Involvement of the Microtubule-Regulated RhoGEF GEF-H1 in the G12 family signaling pathways

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Memòria presentada per Georgina Garrido Antequera per optar

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Als meus pares,

Al Toni,

Croyez ceux qui cherchent la vérité, doutez de ceux qui la trouvent

Creu a aquells que busquen la veritat,

dubta dels que diuen que l'han trobada.

(André Gide)

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ABBREVIATIONS

AGS Activators of G-protein signaling

AKAP A-kinase anchoring protein

BSA Bovine serum albumin

Btk Bruton´s kinase

cAMP cyclic adenosine monophosphate

DH Dbl homology

DMEM Dulbecco's modified Eagle medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DTT DL-Dithiothreitol

EBP50 ERM binding protein 50

EC Extracellular

ECM Cell-extrecellular matrix

E.Coli *Escherichia Coli*

EDTA Ethylene diaminetetraacetic acid

EGF Epithelial grow factor

GAP GTPase-activating protein

GDI Guanosine nucleotide dissociation inhibitors

GDP Guanosine diphosphate

GEF Guanine nucleotide exchange factors

GFP Green fluorescence protein

GPCR G protein-coupled receptor

GST Glutathione S-transferase

GTP Guanosine triphosphate

h Hour

HA Hemagglutinin

HEPES N 2 hydroxyethylpiperazine N 2 ethanesulfonic acid

Hsp90 heat shock protein 90

IP Immunoprecipitation

JNK Jun NH2 terminal kinase

KCl Potassium chloride

KDa Kilodalton

KO knockout

LARG leukemia associated

RhoGEFLbc lymphoid blast crisis

M Molar

MAPK Mitogen-activated protein kinase

min minutes

mRNA Messenger ribonucleic acid

MTOC Microtubule-organizing-center

NaCl Sodium chloride

NP40 Nonidet P-40

p120ctn p120 catenin

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PDZ Postsynaptic density 95, disk large, zona occludens-1

PH Pleckstrin homology

PI-3 K Phosphatidylinositol-4,5-bisphosphate 3-kinase

PKA Protein kinase A

PKC ζ Protein kinase C ζ

PLC Phospholipase C

PMSF Phenylmethylsulfonyl fluoride

PP2A/PP5 protein phosphatase 2A/phosphatase 5

PVDF Polyvinylidene fluoride

PYK2 Protein Tyrosine kinase 2

RGS Regulatory of G protein signaling

RH RGS homology

RNA Rinonucleic acid

rpm Revolutions per minute

RTK Receptor tyrosine kinase

s seconds

SDS Sodium-dodecyl-sulphate

SEM Standard error of the mean

shRNA Short hairpin ribonucleic acid

SRE Serum response element

TE Tris-EDTA

Tec tyrosine-protein kinase Tec

TEMED Tetramethylethylenediamine

TM Transmembrane

Tris Tris(hydroxymethyl)-amino-methane

WDR36 WD repeat-containing protein 36

WD tryptophan-aspartic acid dipeptide

wt Wild type

V volts

ZO 1/2 Tight junction protein 1/2

INTRODUCTION

1. G PROTEIN COUPLED RECEPTOR SIGNALING

Cell signaling is an important process required for the normal growth and development of the cell. All cells have the ability to receive, process and respond to various signaling cues from their environment. Such signals include hormones, growth factors, neuromodulators, light, odorants, lipids, ions and small molecules. These ligands interact with specific receptors on the cell surface, and this binding initiates a cascade of downstream signaling events, resulting in a specific cellular response. One of the most abundant and important families of membrane receptors are the seven transmembrane receptors also called G protein coupled receptors (GPCRs) and its associated partners, the heterotrimeric G proteins. Signal transduction by GPCRs is fundamental for a large spectrum of physiological processes such as cognition, sensory perception of pain, light, odors and taste, metabolism, immunity, inflammation and endocrine secretion (Kobilka, 2007). Consequently, they are involved in many pathophysiological conditions, as is the case of cancer, making the GPCRs superfamily a major target for therapeutic intervention with more than 40% of clinically approved drugs targeting GPCR signaling G protein coupled receptors (Vsevolod *et al.*, 2012).

1.1 Functional and structural features of GPCR

G protein coupled receptors (GPCRs) are integral membrane proteins named for their activation of intracellular heterotrimeric G proteins. Thanks to the sequence of the human genome it is known that there exist more than 800 genes encoding for GPCRs (Pierce *et al.*, 2002). Based on their sequence, GPCRs have

been grouped into four subfamilies: the rhodopsin family, which is the largest family; the frizzled/tasted family; the glutamate family; and the secretin family (Kobilka, 2007). A large fraction of these receptors have unknown physiologic function, named orphan GPCRs.

Members of the GPCR superfamily share the same basic architecture, seven hydrophobic transmembrane (TM) α-helices connected by three extracellular and three intracellular loops. The extracellular regions contain also an N-terminal domain usually glycosylated and are responsible for ligand binding, while the intracellular loops and the C-terminal tail are implicated in the interaction with G proteins, arrestins, other downstream effectors and are responsible for receptor desensitization (Vsevolod *et al.*, 2012). Due to the technical difficulties of obtaining crystals of membrane proteins, however, a few mammalian GPCR structures have been obtained mostly during past six years. These advances have provided useful information for understanding GPCR activation states necessary for the design of reliable pharmaceutical drugs. Moreover, a break through the research has come by several studies pioneered by Robert Lefkowitz demonstrating that ligands acting at the same GPCR can stabilize multiple, distinct, receptor conformations linked to different functional outcomes (for a recent review on the subject see (Costanzi, 2014)). This phenomenon is known as biased agonists. The functional selectivity offers the opportunity to separate on-target therapeutic effects from side effects through the design of drugs that show pathway selectivity.

GPCRs have traditionally been considered monomeric membrane proteins but several lines of evidence obtained during the last years suggest that GPCRs can also form homo- and heterodimers (Pin *et al.*, 2007). Oligomerization can modulate receptor cell surface expression, ligand binding affinity, downstream signaling properties of the receptors and mediating cross-talk between GPCRs pathways. Additionally, it is also known that non-canonical signaling pathways facilitate the cross-talk between GPCR-signaling pathways and receptor tyrosine kinase (RTK)-pathways (Salahpour *et al.*, 2000).

1.2 Heterotrimeric G proteins

Heterotrimeric G proteins consist of two functional signaling units, a guanine nucleotide binding Gα subunit and a Gβγ dimer, and act as molecular switches for the regulation of a large number of cellular responses cycling from a GTP-active to a GDP-inactive state. Ligand-receptor activation induces a conformational change in the Gα subunit that leads to the exchange of bound-GDP for GTP and the dissociation of the Gα subunit from the Gβγ dimer. Subsequently, both subunits activate their downstream effectors. The hydrolysis of the bound-GTP back to GDP can be done by the intrinsic GTPase activity of the Gα subunit, or by the help of GTPase proteins, resulting in the termination of its effector-interaction. The Gα-GDP re-associates with the free Gβγ dimer and the heterotrimer re-enters signaling cycle again (Figure I1).

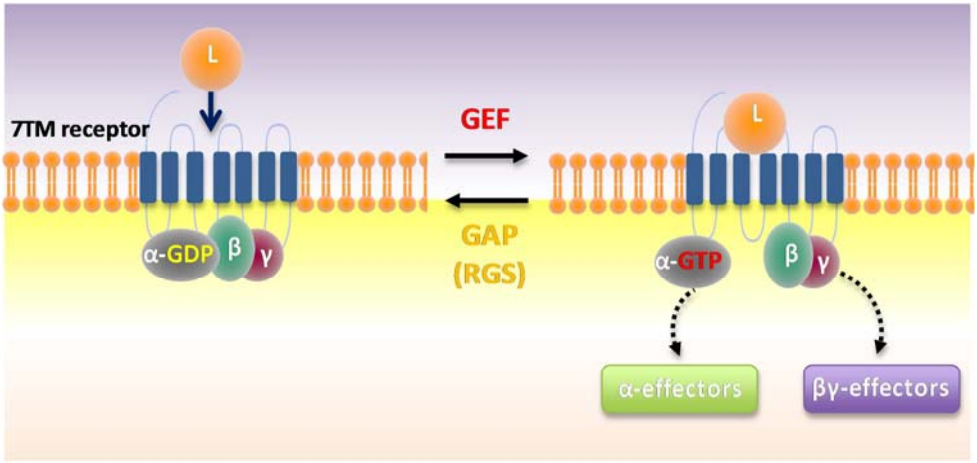


Figure I1. The G-protein cycle. Receptor-mediated signaling. In the absence of ligand, the Gα subunit is GDP bound. Ligand-activated receptor acts as a GEF and promotes exchange of GDP for GTP on the Gα subunit. Gβγ dissociates from Gα·GTP, and both of them signal to their respective effectors. The cycle returns to the basal state when GTP is hydrolyzed to GDP by an intrinsic GTPase activity of the Gα subunit, a process that can be enhanced by the GAP (GTPase-activating) activity of regulators of G protein signaling (RGS) proteins. Adapted from (McCudden *et al.*, 2005).

A parallel mechanism for signal termination is also initiated upon ligand activation of the receptor based on the desensitization of receptor by specific GPCR kinases (GRKs) (homologous desensitization), which induces the binding of

arrestin proteins, blocking further G protein activation with the subsequent internalization of the receptor in clathrin-coated vesicles. Gα subunits may be internalized as well, though this occurs by a separate mechanism (Allen *et al.*, 2005).

Some years ago the GTPase cycle presented timing paradoxes between known GPCR-receptor-mediated physiological responses and the observed activity *in vitro* of the responsible G-proteins, since *in vitro* processes were slower than physiological responses. These questions were answered by the finding of a novel superfamily of proteins, termed Regulator of G-protein Signaling, "RGS", which accelerated the intrinsic GTPase activity of the Gα subunit. The RGS proteins act as a GAP for the Gα subunit, leading to the termination of G protein cycle. More than 20 members of RGS proteins have been identified so far. All these proteins are characterized by housing a common 120 amino acid "RGS box", which binds to Gα-GTP (Siderovski and Willard, 2005). On the other hand, the GTPase cycle can be regulated also by some effectors that act as GAPs for Gα subunits, as the phospholipase C for $G\alpha_q$ (Berstein *et al.*, 1992), or guanine nucleotide exchange factors (GEFs) that activate the Rho family of GTPases stimulating the release of GDP for GTP (Kozasa *et al.*, 1998). In fact, GPCRs act as GEFs for the Gα subunits.

Thanks to resolution of the crystal structure of several Gα subunits in GDP and GTP bound forms, we know many of the features of the Gα subunits (Coleman *et al.*, 1994; Lambright *et al.*, 1994, 1996; Sondek *et al.*, 1994; Tesmer *et al.*, 1997) . The Gα subunit is composed of two domains: 1) a nucleotide binding domain with high structural homology to Ras-superfamily GTPases that also includes sites for binding receptors, effectors, and Gβγ dimer and 2) an alpha-helical domain that, in combination with the Ras-like domain, forms a deep pocket for binding guanine nucleotide (Figure I2) (Sprang, 1997). Three flexible regions named Switch I, II and II change conformation in response to GTP binding and hydrolysis (Coleman *et al.*, 1994; Lambright *et al.*, 1994; Sondek *et al.*, 1994; Tesmer *et al.*, 1997). The N-terminus of Gα subunits are covalently modified by the attachment of the fatty acids myristate and/or palmitate (Wedegaertner *et al.*, 1995; Wedegaertner, 1998). These modifications serve to target these subunits to the membrane and are important for interactions both among the subunits and between the subunits and other proteins.

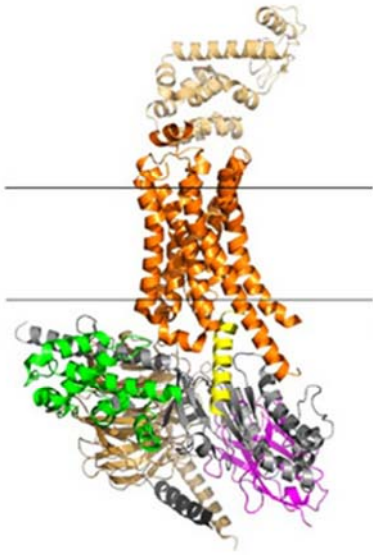


Figure I2. Ribbon diagrams of the crystal structure of the β_2AR-G_s complex. The receptor is shown in orange, the Gα helical domain in green, the Gα GTPase domain in gray, the Gβ subunit in light brown, and the Gγ subunit in black (Alexander *et al.*, 2014).

Recently, it has been solved the crystal structure of the ternary complex composed of nucleotide-free Gs heterotrimer and agonist-occupied monomeric β2-adrenergic receptor. The recent crystal structure of β2ARGαsβγ complex gave profound information on how a GPCR activates G proteins. The principal interactions between the β2AR and Gs involve the amino- and carboxy-terminal α-helices of Gs heterotrimer (Rasmussen *et al.*, 2011).

A given class of heterotrimeric G proteins can be typically recognized by only a subset of GPCRs, and can only interact with one or a few downstream effector targets, ensuring the specificity of signaling from receptor to effector. Likewise, some GPCRs are able to activate more than one G protein subtype. Nevertheless, the interaction between the G protein and the receptor in general does not occur in an absolutely specific or in a completely promiscuous manner (Table I1). So the myriad of combinatorial networks that can be achieved in a cell accounts for the diversity of cellular responses.

