solid compounds and gasses, and adjusting measurement device correctly.

Mant-nucleotides can be used to monitor either slow or fast kinetics of the nucleotide exchange. The fast kinetics is determined with a rapid mixing method using stopped-flow equipment (Guo et al., 2005; Hemsath and Ahmadian, 2005). These methods usually require less protein than the measurement of slow kinetics (from a few μg to several mg) (Eberth and Ahmadian, 2009). A normal fluorescence spectrometer is sufficient to monitor slow kinetics and overall luminescence change, but it gives lower time resolution than stopped-flow equipment. Usually the assay is performed in a low throughput cuvette format, but a microtiter plate can also be used. Luminescence increase over 200% can be

monitored with Ras, but with EF-Tu, for example, the increase is modest (John et al., 1990; Wagner et al., 1995). The normal luminescence change in a GEF induced nucleotide exchange assay is from 40% to 60% at excitation and emission wavelengths of 366 and 450 nm, respectively (Eberth and Ahmadian, 2009). Mant-nucleotides also enable fluorescence polarization (FP) monitoring.

Not all GTPases tolerate modifications of the sugar moiety in the nucleotide (Ostermann et al., 1999). The 8-position modified nucleotides are an alternative, but the GTPase binding affinity is sacrificed. On the other hand, γ-modified nucleotides are not hydrolysable. The most widely used hydrolysis resistant GTP derivatives are 2'(3')-O-(N-methylanthraniloyl)-guanosine-5'-[β,γ-imido]triphosphate (mant-GppNHp) and 2'(3')-O-(N-methyl-anthraniloyl)-guanosine-5'-[γ-thio]triphosphate (mant-GTPγS). These analog are also useful in GTPase-effector interaction determination, because effectors usually bind to active GTP- GTPase (Haeusler et al., 2003). Modifications in the N2-position do not interfere with the hydrolysis or GTPase binding affinity (Noonan et al., 1991; Hoffenberg et al., 1996). N-2-(4"-N-methylanthraniloylaminobutyl)guanosine-triphosphate and N-2-(2"-N-methylanthraniloyl-aminoethyl)guanosine-triphosphate are N2-position labeled nucleotides, whose luminescence increase 25-95% upon binding (Ostermann et al., 1999).
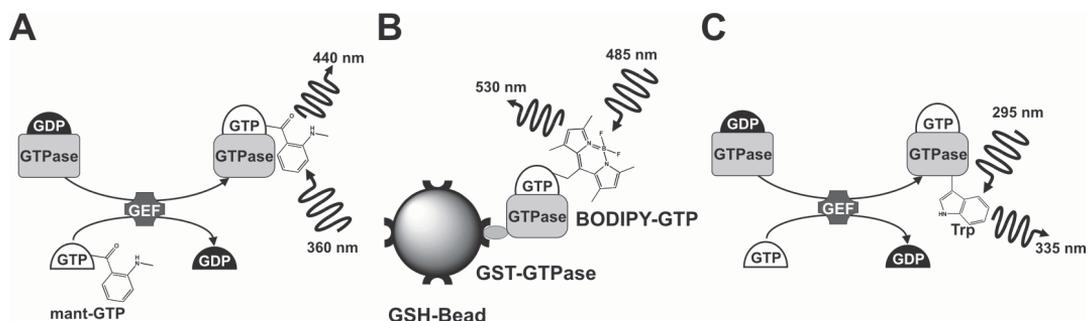
Figure 7. Luminescence based methods for GTPase activation monitoring using environment-sensitive labels. (A) Mant-labels luminescence changes during GTPase binding and the change can be monitored either directly or using anisotropy. (B) BODIPY-label enables similar GTPase assays as mant-label, which can be performed directly in solution or utilizing solid support. (C) Endogenous or introduced tryptophan (Trp) can also act as environment-sensitive reporter.

Mant-nucleotides are usually hydrolysable and can provided indirect data for Ras/GAP reactions, but the mant-GTP does not work for most GTPase/GAP pairs (Ahmadian et al., 1997a; Ahmadian et al., 1997b; Ahmadian et al., 2003). The mant-GTP signaling properties in GTP hydrolysis cannot be predicted from the hydrolysis mechanism (Scheffzek and Ahmadian, 2005). In the Ras/GAP assay with mant, GAP binding rather than GTP hydrolysis is monitored and thus other labels, e.g. tamra, are preferred (Ahmadian et al., 1997a; Ahmadian et al., 1997b; Eberth et al., 2005; Eberth and Ahmadian, 2009).

BODIPY-conjugated nucleotides

Environment-sensitive BODIPY-labels can be used similarly as mant-labels (Figure 7B). However, the nucleotide association induced luminescence increase is usually more prominent than with mant-label (Korlach et al., 2004; Goody, 2014). The most widely used BODIPY-label in GTPase assays is green-luminescent BODIPY FL. Other possibilities for GTPase assays are orange-luminescent BODIPY R6G, and red-luminescent BODIPY TR. In this context, all different color BODIPY-labels are called by the generic name BODIPY.

BODIPY-labels are usually attached to the γ-phosphate, but they can be coupled to 2'- and/or 3'-positions (Willard et al., 2005; Jameson et al., 2006). Conjugation to γ-phosphate has a major effect to the BODIPY-GTP affinity to GTPase, and it interferes with binding more than 2'- and/or 3'-position modifications. Also the γ-labeled BODIPY-GTP activated GTPase cannot interact normally with effectors (Korlach et al., 2004; Goody, 2014). The reduced BODIPY-GTP binding affinity especially complicates cell-based assays where it competes with the high concentration of free GTP (Goody, 2014). In GPCR and G_α studies, BODIPY-GTPγS is more useful, and also its binding affinity is less compromised (McEven et al., 2001; Willard et al., 2005). BODIPY-GDP and unhydrolyzable GTP analogs, BODIPY FL-GMPPNP and BODIPY-GTPγS, have also been used to study GTPases (Korlach et al., 2004; Xiong et al., 2012; Goody, 2014).

Tamra-conjugated nucleotides

A less frequently used luminescent nucleotide analog is 2'(3')-O-(N-ethylcarbamoyl-(5''-carboxytetramethylrhodamine)amide)-GTP (tamra-GTP) (Eberth et al., 2005). Unlike other luminescent analogs, tamra-GTP enables efficient GTP hydrolysis assays sensing the conformational change in the GTPase structure (Eberth et al., 2005; Hemsath and Ahmadian, 2005). Tamra-labels are monitored at higher wavelengths (546 nm excitation and 583 nm emission) than other environment-sensitive labels. In addition, the protein consumption in GTP hydrolysis assays is less than one tenth with tamra-GTP compared to HPLC-based assays (Eberth and Ahmadian, 2009).

Mant-nucleotides cannot provide a prominent luminescence increase in nucleotide exchange with all GTPases, and especially in GTP hydrolysis assays, the luminescence signal decrease is only modest (Ostermann et al., 1999). Tamra-GTP enables kinetic measurements with some of the GTPases that are insensitive to mant (Eberth et al., 2005). Hydrolysis monitoring with mant-nucleotides is based on the change in luminescence when Ras-GAP interaction occurs (Scheffzek et al., 1998; Ostermann et al., 1999). With tamra-GTP, luminescence decrease during the hydrolysis is measurable also with Rho, but not with all GTPases (Eberth et al., 2005). The decrease in tamra's luminescence in GTP hydrolysis is due the lost interaction between tamra and GTPase (Eberth et al., 2005). With Ras or Rho, the decrease in tamra's luminescence is 10-15% (Eberth and Ahmadian, 2009).

Other environment-sensitive labels

Mant, BODIPY, and tamra dominate GTPase assays performed with environment-sensitive labels. However, other nucleotide analogs, e.g. 2'(3')-O-(2,4,6-trinitrocyclohexa-dienylidene)-GTP (TNP-GTP) have also been applied to study GTPases (Hiratsuka, 1985; Hiratsuka, 2003). Like all environment-sensitive labels, TNP enables real-time reaction monitoring based on luminescence signal increase during the nucleotide association to GTPase (excitation 408 nm or 470 nm and emission 530-560 nm). The label is also suitable for Förster resonance energy transfer (FRET) assays (Hiratsuka, 2003).

Other luminescent labels

Not all luminescence based methods rely on environment-sensitivity, even homogeneous methods are not easy to develop with other labels. FP is one of the only platforms enabling this kind of homogeneous assays. Unfortunately, FP-based GTPase assays cannot be easily modified, but when established FP is highly suitable for screening purposes. A FP based assay using GDP-Alexa633 has been used for screening of potential small molecule inhibitors of GAP induced GTP hydrolysis (Huss et al., 2007; Sun et al., 2011). The commercial Transcreener® assay is based on GDP detection using a diphosphate specific antibody. In this assay, GDP and GDP-Alexa633 compete for binding with the antibody, and the reaction is monitored using FP (emission 688 nm). The GDP produced upon hydrolysis can displace the GDP-Alexa633 from the antibody, producing a reduced FP signal of around 2.5-fold with a Z-factor over 0.7. The assay is based on activity measurement, and thus also enables allosteric inhibitor detection. However, due to the single turnover principle of the assay, a relative high concentration of GTP preloaded GTPase is needed (around 100 μM). The antibody used is not GDP but rather diphosphate specific, which delimits the usefulness of the assay only to precisely controlled *in vitro* applications (Sun et al., 2011). The mant-label also enables FP based monitoring (Brownbridge et al., 1993). The change in fluorescence anisotropy reflects the overall molecular size of the complex, in this case nucleotide binding to GTPase or GTPase interactions with effectors. For these reasons the assay is especially useful when affinities between the interaction partners are determined. With mant-nucleotides, FP is often used as a control technique (Phillips et al., 2003; Manor, 2006).

Lanthanide chelates are efficiently used in heterogeneous assay, but not in homogeneous assays for GTPases. Ln^{3+}-chelate labeled GTP derivatives (Eu^{3+}-GTP or Tb^{3+}-GTP) are used to study heterotrimeric- but rarely monomeric G-proteins (Frang et al., 2003; Koval et al., 2010). This is due to the lack of direct homogeneous techniques to measure the proportion between bound and unbound Ln^{3+}-GTP. Usually, Ln^{3+}-based assays for GTPases are based on energy transfer (Vuojola et al., 2009; Martikkala et al., 2011).

2.4.2 Tryptophan modified GTPases

GTP-binding proteins can be studied by observing the changes in the luminescent properties of tryptophan that occurs due conformational changes (Higashijima et al., 1986; Papp and Vanderkooi, 1989). Tryptophan enables monitoring of either nucleotide exchange or GTP hydrolysis with reasonable micromolar sensitivity. Tryptophan serves as an environment-sensitive moiety somewhat similar to luminescent nucleotides (Figure 7C). Tryptophan is most often used as an energy transfer partner to improve the signal-to-background (S/B). Unfortunately, tryptophan cannot be found in all GTPases, although it can be added by mutagenesis (Rensland et al., 1995; Ahmadian et al., 1999). Normally, tryptophan is excited at 290 nm and the emission is monitored at 330 nm, and the change in luminescence is caused by change in emission maxima or intensity (Pan et al., 1995).

Endogenous tryptophan is found in several GTPases, e.g. Rho and Arf. Due to optimal tryptophan positioning, Arfs are the most studied GTPases (Kahn and Gilman, 1986; Antonny et al., 1997; Zeeh et al., 2006). Unfortunately, the change in tryptophan luminescence can be altered for multiple reasons, especially when multiple tryptophan moieties are present. Correctly positioned tryptophan gives a 10-110% change in luminescence during GTPase reactions (Kahn and Gilman, 1986; Higashijima et al., 1986, Antonny et al., 1991). As an alternative to tryptophan, there are nine tyrosines in the Ras structure that also enable activation studies (Antonny et al., 1991). As the intensity of the luminescence from tyrosine is lower than tryptophan, a tryptophan substituted Ras[Y32W] mutant has been created (Skelly et al., 1990; Antonny et al., 1991; Yamasaki et al., 1994; Ahmadian et al., 1999). This method enables GTP hydrolysis monitoring with smaller sample requirements than HPLC (Ahmadian et al., 1999). The link between tryptophan positioning and the change in signal has been studied in greater depth with Rab. With all tryptophan mutants, blue shifted emission spectra were monitored, but usually without notably luminescence change (Pan et al., 1995). Unfortunately, the positioning of tryptophan together with high energy UV-excitation can cause unwanted effects.

Like FP, tryptophan luminescence is frequently selected as a confirmatory method to monitor GTPase state while other reactions are monitored with distinct method (Bill et al., 2011). Direct tryptophan luminescence and FRET between labeled GTPase and an effector is utilized for inhibitor screening. From the FRET signal the PPI can be monitored, and tryptophan residue can report whether GTPase is activated. Based on these signals the position in which inhibitor is affecting can be identified (Bill et al., 2011).

2.4.3 Energy transfer based methods

The energy transfer between two luminophores enables the study of complex GTPase regulator and/or effector systems (Figure 8). Most importantly, these methods enable multiprocess reaction monitoring at once. Energy transfer can also be used with GTPases,

which cannot be studied with environment-sensitive labels. Most of the methods based on energy transfer rely on GTPases modified with luminescent molecules. The downside of methods using luminophores is that GTPase structure modification can affect the GTPase activity and interactions with other molecules. The energy transfer method most widely applied in GTPase studies is FRET, in which there are demand of spectral overlap and short distance between donor and acceptor molecules. Also single-label QRET technology enables GTPase studies.