Table I1. Heterotrimeric G proteins

Class/Type	Gene	Expression	Effector(s)
α-subunits			
$G\alpha_s$ class			
$G\alpha_s$	GNAS	Ubiquitous	AC (all types) ↑
$G\alpha_{sXL}$	GNASXL	Neuroendocrine	AC ↑
$G\alpha_{olf}$	GNAL	Olfactory ephitelium, brain	AC ↑
$G\alpha_{i/o}$ class			
$G\alpha_{i1}$	GNAI1	Widely distributed	AC (types I, III, V, VI, VIII, IX) ↓
$G\alpha_{i2}$	GNAI2	Ubiquitous	
$G\alpha_{i3}$	GNAI3	Widely distributed	
$G\alpha_o$	GNAO	Neuronal, neuroendocrine	VDCC ↓, GIRK ↑ (via Gβγ)
$G\alpha_z$	GNAZ	Neuronal, platelets	AC (e.g V, VI) ↓, Rap1GAP
$G\alpha_{gust}$	GNAT3	Taste cells, brush cells	PDE ↑
$G\alpha_{t\text{-}r}$	GNAT1	Retinal rods, taste cells	PDE 6(γ-subunit rod) ↑
$G\alpha_{t\text{-}c}$	GNAT2	Retinal cones	PDE 6(γ-subunit cone) ↑
$G\alpha_{q/11}$ class			
$G\alpha_q$	GNAQ	Ubiquitous	PLC-β1-4 ↑
$G\alpha_{11}$	GNA11	Almost ubiquitous	PLC-β1-4 ↑
$G\alpha_{14}$	GNA14	Kideny, lund, spleen	PLC-β1-4 ↑
$G\alpha_{15/16}$	GNA16	Hematopoietic cells	PLC-β1-4 ↑
$G\alpha_{12/13}$ class			
$G\alpha_{12}$	GNA12	Ubiquitous	PDZRhoGEF, LARG, Btk, Gap1m, cadherin
$G\alpha_{13}$	GNA13	Ubiquitous	p115RhoGEF, PDZRhoGEF, LARG, radixin
β subunits			
β_1	GNB1	Widely, retinal rods	AC type I ↓
β_2	GNB2	Widely, distributed	AC type II, IV, VII ↑
β_3	GNB3	Widely, retinal cones	PLC-β (β3>β2>β1) ↑
β_4	GNB4	Widely distributed	GIRK1-4 ↑
β_5	GNB5	Mainly brain	Receptor kinases (GRK2 and 3) ↑
γ subunits			
γ_1, γ_{rod}	GNGT1	Retinal rods, brain	PI-3-K, β, γ ↑
γ_{14}, γ_{cone}	GNGT2	Retinal cones, brain	T type VDCC ↓
γ_2, γ_6	GNG2	Widely	$(G\beta_2\gamma 2)$N-,P/Q-,R-type VDCC ↓
γ_3	GNG3	Brain, blood	
γ_4	GNG4	Brain and other tissues	
γ_5	GNG5	Widely	
γ_7	GNG7	Widely	
γ_8, γ_9	GNG8	Olfactory/vomeronasal epithelium	
γ_{10}	GNG10	Widely	
γ_{11}	GNG11	Widely	
γ_{12}	GNG12	Widely	
γ_{13}	GNG13	Brain, taste buds	

AC, adenylyl cyclase; PDE, phosphodiesterase; PLC, phospholipase C; GIRK, G protein-regulated inward rectifier potassium channel; VDCC, voltage-dependent Ca2_ channel; PI-3-K, phosphatidylinositol 3-kinase; GRK, G protein-regulated kinase; RhoGEF, Rho guanine nucleotide Exchange factor. Adapted from (Wettschureck and Offermanns, 2005).

1.3 G protein α-subunits

Despite the size and great diversity of the GPCR superfamily, there are a relatively small number of G proteins to account for the large number of different intracellular signaling cascades. According to current knowledge, in the human genome there are 34 genes encoding G proteins, which 17 genes are for Gα subunits, 5 genes for Gβ subunits and 12 genes for Gγ subunits (Milligan and Kostenis, 2006). The Gα subunits are divided into four subfamilies ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$) and members of each subfamily are structurally similar and have been known for the activation of one main effector.

As such, all the α-subunits belonging to Gs subfamily stimulate adenylyl cyclase, whereas the α-subunits from Gi-subfamily inhibit adelynyl cyclase. On the other hand, the members of the Gq subfamily of proteins couple to phospholipase c-β (PLC-β) and members of the G12 subfamily are involved in the regulation of small GTPases, among other specific functions (Figure I3). For many years those effectors were believed to account for the main actions of these Gα-subfamilies. But the growing list of effectors, regulators and adaptor proteins for each Gα subunit has changed this simplistic linear version of signal transduction. To name some examples it is known that the $G\alpha_q$ protein activates kinases as the Bruton's tyrosine kinase (Bence *et al.*, 1997) or PKCζ (Malhotra *et al.*, 2010) but also binds to adaptor proteins like WDR36 (Cartier *et al.*, 2011) or EBP50 (Rochdi *et al.*, 2002) to name few of them (for more information see review (Sánchez-Fernández *et al.*, 2014)).

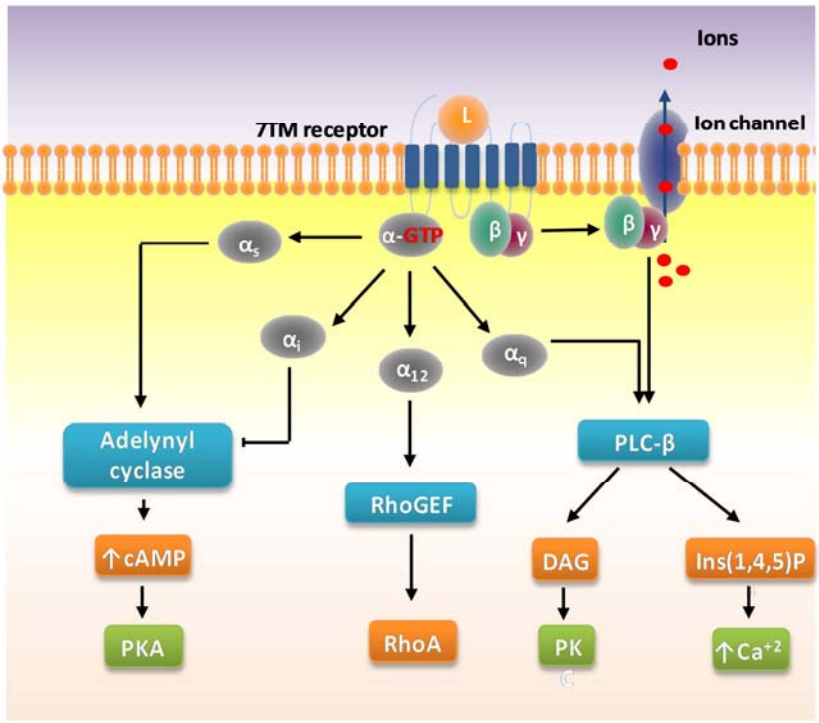


Figure I3. G protein-mediated signaling by GPCR**.** Four families of Gα subunits activate different downstream effectors. Adapted from (Ritter and Hall, 2009)**.**

1.4 The Gβγ dimer

The dimer is composed by two polypeptides, Gβ and Gγ, although functionally is a monomer because the two subunits cannot be dissociated except with denaturants. As mentioned before, in the human genome there are 5 known Gβ subunits and 12 Gγ subunits.

The Gβ subunit is made up of two structurally distinct regions, an N-terminal segment, which is an helix of approximately 20 amino acids, and the remainder of the molecule, which is made up of a seven WD repeats, which allows the protein to be folded in β-strands that are arranged in a ring, forming a propeller structure (Figure I4) (Clapham and Neer, 1997).

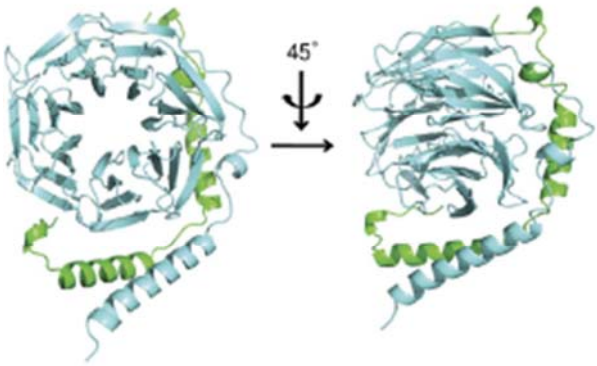


Figure I4. $G\beta_1\gamma_1$ crystal structure. Gβ subunit is colored in aquamarine and Gγ in green. Adapted from (Lin and Smrcka, 2011) .

As mentioned before, the small Gγ subunit binds tightly to the β-propeller. Its amino terminus forms a coiled-coil with the amino terminal non-WD-repeat region of the Gβ subunit. All Gγ subunits are prenylated at their C-terminus post-translationally (Wedegaertner *et al.*, 1995), and this lipid modification is important for the membrane localization of the Gβγ dimer. Initially the Gβγ dimers were thought to be necessary mainly for the inactivation of Gα subunits, thus preventing spontaneous Gα activation in the absence of receptor stimulation (Neer, 1995). However, subsequent studies showed that Gβγ could activate muscarinic potassium channels in the heart (Logothetis *et al.*, 1987). They also could regulate with Gα subunits common effectors, such is the case of the $G\alpha_i$ and Gβγ that can regulate both before and after activation by neurotransmitters the G protein-activated K^+ channel (GIRK) (Berlin *et al.*, 2010). It has been shown that Gβγ can regulate cell-matrix adhesiveness by activating Rap1a-dependent inside-out signals and integrin activation (Ahmed *et al.*, 2010). In the past 20 years, it has become clear that Gβγ subunits can modulate many effectors such as phospholipase C β (Zhang *et al.*, 1996), adenylyl cyclases (Taussig *et al.*, 1994), and voltage-gated calcium channels (Zamponi *et al.*, 1997). Recently identified effectors include, protein kinase D (Jamora *et al.*, 1999), guanine nucleotide exchange factors such as p114-RhoGEF (Niu *et al.*, 2003), PI-3 kinase isoforms (Kerchner *et al.*, 2004), PDZ-proteins (Li *et al.*, 2006), and β-arrestins (Seitz *et al.*, 2014), among others.

2. G12 subfamily

The latest discovered subfamily of heterotrimeric G proteins was the G12 subfamily, which is comprised of only two members, $G\alpha_{12}$ and $G\alpha_{13}$, sharing ~70% amino acid identity. They differ in the N-terminal short sequence and helical domains in which the amino acid identities are 16% and 51%, respectively. $G\alpha_{12}$ and $G\alpha_{13}$ were discovered using a homology-based PCR strategy on a mouse brain cDNA library and are ubiquitously expressed (Strathmann and Simon, 1991; Spicher *et al.*, 1994). The G12 subfamily has been shown to regulate pathways involved in cell growth, oncogenesis and cell shape changes. There has not been many differences found in the coupling of $G\alpha_{12}$ and $G\alpha_{13}$ to different GPCRs, and most of the ligands have the ability to activate both $G\alpha_{12}$ and $G\alpha_{13}$ proteins. However, the results obtained with the knockout of $G\alpha_{12}$ or $G\alpha_{13}$ suggest specific physiological responses for each of them. While $G\alpha_{13}$-deficient mice died at embryonic stage and presented defects in angiogenesis (Offermanns *et al.*, 1997), $G\alpha_{12}$-deficient mice developed normally without any deficiency (Offermanns, 2001). These findings indicate that $G\alpha_{12}$ and $G\alpha_{13}$ cannot compensate each other in physiological significance though both proteins have been shown to activate downstream Rho signaling. As this family of proteins is the main focus of this thesis, I will proceed to a more detailed explanation of their effector pathways and their physiological role.

2.1 Activation of G12 proteins by GPCRs

Understanding the signaling through $G\alpha_{12}$ and $G\alpha_{13}$ is complicated because the activation of these G proteins by GPCRs is rarely demonstrated directly and is instead analyzed from downstream events. According to the current knowledge, more than 30 GPCRs have been reported to couple to G12 subfamily of G proteins. These receptors include serotonin 5-HT(4)R and 5-HT(7)R, angiotensin receptor AT1, endothelin receptors ETA and ETB, galanin receptor GAL2, lysophosphatidic acid receptor LPA, muscarinic M3 receptor, protease activated receptors PAR1, PAR3 and PAR4, sphingosine-1 phosphate S1P(2–5) receptors, and a few others. (Ponimaskin *et al.*, 2002). A striking feature of the receptors that are coupled to

G12 proteins is that they invariably couple to one or more other G proteins, especially to $G\alpha_q$ (Hermans, 2003; Riobo and Manning, 2005).

2.2 Effectors of the $G\alpha_{12/13}$ subunits

The α-subunits of the G12 subfamily of G proteins, $G\alpha_{12}$ and $G\alpha_{13}$, have been linked to cellular events such as cytoskeletal rearrangements (Buhl *et al.*, 1995; Gohla *et al.*, 1999) and cell proliferation (Collins *et al.*, 1997) through the activation of the small GTPase RhoA. $G\alpha_{12}$ and $G\alpha_{13}$ activate Rho principally through direct interaction of the activated Gα subunit with Rho-specific guanine nucleotide exchange factors (RhoGEFs), which are considered their main effectors. In 1998 it was identified the first mammalian downstream binding partner of G12 proteins, p115RhoGEF (Hart *et al.*, 1998; Kozasa *et al.*, 1998). Besides p115RhoGEF, it is now known that other RhoGEFs proteins as LARG and PDZ-RhoGEF are effectors of either $G\alpha_{12}$ or $G\alpha_{13}$ or both. All those RhoGEFs belong to the subfamily of RhoGEF named RH-RhoGEF, characterized by the DH and PH domains and a "RGS-like domain" that binds to the $G\alpha_{12/13}$ proteins. Due to its importance, these subfamily and the Rho proteins will be discussed later with more detail.

Besides the RhoGEFs proteins, a diverse array of proteins has been identified to interact with G12 proteins thanks to new experimental improvements. The binding partners of G12 proteins may serve as direct effectors, adaptor proteins or regulators of G12 signaling (Figure I5).

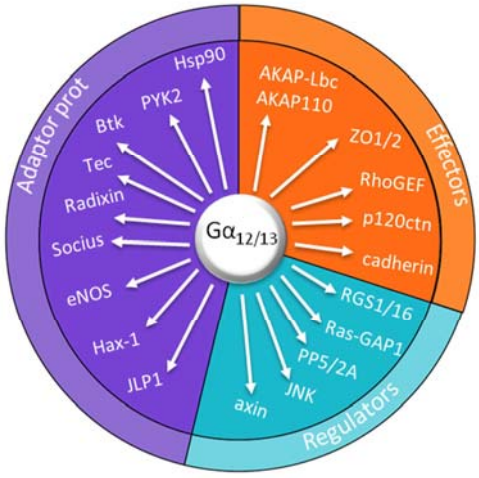


Figure I5. $G\alpha_{12/13}$ signaling. $G\alpha_{12/13}$ versatile binding potential allows for a diverse functional activity. The different binding partners of $G\alpha_{12/13}$ can be classified into three different categories: adaptor proteins, effectors and regulators. Adapted from (Kelly *et al.*, 2007).

Direct effectors for G12 proteins are considered: AKAP-Lbc, AKAP110, ZO1/2, RhoGEFs, p120ctn, and cadherins. It is shown that $G\alpha_{12}$ induces activation of Rho through the non-RGS protein AKAP-Lbc by unknown mechanism (Diviani *et al.*, 2001). AKAP110 significantly potentiated $G\alpha_{13}$-induced activation of PKA (Niu *et al.*, 2001). $G\alpha_{12}$ is concentrated in tight junctions where directly interact with the resident tight junctional proteins zonula occludens-1 and zonula-occludens 2 regulating cell junctions in MDCK cells (Dodane and Kachar, 1996; Meyer *et al.*, 2002, 2003). G12 proteins could also regulate cell adhesion and promote cell migration through the interaction with E-cadherin, neural N-cadherin and cadherin-14 (Meigs *et al.*, 2001) promoting the release of β-catenin from the cadherin tail. On the other hand, p120ctn it has been shown to be a binding partner of G12 proteins, wherein $G\alpha_{12}$ regulates its binding to cadherins, and consequently the cadherin-mediated cell-cell adhesion (Krakstad *et al.*, 2004) .

Other binding partners for G12 proteins could act as regulators proteins. For instance, the phosphatases PP2A and PP5 were reported to be modulated upon G12 protein-binding, which enhances their activity (Yamaguchi *et al.*, 2002; Zhu *et al.*, 2004). Some RGS proteins appear to interact preferentially with either $G\alpha_{12}$ or $G\alpha_{13}$. While RGS1 interact with $G\alpha_{12}$, RGS16 binds to $G\alpha_{13}$ (Moratz *et al.*, 2000; Johnson *et al.*, 2003). Axin, which is a negatively regulator of the Wnt signaling pathway containing an RGS domain, was shown to interact with $G\alpha_{12}$ suggesting a new role for $G\alpha_{12/13}$ proteins in Wnt signaling pathway (Stemmle *et al.*, 2006). G12 proteins have also been reported to interact with Ras-GAP1^{m} to downregulate Ras signaling (Jiang *et al.*, 1998).

Finally, the remaining binding partners of G12 proteins could be classified into adaptor proteins, as can be seen in the figure I5. Thus, the interaction with the chaperone Hsp90 promotes $G\alpha_{12}$ localization to lipid rafts (Waheed and Jones, 2002) or to mitochondria (Andreeva *et al.*, 2008). On the other hand, it is important to note that $G\alpha_{12/13}$ proteins are able to interact with members of different families of non-receptor tyrosine kinases: whereas $G\alpha_{12}$ interact with Bruton's tyrosine kinase (BTK) (Jiang *et al.*, 1998) and Tec (Suzuki *et al.*, 2003), $G\alpha_{13}$ interacts with PYK2 (Shi *et al.*, 2000). These reports suggest that $G\alpha_{12/13}$ proteins regulate tyrosine kinase activity through direct interaction with kinase proteins. $G\alpha_{13}$ interacts with radixin, a member of the ERM (ezrin, radixin, moesin)

family, resulting in neoplasic transformation (Vaiskunaite *et al.*, 2000) and with JLP1 (c-Jun N-terminal kinase(JNK)-interacting leucine zipper protein 1) to regulate JNK activity (Kashef *et al.*, 2005). Other identified adaptor proteins are Socius, eNOS, and Hax-1 (Radhika *et al.*, 2004; Tateiwa *et al.*, 2005; Andreeva *et al.*, 2006).

The list of binding partners of $G\alpha_{12/13}$ proteins and the signaling pathways identified to date is continuously growing. More recently, it was shown that activated $G\alpha_{12/13}$ up-regulate matrix metalloproteinase (MMP)-2 regulating malignant phenotypic conversion of NIH3T3 fibroblast cells (Kim *et al.*, 2011) and that Ric-8 interacts physically with Concertina, the *Drosophila* $G\alpha_{12/13}$ subunit, directing its localization within the cell and regulating Folded gastrulation pathway (Peters and Rogers, 2013) though it has not been proven this association in mammalian cells.

2.3 Deactivation of $G\alpha_{12}$ and $G\alpha_{13}$ by RGS proteins

Like all other heterotrimeric G protein α subunits, $G\alpha_{12/13}$ cycle between GDP-bound inactive state and GTP-bound active state and possess an intrinsic ability to hydrolyze GTP to GDP. Purified $G\alpha_{12}$ demonstrates slower guanine nucleotide kinetics than other Gα subunits (Singer *et al.*, 1994; Kozasa and Gilman, 1995), which is consistent with the preferential role of these molecules in sustained reactions like modulation of cytoskeleton. This deactivation process is accelerated by the GAP activity of RGS proteins. Members of the mammalian RH-RhoGEF family, cited before, have been shown to be the main GAP proteins for G12 subfamily members (Fukuhara *et al.*, 1999; Chen *et al.*, 2005; Tesmer *et al.*, 2005; Kreutz *et al.*, 2006).

2.4 Regulation of G12 proteins

$G\alpha_{12/13}$ proteins undergo post-translational modifications through the addition of lipid molecules. These modifications affect the subcellular localization and the interactions of the Gα subunits with other proteins. Gα subunits are subjected to N-myristoylation and/or palmitoylation, being irreversible and

reversible, respectively and was shown to be needed for the transforming potential of the G12 proteins (Veit *et al.*, 1994; Jones and Gutkind, 1998). Besides the well-known role of G12 proteins at the plasma membrane, it is also reported that $G\alpha_{12}$ is targeted to the mitochondria regulating mitochondrial morphology and motility (Andreeva *et al.*, 2008).

Phosphorylation of Gα subunits is another important modification, which regulates the amplitude and duration of G protein signals. $G\alpha_{12}$ has been reported to be a substrate for protein kinase C (PKC). Although the phosphorylation site has not been mapped, the N-terminal 50 amino acid residues comprise one possible region. It has been demonstrated that phosphorylation blocks the interaction of the $G\alpha_{12}$ subunit with the Gβγ dimer, and Gβγ reciprocally blocks the phosphorylation of $G\alpha_{12}$ by PKC (Kozasa and Gilman, 1996). On the other hand, a recent phosphoproteomic report shown that $G\alpha_{12}$ is phosphorylated during mitosis on serine 67, but nothing is known about this novel phosphorylation function as well as the kinase involved in (Olsen *et al.*, 2010). These results may suggest a putative role for $G\alpha_{12}$ during cell cycle regulation.

MicroRNAs (miRNAs) are endogenously expressed non-coding RNAs that regulate gene expression post-transcriptionally. Recently, large-scale variations in miRNA expression have been implicated in development and progression of cancers (Huang *et al.*, 2011) and G12 proteins have been reported either that could be regulating miRNAs and could be regulated by them. For instance, Kelly and colleagues demonstrated that $G\alpha_{13}$ expression was increased in prostate cancer progression (Kelly, Stemmle, *et al.*, 2006) and that miR-182 and miR-200 family members were regulating the expression of GNA13 post-transcriptionally (Rasheed *et al.*, 2013). By contrast, a recent report shown that $G\alpha_{12}$ repress FOXO1, a member of the forkhead box O subfamily of transcription factors that are considered tumor repressors, and $G\alpha_{12}$ promotes miRNA dysregulation in hepatocellular carninoma (Jung *et al.*, 2014).

3. Biological roles of G12 subfamily

The members of the G12 subfamily are ubiquitously expressed and they are implicated in the regulation of a variety of physiologic and pathophysiologic

processes. Probably the most extensively studied roles of $G\alpha_{12/13}$ are in cell growth, proliferation, and cell migration (Figure I6) because of its involvement in Rho activity and actin structure organization as well as for its importance in cancer processes. I will now proceed to discuss these processes in more detail in the next sections.

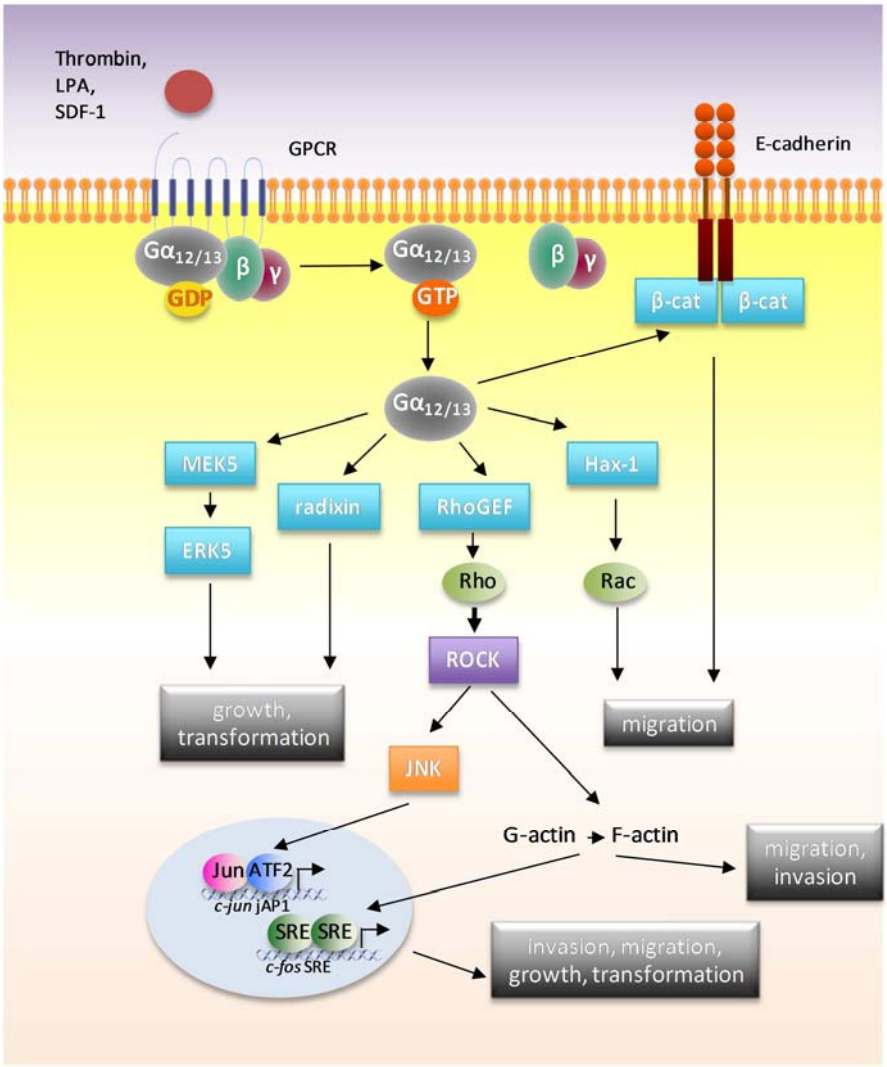


Figure I6. Schematic of $G\alpha_{12/13}$ effectors and signaling pathways impacting cell growth and transformation, migration and invasion. β-cat, beta-catenin; ATF2, activating transcription factor-2; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; jAP1, c-jun AP1-like response element; JNK, c-Jun N-terminal kinase; LPA, lysophosphatidic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; RhoGEF, Rho-specific guanine nucleotide exchange factor; ROCK, Rho kinase; SDF-1, CXC chemokine stromal cell-derived factor-1a; SRE, serum response element; SRF, serum response factor. Adapted from (Juneja and Casey, 2009).

3.1 $G\alpha_{12/13}$ and cell migration

The observation that the deletion of the gene encoding for Concertina, the single ortholog of the $G\alpha_{12}$ and $G\alpha_{13}$ in *Drosophila*, disrupted ventral furrow development suggested that this protein was required for the cellular shape changes and movements that occur during normal gastrulation (Parks and Wieschaus, 1991). Later it was demonstrated that a *Drosophila* DRhoGEF2 protein was located downstream of Concertina regulating *Drosophila* gastrulation (Barrett *et al.*, 1997). Soon it was clear that $G\alpha_{12/13}$ function was linked to the direct modulation of Rho activity, as mentioned before. Changes in cytoskeletal dynamics required for cell migration are coordinated in large part by Rho GTPases (Rac, Cdc42 and Rho), and signaling by G12 proteins is responsible for many of the effects on cell movement that accompany Rho activation. Likewise it has been suggested that G12 proteins regulate lymphocyte and neutrophil migration (Girkontaite *et al.*, 2001; Xu *et al.*, 2003) and deletion of $G\alpha_{13}$ in mice impairs the organization of the vascular system, resulting in lethality at approximately day 10.5 of embryogenesis (Offermanns *et al.*, 1997). Conditional ablation of the genes encoding $G\alpha_{12/13}$ results in neuronal ectopia of the cerebral and cerebellar cortices due to overmigration of cortical plate neurons and cerebellar Purkinje cells, respectively, demonstrating the important role of G12 proteins in the development of the central nervous system (Moers *et al.*, 2008).

Nevertheless, G12 proteins can also promote cell migration in a RhoA-independent way. It has been reported that G12 proteins bind to the cytoplasmatic region of cadherins promoting the release of β-catenin and consequently, activating the β-catenin-mediated transcription (Meigs *et al.*, 2001). The binding of activated G12 proteins to cadherins downregulate the E-cadherin-mediated cell-cell adhesion leading to cancer metastasis (Meigs *et al.*, 2002). On the other hand, it has been shown that $G\alpha_{13}$ physically interacts with Hax-1, a cytoskeleton-associated, cortactin-interacting intracellular protein promoting cell migration. Moreover, co-expression of Hax-1 attenuates $G\alpha_{13}$-stimulated activity of Rho while potentiating $G\alpha_{13}$-stimulated activity of Rac (Radhika *et al.*, 2004). Also it has been shown that activated $G\alpha_{12}$ modulates tight junction-mediated paracellular

permeability through the activation of Src tyrosine kinase pathways (Meyer *et al.*, 2002, 2003).

3.2 $G\alpha_{12/13}$ and cell transformation

Curiously, the first identified function of the G12 subfamily was not the activation of Rho but their ability to promote growth and induce neoplasic transformation (Jiang *et al.*, 1993; Xu *et al.*, 1993). Also, overexpression of known-G12 activating GPCRs as PAR-1 and the M1 muscarinic acetylcholine receptor promotes fibroblast growth. Indeed, results of our group revealed that stimulation of PAR-1 receptor in the 1321N1 astrocytoma cell line induced cell growth in a $G\alpha_{12}$-dependent manner (Aragay *et al.*, 1995). These studies led to the hypothesis that GPCRs may signal through G12 proteins to promote tumorigenesis and tumor cell growth (Radhika and Dhanasekaran, 2001). Many of the growth promoting and transforming effects of the G12 proteins appear to be also mediated by the RhoA family of monomeric GTPases. Stimulation of RhoA downstream of G12 proteins promotes the activation of p38 MAPK (Marinissen *et al.*, 2001), STAT3 (Kumar, Shore, *et al.*, 2006), PDGF-α receptor (Kumar, Ha, *et al.*, 2006), JNK (Marinissen *et al.*, 2004), as well as NF-κB-regulated transcription (Perona *et al.*, 1997), serum response element-regulated transcription (Hill *et al.*, 1995), and expression of COX-2 (Dermott *et al.*, 1999; Slice *et al.*, 1999). Despite of the involvement of RhoA in all these transformation events it has been proposed a system where $G\alpha_{13}$ induces cell transformation in a RhoA-independent manner, through the activation of radixin (Vaiskunaite *et al.*, 2000). Moreover, it was suggested that cell transformation induced by $G\alpha_{12}$ in NIH3T3 cells was mediated through Rac (Tolkacheva *et al.*, 1997).