Förster resonance energy transfer (FRET) based methods

When a mant-nucleotide is used as a GTPase binding ligand, a tryptophan residue can be used as an energy transfer donor (Figure 8A). The mant excitation reaches maximum at 330-350 nm and cannot be excited at the absorption maximum of tryptophan (290 nm) (Hiratsuka, 1983). Energy transfer between tryptophan and mant occurs after nucleotide association to GTPase, substantially increasing the mant emission at 430-445 nm. The low background from the unbound mant-nucleotide enables the usage of high mant concentration (Hiratsuka, 1983). Energy transfer between tryptophan and the mant-label usually increases the S/B ratio about 2-fold, compared to tryptophan or mant *per se* (Rojas et al., 2003; Zeeh et al., 2008).
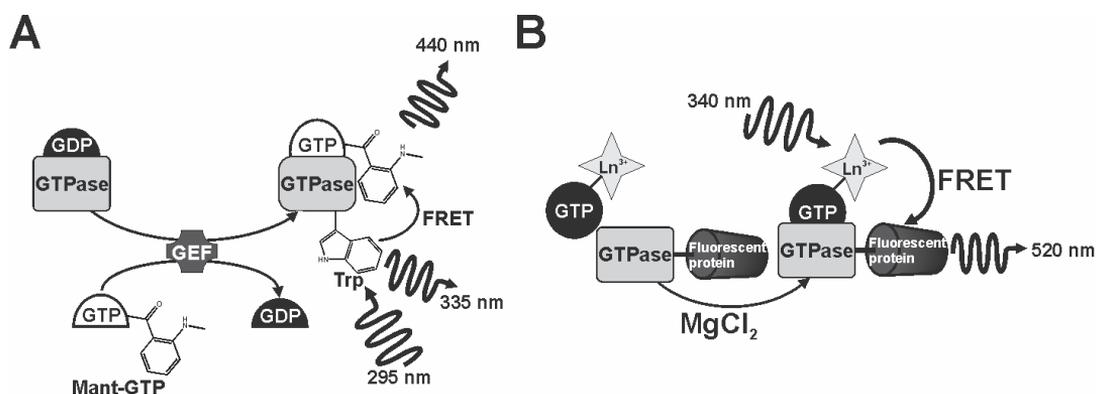
Figure 8. Energy transfer as a tool in GTPase activation monitoring. (A) Tryptophan (Trp) moiety is often used as a Förster resonance energy transfer (FRET) donor. FRET between Trp and mant-GTP occurs after GTPase activation, producing a more prominent luminescence change that either mant- or tryptophan-labels alone. (B) GTPase fused fluorescence proteins provide another FRET signaling platform. Lanthanide (Ln^{3+}) chelate conjugated GTP enables FRET based GTPase activation sensor, using fluorescent protein as an energy transfer acceptor.

Green fluorescent protein (GFP) based FRET is a useful tool to monitor GTPase activation *in vitro* or visualize local activity-changes of GTPases in the living cell (Mochizuki et al., 2001; Nakamura et al., 2005; Vuojola et al., 2009). Fluorescent proteins are usually expressed as a fusion with GTPase. For *in vitro* purposes, FRET is usually too expensive and difficult to construct, due to complicated label positioning. Ln^{3+}-GTP enables more

sensitive GTPase nucleotide exchange detection than normal luminophores, e.g. mant. Nucleotide exchange has been monitored with FRET and nFRET (nonoverlapping FRET) between fluorescent protein and Ln^{3+}-GTP (Figure 8B) (Vuojola et al., 2009). From the two configurations, nFRET between Eu^{3+}-GTP and GFP was more sensitive than FRET between Tb^{3+}-GTP and yellow fluorescent protein (YFP). GFP- and YFP-Rab assays enable the use of only nanomolar protein and Ln^{3+}-GTP concentrations, providing S/B ratios 15 and 75 with FRET and nFRET, respectively (Vuojola et al., 2009).

Quenching resonance energy transfer (QRET) based methods

The quenching resonance energy transfer (QRET) method is based on energy transfer between Ln^{3+}-ligand and soluble quencher molecule (Härmä et al., 2009). The quencher molecule used can be either a non-luminescent molecule or luminophore. In the QRET assay, a high (µM) soluble quencher concentration is used to generate a high local concentration near the Ln^{3+}-chelate. The Ln^{3+}-ligand interaction with the target molecule, relocate the Ln^{3+}-chelate in a different environment near the target surface, which lowers the quencher-Ln^{3+}-chelate interaction (Härmä et al., 2009). The time-gated measurement allows sensitive GTPase monitoring with low protein consumption.

The QRET technique has been used to study Tb^{3+}-GTP binding to H-Ras (Martikkala et al., 2011). As in the other Ln^{3+}-GTP assays, the QRET technique enables the use of nanomolar protein and nucleotide concentrations. Unlike FRET assays, the QRET based nucleotide exchange assay is easy to convert for different GTPases (Vuojola et al., 2009; Martikkala et al., 2011). Unfortunately, the background luminescence increase along with the increased protein concentrations limits the usability of Tb^{3+}-GTP assay and also the sensitivity was lower than with nFRET (Vuojola et al., 2009; Martikkala et al., 2011).

2.4.4 Labeled GTPases

In addition to luminescent nucleotide analogs, labeled GTPases have been used for GTPase cycle monitoring. An environment-sensitive label conjugated to the GTPase structure can serve as a platform to monitor structural changes. However, the same problems in label positioning arise in label conjugation as in the production of fusion proteins for FRET purposes. Usually the correct position needs to be found in a trial-and-error manner. Labels are usually conjugated to cysteine residues in the GTPase structure.

Cdc42GTPase structure conjugated environment-sensitive succinimidyl 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoate (sNBD), has been utilized to study nucleotide exchange (Nomanbhoy et al., 1996). The luminophore discriminates between active and inactive GTPase undergoing luminescence enhancement during GTP association. Similarly, the GTP hydrolysis decreases the luminescence monitored at 545 nm (Nomanbhoy et al., 1996). The sNBD system provides a similar signal change (15-30%) to

those reported using tryptophan luminescence (Leonard et al., 1994; Nomanbhoy et al., 1996). The signal change can be improved 1.5-fold when FRET is monitored between sNBD-GTPase and the mant-nucleotide (Nomanbhoy et al., 1996). Dansyl conjugated GTPase enable assays to be performed in a similar fashion as with sNBD. Dansyl-labeled Rab7:REP-1 has been used to monitor prenylation and can potentially be used to study other posttranslational modifications important for GTPase function (Durek et al., 2004). A dansyl-based FRET sensor using tryptophan as a luminescence donor has been utilized to determine GTPase-effector interaction. This assay can be performed using nanomolar protein concentrations providing S/B ratio from 3 to 4 (Durek et al., 2004). With Iaedans-Ras, label in positions 32 and 86 were found to provide evident luminescence change (30-70%) in GTP hydrolysis assay, monitored at emission wavelength 480 nm (Kraemer et al., 2002). The measurement of Ras is easy with most of the methods, and therefore the assay was tested with more problematic GTPases. Iaedans-Rap (A86C) assay was developed to give evidence of the wide suitability of the GTPase conjugated labels. Unfortunately, the cysteine residue needs to be mutated in the GTPase structure. The optimal point for mutation varies between GTPase and might also affect the binding properties. These features limit the usefulness of environment sensitive labels conjugated to GTPases (Kraemer et al., 2002).

2.4.5 Special methods for GTP hydrolysis detection

HPLC can provide GTP hydrolysis data but without the kinetic information. Luminescence based GTP hydrolysis monitoring with GTPases is usually based on hydrolysis product, GDP or P_i, monitoring (Figure 9). Unfortunately, the P_i detection is predisposed to the effects of contaminants and the GDP detection suffers from the lack of specific binders.

Luminescently labeled bacterial actin homologue (ParM) was originally developed to monitor ADP, but the sensor also enables GDP monitoring because it can bind all nucleoside diphosphates (Kunzelmann and Webb, 2009; Kunzelmann and Webb, 2010; Kunzelmann and Webb, 2011). ParM based GDP detection relies environment-sensitive MDCC (N-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamide) or tetra-methylrhodamine. In the GTP hydrolysis assay, the produced GDP binds to ATR-ParM inducing conformational change in the ParM structure and a 10-fold signal increase, which can be monitored in real-time (Figure 9A) (Kunzelmann and Webb, 2011). GDP monitoring using GDP binding antibodies, like in the FP based Transcreener® assay, is also applicable for GTP hydrolysis monitoring (Kleman-Leyer et al., 2009; Zielinski et al., 2009). Both the ParM protein and GDP antibody have over 100-fold higher affinity to GDP over GTP. Similarly, nucleoside diphosphate kinase (NDPK) enables diphosphate sensor development (Brune et al., 2001). IDCC (N-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide) labeled NDPK (DCC-NDPK) has low substrate specificity and only the number of phosphates signify in the binding. Using NDPK-IDCC,

signal-on and signal-off sensors were designed based on the phosphorylation status of NDPK. In signal-on and signal-off assays, a 4-fold signal change (emission 455 nm) was recorded. The assay can be performed jointly with P_i sensors (Brune et al., 2001).

Most often the GTP hydrolysis detection is performed monitoring P_i concentration. In these methods, increase in P_i concentration can induce increase in luminescence (signal-on) or P_i can work as a quencher (signal-off). Signal-on methods utilized luminescently labeled phosphate binding proteins (PBP) (Brune et al., 1994; Nixon et al., 1995; Brune et al., 1998; Hirshberg et al., 1998). The MDCC attached to the PBP changes the conformation during P_i binding, moving the MDCC-label into a more hydrophobic environment and providing a 7-fold signal increase (Shutes and Der, 2005). In addition to MDCC, other environment-sensitive labels, e.g. IDCC, can also be similarly used. When the label is in favorable environment, a change in circular dichroism or more preferably an increase in luminescence signal can be monitored. The P_i sensitivity of the MDCC-PBP is sufficient to monitor GTP hydrolysis (Shutes and Der, 2005). The rapid kinetics and high P_i binding affinity (K_d 100 nanomolar) enables MDCC-PBP based GTP hydrolysis assays. However, free P_i, which is often present in the solution, can cause problems and thus buffer controls are of high importance (Shutes and Der, 2005).
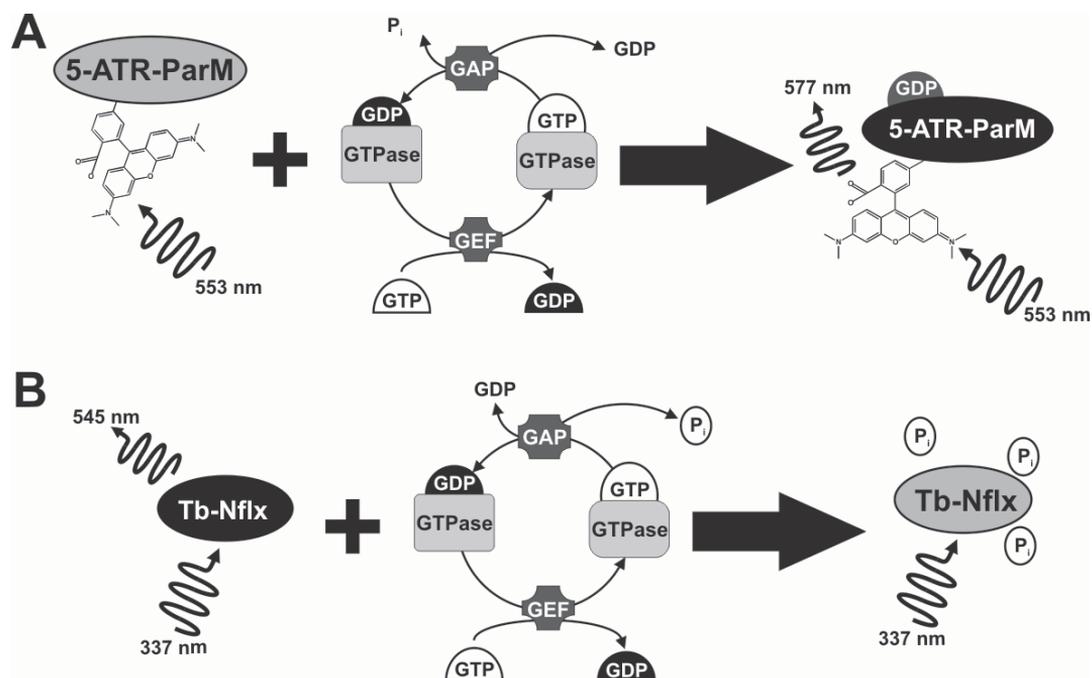
Figure 9. Luminescence based methods for GTP hydrolysis monitoring. In GTP hydrolysis, GDP and inorganic phosphate (P_i) is formed. (A) The concentration of GDP formed can be monitored using a luminescently labeled GDP binding molecule, e.g. bacterial actin homologue (ParM). ATR-ParM binds only GDP, which induces the luminescence change. (B) P_i sensitive probe, e.g. Tb^{3+}-labeled norfloxacin, is luminescent in the absence of P_i, but the signal is quenched after hydrolysis.

Methods based on P_i induced luminescence signal quenching are not as sensitive as P_i detection using the signal-on principle (Spangler et al., 2009). Advantageously, these sensors are also less sensitive to contaminants. Using the signal-off platform, GTP hydrolysis has been monitored using a modified ATP detection probe based on Tb^{3+}-norfloxacin (Tb^{3+}-Nflx) (Miao et al., 2006; Spangler et al., 2008). The GTP hydrolysis cause changes in P_i/GDP/GTP proportion and thus Tb^{3+}-luminescence changes (Spangler et al., 2009). Based on the P_i induced Tb^{3+}-luminescence quenching, GTP hydrolysis can be monitored more robustly than with the previously described MDCC-PBP sensors (Shutes and Der, 2005; Spangler et al., 2009) (Figure 9B). However, the protein and nucleotide concentrations needed are in micromolar level.

Majority of methods developed to monitor GTP hydrolysis rely on environment-sensitive labels. These labels are, however, usually conjugated to external reporter protein which is not involved in the GTPase cycle studied. This enables the use of the same sensor to monitor different hydrolysis reactions, in addition to GTP hydrolysis, without further modification. This is an important feature that helps to provide reliable and comparable knowledge about the GTP hydrolysis. However, similar problems occur with all systems based on GDP and P_i detection. In the detection of GDP, the ParM, NDPK, or GDP antibody cannot distinguish nucleoside diphosphates from each other. On the other hand, this lack of specificity enables the use of the same assay system to monitor either ATP or GTP hydrolysis. P_i sensors cannot resolve the source of P_i. For these reasons, either the GDP or P_i sensor cannot be used in cell based assays but only in *in vitro* (Webb, 2007).

2.5 Cell based assays

There are three main limitations with the *in vitro* assay; 1. multi-domain proteins are difficult to produce and use, 2. post-translational modifications are not present, 3. subcellular localization induced effects cannot be monitored (Casanova, 2012). Cell- and tissue-based assays possess certain advantages over cell-free systems, e.g. reaction monitoring and regulation in the context of the cell. For more than ten years there has been a trend towards cell-based assays (Mochizuki et al., 2001). The first assays were performed in cell lysate, but later whole cell assays have been progressively used. In this section the most important methods to study GTPase related reactions are briefly introduced.

2.5.1 Assays in cell lysate

The two general categories of cell-based assays are biochemical assays and morphological *in situ* assays. Pull-down (affinity precipitation) assays are the largest group of biochemical assays. In the early days, GTPase activity was monitored from cell lysates using GTPase binding antibodies and radiolabeled nucleotides. Immunoprecipitation based methods are difficult and the lack of proper antibodies limits their usefulness. More recently pull-down assays have still been performed from cell lysates, but using tagged effector proteins (van

Triest and Bos, 2004). These assays make use of the increased affinity of the effectors to activated GTPase. Also luminescence based pull-down sensors have been developed.

The GST-tag is suitable for both purification and also as a fusion tag in pull-down assays (Taylor and Shalloway, 1996; de Rooij and Bos, 1997). The first pull-down assay for Ras was performed using GTP-Ras binding GST-tagged RBD (Ras binding domain) from Raf-1. The pull-down assay can be performed similarly for all GTPases using a suitable effector molecule fragment, e.g. p21-binding domain (PBD) or Rho-binding domain (Ren et al., 1999). All the pull-down assays are time-consuming and laborious to perform (Taylor and Shalloway, 1996). Usually pull-down assays are performed using non-selective effector fragments that bind multiple GTPases. To enable more precise GTPase activation data, GTPases can be further analyzed using specific antibodies (Benard and Bokoch, 2002; Jian et al., 2010). Nowadays, polyHis- and biotin-tag based pull-down assays are also available. Regardless of the effector, the fusion protein traps the active GTPase and links it to the detection matrix. In the case of GST, the detection is performed using glutathione sepharose gel or beads. Affinity precipitation gives a general view of GTPase activation although cannot be used to observe low or localized changes. Fluorescent proteins conjugated to the effector binding fragment have been developed to overcome these problems (Chiu et al., 2002; Kiyokawa et al., 2011). Using GFP-RBD, the accumulation of the fluorescent protein to the area of activation can be monitored with fluorescence microcopy. Unfortunately, the low RBD/GTP-Ras interaction affinity limits the usefulness of this technique (Chiu et al., 2002; Augsten et al., 2006). When pull-down methods are compared to intracellular monitoring techniques, like total internal reflection fluorescence (TIRF) microscopy, the difference in data quality is immense. TIRF enables single-molecule imaging of GTP-activation in time and space.

2.5.2 Energy transfer based methods

The use of luminescent reporters enables the location specific interaction monitoring within a living cell. These assays are usually based on energy transfer between donor and acceptor luminophores or protein-fragment complementation (PCA). Energy transfer assays often use FRET signaling between a donor tagged GTPase and acceptor conjugated to the effector protein (Wang and Wang, 2009; Aoki et al., 2013). This is a common form in bi-molecular FRET biosensors. In unimolecular (intramolecular) biosensors, luminescently tagged GTPase and effector are connected through a flexible linker (Mochizuki et al., 2001; Aoki and Matsuda, 2009; Aoki et al., 2012). This design enables equivalent expression of GTPase and effector in the same cell compartment, which rarely reflects the natural situation. In addition to these FRET sensors between fluorescent proteins, luminescently labeled nucleotides are applicable (Murakoshi et al., 2004). The most important cellular GTPase monitoring assays are depicted in Figure 10.
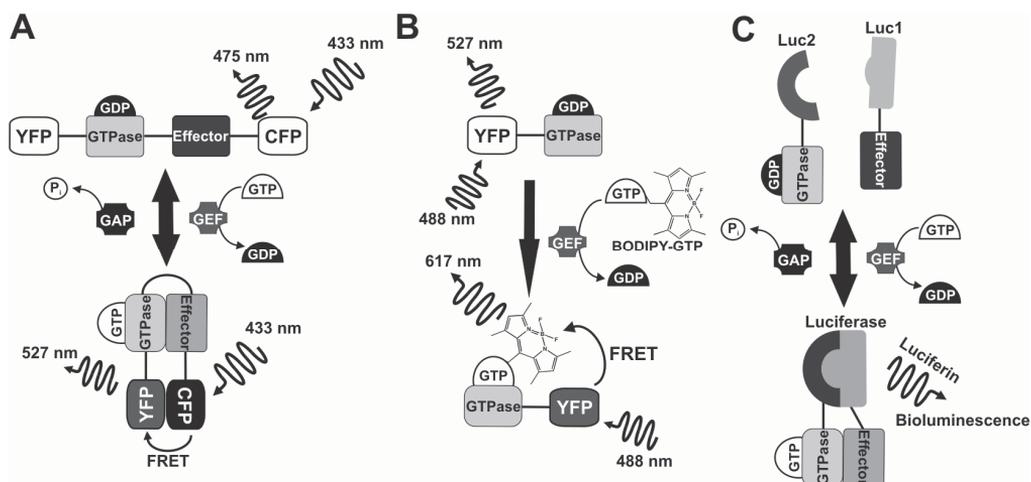
Figure 10. Cell-based GTPase monitoring platforms using luminescence. (A) Förster resonance energy transfer (FRET) between two fluorescent proteins can be used to monitor GTPases. In a unimolecular Raichu-sensor, GTPase, effector, and luminescence reporters are all linked as a one structure. As a result of GTPase activation, FRET signaling occurs. (B) Luminescently labeled GTP can also be microinjected into a cell providing a FRET sensor for GTPase activation together with fluorescent protein GTPase fusion protein. (C) The protein-fragment complementation (PCA) assay is based on a divided enzyme or fluorescent protein whose activity is restored when GTPase is activated. The complemented luciferase is spontaneously formed when two portions of luciferase are brought into close proximity.

Different GFP variants and other luminescent molecules encoded in the genome are widely used in cell-based FRET biosensors (Mochizuki et al., 2001; Wang and Wang, 2009; Aoki et al., 2013). The most frequently utilized GFP variants are YFP and cyan fluorescent protein (CFP), which display bright luminescence and low photobleaching (Nguyen and Daugherty, 2005; Goedhart et al., 2010). Red fluorescent proteins (RFPs) on the other hand are useful in two-photon microscopy (Kotera et al., 2010). Fluorescent protein pair selection has a major impact on the sensitivity and dynamic range of the assay (Kiyokawa et al., 2011). In all interaction sensors, the FRET signal is produced when the tagged GTPase and effector molecule are in close vicinity. The label positioning is especially difficult in single-domain type intramolecular FRET probes (Kiyokawa et al., 2011).

The first example of a unimolecular FRET design was the so called Raichu-sensor (Ras and interacting protein chimeric unit) (Mochizuki et al., 2001). This design set the trend towards whole cell assays. In the Raichu-sensor, FRET between luminescent proteins occurs when GTPase is activated (Figure 10A). Nowadays, there are many variations of the original Raichu-design, e.g. single-domain type sensor for Ras (Kotera et al., 2010; Kiyokawa et al., 2011). In the single-domain assay, a decrease in FRET is monitored after GTP-Ras interact with a double labeled GTPase binding protein. This interaction separates the fluorescent proteins, thus causing decreased FRET (Kotera et al., 2010). Usually, inter-

and intramolecular sensors are used in different applications (Nakamura et al., 2005). Unfortunately, microscopy-based whole cell approaches are slow, but throughput can be increased using cytometry-based monitoring (Abankwa and Vogel, 2007; Köhnke, 2012). FRET biosensors also suffer from the requirement of a high or even toxic level of exogenous protein expression. FRET sensors can also be linked to conventional pull-down assays (Aoki and Matsuda, 2009).

Not all FRET assays utilize energy transfer between two fluorescent proteins, but use common small luminophores instead. The first bimolecular design was performed with GFP-Rac1 and PBD-Alexa546 (Kraynov et al., 2000). This sensor was named the fluorescence activation indicator for Rho protein (FLAIR). The sensor suffers from the high background induced by unbound PBD, which could be reduced using nucleotide conjugated luminophores (Kraynov et al., 2000; Murakoshi et al., 2004). Like the PBD-Alexa probe, BODIPY-GTP can also be microinjected to the cell, working as an acceptor for the YFP donor (Figure 10B). The major benefit of this is the possibility to use single-molecule FRET in the visualization due to improved FRET sensitivity (Ha et al., 1996; Murakoshi et al., 2004). One FRET complex can be measured at a time with BODIPY-GTP, but not with two fluorescent proteins (Mochizuki et al., 2001; Murakoshi et al., 2004). This single-molecule FRET has been used to study GTPase signal-transduction mechanism and track the GTPase movement during activation.

Environmental sensitive merocyanine dyes

Environment-sensitive labels, like mant and MDCC, are in everyday use *in vitro*, but rarely within cell-based assays due to their modest brightness. Environment-sensitive merocyanine dyes are brighter than conventional labels enabling *in vivo* applications (Toutchkine et al., 2003). The first biosensor for Cdc42 activation was employed using a solvatochromic dye with a 3-fold increase in fluorescence intensity upon binding. Later, improved merocyanine dyes have produced a 15-fold luminescence increase in the Cdc42 activation assay (MacNevin et al., 2013). Merocyanine dyes are advantageous due to their bright, long wavelength (excitation >590 nm) luminescence. Small merocyanine dyes also have only a small effect on the GTPase activity, compared to large fluorescent proteins (Toutchkine et al., 2003). Merocyanine dyes are constructed from a donor and an acceptor, utilizing intramolecular energy transfer. The dye properties can be modified by changing either part of the dye (MacNevin et al., 2013). Unfortunately, labeled GTPases needs to be microinjected to the cell.

2.5.3 Protein-fragment complementation (PCA)

Unlike in FRET assays, PCA-based methods use a single divided reporter, which can be a luminescent molecule or component essential to the cell (Campbell-Valois and Michnick, 2005; Westwick and Michnick, 2006; Stynen et al., 2012). Toxic effect is avoided due to

lower protein expression in PCA methods, compared to fluorescent protein based assays (Yu et al., 2004). PCA is already an old method, and it has been traditionally used to study molecule-molecule interactions (Johnsson and Varshavsky, 1994). PCA is highly distance dependent and spontaneous refolding of the protein can occur after two parts are brought within close proximity. Like FRET, PCA enables assays in the living cell, giving information about protein complex formation and subcellular localization. The high precision of the signal positioning is the main advantage of this method. The signal can be monitored using microscopic systems, flow cytometer or a plate reader. The method is thus also HTS compatible, but the setup is usually too expensive to use and compile (Westwick and Michnick, 2006).

The split luciferase assay is one of the most sensitive assays, needing only nanomolar protein concentrations, and enabling assays in cell lysate or intracellularly (Andrerson and Hamann, 2012). The active luciferase is spontaneously formed when the two portions are brought into close proximity (Ozawa et al., 2001; Paulmurugan et al., 2002; Andrerson and Hamann, 2012; Leng et al., 2013). In GTPase PPI studies, the luciferase portions are conjugated to the GTPase and effector. The active luciferase is formed after GTPase activation (Figure 10C) (Andrerson and Hamann, 2012). The assay enables the detection of GEF induced nucleotide exchange, GAP induced hydrolysis, and GTPase-effector interactions. Unfortunately, the indirect signal formation does not enable reaction kinetic determination (Andrerson and Hamann, 2012). To enable cell-based assays, the split luciferase signaling construct is microinjected into the cell or animal to enable *in vivo* imaging of GTPases (Paroo et al., 2004; Luker et al., 2008; Leng et al., 2013). In live cell imaging, the signaling construct fusion protein cell line is created (Luker et al., 2008; Leng et al., 2013). The 2- to 13-fold signal increase over the baseline is usually monitored *in vivo* (Leng et al., 2013).

Split-fluorescent proteins (split-FPs) also enable intracellular assays (Magliery et al., 2005; Stynen et al., 2012). Usually, GFP variants are used in split-FP assays, enabling multicolor detection platform development. Split-FP has the tendency to give high numbers of false positive signals, and the luminescence properties are also limited (Stynen et al., 2012; Wong et al., 2013).The split-ubiquitin system relies on the same principle as luminescence based assays (Johnsson and Varshavsky, 1994). Instead of a luminescent reporter, the GTPase-effector interaction forms a protease cleavage site, and the cleavage releases the luminescent protein. The split-ubiquitin system enables PPI studies in different time and space (Moreno et al., 2013). PCA methods can also utilize split molecules essential to cell survival or colocalization, e.g. split-lactamase. These methods cannot be used directly to monitor GTPases but linked signaling system needs to be used (Stynen et al., 2012).

Two-hybrid system

Yeast two-hybrid system is widely used to study PPIs and finding new interaction partners. The system is based on modular transcription factor design where binding and activating parts are separated. Despite the name, two-hybrid system is not limited to yeast cells (Stynen et al., 2012). In PCA, the detected signal is provided due to reporter refolding, but in the two-hybrid system the colocalization of two protein domains serves as reporter. Two-hybrid system is not based on protein refolding due to interaction (Field and Song, 1989). However, the system suffers from high false positive rate. To study GTPases, both GTPase and GEF recruitment assays have been used (Aronheim, 1997; Maroun and Aronheim, 1999; Hubsman et al., 2001; Stynen et al., 2012). In these assays the GTPase system was used more as a reporter system than the object of interest. GTPases have also been studied directly, distinguishing novel interaction partners (Serebriiskii et al., 1999).

2.6 Summary and future trends of GTPase research

Ras genes were identified thirty years ago and since then the urgency of devising strategies for treating Ras-driven cancers has only increased. Defects in GTPase regulation are linked to a continually increasing number of diseases. During the last decade, major leaps have been made for understanding GTPase structural and biochemical mechanisms. The increased knowledge of GTPases has led us to focus more on regulation and the dynamics of these proteins e.g. autoinhibition and intramolecular interactions.

GTPases have been a challenging target to drug discovery due to their extensive binding surface and nucleotide-specific interactions with regulators, effectors, and membrane. To enable these interactions, the GTPase structure is highly flexible especially in the switch I and switch II areas. This further challenges the modulation of GTPase function using small molecules, and also imposes upon the assay methods used for inhibitor selection in regard to GTPase structural considerations, processing, and membrane localization. Only a few inhibitory compounds directly for GTPases have been described in recent years, although without any major breakthroughs at the clinical level. On the contrary, major disappointment in the clinical trials has led to a necessary reassessment on the field, for which new assay methods are needed. There is also an ongoing debate in which molecules in GTPase cycle have the potential to serve as a drug target (Bos et al., 2007; Vigil et al., 2010). The prevailing view is that rather than a single inhibitor, multiple inhibitors for different target are needed.