3.3 $G\alpha_{12/13}$ in oncogenic transformation and cancer

$G\alpha_{12/13}$ are suggested to be involved in the development of cancer processes as they are found up-regulated both in aggressive cancer cells and in advanced cancer tissues. Kelly and co-workers demonstrated that G12 proteins were up-regulated in tissue specimens from patients with adenocarcinoma of the breast

and prostate; however, G12 did not promote tumor cell growth (Kelly, Moeller, *et al.*, 2006; Kelly, Stemmle, *et al.*, 2006). Likewise, $G\alpha_{13}$ was found to be highly expressed in the most aggressive cancers cell lines. In particular, in prostate cancer $G\alpha_{13}$ was reported to be essential for cancer cell invasion and migration (Rasheed *et al.*, 2013). Other study shown that LPA promotes ovarian cancer cell migration *in vitro* with G12 proteins as key regulators of the process (Bian *et al.*, 2006) and $G\alpha_{12}$ was also reported to be up-regulated in oral squamous cell carcinoma (OSCC) (Cheong *et al.*, 2009). By contrast, stimulation of sphingosine-1-phosphate receptor-2 (SP1) in gliobastoma cell lines inhibits cell migration through a Rho-dependent pathway likely mediated by G12 proteins (Lepley *et al.*, 2005). In all of these studies, the activation of Rho appeared to be critical for these G12-promoted events. Interestingly, a recent report found that $G\alpha_{12}$ was up-regulated in patients with hepatocellular carcinoma (HCC) in association with malignancy due to the repression of the expression of FOXO1, a tumor suppressor, and the dysregulation of miRNAs (Jung *et al.*, 2014). Activated $G\alpha_{13}$ was also shown to impair cell invasiveness through the inhibition of RhoA in human melanoma cells (Bartolomé *et al.*, 2008). These findings suggest a dual role for G12 proteins inducing and repressing malignancy, which could be explained if cell type-specific differences exist in the molecular signals.

4. Rho GTPases

All eukaryotic cells contain RhoGTPases and, as mentioned, they are involved in the regulation of many biological processes, from adhesion and motility to gene expression and differentiation (Etienne-Manneville and Hall, 2002). Rho is a member of the Ras superfamily of small GTP-binding proteins and like Ras, cycles between an inactive GDP-bound state to an active GTP-bound state. As for the heterotrimeric G proteins, the cycle is regulated by guanine-nucleotide exchange factors (GEFs), and by GTPase-activating proteins (GAPs). The GDP-bound inactive form of Rho is located in the cytosol in a complex with RhoGDI, which stabilizes the GDP-Rho complex and inhibits the exchange of GDP for GTP (Figure I7).

The Rho family of monomeric GTPases is composed by three subfamilies: Rho (A, B, and C), Rac (1 and 2), and Cdc42. All of them promote reorganization of

actin cytoskeleton, but have distinct effects on cell shape and movement (Hall, 1998; Schmitz *et al.*, 2000). RhoA promotes actomyosin contractibility and, consequently, the formation of stress fibers and focal adhesions, regulating cell shape, attachment and motility. Rac1 is implicated in actin polymerization and the formation of lamellipodia, which consists in actin projection on the mobile edge of the cell. Cdc42 promotes formation of filopodia, which are thin, finger-like cytoplasmatic extensions that contains tight actin bundles and might be involved in the recognition of the extracellular environment. The multitude of effectors identified for RhoA, Rac1, Cdc42 and other family members reflects the complexity and diversity of these proteins.

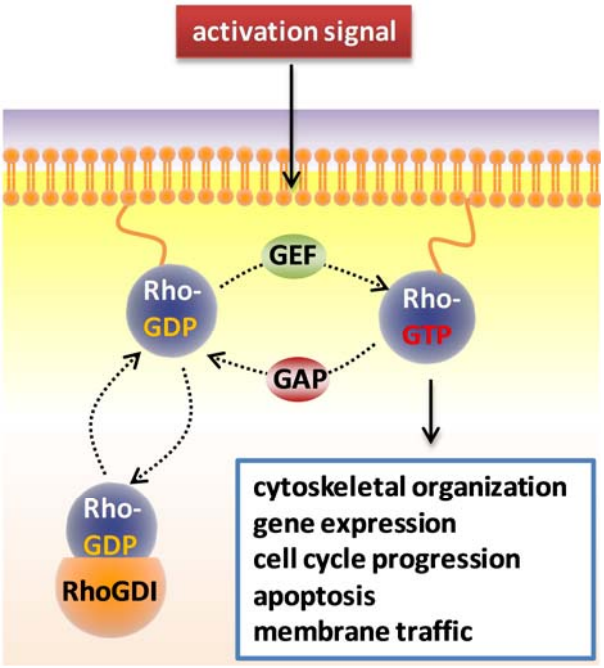


Figure I7. The Rho GTPase switch. Cycling between the inactive (GDP-bound) and active (GTP-bound) forms is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Guanine nucleotide dissociation inhibitors (GDIs) inhibit nucleotide dissociation and control cycling of Rho GTPases between membrane and cytosol. Active, GTP-bound GTPases interact with effector molecules to mediate various cellular responses. Upstream activation of the GTPase switch occurs through activation of GEFs. Adapted from (Schmidt and Hall, 2002).

4.1 Activation of Rho proteins

For the 20 human Rho GTPases there are 83 GEFs and 67 GAPs, and a subset of Rho GTPases are not likely to be regulated by GEFs and GAPs (such as RND3; also known as RHOE). Rho GTPases are activated by distinct RhoGEF families: Dbl family RhoGEFs (of which there are 68); dedicator of cytokinesis (Dock) family RhoGEFs (of which there are 11); switch-associated protein 70 (SWAP70); and SLAT (also known as DEF6) (Vigil *et al.*, 2010). The largest and best characterized RhoGEF family is the Dbl-family, which shares a common structural motif consisting of a 250-amino acid stretch of sequence similarity with Dbl, known as DH domain, adjacent to a pleckstrin homology domain (PH) (Van Aelst and D'Souza-Schorey, 1997; Whitehead *et al.*, 1997). DH domain is responsible for nucleotide exchange activity toward GTPases of the Rho family and is highly conserved within the members of this family, whereas PH domain has been implicated as mediator of protein-protein and protein-lipid interactions. In most cases the DH–PH module is the minimal structural unit that can promote nucleotide exchange *in vivo*. Other conserved motifs were found in the structure of RhoGEFs proteins, which include SH2, SH3, Ser/Thr or Tyr kinase, Ras-GEF, Rho-GAP, Ran-GEF, PDZ, or additional PH domains. These are likely to be involved in coupling RhoGEFs to upstream receptors and signaling molecules.

5. RH-RhoGEFs

As mentioned before $G\alpha_{12/13}$ activate three mammalian RhoGEFs: p115RhoGEF, PDZ-RhoGEF and LARG (Kozasa *et al.*, 1998; Fukuhara *et al.*, 1999), which are considered as a subfamily of RhoGEFs named RH-RhoGEFs. Soon thereafter, AKAP-Lbc and proto-Lbc, two splice variants of Lbc protein, were found to be specifically stimulated by G12 proteins (Diviani *et al.*, 2001; Dutt *et al.*, 2004). However, more recent evidence shows that Lbc is also implicated in mitogenesis most likely through the cooperation with $G\alpha_q$ (Bear *et al.*, 2010).

5.1 Structural features of RH-RhoGEFs

All three mammalian RhoGEFs proteins regulated by $G\alpha_{12/13}$ belong to Dbl-RhoGEF family and contain a domain that shares remote sequence similarity to the RGS domain of the Regulators of G-protein Signaling (RGS) proteins. The canonical RGS domain is characterized by a conserved 120 residues helical folded (the "RGS-box") and is responsible of the interaction with Switch I and II regions of Gα subunits, accelerating its GAP activity. In contrast, the RH domains of p115RhoGEF and LARG, also termed "rgRGS" domain (Chen *et al.*, 2001, 2003), shares remote sequence similarity (between 10 and 15%) to the RGS domain. The RH domains are present in their N-terminal regions and do not present GAP activity. PDZ-RhoGEF and LARG additionally contain a PDZ domain in their N-terminal region that allows coupling to cell surface receptors such as plexins, insulin-like growth factor receptor or GPCRs (Siehler, 2009).

The lysine 204 of $G\alpha_{13}$ located in its Switch I region, was demonstrated to be important for the interaction with the RH domains of both p115RhoGEF and LARG (Nakamura *et al.*, 2004; Grabocka and Wedegaertner, 2005). The crystal structure of $G\alpha_{13}$ in complex with p115RhoGEF and PDZ-RhoGEF allowed to determine the specific regions of $G\alpha_{13}$ responsible for the binding to these RhoGEFs (Figure I8) (Chen *et al.*, 2008; Hajicek *et al.*, 2011).

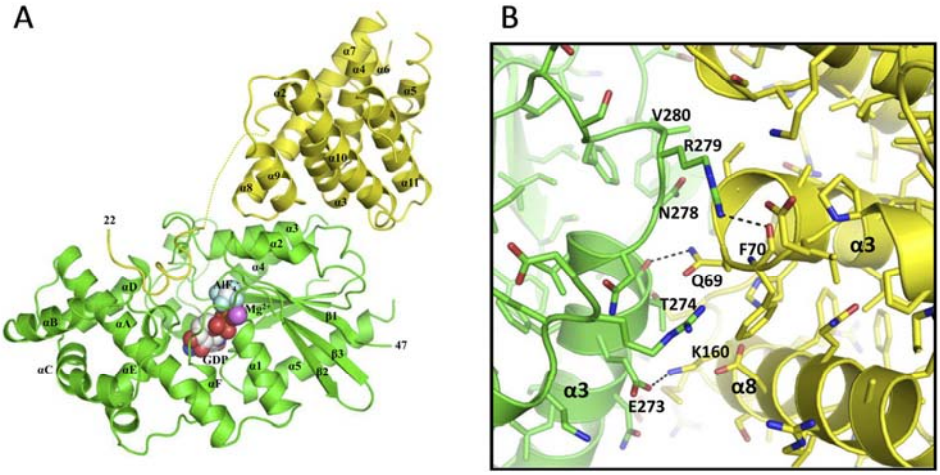


Figure I8. Structure of the $G\alpha_{13}$-p115 RH complex. **(A)** The complex is shown as a ribbon diagram, with $G\alpha_{13}$ colored in green and the RH domain of p115RhoGEF in yellow. GDP, Mg^{+2}, and AlF_4^- are shown as space-filling spheres. Carbon, oxygen, nitrogen, phosphate, magnesium, aluminum, and fluoride atoms are colored white, red, dark blue, orange, purple, light gray, and light blue, respectively. The disordered region in p115RhoGEF between the GAP interface and the RGS box is shown as a dashed yellow line. **(B)** A detailed view of the $G\alpha_{13}$-p115RhoGEF RH domain effector interface. Adapted from (Hajicek *et al.*, 2011).

The Lbc-RhoGEF has a C-terminal region sharing 39% amino acid identity to the consensus RH domain. Thus, there is a discrepancy about the existence of the RH domain within the Lbc-RhoGEF sequence and it is known as "RGS-like" domain (Figure I9).

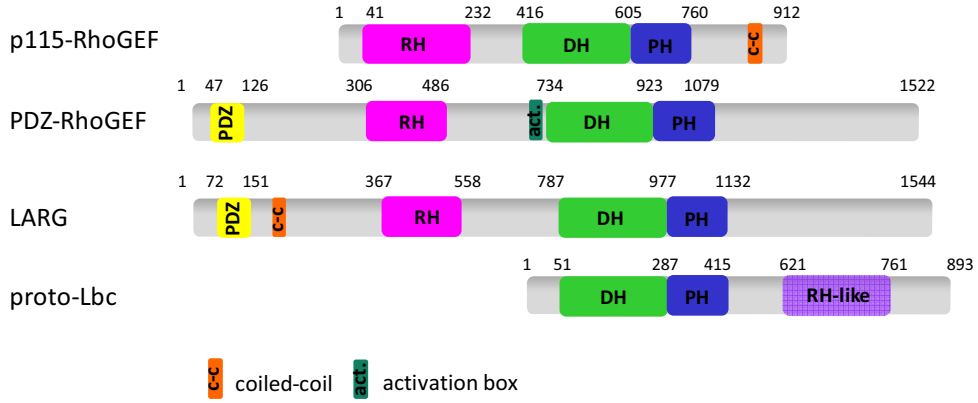


Figure I9. Domain structure of RhoGEFs reported to be directly regulated by heterotrimeric G proteins. The amino acid numbers shown above each protein correspond to the human ortholog. Adapted from (Aittaleb *et al.*, 2010)

5.2 Localization of RH-RhoGEFs

RH-RhoGEFs are large proteins ranging from 900 to 1600 amino acids approximately and are widely expressed in mammals. p115RhoGEF can be found in hematopoietic cells and localizes throughout the cytosol. p115RhoGEF is translocated to the plasma membrane upon its activation by G12 proteins. High transcript levels of PDZ-RhoGEF are present in the central nervous system but depending on cell type can be localized in the cytosol, at the cell periphery at or near the plasma membrane. Nevertheless, the activation by $G\alpha_{12/13}$ also induces its

translocation to the plasma membrane. On the other hand, LARG is ubiquitously expressed and distributed throughout the cytoplasm in most cell types, except in MDCK cells, in which LARG is localized at laterals membranes. In fibroblasts, LARG was reported to localize to the microtubule-organizing center and along microtubule tracks to contribute cell polarity (Goulimari *et al.*, 2005). Similarly to the other RH-RhoGEFs, upon its activation by G12 proteins LARG is translocated to the plasma membrane. The Lbc-RhoGEF splice variant A-kinase anchoring protein (AKAP)-Lbc is highly expressed in the heart and redistribution from the cytosol to the plasma membrane was reported upon its activation by $G\alpha_{12/13}$ (Diviani *et al.*, 2001, 2004).