A multitude of different assays have been developed since GTPases were discovered. These assays have provided a large amount of data for structure and function, but the challenge has been how to correlate the data from different sources. Until recently, GTPase functions were mostly studied with nucleotide association and hydrolysis assays utilizing radioactive labels or luminescence. On the other hand, GTPase interactions with effectors

and regulators have been studied using pull-down assays and immunoblotting. These methods are still in use, but NMR and cellular assays have attracted growing interest. Assays which can be used in the context of the cellular milieu could close the gap between drug screening campaigns and disappointment in clinical trials. NMR brings another important aspect, combining structural changes to the nucleotide binding.

In vitro GTPase assays have been developed towards simpler and more sensitive assays suitable for drug molecule screening. In drug screening, the key factors are robustness, sensitivity, costs, and automation. In primary screening, traditional nucleotide exchange assay retain their place but in an improved form. To enable larger molecule library screens for both novel inhibitors and activators, new assay methods are needed. Much improvement is also needed to find better congruence between primary screening and real inhibitors that could act also into clinic. This common problem is not just related to GTPases, but screening in general. In the case of GTPases, this mostly means that inhibitor specificity and affinity needs to be high, but due to the lack of clear binding pockets in the GTPase surface this has been problematic. Interfacial inhibitors have raised promises, potentially offering some solutions to tackle these problems. However, most of the current assay strategies are designed to discover molecules that decrease interaction between interaction partners (competitive inhibitors). Thus they cannot detect interfacial inhibitors, which increase the interaction between proteins.

In addition to the development in assay design, the development of the reporter material is also highly important. With the current luminescence based *in vitro* assays, the sensitivity is usually weaker than with radioactive labels. The sensitivity problems encountered with homogeneous assays with environment-sensitive labels have increased the popularity of the energy transfer based applications. However, the problem in almost all luminescence based assays is the need for modifications in the nucleotide and/or GTPase. These modifications can change the GTPase activity and interactions unpredictably (Mazhab-Jafari et al., 2010). Also in the cellular milieu, the label technology is the limiting factor. With fluorescent proteins, the luminescence properties do not enable the use of microscopy to its full capacity. With microinjection, luminescent labels with better luminescent properties can be used. Unfortunately, the use of externally modified components might change the natural network inside the cell, which is the ultimate object of interest.

All in all, there is still a need for luminescence based methods and especially new methods with improved properties. There is plenty of room to improve the current methods to study GTPases and especially methods enabling inhibitor screening. New improved luminescent labels could enable the more efficient study of GTPase both *in vitro* and *in vivo*. Most importantly, if the same method could be used for multiple purposes and for all GTPases from different families, the comparativeness of the results could be improved dramatically.

In conclusion, there is still a lot to be improved in GTPase assays. Also there is room for improved luminescence as well as sophisticated non-luminescence based methods.

The single-label time-resolved luminescence (TRL) based QRET technique was invented to improve and simplify the primary screening process (Härmä et al., 2009). The QRET technique is based on the separation between target bound and non-bound lanthanide (Eu^{3+} or Tb^{3+}) chelate conjugated ligand. The labeled small molecule ligand could be for example, a DNA-fragment, peptide, nucleotide or a small hormone, which are small enough that they cannot itself protect the label (Rozwandowich-Jansen et al., 2010; Huttunen et al., 2011; Martikkala et al., 2011). The QRET method has an exceptional property that enables the detection of the targeted reaction whether the assay is performed with purified proteins, membrane or intact cells. The robustness of the QRET method enables the use of the method in compination with other signaling techniques as well as ensures the assay performance in the presence interfering buffer components e.g. high dimethyl sulfoxide concentration (Kopra et al., 2013a; Kopra et al., 2013b). Due to TRL detection, the assay sensitivity is higher than with conventional luminophores. Together with FP, the QRET technique serves as single-label platform (Härmä et al., 2010). With only single luminophore conjugation, possible interferences from multiple labels and multiple conjugation reactions can be avoided. At the beginning, the QRET method was developed to enable screening by monitoring interactions. Today, the QRET method provides a fast-growing set of assays for different targets and assay purposes (Kopra and Härmä, 2015). The QRET technique enables not only interaction and drug molecule screening, but also protein quantification and reaction monitoring in real-time.

3 AIMS OF THE STUDY

The overall aim of this study was to evaluate the potential of the QRET technique in a diverse range of assays and to develop them to monitor GTPase related reactions. The separation free single-label QRET technique was previously demonstrated to enable sensitive detection or various analytes also in the cellular milieu (Härmä et al., 2009). The purpose of this study was to improve the QRET method to enable a larger variety of different types of assays and to enable kinetic monitoring of the reaction. A major theme was to observe different GTPase reactions e.g. nucleotide exchange and GTP hydrolysis, and to study the advantages that the lanthanide chelates and the QRET technique can offer.

More specifically the aims were:

I   To design universal **QRET assay enabling direct protein detection and monitoring of the reaction kinetics**, using single Eu^{3+}-labeled DNA-aptamer as a signaling and a detection molecule.

II   To improve GTPase activation assay sensitivity and to enable the **kinetic analysis of the GTPase nucleotide exchange reaction** using different GTPases.

III   To study the novel **nanoclustering related switch III region in Ras proteins using mutant analysis, kinetic profiles, and differences in effector molecules binding** to study a new mechanism that contributes to the overactive Ras in cancer.

IV   To select and produce **specific GTP binding antibody** to enable the study of **GTP hydrolysis reaction monitoring with GTPases**.

4 MATERIALS AND METHODS

This chapter presents a short summary of the materials and methods employed in this study. A detailed description of materials and methods can be found in the original publications (**I-IV**).

4.1 Labels and quencher molecules

All the assays in this study were made using Ln^{3+}-chelate based time-resolved luminescence (TRL) detection. In all the homogeneous QRET studies (**I-III**), an unspecific quenching based method with Ln^{3+}-chelate and a soluble quencher molecule were used. In publication **IV**, the luminescence signal was monitored directly after a separation step (from the surface) or after the Eu^{3+}-ion was released and re-chelate with a europium luminescence intensifier (EFI), as previously shown by Hemmilä et al., 1984.

4.1.1 Lanthanide chelates

The Ln^{3+}-chelate used in publications **I-III** was {4-[2-(4-isothiocyanatophenyl)-ethynyl]-2,6,-bis{[N,N-bis(carboxymethyl)-amino]methyl}pyridine}-europium(III) with an additional iminodiacetate coordinating arm (Wang et al., 2013). The Ln^{3+}-chelate used in publication **IV** was {2,2′,2″,2‴-{4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]-bis(methylenenitrilo)}tetrakis(acetato)}-europium(III) (Mukkala et al., 1993). Ln^{3+}-chelates were synthesized following the protocols described earlier (Mukkala et al., 1993; Wang et al., 2013). The excitation and emission maximum of the Eu^{3+}-chelates are approximately 340 nm and 615 nm, respectively. In the homogeneous QRET assays, the Eu^{3+}-chelate was conjugated to vascular endothelial growth factor (VEGF) binding aptamer (VBA) and basic fibroblast growth factor (bFGF) binding aptamer (FBA) (**I**) or to GTP-analog (**II-IV**). In the publication **IV**, the Eu^{3+}-chelate was also conjugated to streptavidin (SA).

4.1.2 Quenchers, luminophores, and fluorescent proteins

In publications **I-III**, quencher molecules were chosen according to the absorption spectra. In all assays, quencher suitability was tested and confirmed in the selected buffer. All the soluble quencher molecules were stored in DMF and purchased from the QRET Technologies (Turku, Finland). In publications **III-IV**, mant-label conjugated GDP, GTP, and GTPγS was used in the fluorescence anisotropy measurements. The excitation and emission maximum of the mant-luminophore are approximately 370 nm and 450 nm, respectively. In publication **III**, fluorescent proteins, mGFP, mCherry, mRFP, and mCFP, were used to study nanoclustering and effector recruitment. In these FRET assays, fluorescent proteins were used as Ras, C-Raf-RBD, and Gal-1 fusions.

4.2 Instrumentation and instrument settings

4.2.1 Plate reader for TRL-signal measurements

In the QRET assays (**I-III**), the TRL-signal from the Eu^{3+}-chelate was monitored with a Victor 1420 multilabel counter (PerkinElmer, Wallac, Turku, Finland). To reduce autofluorescence and background signals, measurements were performed in black Optiplate 384F (PerkinElmer, Groningen, Netherlands). The default settings for Eu^{3+} (excitation 340 nm, emission 615 nm, delay 0.4 ms, and gate time 0.4 ms) were used. In the heterogeneous assay (**IV**), the TRL-signal from Eu^{3+}-chelate was measured similarly as in homogeneous assays, but the assay was performed in 96-well plate format.

4.2.2 Fluorescence spectroscopy and fluorescence anisotropy

Spectral measurements in publications **I-III** were performed with a Varian Cary Eclipse fluorescence spectrophotometer using a red-sensitive photomultiplier tube (Varian Scientific Instruments, Agilent Technologies, Santa Clara, CA). In publication **I**, the excitation spectra's for Eu^{3+}-aptamer and Quench V were measured from 200 nm to 500 nm using a 20 nm slit, 0.4 ms gate time, 0.1 ms delay time, and 615/5 nm emission wavelengths. Emission spectra were measured from 550-750/5 nm, using a 0.4 ms gate time, 0.1 ms delay time, and 340/20 nm excitation wavelength. Emission lifetimes for the Eu^{3+}-aptamer, with and without the complex with the target protein and quencher, were measured using a 340/20 nm excitation wavelength, 615/20 nm emission wavelength, and a gate time of 0.005 ms. In publications **II** and **III**, the excitation and emission spectra for quencher molecules were measured from 200 nm to 750 nm. All the spectra's were measured using 0.4 ms gate time and 0.1 ms delay time using 615/10 nm emission and 340/20 nm excitation wavelengths.

In publication **III**, nanoclustering-FRET was monitored using fluorescence lifetime microscopy (Lambert Instruments, Roden, Netherlands). In these assays, an mGFP-Ras donor was used with an mRFP-C-Raf and mRFP-Gal-1 acceptor. The fluorescence lifetime of mGFP was monitored on an inverted microscope (Zeiss AXIO Observer D1) and filter set 38 (excitation: BP 470/40, beam splitter: FT 495, emission: BP 525/50).

In publications **III** and **IV**, the fluorescence anisotropy was monitored using a Synergy H1 platereader (BioTek, Winooski, VT, USA) and a polarization filter cube with 340/30 nm excitation and 485/20 nm emission filters. In publication **III**, measurements to monitor C-Raf and H-Ras interaction were made with 100 nM mant-GTPγS or 500 nM mant-GDP-loaded H-Ras, as previously described (Guzmán et al., 2014). In publication **IV**, mant-GTP (2'/3'-O-(N'-methylanthraniloyl)guanosine-5'-O-triphosphate) was used to monitor $2A4^{GTP}$ antibody binding affinity. The assay was performed using an increasing concentration of the $2A4^{GTP}$ antibody (0-500 nM) and a constant mant-GTP (100 nM) concentration.

4.3 Reagent preparation

4.3.1 Expression and purification of GTPases and regulator proteins

The expression and purification of the recombinant proteins are described in more detail in publications **II-IV**. All GTPase and effector proteins were produced using *E. coli* strains, M15 (Ras), BL21 (SOS, p120GAP, and NF-1), and DH5α (Rho and Ect). Bacteria were dispersed by freeze/thawing followed by sonication. Intact cells and debris were removed by centrifugation before purification. His-tagged proteins (Ras and SOS) were purified using a HiTrap nickel nitrilotriacetic acid column (GE Healthcare Life Sciences, Buckinghamshire, UK), and GST-tagged proteins (Rho, NF-1, p120GAP, and Ect) were purified using glutathione beads, after which the tags were removed. All GTPase and GEF proteins were snap-frozen and stored in -80 °C. Unnecessary freeze/thaw cycles were avoided by creating small sample aliquots.

4.3.2 Antibody selection and production

The antibody selection and purification are described in more detail in publication **IV**. GTP antibodies were selected from a ScFvM-library using the phage display technique (Huovinen et al., 2013). Three rounds of selection were performed and individual clones were tested using enzyme-linked immunosorbant assay. Plasmid DNA from the positive clones was extracted according to the GeneJET plasmid miniprep kit instructions (Thermo Scientific) and clones were sequenced. After antibody selection, Fab (fragment antigen-binding) fragments were cloned in periplasmic expression vector (pAK400) to provide oxidizing environment which enables correct Fab folding. Fab fragments were constructed using PCR-based cloning and standard molecular biology procedures, in which constant domain was added to the single-chain variable fragment (ScFv) fragment. After Fab construction, heat shock transformation to CaCl$_2$–competent cells was performed and antibody production was made in isopropyl β-D-1-thiogalactopyranoside induced glucose free medium. Cells were collected and pelleted cells were lysed using sonication. After centrifugal debris removal, Fab containing supernatant was collected and filtered through 0.45 μm filter before further purification using HisTrap-columns with 0.5 ml/min flow rate (GE Healthcare Life Sciences, UK). Buffer was further changed to Fab storage buffer (20 mM Hepes, pH 7.5, 20 mM NaCl) using NAP-5 column (GE Healthcare Life Sciences).

4.3.3 Preparation of Ln^{3+}-chelate and biotin conjugates

In publication **I**, the Eu^{3+}-chelate was conjugated to either the 3'- or 5'-end of the two DNA-aptamers. In all the label conjugations, the isothiocyanate group of the Eu^{3+}-chelate reacted with the amino group of the aptamer. In publications **II-IV**, the Eu^{3+}-chelate was similarly conjugated to 2'-/3'-O-(6-aminohexyl-carbamoyl)guanosine-5'-O-triphosphate (2'/3'-AHC-GTP) and 8-(6-aminohexylthio)-guanosine-5'-O-triphosphate (8-AHT-GTP)

derivatives (BIOLOG Life Science Institute, Bremen, Germany). In publication **IV**, the EZ-link NHS-SS-PEG$_4$-biotin (Thermo Scientific, Pierce Biotechnology, Rockford, IL) was conjugated to 2'/3'-AHC-GTP and 8-AHT-GTP. Conjugates were isolated with reverse-phase HPLC (Dionex ultimate 3000 LC system from Thermo Scientific, Dionex, Sunnyvale, CA) using Ascentis RP-amide C18 column from Sigma-Aldrich, Supelco Analytical (St. Louis, MO). All the HPLC isolations were performed using 20 mM triethylammonium acetate buffer (TEAA) and an acetonitrile gradient. Conjugates were further analyzed with an ion trap mass spectrometer (Esquire HCT, Bruker Daltonik GmbH, Germany). In publication **IV**, the heptadentate Eu^{3+}-chelate was conjugated to streptavidin (SA) (BioSpa Division, Milan, Italy) using 60-fold excess of Eu^{3+}-chelate.