5.3 Regulation of RH-RhoGEF proteins

Although $G\alpha_{12}$ and $G\alpha_{13}$ are the most homologous among Gα subunits and share similar biochemical properties, their signaling functions are clearly different. $G\alpha_{13}$, but not $G\alpha_{12}$, was found to stimulate the GEF activity of p115RhoGEF and PDZ-RhoGEF. Whereas $G\alpha_{13}$ enhances weakly the GEF activity of LARG, $G\alpha_{12}$ is reported to activate LARG only when it is phosphorylated (Suzuki *et al.*, 2003). Phosphorylation events are important regulators for RH-RhoGEFs proteins. PDZ-RhoGEF and LARG were reported to be tyrosine phosphorylated by FAK or Tec (Fukuhara *et al.*, 2001; Chikumi *et al.*, 2004), while protein kinase C (PKC) α phosphorylates p115RhoGEF on serine residues. On the other hand, p21-activated kinase 1 (PAK1) can bind to the DH/PH domains of p115RhoGEF (but not of LARG or PDZ-RhoGEF), and mediate Rac-induced inhibition of RhoA activation (Rosenfeldt *et al.*, 2006).

Many members of the Dbl family of GEF are maintained in a basal inactive conformation by intramolecular interactions involving the DH and PH domains as well as other regulatory sequences. Such interactions have been proposed to block the access of Rho GTPases to the catalytic module and/or suppress the GEF activity of the exchange factors (Zheng, 2001). Recent studies demonstrate that the activity of Dbl family members can also be regulated through oligomerization. In line with these observations p115RhoGEF, PDZ-RhoGEF, LARG and AKAP-Lbc have been reported to homo- and heterodimerize through their coiled-coil motif in the C-

terminal region. Indeed, oligomerization of RH-RhoGEFs seems to negatively regulate its activity, as deletion of the C-terminus increases the ability of RH-RhoGEFs to stimulate the formation of Rho-GTP in cells under basal conditions (Eisenhaure *et al.*, 2003; Chikumi *et al.*, 2004; Baisamy *et al.*, 2005). In turn, how the C-terminal oligomerization inhibits the function of these RhoGEFs is still unknown because C-terminal does not bind the DH-PH domain or any other N-terminal regulatory region. Furthermore, the binding of G12 proteins to RH domain does not disrupt their oligomerization (Chikumi *et al.*, 2004). A summary of the most significant characteristics of the human p115RhoGEF, PDZ-RhoGEF, LARG, and Lbc-RhoGEF are described in table I2.

Table I2. Characteristics of the four G12-regulated RhoGEFs.

	p115RhoGEF	**PDZ-RhoGEF**	**LARG**	**Lbc-RhoGEF**
Expression	Blood cells, wide at low levels	CNS, wide at low levels	Ubiquitous	Wide (AKAP-Lbc in heart)
Size (human)	927 aa	1522 aa	1544 a	Variable (splice variants)
RGS domain	Yes	Yes	Yes	RGS-like (C-terminal)
Localization inactive form	Cytoplasm	Cytoplasm or PM (cell type)	Cytoplasm or PM (cell type)	Cytoplasm
Localization active form	Plasma membrane	Plasma membrane	Plasma membrane	Plasma membrane
$G\alpha_{12}$-induced GEF activation	No	No	Yes	Yes
$G\alpha_{13}$-induced GEF activation	Yes	Yes	Yes	Yes
Regulation of GEF activity	PKC (+), PAK (-)	Tec, FAK (+)	Tec, FAK (+)	PKA (-) for AKAP-Lbc
Oligomerization	Homo-oligomers	Homo- and hetero-oligomers	Homo- and hetero-oligomers	Homo-oligomers (AKAP-Lbc)

Adapted from (Siehler, 2009).

5.4 Other members of the RhoGEF family

Homology comparison of the DH and PH domains has identified another family closely related to the RH-RhoGEF group (Sternweis *et al.*, 2007). This family includes: AKAP-Lbc, p114-RhoGEF, p190RHoGEF, and GEF-H1 (Figure I10) (Appendix I). It has been shown that G proteins can stimulate some members of this family. Whereas the splice variant AKAP-Lbc is stimulated only by $G\alpha_{12}$, both $G\alpha_{12}$ and $G\alpha_{13}$ enhance the GEF activity of the shorter form Lbc-RhoGEF splice variant through triggering the release of PKA, which inhibits its GEF activity by phosphorylation and concomitant recruitment of 14-3-3 (Diviani *et al.*, 2001, 2004). Another member, p114-RhoGEF, can be activated by GPCRs via stimulation of LPA, causing RhoA activation (Niu *et al.*, 2003). p114-RhoGEF activity is regulated by Gβγ but not by Gα subunits, through its direct interaction stimulating both RhoA and Rac1. However, other reports suggested that it is selective for RhoA (Nagata and Inagaki, 2005). The two last related proteins p190RhoGEF and GEF-H1 have not been identified as targets of the G proteins signaling pathways. p190RhoGEF is implicated in normal and colorectal cell motility, tumor progression and facilitating gastrin-CCK2 stimulating signaling events (Lim *et al.*, 2008; Yu *et al.*, 2011; Miller *et al.*, 2012). It has also been implicated in several other biological functions. In neuronal cells, p190RhoGEF possesses anti-apoptotic activity (Wu *et al.*, 2003); it is implicated in the pathogenesis of motor neuron degeneration (Lin *et al.*, 2005); and inhibits axonal branching and synapse formation downstream of focal adhesion kinase (FAK)(Rico *et al.*, 2004); and δ-catenin induces dendrogenesis and spine morphogenesis by inhibiting Rgnef activity (Kim *et al.*, 2008). However, recent results of our group suggest that p190RhoGEF is a novel downstream effector of $G\alpha_{13}$ ((Masià *et al.*, 2014) submitted). The properties and functions of the GEF-H1 will be extensively discussed below.

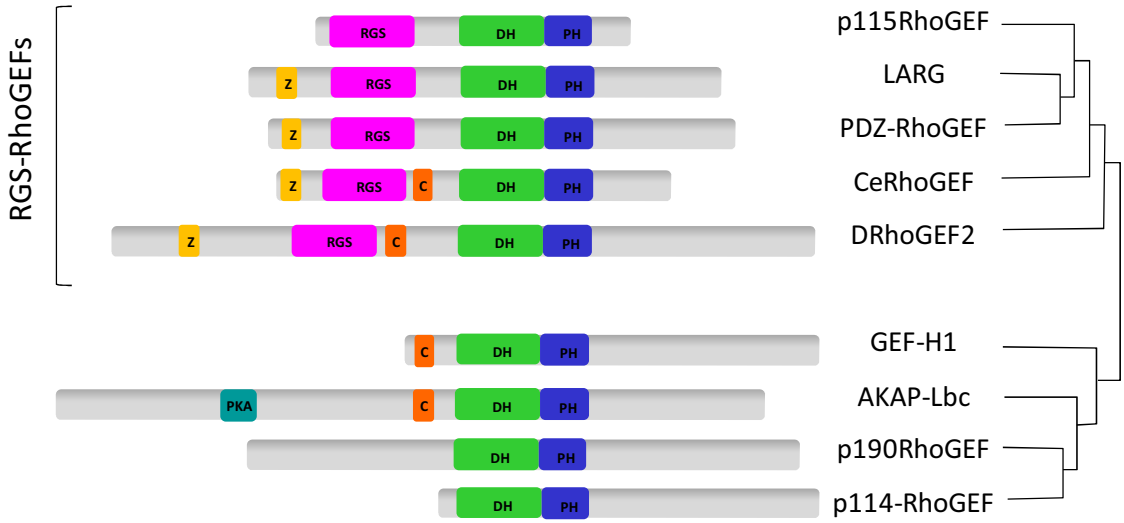


Figure I10. Dendrogram obtained from homology comparison of the DH and PH domains of RH-RhoGEFs with other related members of the Lbc subfamily. DRhoGEFGEF2 and CeRhoGEF are the homologous proteins of the human p115RhoGEF in *Droshophila melanogaster* and *Caenorhabditis elegans*, respectively. Unlike human RHRhoGEFs, CeRhoGEF and DRhoGEF2 each contain one C1 homology domain. Domains: RGS, regulator of G protein signaling; DH, dbl homology; PH, pleckstrin homology; Z, PDZ; C, C1 homology domain; PKA, binding site for protein kinase A. Image adapted from (Sternweis *et al.*, 2007).

6. GEF-H1

GEF-H1 has the peculiarity to be associated to microtubules and regulated by microtubule binding (Ren, 1998; Krendel *et al.*, 2002). However, the mechanism that promotes its binding and its release from them is not fully understood. It has been suggested that GEF-H1 is released from microtubules upon their state of polymerization. Thus, GEF-H1 regulation by microtubules would provide a link between microtubule dynamics and RhoGTPase activation in a variety of normal biological situations.

As mentioned before, although GEF-H1 has not been considered as a downstream effector of $G\alpha_{12/13}$, our studies have found evidences supporting direct interaction and activation of GEF-H1 through G12 proteins. Therefore, a description of its structure and function is detailed below.

6.1 Structure features of GEF-H1

GEF-H1 is expressed in wide range of human tissues. Whereas human GEF-H1 is a 4,4-kilobase pair mRNA transcript, the mouse Lfc displayed three transcripts with lengths of 4.5-, 3.7-, and 3.3-kilobase pair (Ren, 1998). GEF-H1 and Lfc share 88% amino acid identity and share a similar domain structure (Glaven, 1999) (Figure I11). Lfc was originally identified in its C-terminally truncated form as a gene capable to promoting transformation of NIH3T3 cells when overexpressed (Whitehead *et al.*, 1995). Human GEF-H1 is composed of 986 amino acids. At the N-terminal region there is a C1 domain, which contains a zinc-finger motif similar to the C1 diacylglicerol (DAG) binding domain of the atypical protein kinase C (PKC) family. As a member of the subfamily of Dbl-RhoGEFs, GEF-H1 possesses a DH domain, which confers its catalytic activity. Whereas GEF-H1 can physically interact through the DH domain with both RhoA and Rac1, GEF-H1 selectively stimulates RhoA activity. Adjacent to the DH domain there is a PH domain that is involved in microtubule binding and targeting of the protein to different subcellular compartments, such tight junctions. It has been demonstrated that the PH domain is necessary for the activity of GEF-H1 since deletion of PH domain eliminated the transforming ability of GEF-H1 in NIH3T3 cells (Whitehead *et al.*, 1995). GEF-H1 has a coiled-coil domain at C-terminal, which is involved in protein-protein interaction. Indeed, GEF-H1 interacts with neurabin and spinophilin through its coiled-coil motif (Ryan *et al.*, 2005). Additionally, a C-terminally truncated GEF-H1 lost its ability to interact with microtubules suggesting that the coiled-coil motif also plays a role in GEF-H1 microtubule localization (Ren, 1998).

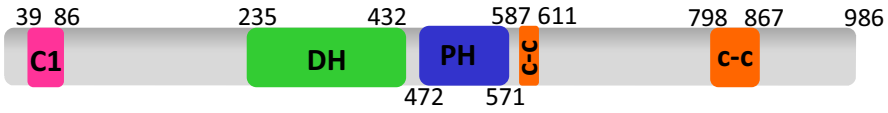


Figure I11. Schematic representation of GEF-H1 structure. C1, zinc finger-containing C1 domain; DH, Dbl homology domain; PH, pleckstrin homology domain; c-c; coiled-coil motif.

6.2 Regulation of GEF-H1 by microtubules

As mentioned before, the most characteristic feature of GEF-H1 is its regulation by microtubules. Whereas microtubule binding is associated with low exchange activity of GEF-H1 toward RhoA, the release from microtubules leads to an activation of GEF-H1 and consequently, RhoA activation. Early studies have been demonstrated that a single amino acid substitution (C53) at the zinc-finger located in the C1 domain is sufficient to induce the loss of microtubule localization of the protein (Krendel *et al.*, 2002). Moreover, the expression of GEF-H1 constructs that are deficient in microtubule binding promote changes in cell morphology. Thus, the loss of microtubule binding induces activation of GEF-H1 (Krendel *et al.*, 2002). A recent study suggest that Tctex-1, a light chain component of the dynein motor complex, is the factor that links GEF-H1 to microtubules maintaining the protein in a inhibited state (Meiri *et al.*, 2012).

6.3 Regulation of GEF-H1 by phosphorylation

Several studies reported a complex regulation by phosphorylation of GEF-H1 through the action of different kinases. Thereby, GEF-H1 is phosphorylated by p21-activated kinase 1 and 4 (PAK1/4) (Zenke *et al.*, 2004; Callow *et al.*, 2005), by the mitotic kinases Aurora A/B and Ck1/Cyclin B (Birkenfeld *et al.*, 2007), by ERK1/2 (Fujishiro *et al.*, 2008), by protein kinase A (PKA) (Meiri *et al.*, 2009), and by partitioning-defective 1 (PAR1) kinase (Yamahashi *et al.*, 2011).

The carboxyl terminus of GEF-H1 contains a region, where are located the main phosphorylation residues, that is necessary for suppression of GEF-H1 exchange activity. Mutants lacking amino acids from 573 to 985 showed up-regulated GEF activity. In fact, GEF-H1 can be phosphorylated at serine 885 by PAK1 and PKA and this phosphorylation induces 14-3-3 binding to GEF-H1/Lfc. Moreover, it has been suggested that this phosphorylation has an inhibitory role because promotes the re-localization of GEF-H1 to microtubules. The authors proposed a model in which 14-3-3 proteins may force GEF-H1 into a closed conformation that precludes an interaction with Rho, since 14-3-3 has the ability

to interact with GEF-H1 through its dominant site S885 and also a second ancillary site at T114, upon GEF-H1 phosphorylation (Zenke *et al.*, 2004; Meiri *et al.*, 2009). In line with these observations the polarity-regulating serine/threonine kinase PAR1b also induces phosphorylation on S885 and S959 of GEF-H1 thus, inhibiting its exchange activity. Additionally, phosphorylation of GEF-H1 by PAK4 blocks GEF-H1-dependent stress fiber formation and endogenous PAK4-GEF-H1 complex is found to be associated to microtubules (Callow *et al.*, 2005). On the other hand, ERK1/2 induces phosphorylation on threonine 678 of GEF-H1 enhancing its enzymatic activity of the exchange factor toward RhoA (Fujishiro *et al.*, 2008). Early in mitosis, Aurora A/B and Cdk1/Cyclin B have been reported to phosphorylate GEF-H1 on serine 885 and serine 959, respectively, therefore inhibiting GEF-H1 catalytic activity. Dephosphorylation of GEF-H1 during telophase triggers RhoA activation, which provokes cleavage furrow formation and ingression during cytokinesis (Birkenfeld *et al.*, 2007). These observations suggest that S885 and S959 may act as inhibitory switches of GEF-H1, which is targeted by multiple distinct kinases in a context-dependent manner.

6.4 GEF-H1 in mitosis

A role for GEF-H1 in regulating localized activation of RhoA during different stages of normal cell mitosis has been established. Different studies showed that GEF-H1 is involved in RhoA activation during prometaphase and telophase and/or cytokinesis. An earlier study reported that Lfc is required for the formation of the mitotic spindle during prophase and prometaphase since cells lacking Lfc were not able to assemble properly a functioning spindle (Bakal *et al.*, 2005). Similarly, a key role for GEF-H1 during cytokinesis was demonstrated in HeLa cells, where perturbation of GEF-H1 function induced mitotic aberrations. GEF-H1 activity during cytokinesis is controlled by the mitotic kinases Aurora A/B and Cdk1/Cyclin B, as commented above. These phosphorylations negatively regulate the catalytic activity of GEF-H1 toward RhoA. Besides ECT2, a RhoGEF essential for RhoA localization at the equatorial cortex and a key regulator of mitosis (Tatsumoto *et al.*, 1999), GEF-H1 is also a key player involved in cytokinesis (Birkenfeld *et al.*, 2007).

6.5 Physiological functions of GEF-H1

Besides its role in mitosis, GEF-H1 has been implicated in other biological processes. For example, GEF-H1 has been shown to regulate epithelial cell permeability inducing the disassembly of the epithelial cell barrier (Samarin *et al.*, 2007) and it is also a component of MDCK cell epithelial tight junctions (Benais-Pont *et al.*, 2003). GEF-H1 depletion attenuates the increase in vascular permeability seen upon microtubule depolymerization (Birukova *et al.*, 2006). Likewise, GEF-H1 has been reported to bind directly to the adaptor protein cingulin, and this binding has an inhibitory role on GEF-H1 activity (Aijaz *et al.*, 2005). Similarly, GEF-H1 is also implicated in cell polarization and motility through the microtubule-regulated aspects of cell motility. Thus, overexpression of Lfc induces the assembly of stress fibers (Krendel *et al.*, 2002) while the stimulation with thrombin, lysophosphatidic acid or the microtubule depolymerizing agent nocodazole in depleted GEF-H1 cells remarkably attenuates stress fiber formation (Birukova *et al.*, 2006). On the other hand, GEF-H1 is also a key regulator of the exocytic pathway. A recent study shows that GEF-H1 is involved in the regulation of vesicles trafficking pathways of endocytic recycling and exocytosis. In fact, GEF-H1 is able to interact directly with Sec5, a component of the exocyst complex, in a RaI GTPase-dependent manner. This interaction leads RhoA activation, which in turn regulates exocyst assembly/localization and exocytosis (Pathak *et al.*, 2012).

7. Cell cycle

"Where a cell arises, there must be a previous cell, just as animals can only arise from animals and plants from plants". The German pathologist Rudolf Virchow proposed this *cell doctrine* in 1858. Thereby, cell division consists mainly in two processes that imply DNA replication and segregation of replicated chromosomes into two separate cells. DNA replication occurs in the S phase, which requires 10-12 hours and occupies about half of the cell-cycle time in a typical mammalian cell. After that, the segregation of replicated chromosomes occurs during the M phase, or mitosis, which requires less than an hour in mammalian cells. Between the S and the M phases, there are two more phases: G_1 phase, in

which cells prepare to DNA synthesis; and G_2 phase, in which cells prepare to mitosis. Therefore cell cycle is composed by four phases (Figure I12). Cells in G_1 can, before to DNA replication, enter a resting state called G_0. Cells in G_0 account for the major part of the non-growing, non-proliferating cells in the human body.

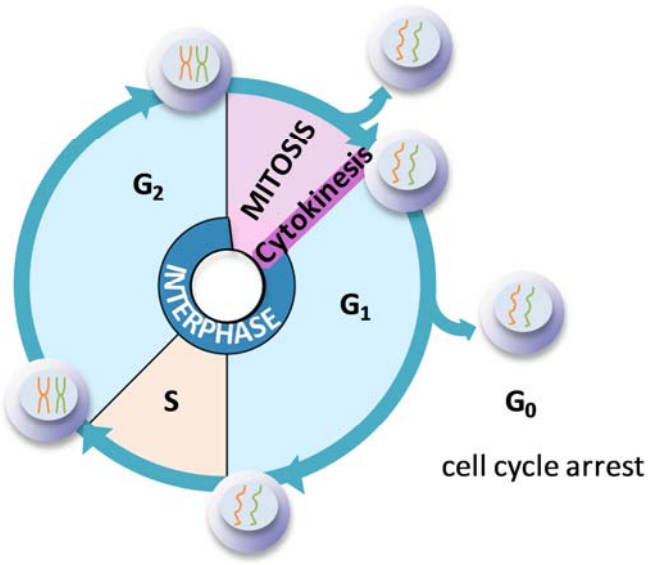


Figure I12. The phases of the cell cycle. The cell grows continuously in interphase, which consists of three phases: DNA replication is confined to S phase; G_1 is the gap between M phase and S phase; while G_2 is the gap between S phase and M phase. In the M phase, the nucleus and the cytoplasm divide.

7.1 Cell cycle regulation

Different cellular proteins regulate the transition from one cell cycle phase to another. The core of the control system is the cyclin-dependent kinases (CDK), a family of serine / threonine protein kinases that are activated at specific points of the cell cycle. When activated, CDK induce downstream processes by phosphorylating selected proteins (Morgan, 1995; Pines, 1995). Moreover, they are inactivated by various Cdk inhibitory proteins (CKIs) and by degradation of the cyclin subunits at specific stages of the cycle.

Alterations in genetic control of cell cycle lead to the development of cancer processes, resulting in an unrestrained cell proliferation. Cell cycle deregulation associated with cancer occurs through mutation of proteins important at different levels of the cell cycle. Thereby, mutations have been observed in genes encoding CDK, cyclins, CDK-activating enzymes, CKI, CDK substrates, and checkpoint proteins. Thus, an accurate regulation is necessary to coordinate division events.

7.2 Mitosis

The M phase is the culmination of the cell cycle and includes the various stages of nuclear division (mitosis) and cytoplasmatic division (cytokinesis). The content of the parent cell, which was doubled during earlier phases of the cycle, is partitioned into two daughter cells. The mitosis is divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase, which occur in strict sequential order, while cytokinesis begins in anaphase and continues through telophase. During prophase, the replicated chromosomes condense in step with the reorganization of the cytoskeleton and the nuclear envelope breaks. In prometaphase the chromosomes become attached to and positioned on the mitotic spindle as it forms. Once all chromosomes are aligned near the spindle equator the cell is considered to be in metaphase. At anaphase the sister chromatids are segregated and move toward the opposing spindle poles. Finally, during telophase adjacent chromosomes fuse to form the two daughter nuclei, and a microtubule-based midbody assembles near the original spindle equator and participates in cytokinesis. Cytoplasmatic division is complete by the end of telophase, and the nucleus and cytoplasm of each daughter cell then return to interphase, signaling the end of M phase.

The progression and execution of mitosis is under post-translational control by several protein kinases. Cyclin-B/Cdk-1 activity is the driving force that promotes mitotic entry and proper mitotic progression. Several other kinases like the Plks, the NIMA kinases and the Aurora kinases play essential roles during mitosis.

7.3 Cytokinesis

Animal cell cytokinesis is initiated during anaphase, when the mitotic spindle reorganizes to form a dense array of antiparallel microtubules midway between the two centrosomal asters, which is called the central spindle (or spindle midzone). Together with microtubules from the spindle asters, the central spindle defines the position of the division plane between the segregated chromosomes. This spatial signal is transmitted through a pathway involving the small GTPase

RhoA, leading to the assembly of an actomyosin ring at the equatorial cell cortex. Contraction of the actomyosin ring results in ingression of the attached plasma membrane to form a cytokinetic furrow, which partitions the cytoplasm into two domains. At this stage, sister cells remain connected by a narrow intercellular bridge containing dense antiparallel bundles of microtubules that overlap at a central region termed the midbody (or Flemming body). Physical separation of the emerging sister cells is finally accomplished by plasma membrane fission at the intercellular bridge. Chromosome segregation and cytokinetic furrow ingression is initiated by the E3 ubiquitin ligase anaphase complex (APC). In this step CDK1 is inactivated since its coactivator cyclin B is targeted to degradation. Consequently, many CDK1 substrates undergo dephosphorylation, which promote cytokinetic furrow ingression and mitotic exit. In the last step of mitosis, Aurora B kinase is the responsible to coordinate the signaling pathway to promote completion of chromosome segregation and abscission.

RhoA is the key player involved in the spatio-temporal control required for cytokinesis. It is through the regulation in the interplay between RhoGEFs and RhoGAPs that RhoA translocates to the equatorial cortex and initiates the assembly and ingression of the contractile ring (Mishima *et al.*, 2002; Bement *et al.*, 2005; Yüce *et al.*, 2005). Different RhoGEFs are involved during mitosis to ensure proper assembly and ingression of cytokinetic furrow (Figure I13). MyoGEF has been shown to interact with the centrosome/spindle pole-associated protein (CSPP) and is necessary for recruitment of ECT2, RhoA and non-muscle myosin II to the central spindle (Asiedu *et al.*, 2009). ECT2 is recruited to the central spindle and equatorial cortex and is necessary for the cortical localization and activation of RhoA, a critical step in the establishment of the cleavage furrow at the equatorial cortex (Su *et al.*, 2011). GEF-H1 is also implicated since is important directing the activation of RhoA during cleavage furrow ingression and may provide essential communication between the microtubule network responsible for segregating genomic material and the actomyosin driven furrow ingression process (Birkenfeld *et al.*, 2007). Furthermore, LARG has been reported to be a novel and temporally RhoGEF required for the completion of abscission, the final stage of cytokinesis (Martz *et al.*, 2013).

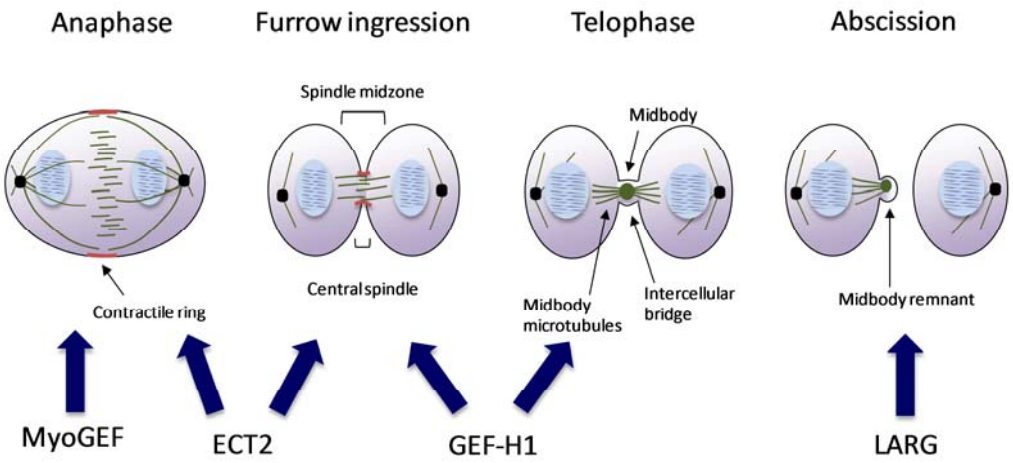


Figure I13. Cartoon of the RhoGEFs implicated in the late stages of mitosis. Multiple RhoGEFs exhibit temporally distinct functions for the proper completion of mitosis.

OBJECTIVES

Heterotrimeric G proteins are master regulators of cell homeostasis. They are implicated in a myriad of cellular processes. Some Gα subunits were reported to regulate mitosis, but nothing is known about $G\alpha_{12}$. As mitosis is a process regulated by the small GTPase Rho; the goals of this study were:

1. Study the role of $G\alpha_{12}$ during cell division.

2. Identification of novel effectors for G12 signaling pathways.

3. Study and characterization of the interaction between $G\alpha_{12}$ and GEF-H1.

4. Study the mechanism of interaction between $G\alpha_{12}$ and GEF-H1.

5. Identification of novel cellular roles for GEF-H1 and its implication in signaling pathways regulated by $G\alpha_{12}$.