4.4 Assay principles

In this section, the homogeneous QRET method used in publication **I,** and the competitive QRET method used in publications **II-III** are introduced. The heterogeneous method used in publication **IV** is also introduced. The TRL-based assay specifications are summarized in Table 3. Some additional methods used in publication **III** are also introduced.

Table 3. Summary of the QRET assay specifications in the original publications I-IV.

Original Publication	I	II	III	IV
Assay type	Homogeneous QRET	Competitive homogeneous QRET	Competitive homogeneous QRET	Heterogeneous
Signal reporter	Eu^{3+}-VBA or Eu^{3+}-FBA	Eu^{3+}-GTP	Eu^{3+}-GTP	Eu^{3+}-SA and biotin-GTP or Eu^{3+}-GTP
Quencher	Quench V	Quench II	Quench II	-
Plate format	black 384-well plate	black 384-well plate	black 384-well plate	anti-mouse IgG 96-well plate
Reaction volume	50 µl	50 µl	50 µl	60 µl

Abbreviations: QRET, quenching resonance energy transfer; FP, fluorescence polarization; VBA, vascular endothelial growth factor binding aptamer; FBA, fibroblast growth factor binding aptamer; GTP, guanosine triphosphate; SA, streptavidin; IgG, immunoglobulin G

4.4.1 QRET method for growth factor detection

In publication **I**, a direct QRET assay for growth factor (GF) detection was constructed by using the Eu^{3+}-aptamer/protein interaction inducing Eu^{3+}-chelate protection from soluble quencher. The QRET assay method was used to detect VEGF and bFGF, using previously selected specific aptamers which was now labeled (Eu^{3+}-VBA and Eu^{3+}-FBA) (Kaur and Yung, 2012; Golden, 2000). The assay principle is presented in Figure 11.
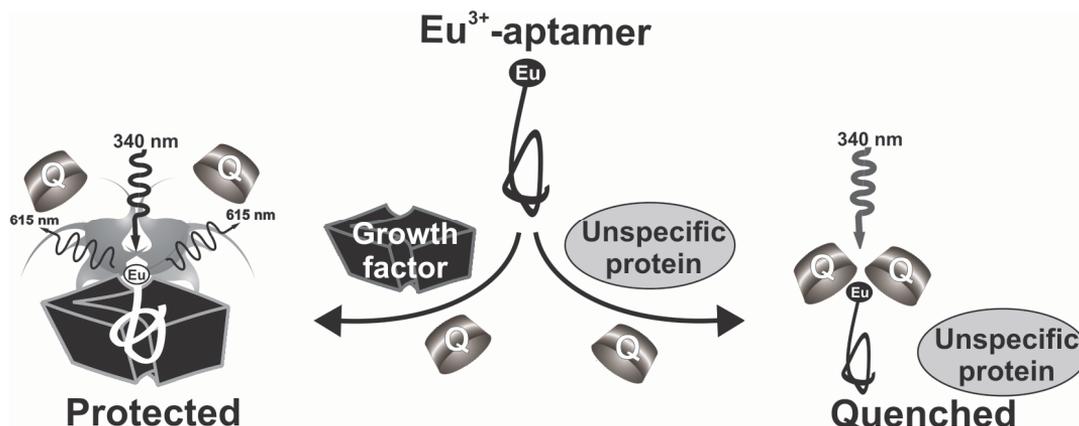
Figure 11. The principle of the homogeneous growth factor assay using the quenching resonance energy transfer (QRET). In the presence of a specific growth factor, the Eu^{3+}-chelate conjugated to aptamer is in close vicinity to the protein surface and high time-resolved luminescence (TRL) signal is monitored. In the presence of an unspecific protein or without any target protein, the aptamer stays in the solution and the TRL-signal is quenched due to the soluble quencher. Figure is modified from publication I.

The homogeneous GF assay was performed in a buffer based on the aptamer selection buffers, 0.11 mM KH_2PO_4, 0.56 mM Na_2HPO_4, 15.4 mM NaCl, 0.01% Triton-X 100, 1 mM $MgCl_2$ (pH 7.4) (Golden, 2000; Kaur and Yung, 2012). In the assay, Eu^{3+}-VBA or Eu^{3+}-FBA (0.5-10 nM) and VEGF or bFGF target (0-750 nM) were incubated for 10 min. The TRL-signal from Eu^{3+}-aptamer was measured before and after Quench V (1.4-2.3 μM) addition. TRL-signal was measured as described in section 4.2.1. In kinetic measurements, the Eu^{3+}-aptamer and Quench V were mixed and the increased TRL-signal was monitored after GF addition. All fitting functions were performed using Origin 8 software (OriginLab, Northampton, MA, USA). Sigmoidal dose response curves were fitted using logistic function and kinetics using an exponential decay function.

4.4.2 QRET technique for GTPase nucleotide exchange detection

In publications **II** and **III**, a single-label QRET assay to monitor nucleotide exchange with GTPases was constructed. The GTPase ability to protect bound Eu^{3+}-GTP from soluble quencher was used to monitor Eu^{3+}-GTP association and dissociation kinetics with different GTPases. All QRET assays in publications **II** and **III** were performed using 2'/3'-GTP-Eu^{3+}. The principle of the assay is presented in Figure 12.

The homogeneous QRET assay for kinetic analysis of the GTPase nucleotide exchange reactions were performed with Quench II and in an optimized buffer, 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 3 mg/ml Triton-X 100, 0.1 mg/ml γ-globulins (Martikkala et al., 2011). Eu^{3+}-GTP (10 nM), Quench II (22 μM), and GTPase (200 nM Ras or 500 nM RhoA) were incubated with slow shaking for 5 min. The quenched TRL-signal was measured as

described in section 4.2.1. The GEF (200 nM SOS^{cat} or 500 nM Ect2) was added and the Eu^{3+}-GTP association induced TRL-signal increase was monitored (1500 s). Eu^{3+}-GTP dissociation was monitored from the decreasing TRL-signal (1500 s), occurring after the addition of free GTP (100 μM). Before Eu^{3+}-GTP dissociation, Eu^{3+}-GTP (10 nM), Quench II (22 μM), GTPase (200 nM Ras or 500 nM Rho), and GEF (200 nM SOS^{cat} or 500 nM Ect2) were incubated together for 20 min. In publication **II**, the kinetic analysis for H-Ras^{Wt}, H-Ras^{Q61G}, K-Ras^{Wt}, and RhoA^{Wt} were performed. In publication **III**, the kinetic analysis for H-Ras^{Wt} and mutants (R169A/K170A, R161A/R164A, D47A/E49A, R128A/R135A, G48R, G48R/D92N) were performed in 1:1 GTPase/GEF ratio (200 nM). All fitting functions were performed using an exponential decay function (OriginLab).
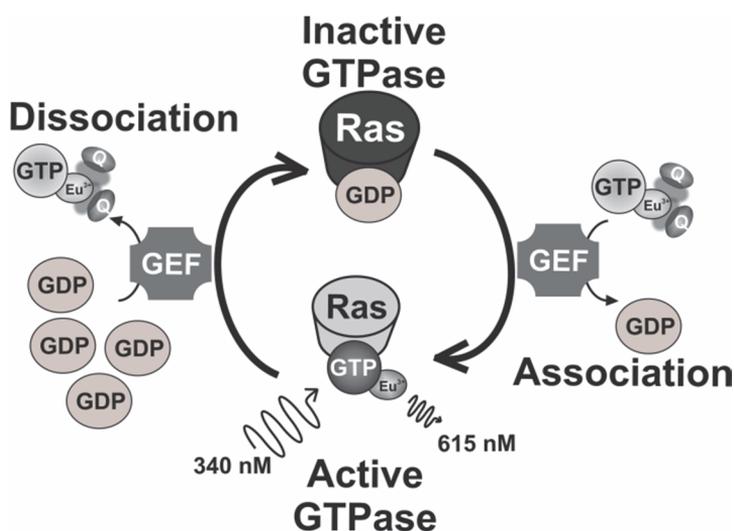
Figure 12. The principle of the quenching resonance energy transfer (QRET) assay for monitoring the kinetics of the nucleotide exchange reaction. In the presence of the guanine nucleotide exchange factor (GEF), the GTPase bound GDP is dissociated enabling Eu^{3+}-GTP association. The Eu^{3+}-GTP association increases the time-resolved luminescence (TRL) signal observed. Eu^{3+}-GTP dissociation occurs when an excess of GDP (or GTP) is added. After dissociation of the Eu^{3+}-GTP, the Eu^{3+}-chelate is exposed to soluble quencher and a decreased TRL-signal is observed.

4.4.3 Cancer associated Ras switch III mutation analysis and nanoclustering

In publications **III**, the activity of Ras mutants were studied in effector-recruitment and nanoclustering FRET assays (Figure 13) (Abankwa et al., 2008). These assays were performed in baby hamster kidney cells using Eugene 6 (Promega, Fitchburg, WI) transfection. The mGFP-tagged Ras was used as a control. In the RBD-recruitment FRET assay (Figure 13A) and in the nanoclustering FRET assay (Figure 13B), mRFP-C-Raf-RBD and mCherry-Ras were used as acceptors, respectively. Effector-recruitment and nanoclustering were modulated using Gal-1 co-transfections to change the Gal-1 level. Fluorescence lifetime was monitored using 48 h growth cells fixed (approximately 60

cells) with 4% PFA and mounted with Mowiol 4-88 (Sigma-Aldrich). The fluorescent lifetime was monitored using three biological repeats, and with fluorescein (10 μM) as a lifetime standard. Ras subcellular colocalization was assured using mCherry-Ras mutants and mGFP-Ras[Wt].

C-Raf-RBD binding to H-Ras was studied using fluorescence anisotropy (Guzmán et al., 2014). Anisotropy was used to monitor C-Raf-RBD/Ras interaction with Ras mutants. Measurements were performed in an assay buffer of 25 mM HEPES (pH 7.2), 100 mM NaCl, and 3 mM MgCl$_2$. In the assay, 100 nM mant-GTPγS or 500 nM mant-GDP-loaded H-Ras were used. K$_d$ values were determined using a global fitting function (GraphPad Prism 6, GraphPad, La Jolla, CA, USA).
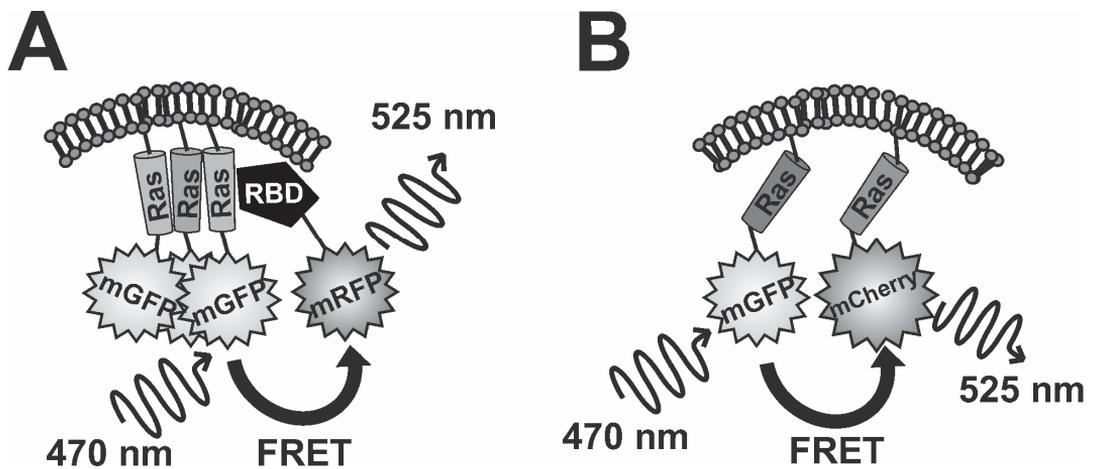
Figure 13. Principles of the effector recruitment and nanoclustering assays. (A) Recruitment of the effector, Ras binding domain (RBD) from C-Raf, to Ras was monitored with Förster resonance energy transfer (FRET) assay. RBD-recruitment to the switch III Ras mutants was monitored using FRET between green fluorescent protein (GFP) tagged Ras and red fluorescent protein (RFP) tagged RBD. (B) Ras mutants were also studied monitoring the FRET signal occurring due the packing of mGFP- and mCherry-tagged Ras proteins in nanoclusters.

GAP induced GTP hydrolysis was monitored using a GST-RBD pull-down assay and Western blotting (Cirstea et al., 2010). The assay was performed in baby hamster kidney cells transfected (JetPRIME Polyplus-transfection, NY) with fluorescent Ras fusion-proteins. Cells were serum-starved for 4 h before 5 min EGF (100 ng/ml) stimulation and Ras-GTP level determination using glutathione beads (Thermo Scientific, Pierce). Lysed cells were incubated for 30 min (+4 ºC) in Tris (pH 7.5), 100 mM NaCl, 2 mM MgCl$_2$, 1% Nonidet-40, 10% glycerol inhibitor cocktail, with or without NF1 (10 μg) and with GST-RBD coupled glutathione beads. After washing in lysis buffer, western blotting in 15% SDS-PAGE was performed using anti-HAtag antibody (Cell Signaling Technology, Danvers, MA).

4.4.4 GTPase activation cycle detection using GTP-specific Fab fragment

In publication **IV**, a heterogeneous assay for GTP was constructed to monitor the GTP hydrolysis reaction. The 2A4^{GTP} Fab fragment was selected in this study from ScFvM-library (Huovinen et al., 2013). The 2A4^{GTP} ability to differentiate between GTP and GDP was utilized to monitor 2'/3'-GTP-biotin (or 2'/3'-GTP-Eu^{3+}) hydrolysis in the presence of H-Ras, GEF, and GAP. Both 2'/3'-GTP-biotin and 2'/3'-GTP-Eu^{3+} performs equally in H-Ras activation/de-activation assays, but Eu^{3+}-SA-biotin-GTP-complex is more highly luminescent than Eu^{3+}-GTP in the heterogeneous assay. The principle for heterogeneous GTP detection (**IV**) is presented in Figure 14. Additionally, the principle of the developed QRET based homogeneous GTP detection assay is presented in Figure 15. The QRET assay also was performed using 2'/3'-GTP-Eu^{3+} and 2A4^{GTP} Fab fragment.
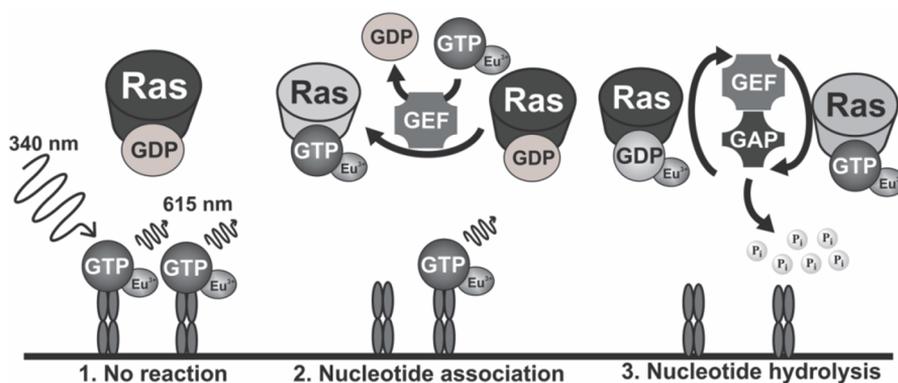
Figure 14. The principle of the heterogeneous GTP detection assay. (1) In the reaction without guanine nucleotide exchange factor (GEF) the Ras cannot be activated and all Eu^{3+}-GTP binds to the Fab fragment (2A4^{GTP}) surface created on an anti-mouse IgG 96-plate. With this setup, the high time-resolved luminescence (TRL) signal is monitored. (2) In the presence of GEF, part of the Eu^{3+}-GTP is associated to Ras and part can bind to the 2A4^{GTP} surface, producing a moderate TRL-signal after the separation step. (3) In the GTP hydrolysis assay, GEF and GTPase activating protein (GAP) enables a multiple turnaround GTP hydrolysis cycle. In the hydrolysis, the Eu^{3+}-GTP is cleaved producing Eu^{3+}-GDP and inorganic phosphate (P$_i$). These compounds cannot bind to the 2A4^{GTP} Fab fragment surface. If GTP hydrolysis occurs, no TRL-signal is monitored because no Eu^{3+}-GTP is present. Instead of Eu^{3+}-GTP, biotin-GTP and Eu^{3+}-streptavidin can be used.

The heterogeneous assay in publication **IV**, was performed in the two same buffers used in publications **II**, **III** (Martikkala et al., 2011). In the first step, the GTPase hydrolysis reaction was performed in a separate tube. In the second step, the hydrolysis reaction mixture was introduced to the 2A4^{GTP} surface (3.0 µg/ml) created in anti-mouse IgG 96-plates (Kaivogen, Turku, Finland). After separation step, the TRL-signal from Eu^{3+}-SA-biotin-GTP-complex or Eu^{3+}-GTP was measured as described in section 4.2.1. In the GTPase hydrolysis step, biotin-GTP or Eu^{3+}-GTP (15-100 nM), H-Ras^{Wt} or H-Ras^{Q61G} (1.2 µM), SOS^{cat} (250 nM), and p120GAP (0-170 µM) or NF-1 (0-6.5 µM) were incubated 0-120 min. GDP (8 µM) was added to stop the hydrolysis reaction (optional step). The

GTPase hydrolysis reactions were added in $2A4^{GTP}$ surface together with Eu^{3+}-SA (15-65 nM, only when biotin-GTP), and reactions were incubated for 30 min. The TRL-signal was monitored after the separation step. Sigmoidal dose response curves were fitted using logistic function and kinetics using exponential decay function (OriginLab).

In publication **IV**, ScFvM-library screening using phage display was performed to find a GTP-specific antibody (Huovinen et al., 2013). Additionally, the heterogeneous GTP detection platform using $2A4^{GTP}$ Fab fragment has been converted to enable homogeneous GTP detection using the QRET technique. The principle of the developed homogeneous GTP detection platform and its ability to monitor GTP hydrolysis and hydrolysis inhibition is depicted in Figure 15.
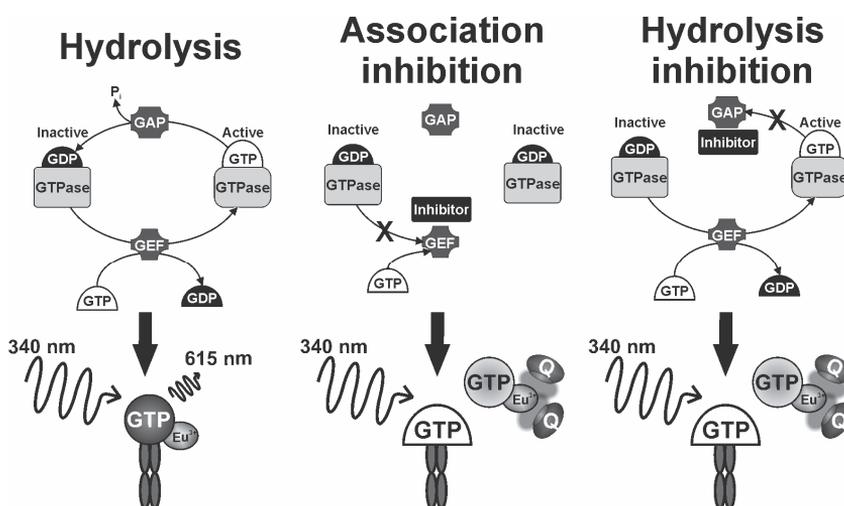
Figure 15. The principle of the competitive antibody based quenching resonance energy transfer (QRET) for homogeneous GTP detection. In the presence of GTPase, guanine nucleotide exchange factor (GEF), and GTPase-activating protein (GAP) the unlabeled GTP is hydrolyzed and the Eu^{3+}-GTP reporter can bind to the $2A4^{GTP}$. Fab fragment bound Eu^{3+}-GTP is protected from quenching. When GTP hydrolysis is disturbed in the presence of an inhibitor, the GTP cannot be hydrolyzed and it binds to $2A4^{GTP}$ preventing Eu^{3+}-GTP binding. In solution, Eu^{3+}-GTP is exposed it to quencher and low time-resolved luminescence signal is monitored.

Homogeneous assay to monitor GTP was performed using two stepped competitive homogeneous protocol, where Eu^{3+}-GTP-$2A4^{GTP}$ based detection was performed after GTP hydrolysis in coupled enzymatic reaction (H-Ras, SOS^{cat}, p120GAP). Two stepped protocol was selected to protect $2'/3'$-GTP-Eu^{3+} from unwanted hydrolysis. In the QRET assay, high TRL-signal is monitored when GTP is efficiently hydrolysed, and cannot compete with Eu^{3+}-GTP-$2A4^{GTP}$-complex. However, when the inhibitor molecule blocks either GEF catalyzed nucleotide exchange or GAP induced GTP hydrolysis, GTP stays unhydrolysed can bind to $2A4^{GTP}$. When $2A4^{GTP}$ is blocked by free GTP, low TRL-signal is monitored because Eu^{3+}-GTP form complex with the soluble quencher.

5 RESULTS AND DISCUSSION

In this chapter, a brief summary of results and discussion is presented with some additional information. A detailed description of results and discussion employed in this study can be found in publications **I-IV**.

5.1 Homogeneous QRET technique for kinetic reaction monitoring

5.1.1 QRET technique (I)

There has been an increased interest towards Ln^{3+}-chelates due to their advantageous properties, e.g. long emission lifetime and sharp emission and excitation peaks. These properties enable sensitive TRL-signal detection even in a complex matrix. Regardless of the advantages of the Ln^{3+}-chelates, homogeneous assays often rely on TR-LRET. In publication **I**, the direct QRET assay for VEGF and bFGF was constructed using Eu^{3+}-aptamers. The Eu^{3+}-chelate was conjugated at either the 3'- or 5'-end of the DNA-aptamer, which can then work both as a binding (aptamer) and signaling (Ln^{3+}-chelate) molecule. The signal difference between target bound and unbound Eu^{3+}-aptamer is created with soluble quencher molecule.
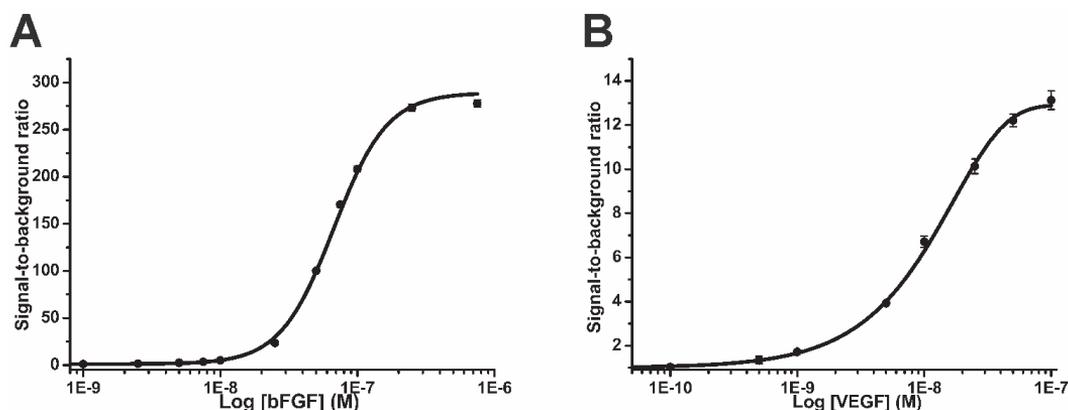
Figure 16. The QRET assay calibration curves for two human growth factors (GFs). The signal-to-background ratios in the QRET assay at increasing basic fibroblast growth factor (bFGF) (A) and vascular endothelial growth factor (VEGF) (B) concentration. Assays were performed using 10 nM Eu^{3+}-aptamers, 0-750 nM GFs, and 2.3 µM Quench IV. The results are shown as means ± SD of four replicates. Figure is modified from publication I.

In the QRET format, a higher analyte concentration produces a more intense TRL-signal. The linear ranges with 2 nM Eu^{3+}-aptamer for the VEGF and bFGF were 0.5-10 nM and 1-50 nM, respectively. The limit-of-detections (LOD) were 238 pM and 725 pM for VEGF and bFGF, respectively. Similarly the S/B ratios were 6.7 and 62 for VEGF and bFGF, respectively. Regardless of that the assay buffer was totally optimized for VEGF, the bFGF

assay produced higher S/B ratio but lower LOD than VEGF. In the assay, the TRL-signal from unbound Eu^{3+}-aptamer was quenched, which enables an increase of the dynamic range by increasing the aptamer concentration without sacrificing the LOD. The linear range, LOD, and S/B ratio with 10 nM Eu^{3+}-VBA was 0.5-25 nM, 450 pM, and 13.1, respectively (Figure 16A). With Eu^{3+}-FBA, the linearity, LOD, and S/B were 2.5-100 nM, 998 pM, and 278, respectively (Figure 16B). The method also enables the GF detection from diluted serum. The ability to monitor PPIs was also proved by monitoring growth factor interaction with heparin (K_i 0.17 ± 0.14 nM).
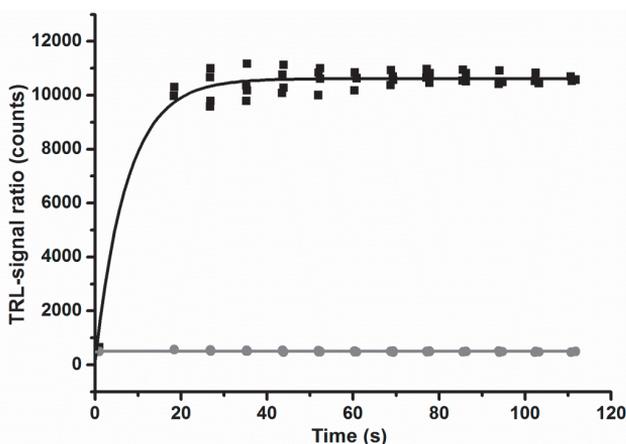
Figure 17. Luminescently labeled basic fibroblast growth factor binding aptamer (Eu^{3+}-FBA) interaction kinetics. The Eu^{3+}-FBA binding kinetics was monitored using 10 nM Eu^{3+}-FBA, 25 nM bFGF (black), and 2.3 µM Quench IV using the non-competitive QRET assay. Control reaction was performed without bFGF (grey). The results are shown as signals from four measurements using nine second measurement interval after a 18 s lag phase. Figure is modified from publication I.