MATERIALS AND METHODS

1. MATERIALS

1.1 Buffers and solutions

Buffer	Composition	Comments
DNA sample buffer 6X	10 mM tris-HCl pH 7.6 0.15% (w/v) bromophenol blue 0.15% (w/v) xylen cyanol 60% (v/v) glycerol 60mM EDTA	store at -20 °C
GTB buffer	80 mM PIPES pH 7 1 mM EGTA 1 mM $MgCl_2$	store at 4 °C for less than 3 months
	1% NP-40 1 mM EDTA pH 8 1 mM Na_3VO_4 50 mM NaF 1 mM PMSF 5 µg/ml aprotinin 5 µg/ml leupeptin	added fresh
HBS 2x	250 mM NaCl 9 mM KCl 1.5 mM Na_2HPO_4 10 mM glucose 50 mM HEPES	adjust pH 7.12 filter and keep at 4 °C
HBS (RhoG17A pull-down)	20mM HEPES pH 7.5 150mM NaCl	
LB broth	1% NaCl 1% (w/v) bacto-tryptone 0.5% (w/v) bacto-yeast extract	autoclaved
LB-agar	LB broth 1.5% (w/v) agar	autoclaved
PBS (1X)	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO4	adjust pH 7.4
Polyacrylamide gel	8-12% acrylamide/bisacrylamide	

	0.375 M Tris-HCl pH 8.8 0.1% SDS 0.1% APS TEMED	
Ponceau staining solution	10% (v/v) acetic acid 2% (w/v) Ponceau-S	
RhoA(G17A) lysis buffer	20 mM HEPES pH 7.5 150 mM NaCl 5 mM $MgCl_2$ 1% Triton X-100	store at 4 °C for less than 3 months
	1 mM DTT 1 mM PMSF 10 µg/ml aprotinin 10 µg/ml leupeptin	added fresh
RIPA buffer (1X)	300 mM NaCl 50 mM Tris pH 7.4 0.1% SDS 0.5% DOC	store at 4ºC for less than 3 months
	1 mM EDTA pH 8 1 mM Na_3VO_4 50 mM NaF 1% *n*-dodecyl β-D-maltoside 1 mM PMSF 5 µg/ml aprotinin 5 µg/ml leupeptin	added fresh
RIPA wash buffer (1X)	300 mM NaCl 50 mM Tris pH 7.4 0.1% SDS 0.5% DOC	store at 4 °C for less than 3 months
	1 mM EDTA pH 8 1 mM Na_3VO_4 50 µM NaF 0.01% *n*-dodecyl β-D-maltoside 1 mM PMSF	added fresh
SDS-running buffer (10X)	0.21 M Tris base 1.92 M glycine 0.035 M SDS	
SDS-sample buffer (5X)	250 mM Tris-HCl pH 6.8 500 mM DTT 10% SDS	add DTT in cold buffer

	50% (v/v) glycerol 0.5% (w/v) bromophenol blue	
SOB	2% Bacto Tryptone 0.5% Bacto Yeast Extract 8.6 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$	autoclave and add sterile: 10 mM $MgCl_2$ 10 mM $MgSO_4$
TAE	40 mM Tris-acetate pH 7.6 1 mM EDTA	
TBS (10X)	0.1 M Tris base 1.5 M NaCl	adjust pH 7.4
TBS-Tween (1X)	TBS 1X 1% (v/v) Tween-20	
Transfer buffer (10X)	25 mM Tris base 192 mM glycine 10% methanol	

1.2 Oligonucleotides

Name	**5'→ 3'**
HA-GEF-H1-Nt KpnI forward	CGC GGT ACC ATG TAT CCT TAT GAC GTT CCT GAC TAT GCC TCT CGG ATC GAA TCC CTC ACG CGG
HA-GEF-H1-Nt EcoRV reverse	TGC TGC GAT ATC CGT CGC TCA GCG CAC GCT CTG CTG AAT GAC CCG
HA-GEF-H1-Ct KpnI forward	CGC GGT ACC ATG TAT CCT TAT GAC GTT CCT GAC TAT GCC ACA TGC CCA TCC AGG GAG GAC TTC
HA-GEF-H1-Ct EcoRV reverse	TGC TGC GAT ATC GTC GCG TTA GCT CTC GGA GGC TAC AGC CTC CCC

1.3 Short hairpin RNA

shRNA	**Specie**	**Clone Id**	**Target gene**	**Vector**	**Target sequence**
GEF-H1(1)	human	TRCN0000003174	ARHGEF2	pLKO.1	CGCTCTGTCCATCGAAACTTT
GEF-H1(2)	human	TRCN0000003175	ARHGEF2	pLKO.1	CGATGCCCTGTACTTGAGTTT

GEF-H1(3)	human	TRCN0000003176	ARHGEF2	pLKO.1	CCCAACCTGCAATGTGACTAT
GEF-H1(4)	human	TRCN0000010764	ARHGEF2	pLKO.1	GCGGCGAATTAAGATGGAGTT
$G\alpha_{12}$(1)	human	TRCN0000036756	GNA12	pLKO.1	GCACGAGATAAGCTTGGCATT
$G\alpha_{12}$(2)	human	TRCN0000036758	GNA12	pLKO.1	CCATGCTGTGAAAGACACCAT
GEF-H1(5)	mouse	TRCN0000109985	ARHGEF2	pLKO.1	GCAGGAGATTTACAACCGAAT
GEF-H1(6)	mouse	TRCN0000109986	ARHGEF2	pLKO.1	CCTGAGATGTATGAGGTACAT
GEF-H1(7)	mouse	TRCN0000109987	ARHGEF2	pLKO.1	CGACGCTTTATACTTGAGCTT
GEF-H1(8)	mouse	TRCN0000109988	ARHGEF2	pLKO.1	CCCTCATTTGTCCTACATGTA
GEF-H1(9)	mouse	TRCN0000109989	ARHGEF2	pLKO.1	TCAGTGACATTCACACACGTT

1.4 Plasmids

Plasmid	**Species**	**Vector**	**Source**
14-3-3 γ-Flag	human	pCDNA3.1	Dr. T. Pawson. Mount Sinai Hospital, Canada.
mtDSRed	human	pCDNA3.1	Generated in the laboratory.
pSRE-Lmut			Dr. D. Toksoz. Tufts Medical Center. Boston.
pRL-TK			Dr. J.M. Therme. IBMB-CSIC. Barcelona.
RhoAG17A		pGEX-2T	Dr. K. Burridge. Universtiy of North Carolina (UNC). Chapel Hill, NC.
$G\alpha_{12}$	human	pCDNA3.1	Guthrie Research Institute, Sayre, PA.
$G\alpha_{12}$Q231L	human	pCDNA3.1	Guthrie Research Institute, Sayre, PA.
$G\alpha_{13}$	human	pCDNA3.1	Guthrie Research Institute, Sayre, PA.
$G\alpha_{12}$-GFP	mouse	pCDNA3.1	Dr. T.E. Meigs, University of North Carolina, Asheville.
$G\alpha_{12}$Q229L-GFP	mouse	pCDNA3.1	Dr. T.E. Meigs, University of North Carolina, Asheville.
Myc-$G\alpha_{12}$Q229L	mouse	pCDNA3.1	Dr. T.E. Meigs, University of North Carolina,

			Asheville.
Myc-$G\alpha_{12}$Q229LEEE	mouse	pCDNA3.1	Dr. T.E. Meigs, University of North Carolina, Asheville.
Myc-$G\alpha_{12}$Q229LMM	mouse	pCDNA3.1	Dr. T.E. Meigs, University of North Carolina, Asheville.
$G\alpha_{12}$G228A	mouse	pCIS	Dr. M. Simon, California Institute of Technology.
HA-GEF-H1	human	pCDNA3.1	Dr. G.M. Bokoch. The Scripps Research Institute, La Jolla.
HA-GEF-H1-Nt	human	pCDNA3.1	Generated as part of this thesis
HA-GEF-H1-Ct	human	pCDNA3.1	Generated as part of this thesis
HA-GEF-H1(DH-PH)	human	pCDNA3.1	Generated as part of this thesis
EGFP-GEF-H1	human	pCMV5	Dr. G.M. Bokoch. The Scripps Research Institute, La Jolla.
EGFP-GEF-H1C53R	human	pCMV5	Dr. G.M. Bokoch. The Scripps Research Institute, La Jolla.
EGFP-GEF-H1S885/959A	human	pCMV5	Dr. G.M. Bokoch. The Scripps Research Institute, La Jolla.
EGFP-GEF-H1S885/959D	human	pCMV5	Dr. G.M. Bokoch. The Scripps Research Institute, La Jolla.
HA-GEF-H1	human	pCEFL	Generated as part of this thesis.
VSV-G	retroviral	pCMV	Dr. A. Jordan, IBMB-CSIC. Barcelona.
ΔR8.91	retroviral	pCMV	Dr. A. Jordan, IBMB-CSIC. Barcelona.
scramble		pLKO.1	Sigma (Functional Genomics Service-PCB).
$G\alpha_{12}$-sh1	human	pLKO.1	Sigma (Functional Genomics Service-PCB).
$G\alpha_{12}$-sh2	human	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh1	human	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh2	human	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh3	human	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh4	human	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh5	mouse	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh6	mouse	pLKO.1	Sigma (Functional Genomics Service-PCB).

GEF-H1-sh7	mouse	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh8	mouse	pLKO.1	Sigma (Functional Genomics Service-PCB).
GEF-H1-sh9	mouse	pLKO.1	Sigma (Functional Genomics Service-PCB).

1.5 Primary antibodies

Antigen	**Species**	**Supplier**	**Reference**
58K Golgi protein [58K-9]	mouse	Abcam	ab6284
pan 14-3-3 (K-19)	rabbit	Santa Cruz Biotechnology	sc-629
Aurora-A kinase	mouse	BD, Transduction Laboratories™	610938
β-catenin	mouse	BD, Transduction Laboratories™	51-9001921
caveolin-1	mouse	Zymed	03-6000
Flag M2	mouse	Sigma-Aldrich	F4049
$G\alpha_{12}$	rabbit	Abcam	ab35016
$G\alpha_{12}$ (S-20)	rabbit	Santa Cruz Biotechnology	sc-409
$G\alpha_{13}$ (S-20)	rabbit	Santa Cruz Biotechnology	sc-410
GEF-H1 [B4/7]	mouse	Abcam	ab90783
GEF-H1 (55B6)	rabbit	Cell Signalling Technology®	4076
GFP	rabbit	From Sebastià Pons, (IIB-CSIC)	
HA high affinity	rat	Roche	11867423001
HA agarose conjugate	mouse	Sigma-Aldrich	A2095
Lfc (Y-20)	goat	Santa Cruz Biotechnology	sc-49684
Mannosidase II	mouse	Covance Research Products	MMS-110R
Myc clone 4A6	mouse	Millipore	05-724
N-cadherin	mouse	BD, Transduction Laboratories™	51-9001943
pericentrin (E-17)	goat	Santa Cruz Biotechnology	sc-28142
Alexa Fluor® 488		Invitrogen	A12379

Phalloidin			
Alexa Fluor® 555 Phalloidin		Invitrogen	A34055
Rab1	mouse	Bruno Goud. Curie Institute, Paris.	
Rab11	mouse	BD, Transduction Laboratories™	610656
α-tubulin (DM1A)	mouse	Sigma-Aldrich	T9026
β-tubulin	rabbit	Sigma-Aldrich	T2200

1.6 Secondary antibodies

Name	**Supplier**	**Dilution**
Alexa Fluor 488 conjugated donkey anti-mouse IgG	Invitrogen	1:1000
Alexa Fluor 488 conjugated donkey anti-rabbit IgG	Invitrogen	1:1000
Alexa Fluor 555 conjugated goat anti-mouse IgG	Invitrogen	1:1000
Alexa Fluor 555 conjugated goat anti-rabbit IgG	Invitrogen	1:1000
Alexa Fluor 568 conjugated donkey anti-goat IgG	Invitrogen	1:1000
Alexa Fluor 647 conjugated goat anti-rabbit IgG	Invitrogen	1:1000
IRDye 680 goat anti-rabbit IgG	LI-COR	1:20000
IRDye 800 goat anti-rabbit IgG	LI-COR	1:20000
IRDye 800 goat anti-mouse IgG	LI-COR	1:20000

1.7 Cell lines

Cell line	**Origin**	**From**
COS-7	African green monkey kidney	ATCC
HEK-293	Human embryonic kidney cells stably expressing the Epstein Barr Virus (EBV) EBNA-1 gene from pCMV/EBNA	Invitrogen
HEK-293T	Human embryonic kidney cells containing Adeno and SV-40 viral DNA sequences	ATCC

HeLa	Human epithelial cells containing human papilloma virus	ATCC
MDCK	Madin-Darby canine kidney	ATCC
MEF	Mouse embryonic fibroblast	Zorzano, A.
MEF $G\alpha_{12/13}$(-/-)	Mouse embryonic fibroblast KO for $G\alpha_{12/13}$	Offermanns, S.
NIH3T3	Mouse embryonic fibroblast	ATCC
TREX-2T ($G\alpha_{12}$Q231L) HEK-293	HEK-293 cells with inducible expression of $G\alpha_{12}$Q231L	Obtained in the laboratory.

2. Methods

2.1 DNA manipulations

2.1.1 Competent cells preparation

All the plasmids used containing the different DNA constructs were amplified in the *Escherichia Coli* strain DH5α or DH10B transformed by heat shock or electroporation, respectively.

- *Heat shock competent cells preparation*

To prepare heat shock competent *E.coli* cells (DH5α strain), one colony from a LB-agar plate was inoculated in 250 ml of SOB and was incubated at 37 °C with vigorous shaking until reached an OD_{600} between 0.4-0.6. After placing the cultures on ice for 5-10 minutes to stop the growth, bacteria were pelleted at 4000 rpm for 10 minutes at 4 °C. Pellet was resuspended in 100 ml of ice-cold 50 mM $CaCl_2$ and stored on ice for 10 minutes. Bacteria were recovered by centrifugation and resuspended in 16 ml of ice-cold 50 mM $CaCl_2$-15% glycerol solution. Finally with a pre-chilled pipette tip cells were aliquoted in 200 µl and stored at -80 °C.

- *Electrocompetent cells preparation*

To prepare electrocompetent cells (DH10B strain), one colony was picked from a LB-agar plate and inoculated to 50 ml of SOB, incubated overnight at 37 °C

while shaking at 250 rpm. Next day 0.5 ml was taken and added to 500 ml of SOB and incubated at 37 °C while shaking until an OD_{550} of approximately 0.8 was reached. The cells were kept on ice for 15 minutes and centrifuged for 10 minutes at 5000 rpm at 4 °C. The pellet was washed three times with 500 ml of ice-cold 10% glycerol. After the last centrifugation at 4 °C, cells were resuspended in 0.5 ml of 10% glycerol and 30 µl aliquots were kept at -80 °C until use.

2.1.2 Bacterial transformation

- *Heat shock*

For heat shock, 2 µl of circular DNA was added into 100 µl of competent DH5α cells and incubated on ice for 20 minutes. Next, the mixture was placed into a water bath at 42 °C for 90 seconds and back on ice for 2 minutes, to which 1 ml of 37 °C warmed LB (with no antibiotic added) was added and incubated for 30 minutes at 37 °C. About 200 µl of the resulting culture was spread on pre-warmed LB plates with the appropriate antibiotic and grown overnight at 37 °C. Next day, colonies were picked and placed in LB medium for overnight culture at 37 °C.

- *Electroporation*

The bacteria transformation was performed incubating, for 10 minutes on ice, 1 µl of DNA with 30 µl of DH10B electrocompetent cells. Then, bacteria were transferred to electroporation cuvettes and electroporated using the GENE PULSER II from BioRad at 200 Ohms, 25 mF and 2.5 V. Following electroporation, 500 µl of LB medium was added and the bacteria mix was incubated at 37 °C for 30 minutes while shaking at 250 rpm. From that bacteria mix, 100 µl was plated on the LB plates containing the appropriate antibiotic and incubated overnight at 37 °C. Colonies from overnight cultures were picked the following day for plasmid preparation.

2.1.3 DNA extraction from bacterial cells

To purify plasmidic DNA from bacteria cultures Mini and Maxi-Prep kits were used. Small-scale purification of plasmid DNA was carried out with the

Minipreps DNA Purification System Protocol (Fermentas), following manufacturer's protocol. Large-scale purification of plasmid DNA was performed using Maxi-prep kits (QIAGEN), according to the manufacturer´s instructions. Both protocols are based in the alkaline lysis method. The purified DNA was eluted in TE buffer or water. The concentration of DNA samples was measured with the ND-1000 Nanodrop spectrophotometer and software (NanoDrop Technologies).