End-point detection strategy has been used in all previous QRET assays. When the quencher is added before the target molecule, the kinetics of the aptamer-target interaction could be monitored (Figure 17). However, in the case of aptamers the interaction kinetics is too fast for the TRL-device to be monitored reliably (estimated k_{on} rate of $1.35*10^{-3}$ M^{-1} s^{-1}). The assay underlines the possibility to monitor enzyme reaction kinetics, for example. The real-time detection method also enables a reduction of the overall detection time from 20 min to ≥ 3 min. The luminescence lifetime for the Ln^{3+}-chelate is traditionally the in millisecond range, enabling time-gated measurement. The luminescence lifetime monitored for the Eu^{3+}-aptamer was reduced from 1.38 ms to nearly zero when the quencher molecule was introduced (Figure 18A). When Eu^{3+}-FBA is bound to FGF, two luminescence lifetimes for the quenched and for the protected Eu^{3+}-aptamer populations were monitored. Not all Eu^{3+}-FBA was bound to bFGF and thus the quenched population was monitored. The Eu^{3+}-FBA/bFGF-complex formation increases the luminescence lifetime to 0.58 ms, thus enabling the TRL detection. The quencher has no effect on the shape of the luminescence spectra monitored (Figure 18B).
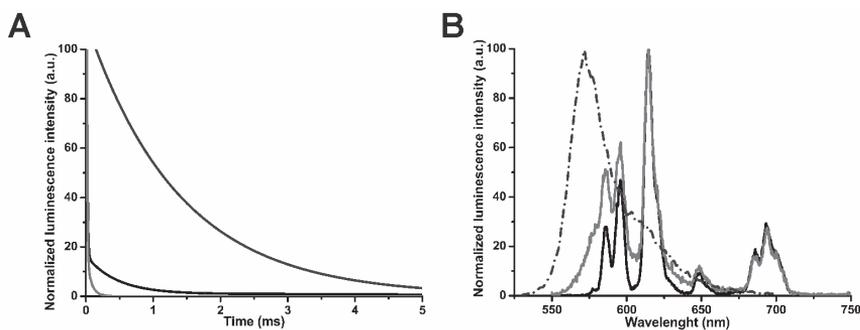
Figure 18. Normalized luminescence lifetime and emission spectra's for Eu^{3+}-aptamer. (A) Luminescence lifetimes for aptamer-Eu^{3+} in the presence of Quench V (light grey), protected bFGF-aptamer-Eu^{3+}-complex in Quench V solution (black), and for protected aptamer-Eu^{3+} without quencher (dark grey). (B) The emission spectra's for Quench V (dashed dot), protected bFGF-aptamer-Eu^{3+}-complex in Quench V solution (grey), and for bFGF-aptamer-Eu^{3+}-complex without quencher (black). Figure is modified from publication I.

In publication **I**, a direct non-competitive QRET assay was used to quantify growth factors using single Ln^{3+}-chelate conjugated DNA-aptamer. This assay was used not only for growth factor quantification but also to study the QRET signaling. QRET is known to enable robust assays with cells and cell membrane, but the performance in plasma or serum was previously unknown (Härmä et al., 2009). In the assay, quencher molecules were found to be the limiting component. Unspecific interaction between plasma/serum proteins and quencher molecule weaken the sensitivity of the assay. We can conclude that with current quencher molecules the QRET assay is not applicable for clinical testing. This was even the Eu^{3+}-FBA showed exceptional Ln^{3+}-chelate protection when bound to bFGF. Approximately 50% of the TRL-signal monitored before quencher addition could be observed after Eu^{3+}-FBA/bFGF-complex formation in the presence of quencher. This enabled, for the first time, reliable luminescence lifetime monitoring for Eu^{3+}-FBA in the QRET assay (Fig. 18). The most crucial observation, however, was that quencher molecule do not interfere Eu^{3+}-aptamer interaction with the target protein. This enables monitoring of reaction kinetics, which is highly important when for example enzymatic reactions are studied. Until now, QRET reaction has been performed only by using endpoint reaction monitoring (Kopra and Härmä, 2015). In publication **I**, the possibility to monitor binding kinetics was proved by adding the soluble quencher before reaction initiation. These observations from publication **I** was vital for the further development of the QRET assay.

5.1.2 The kinetic determination of GTPase nucleotide exchange reaction (II)

GTPases works as intracellular molecular switches regulating cellular processes (Colicelli, 2004). Current methods for GTPase research are based on luminescent nucleotide analogs. The QRET technique with sensitive Ln^{3+}-chelate based detection provides a novel tool for GTPase research (Martikkala et al., 2011). To enable kinetic monitoring of the GTPase reaction, a new 2'/3'-GTP-Eu^{3+} molecule was constructed (publication **II**). In addition, the assay protocol was modified based on the observations in publication **I**. In publication **II**, a

homogeneous QRET assay for Eu^{3+}-GTP association and dissociation monitoring was constructed. The assay was constructed based on the knowledge from publication **I** and the previous GTPase assay (Martikkala et al., 2011). In this study, the nucleotide exchange kinetics was monitored for H-Ras^{Wt}, H-Ras^{Q61G}, K-Ras^{Wt}, and RhoA^{Wt}. Both GTP association and dissociation were monitored in the presence of a specific GEF molecule (SOS^{cat} for Ras and Ect2 for Rho) (Figure 19). Also GEF independent Eu^{3+}-GTP association with H-Ras^{Wt} was monitored, using Mg^{2+}/EDTA-chelation strategy. The nucleotide association and dissociation rate monitored using the QRET technique, were in the order K-Ras^{Wt} > H-Ras^{Wt} > H-Ras^{Q61G} > RhoA^{Wt}. The k_{on} for H-Ras^{Wt} with Mg^{2+}/EDTA-chelation was $4.04*10^5$ $M^{-1}s^{-1}$, which is nearly identical for the k_{on} $3.76*10^5$ $M^{-1}s^{-1}$ in GEF dependent assay. The k_{on} value was in accordance with the literature value (John, et al., 1990). The K_d values calculated based on association (k_{on}) and dissociation (k_{off}) monitored for K-Ras^{Wt}, H-Ras^{Wt}, H-Ras^{Q61G}, and RhoA^{Wt} were 6.0 ± 0.3 nM, 9.1 ± 0.4 nM, 14.7 ± 0.5 nM, and 12.5 ± 0.9 nM, respectively. All assays were performed using nanomolar protein and nucleotide concentrations.
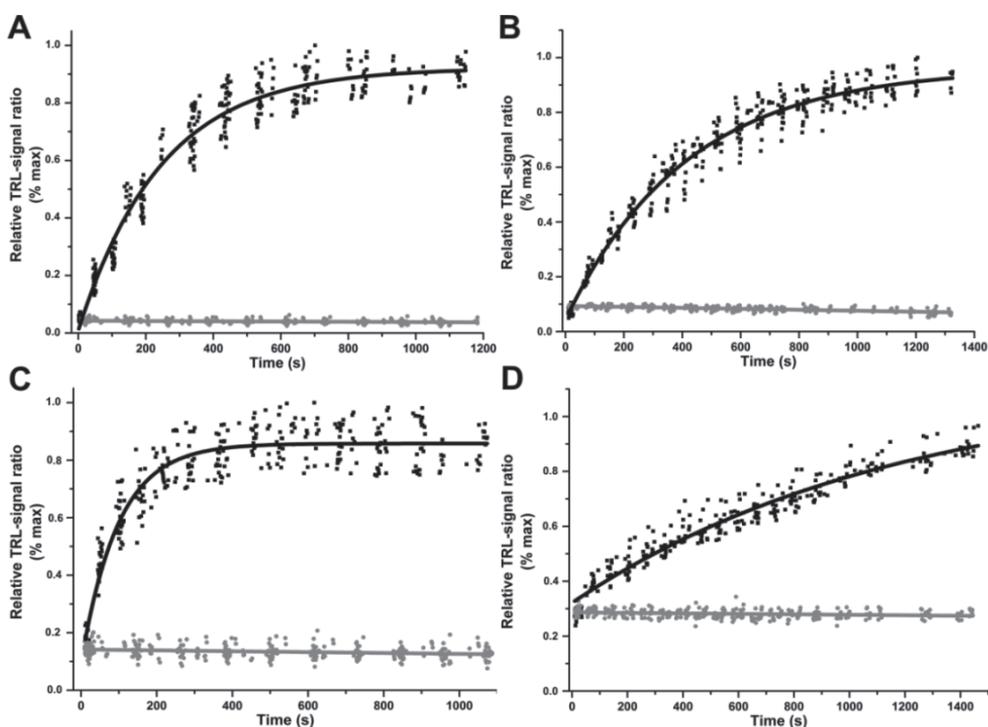
Figure 19. The guanine nucleotide exchange factor (GEF) induced Eu^{3+}-GTP association kinetics with different GTPases. The association kinetics for 10 nM Eu^{3+}-GTP using 22 μM Quench II, 200 nM H-Ras^{Wt} (A), H-Ras^{Q61G} (B), K-Ras^{Wt} (C), with (black) or without (gray) 200 nM GEF, respectively. The RhoA^{Wt} (D) association kinetics was determined with 500 nM Ect2^{cat} and 500 nM RhoA^{Wt}. Dots represent data points obtained from individual reactions, measured on three different days. Altogether 360 individual data points during 1500 s was monitored. Figure is modified from publication II.
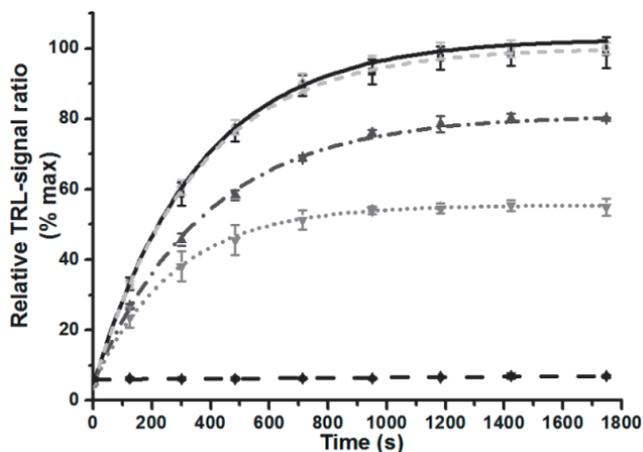
Figure 20. Peptide based blocking of the SOS^{cat} catalyzed Eu^{3+}-GTP association with H-Ras^{Wt}. The inhibition of SOS^{cat} (200 nM) catalyzed Eu^{3+}-GTP (10 nM) association to H-Ras^{Wt} (200 nM) was performed using 0 µM (solid), 5 µM (dashed), 50 µM (dashed dot) or 500 µM (dotted) SOS$^{980-989}$ peptide. The control reaction was performed with 500 µM SOS$^{980-989}$, but without SOS^{cat} (long dashed). The results are shown as means ± SD of triplicate reactions during 0-1800 s. Figure is modified from publication II.

The signal change in the QRET assay progressed over a time scale of minutes, compared to a time scale of seconds in stopped-flow measurements. Thus only slow nucleotide exchange kinetics can be monitored. Already the use of TRL-signal detection precludes the measurement of fast kinetics. On the other hand, the assay allows the monitoring of exchange kinetics in a HTS compatible 384-well plate format using a standard TRL-plate reader. The robust QRET technique could serve as a method of choice for efficient small molecule inhibitor screening. As proof-of-principle inhibitor screening is demonstrated in Figure 20, using a peptide fraction from the SOS^{cat} protein (SOS$^{980-989}$). Both inhibition data and robust kinetic data can be generated simultaneously using 1:1 H-Ras/SOS^{cat} ratio.

The QRET assay developed in publication **II** is one of the most sensitive homogeneous assays to monitor GTPase nucleotide exchange kinetics known. The QRET assay provides a multifunctional platform to monitor nucleotide exchange kinetics (slow), perform mutation analysis (**III**), and to select potential inhibitors (unpublished). Most of the current luminescence based nucleotide exchange assays are performed in cuvette using high protein concentrations and reaction volumes (around 500 µl) (Eberth and Ahmadian, 2009). The throughput of these assays is highly limited, when compared to the developed QRET assay, which can be performed in a 384-well plate using nanomolar protein amounts in 50 µl or even 10 µl reaction volumes. Thus the protein consumption can be decreased by up to 250-fold compared to a conventional mant-label based assay (Eberth and Ahmadian, 2009). The QRET system can be used at least with Ras- and RhoGTPases to select potential inhibitors affecting the nucleotide exchange. No changes are needed when the assay is used for screening or kinetic measurements with GTPases.

5.2 Functional assays for GTPase cycle monitoring

5.2.1 Structural and functional analysis of H-Ras mutation (III)

GTPases are frequently mutated in cancer and other diseases, which makes them attractive drug targets (Karnoub and Weinberg, 2008). Until now, cancer related mutations in Ras have been linked to three loci, Gly12, Gly13, and Gln61 at P-loop, and II regions (Prior et al., 2012). In publication **III**, the role of nanoclustering in Ras signaling was studied. Clustering associated mutations at a novel switch III region, and conformer stabilizing elements (α4 and hypervariable region (hvr)) were also characterized (Figure 21).
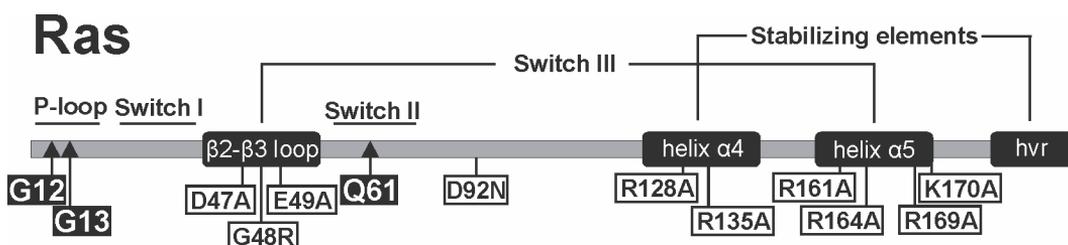
Figure 21. Schematic representation of the linear Ras structure. Critical functional regions for Ras structure are annotated in linear protein structures. Mutational hotspot codons (G12, G13, and Q61) in P-loop and switch II region are marked as triangles. Here studied H-Ras mutations in switch III region and in stabilizing elements are marked in boxes. Figure is modified from publication III.

In publication **III**, the first examples of Ras nanoclustering as a cancer driven feature were presented. The mutations studied in the Ras structure were located in the switch III region (β2-β3-loop and helix α5) and the conformer stabilizing element (helix α4 and hvr) (Figure 21). The effect of different mutations was studied with purified proteins and in cells with different Ras subfamilies. Mutant Ras activity was studied using an effector-recruitment FRET assay in mammalian cells and using the homogeneous QRET technique in solution. A few of the studied single- and double-mutants showed significantly increased nanoclustering and increased H-Ras activity. Mutants G48R and G48R/D92N revealed unaltered GTP binding affinity and GAP-induced GTP hydrolysis, but increase in Gal-1 dependent nanoclustering and RBD recruitment was monitored (Table 4). Mutants G48R/D92N, but not G48R, also showed minor increased SOS-mediated activation. On the other hand, some of the activating mutants could be neutralized by the inactivating mutations. The same observations were found with mutated H-Ras, N-Ras, and K-Ras. In all cases, the mutations did not alter the subcellular distribution of Ras or their GTP affinity. Also, in case of all Ras, increased clustering was followed by higher effector recruitment. Due to clustering, Ras activity was found to be increased 2-3-fold.

Table 4. Summarized differences in guanine-nucleotide exchange factor dependent nucleotide exchange, GTP dissociation constant, and effector binding between H-Ras mutants. Table is modified from publication III.

H-Ras	k_{on} (normalized) (Eu^{3+}-GTP) (GEF)	k_{off} (normalized) (Eu^{3+}-GTP) (GEF)	K_d (normalized) (Eu^{3+}-GTP) (GEF)	K_d (normalized) (Mant-GTPγS) (EDTA)	K_d (normalized) (Mant-GTPγS) (RBD)
Wt	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.13	1.00 ± 0.18
R169A/K170A	0.89 ± 0.04	1.32 ± 0.05	1.48 ± 0.08	0.20 ± 0.03	0.79 ± 0.16
R128A/R135A	1.05 ± 0.04	1.38 ± 0.04	1.32 ± 0.07	0.10 ± 0.02	1.10 ± 0.22
G48R	1.00 ± 0.04	0.91 ± 0.03	0.91 ± 0.05	1.21 ± 0.14	1.28 ± 0.24
R161A/R164A	0.67 ± 0.02	0.19 ± 0.02	0.28 ± 0.03	3.73 ± 0.52	0.89 ± 0.16
D47A/E49A	1.14 ± 0.06	0.41 ± 0.04	0.36 ± 0.04	4.13 ± 0.65	1.53 ± 0.30
G48R/D92N	0.89 ± 0.03	0.65 ± 0.03	0.73 ± 0.04	2.12 ± 0.30	0.98 ± 0.23

Abbreviations: k_{on}, association kinetics; GEF, guanine-nucleotide exchange factor; k_{off}, dissociation kinetics; K_d, dissociation constant; EDTA, ethylenediaminetetraacetic acid; RBD Ras-binding domain.

Mutation frequency in orientation-switch III region and conformer stabilizing elements were studied by running a database search using H-Ras, N-Ras, and K-Ras. Three public cancer databases (COSMIC, cBio, and ICGC) were used, and in these databases approximately 10% of unique mutations occur in the regions studied. In the case of all the Ras subfamilies, the highest number of cancer associated mutations was found in colorectal cancer. Mutations in switch III were also found in several Rasopathies.

Hotspot mutations (G12, G13, and Q61) have a high impact on Ras activity, and these mutations are frequently linked in cancer. Recently described switch III-mutations have a lower Ras activation capability and frequency in cancer than hotspot mutants, but they have major impact to nanoclustering and effector recruitment. This new mechanism of activation showed the importance of the Ras conformation in the membrane. These findings could be important when novel drugs to block Ras over-activity are selected. Nanoclustering could have significance not only in the case of Ras signaling, but also as a more general regulator with a multitude of membrane anchored signaling proteins.

### 5.2.2 Heterogeneous GTPase activation cycle monitoring (IV)

Hydrolysis is one of the basic reactions involved in cell energy metabolism and intracellular signaling. GTPases work as intracellular molecular switches and the GAP induced GTP hydrolysis causes the GTPase transition from active state to inactive state. In this reaction, the γ-phosphate of the GTP is hydrolyzed forming GDP and P_i. These molecules are used in conventional methods to monitor GTP hydrolysis. These methods suffer problems caused by the lack of specificity. All P_i sensing methods are sensitive for all free P_i. GDP detection methods, on the other hand, are only diphosphate specific.
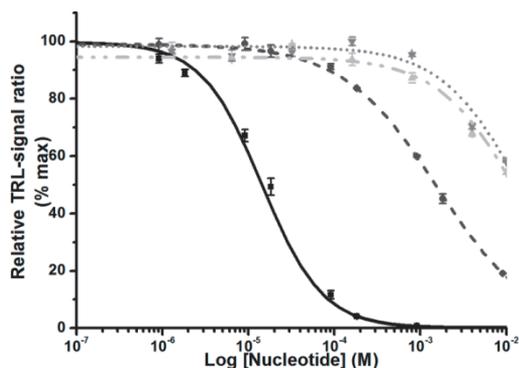
Figure 22. Heterogeneous competitive nucleotide binding assays. Competitive 2A4[GTP] Fab fragment specificity assay using biotin-GTP/streptavidin-Eu^{3+}-complex based TRL-signaling and GTP (solid), GDP (dashed), ATP (dashed dot), and CTP (dotted) competition. Nucleotides were titrated from 0 to 100 mM, and the results are shown as means ± SD of triplicates.

The heterogeneous assay was performed with H-Ras (H-Ras^{Wt} and H-Ras^{Q61G}) and two GAP proteins (p120GAP and NF-1). The half maximal effective concentration (EC$_{50}$) values (concentration giving 50% of the maximum effect) for p120GAP were 0.27 ± 0.03 µM and 9.90 ± 0.91 µM for H-Ras^{Wt} and H-Ras^{Q61G}, respectively. The EC$_{50}$ values for H-Ras^{Wt} and H-Ras^{Q61G} with NF-1 were 35.83 ± 7.28 nM and 77.89 ± 37.52 nM, respectively. H-Ras^{Q61} is one of the three most frequent mutations in mammalian Ras, and the mutant reduces binding to GEF and GAP (Der et al., 1986; Ford et al., 2006; Prior et al., 2012). Previous finding with H-Ras^{Q61G} were in accordance with the results found with p120GAP, but not totally with NF-1. The difference could be due to 2'/3'-GTP-biotin (or 2'/3'-GTP-Eu^{3+}) used as a hydrolysable substrate. Also NF-1 has a higher affinity to H-Ras than p120GAP, which can partly cause the observed effect (Figure 23).
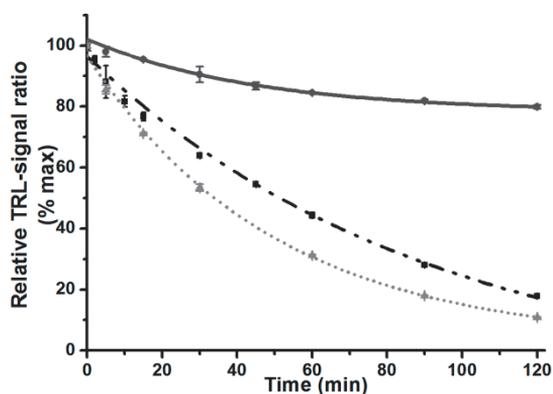
Figure 23. GTPase activating protein (GAP) induced GTP hydrolysis and spontaneous hydrolysis rates. The hydrolysis of 100 nM biotin-GTP was monitored in the presence of p120GAP (dashed dot), NF-1 (dot), and without GAP (solid). Individual reactions were stopped in certain time points during 120 min incubation by addition of 8 µM GDP and ice. The detection was performed with plate conjugated 2A4[GTP] Fab fragment. The results are shown as means ± SD of triplicates. Figure is modified from publication IV.

Heterogeneous assays are advantageous when an efficient separation step is needed. However, for the same reason part of the assay information and materials are wasted. Homogeneous assays usually consume less material and can monitor reactions in a real-time or nearly real-time fashion. To provide a homogeneous platform to monitor the GTPase cycle, GTP detection using a 2A4^{GTP} and the QRET technique was developed. In the competitive QRET assay using 500 nM GTP and 5 nM 2'/3'-GTP-Eu^{3+}, S/B ratio of approximately 6 was monitored in the presence of 7 nM 2A4^{GTP} Fab fragment and 2.5 µM Quench III (Figure 24). This proves that it is possible to monitor the GTPase cycle in a homogeneous assay format, using competitive GTP monitoring. The assay is applicable for assays in 384-well plate and it can be used for HTS-type inhibitor screening (unpublished).
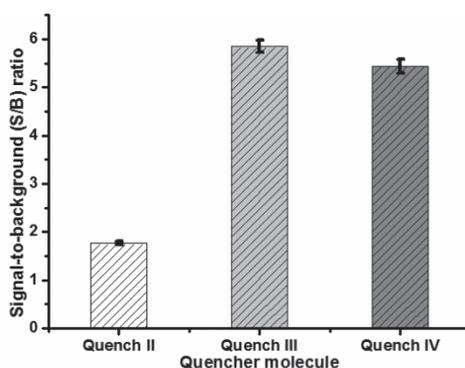
Figure 24. Homogeneous QRET assay for GTP detection with different quenchers. Three quenchers, Quench II (5.0 µM), Quench III (2.5 µM), and Quench IV (2.7 µM) were tested in the QRET assay. The GTP detection (0 nM or 500 nM) was performed in the presence of 2'/3'-GTP-Eu^{3+} (5 nM) and 2A4^{GTP} Fab fragment (7 nM). The results are shown as means ± SD of triplicates.

Currently the methods used to study GTP hydrolysis are based on the detection of the hydrolysis products or GTP/GDP ratio. Compared to these methods, the introduced GTP monitoring platform can be used with similar protein concentrations, but with less possible interference from the matrix compound, e.g. free P$_i$. Unfortunately, the introduced GTP monitoring method is heterogeneous, and thus real-time monitoring cannot be performed. However, Fab fragment based method can be converted to enable homogeneous GTP detection using the QRET technique. A homogeneous assay improves the usefulness of the GTP detection platform, enabling assays which are less material- and time-consuming. More importantly the assay can be performed using competitive assay format in which unlabeled GTP is used as a hydrolyzed substrate. The homogeneous QRET method is HTS compatible and enables inhibitor screening to block either GTPase activation or GTP hydrolysis. However, the properties of the 2A4^{GTP} Fab fragment are not fully known, and the suitability for different assay conditions needs to be further studied. Potentially, the 2A4^{GTP} can be used to monitor GTP concentration not only with purified proteins but also with membrane G proteins. There are no known GTP detection assays and the 2A4^{GTP} based platform thus gives a unique possibility to monitor GTP related reactions.

6 SUMMARY AND CONCLUSIONS

Although GTPases have been under focus of intensive investigation over the past decades, there are still many open questions about the exact working mechanism of the GTPases and their importance as druggable targets. Problems with inhibitor screening are mostly due to limitations in assay methodologies, as well as the small differences in GTPase working mechanisms between subfamilies. GTPase assays can be divided into cell based and *in vitro* assays performed with purified proteins. The use of purified proteins simplifies the assay protocol and interpretation of the results, but the GTPase working mechanism is also simplified. There are a wide range of known or proposed molecules which can interact with GTPases. However, the final confirmation about the exact influence of these molecules is still under debate and awaiting more advanced research tools.

In this study, a range of different TRL-based assays were constructed. TRL detection enables the elimination of assay matrix-induced short-term autoluminescence. In all assays, Eu^{3+}-chelate was used as a signaling donor and in publications **I-III**, the homogeneous QRET technique was used. With the exception of publication **I**, all original publications were GTPase related, introducing methods to study GTPase activation/de-activation cycle. Homogeneous assays format together with a single reporter platform, enables development of sensitive methods with low material consumption. In this study, novel QRET technique was developed to enable kinetic measurement (**I**). This ability was then utilized to study nucleotide exchange (**II**, **III**) for GTPases. Additionally, GTP-specific Fab fragment was selected using phage display technique. This Fab fragment was then used to study RasGTPases in a heterogeneous assay format (**IV**). GTP monitoring in homogeneous QRET format was also studied. Assay to study either GTPase activation or de-activation are applicable for HTS-type small molecule inhibitor screening.

The main conclusions based on the original publications are:

I   Only very few single-label based assay techniques has been developed. The homogeneous QRET technique has been previously utilized only in competitive endpoint assays. In this article, we proved that the technique enables non-competitive protein detection. The QRET method is based on differences in Ln^{3+}-chelate interaction with soluble quencher. For the first time, quenching induced effect to luminescence lifetime could be monitored due the strong protective effect during Eu^{3+}-FBA-bFGF interaction. In the QRET assay, Ln^{3+}-aptamers can serve both as binding and recognition elements. This property could enable assay development to detect any target with a known aptamer. Most importantly, single-step assay platform, where also quencher is present throughout the reaction, enables kinetic reaction monitoring which is now demonstrated for the first time.

II   GTPase research suffers from the need of large amounts of purified proteins, due to lack of efficient research tools. In this article, the potential of the QRET technique to use low nanomolar protein concentrations was combined with the ability to measure reaction kinetics. The Eu^{3+}-GTP reporter provides a strong and robust TRL-signal, which was utilized to monitor nucleotide exchange reactions with Ras- and RhoGTPases. The developed platform enables kinetic monitoring for both GTP association and dissociation. The assay can be used with different GTPases without complicated optimization, only by adjusting the Eu^{3+}-GTP and protein concentrations. QRET provides a higher S/B ratio with 10- to 100-fold lower protein concentrations than method using environment-sensitive labels. A HTS compatible assay was performed in a 384-well plate, which is especially important when used to perform large assay panels, e.g. inhibitor screening.

III   Single point mutations in RasGTPases are very common in different type of cancers and developmental disorders. So far, most of the effort has been to study so called hotspot mutations and their effects to cancer. We have now shown that the switch III region has a major effect on Ras nanoclustering and effector recruitment. We have shown, for the first time, examples of disease associated Ras mutations with altered nanoclustering features. Mechanistically uncharacterized Ras mutants were studied using biochemical, bioinformatical, and cell-biophysical methods to reveal the importance of nanoclustering.

IV   GTPases work as molecular switches shifting between active and inactive conformation. To study the whole GTPase cycle, assays for both GEF dependent GTP association and GAP catalyzed GTP hydrolysis are needed. Currently, both reactions are monitored using different assays. Here we selected a GTP-specific Fab fragment from a large phage display library. For the first time, an antibody fragment specific to GTP was selected. This unique Fab fragment has over 100-fold higher affinity to GTP over its closest relatives GDP, ATP or CTP. Using this Fab, we have developed a heterogeneous detection method that enables both nucleotide association and GTP hydrolysis detection. For the first time, GTP concentration was directly used to monitor GTP hydrolysis.

The QRET technology has quickly evolved from a screening tool to functional bioanalytical technique. The method can be customized in a multitude of different type of assays. To enable efficient GTPase regulator and effector molecule research, fast, simple, and robust methods are needed. The need for large amounts of purified proteins makes GTPase research with the current methods and especially screening type of assays, expensive. New HTS compatible techniques are especially of high importance. The advances in the TRL monitoring and the QRET technology hold high promise to fulfill these requirements in GTPase research. The presented panel of GTPase assays covers the main assemblies and reactions involved in GTPase research.

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