2.1.4 DNA agarose gel electrophoresis

The analysis, size identification and isolation of DNA plasmids and fragments were performed by agarose gel electrophoresis. Between 0.8% and 1% (w/v) of electrophoresis grade agarose (Invitrogen) and SYBR® Safe (1:10000) DNA gel stain (Invitrogen) were added in TAE buffer and heated in the microwave until molten. DNA samples were loaded into the wells in DNA loading buffer and run along-side of 5 µl of 2-Log DNA ladder (BioLabs). DNA was visualized using GeneGenius image system (SynGene). To visualize and to cut the bands for cloning, the Safe Imager 2.0 Blue Light Transilluminator (Invitrogen) was utilized.

2.1.5 DNA purification from agarose gels

PCR products and digested DNA fragments were excised from agarose gels using a sterile scalpel blade and DNA was extracted utilizing the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare) following manufacturer's instructions.

2.1.6 Polymerase chain reaction (PCR)

Based on the base-pairing principle, specific primers complementary to the flanking regions of the target DNA were designed. The primers contained in addition restriction endonuclease cleavage sites, facilitating subsequent cloning. The Phusion® High-Fidelity DNA polymerase (New England Biolabs) was used to amplify the DNAs. The PCR reaction mixture was prepared by adding 50 ng of DNA template in 1xPCR buffer (Phusion® HF buffer), 10 mM dNTPs (Stratagene), 2.5

µM of each primer and 1 U of Phusion® polymerase (Biolabs) and autoclaved Milli-Q water up to 50 µl. The reaction was carried out in the Thermal Cycler MJMini from Bio-Rad with the conditions described in Table M1. The PCR fragments were run in 1% agarose gel to check correct DNA amplification.

Table M1. PCR conditions.

Step	Temperature	Time	Comments
Hot start	98 °C	30 s	
Denaturation	98 °C	10 s	
Annealing	40-65 °C	30 s	Different annealing temperature was used depending on the primers
Elongation	72 °C	30 s	Time varies on fragment DNA (1 kb/min)
Cycles			Steps 2, 3 and 4 were repeated 30 times
Final elongation	72 °C	10 min	

2.1.7 DNA cloning

Different plasmids were generated along this study. For that the main strategy used was the cloning of fragments obtained by the polymerase chain reaction (PCR) with the adequate restriction enzymes. In the Materials section there is a list of all the plasmids utilized in this study, generated in our group or obtained from other scientists.

- *DNA digestion and 5'dephosphorylation*

The purified PCR products or plasmids containing a DNA to be cloned (insert or vector) were digested with the appropriate restriction endonucleases (Biolabs). In a 50 µl final volume reaction, approximately 1 µg of plasmid DNA or the whole PCR DNA preparation was digested with 2 U of the restriction enzyme in the corresponding buffer recommended for the supplier's catalogue for 2 hours at 37 °C. Some restriction enzymes have a much lower activity when digesting the end-sides of linear DNA (in our case the PCR products) and therefore were digested for 16 hours at 37 °C. The digested products, insert and vector, were

separated by agarose gel electrophoresis and purified using Gel Band Purification Kit from GE Healthcare.

Prior to ligation and if its appropriated, dephosphorylation of 1 µg of linearized DNA vector was performed with the addition of 1 U of Alkaline Phosphatase (shrimp from Roche) in 20 µl of 1x dephosphorylation buffer at 37 °C for 30 minutes. The phosphatase was inactivated at 65 °C during 30 minutes. The products, insert and vector, were separated by agarose gel electrophoresis and purified with the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare) before ligation.

-Ligation

Ligation of vector and insert was performed using T4 DNA ligase (New England Biolabs). The ligation reaction was set estimating the molar ratio between Insert (I) and Vector (V): pbV x I (mg/ml) = pbI x V (mg/ml). The reaction was done with 50 ng of vector in 20 µl final volume, adding 1 U of T4 DNA ligase and incubating overnight at 16 °C in the thermo cycler. Following ligation, the plasmids were transformed by electroporation and plated on agar plates with the proper antibiotic selection, which were incubated at 37 °C overnight. The next day several colonies were picked for mini-prep. To check which clones contained the desired insert, each DNA (3-5 µl) was digested for 2 hours at 37 °C with the correct restriction enzymes (usually the same ones used for the cloning) and run in agarose gel. The plasmids containing the right inserts were then sequenced to verify that they contained the expected fragments.

- DNA Sequencing

All the plasmids cloned in this work were sent for sequencing in the Sequencing Capillary Service of CRAG (Centre de Regulació Agrogenòmica - Bellaterra, Barcelona). The kit utilized was BigDye v1.1 and the Sequencer 3730 DNA Analyzer from Applied Biosystems.

2.2 Cell Culture

2.2.1 Maintaining and subculturing the cells

Commonly used cell lines (specified above) were grown in DMEM (Dulbecco's Modified Eagle's Medium) (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Invitrogen) and 100 U Penicillin/Streptomycin (Lonza). All cells were manipulated in a laminar flow cabinet (ESCO Labculture) and grown in a humidified atmosphere of 5% CO_2 in air at 37 °C (Core Scientific Services CSS-PCB). In order to subculturing the cells, at 80-90% confluence cells were passed, washing first with pre-warmed PBS and incubating with 1 ml of Trypsin/EDTA (Invitrogen) at 37 °C until cells detached (approximately 1-2 minutes). Afterwards, cells were resuspended in fresh media. The appropriate passage of cells was performed, usually ranging between 1:10 or 1:20 depending on the case.

In the case of TREX-2T($G\alpha_{12}$Q231L) 293 cells 7.5 μg/ml of blasticidin and 50 μg/ml of zeocin should be supplemented to the media for subculturing the cells, and the induction of protein expression was achieved with 0.75 μg/ml of tetracycline for 4 hours.

2.2.2 Storage and freezing/thawing cells

Cells were kept no longer than two months in culture and after that a new batch of frozen cells was thaw. Cells from a confluent 10 cm dish were collected and resuspended in 1.5 ml of complete DMEM and kept on ice. One volume of double concentrated freezing medium (20% DMSO, 50% FBS and 30% complete DMEM) was added drop-by-drop to the cell suspension and 1 ml of cell suspension was transferred to a cryo-tube (Nunc). Cells were first stored in an isopropanol-filled freezing container at -80 °C for 1-2 days to provide the critical and repeatable -1 °C/minute cooling rate required for successful cell cryopreservation and recovery. Then, cells were transferred to liquid N_2 tank for long term storage.

Cells were quickly thawed in a 37 °C water bath, diluted with 9 ml of pre-warmed growth medium and centrifuged at 1200 rpm for 5 minutes. The cell pellet was resuspended in fresh medium and plated in a dish.

2.2.3 Transient transfections

- *FuGene or Metafectene transfection*

Transient transfections were performed on 60-70% confluent monolayers by using FuGENE® 6 (Roche) or METAFECTENE® Pro (Biontex) according to the manufacturer´s instructions (see Table M2). Expression of the proteins was conducted for 24 hours (see legend figures for more details).

Table M2. Transfections reagents and parameters optimized for this work.

	FuGene		**Metafectene**	
Culture plate diameter (mm)	22 (p12-well plate)	p60	22 (p12-well plate)	p60
DNA (µg)	1	4	0.8	4
Transfection reagent (µl)	3	12	2	12
Dilution volume of DNA and reagent (µl)	50	195	50	300
Total volume (ml)	1.05	5.2	1.1	5.6

- *Calcium phosphate transfection*

Cells were plated in a 10 cm dish and the next day cells were transfected. First, 10 µg of the corresponding DNA was pipetted into 1.5 ml tube and resuspended in 375 µl of sterile Milli-Q H2O. Then 125 µl of 1 M $CaCl_2$ was mixed and with vortexing 500 µl of HBS 2x was added drop-by-drop into the mixture to obtain a precipitate with small particles. After 30 minutes the calcium phosphate-DNA suspension was added to the cells.

2.2.4 Stable knockdown cell lines

Utilizing MISSION shRNA human system (Sigma-Aldrich), for GEF-H1 and $G\alpha_{12}$, and MISSION shRNA mouse system (Sigma-Aldrich) for GEF-H1, short hairpin-interfering RNAs (shRNA) targeted against the coding region of the human and murine genes were used for stable knockdown of endogenous proteins expression in different cell lines. The oligonucleotides for proteins shRNA were obtained from Sigma at 0.05 μM scale. The target sequences tested for each protein are in the Materials section.

-*Lentivirus particles production*

$2.5x10^6$ HEK-293T packaging cells were seeded in a 10 cm diameter dish for each lentivirus coding for the different mRNA sequences or an empty vector (control) the day before transfection. Co-transfection, of pLKO.1 vectors, containing the shRNAs with pCMVΔR8.91, which expresses HIV gag and pol, and pVSVG (necessary for envelope and particle formation) at proportion 4:3:1, respectively, was done utilizing calcium phosphate. Medium was collected every 24 hours for 2 days and filtered through 0.45 μm MCE filter (Millipore). Viral particles were stored at –80 °C.

-*Viral transduction*

To infect cell by spinoculation, $5x10^4$ cells were seeded in a 6-well plates. After 24 hours, 1 ml of the viral-particle supernatant was added and plates were centrifuged at 1200xg for 2 hours at room temperature. The day after, infected cells were selected adding 3 μg/ml of puromycin (Sigma Aldrich) for HeLa and NIH3T3 cells and 1.5 μg/ml of puromycin for MEF and HEK-293 cells to the fresh medium. Efficient selection process takes about 3-6 days. Prior to selection process, appropriated puromycin concentration was measured by tritation corves in the different cell lines. To keep the selective pressure in the stable knockdown cell line, puromycin (1μg/μl) was continuously added to the media. The efficiency of shRNA knocking down was confirmed by SDS-PAGE and western blot analysis of protein extracts with the adequate antibodies.

2.2.5 Inducible protein expression using T-REx™ System

The T-REx™ System is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn10-encoded tetracycline (Tet) resistance operon. First, $G\alpha_{12}$Q231L was cloned into the pCDNA4-TO plasmid (Invitrogen) and HEK-293 cells were transfected using calcium phosphate method with pcDNA4-TO-$G\alpha_{12}$Q231L and pCNDA6-TR (Invitrogen) in a 1:6 proportion. After transfection cells were treated with 7.5 μg/ml of blasticidin and 50 μg/ml of zeocin to select transfected cells. In order to induce $G\alpha_{12}$Q231L expression, cells were treated with 0.75μg/ml of tetracycline for 4 hours.

2.2.6 Cell treatments

Cell lines were treated with different drugs during the indicated time periods (see legend figures for mores details).

Table M3. Cell treatments.

Drug	**Concentration**	**Time**	**Assay**
Tetracycline	0.5 μg/ml	4 h at 37 °C	Inducible expression of $G\alpha_{12}$ in TREX-2T ($G\alpha_{12}$) HEK-293 cells
Brefeldin A	5 μg/ml	30 min at 37 °C	Golgi disassembly
Nocodazole	10 μg/ml	30 min on ice, 30 min at 37 °C	Microtubule depolymerization
	200 ng/ml	16 h at 37 °C	Cell synchronization

2.2.7 Cell synchronization

Asynchronous cultures were supplemented with 200 ng/ml nocadazole (Noc) (Sigma Aldrich). Mitotic cells were washed six times with PBS and cells were allowed to progress through mitosis/cytokinesis for two hours. Then, cells were fixed and processed for immunostaining.

2.2.8 Preparation of cell lysates

To prepare cellular lysates cells were washed twice with cold PBS before they were lysed on ice in 200-700 µl of ice-cold lysis buffer (different lysis buffers were used: RIPA, Passive Lysis Buffer, RhoAG17A lysis buffer and GTB buffer). The cells were then harvested with the help of a scraper and transferred to a precooled 1.5 ml tubes. The lysis was allowed to proceed for 30 minutes in a rotating wheel at 4 °C before centrifugation at 13000 rpm for 15 minutes at 4 °C. The cleared lysates were transferred to fresh precooled 1.5 ml tube and were ready for the assay or stored at -80 °C.

2.2.9 Determination of protein concentration

The protein concentration in cellular lysates was determined using the BioRad Protein Assay (BioRad). Between 2-4 µl of cellular lysates were mixed with 798-796 µl of H_2O and 200 µl of Bradford reagent. Absorbance at 595 nm was measured in spectrophotometer. Protein concentration was determined with respect to the absorbance of known concentrations of BSA protein.

2.3 Protein Electrophoresis

2.3.1 SDS-PAGE Electrophoresis

To separate proteins, polyacrylamide-SDS gel electrophoresis was used following the method described by Laemmli (1970). In this work we have utilized Mini-Protean Tetra Cell System (Bio-Rad) for electrophoresis with polyacrylamide in denaturing conditions (SDS-PAGE). For this, 1.5 mm thickness gels with 10-12% of acrylamide for the resolving gels and with 5% of acrylamide for the stacking gel were used. The samples were prepared adding 5xSDS loading buffer and heating for 5 min at 95 °C to denaturalize the proteins. Electrophoresis was run at room temperature in 1X SDS-PAGE running buffer at 100-115 V for approximately 1-2 hours until the samples reached the desired separation, visualized by the

PageRuler™ prestained protein ladder (Fermentas). The gels were then electrotransferred and analyzed by western blot.

2.3.2 Western blot

Proteins were transferred from the acrylamide gel to a Immobilon-FL PVDF membranes (Millipore) by electroblotting utilizing Mini Trans-Blot system (BioRad). The transference was done in 1X Transfer buffer, at room temperature with constant voltage (60 V) for 180 minutes.

2.3.3 Immunodetection

The infrared detection used in this work has the advantage that has a wider linear dynamic range that the chemiluminescence method does not offer. The Odyssey equipment is equipped with two infrared channels for direct fluorescence detection on membranes. Protein detection was performed by the sequential incubation of the membrane with a specific primary antibody and a subsequent secondary antibody conjugated to IRDye (680 and 800 CW). First, the membranes were blocked for 1 hour with TBS-Tween supplemented with 3% BSA at room temperature in a rocking platform. Next, the membrane was washed three times with TBS-Tween and incubated with the primary antibody overnight at 4 °C. The primary and secondary antibodies were diluted in TBS-Tween with 0.15% BSA. Afterwards, the membrane was washed three times with TBS-Tween for 10 minutes and incubated with secondary antibody for 1 hour at room temperature (see Materials for antibody dilution). After three final washes with PBS in absence of Tween, the membrane was analyzed utilizing the Odyssey System equipment from LI-COR. If the blot was analyzed for additional proteins, the membrane was stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) for 15 minutes at 37 °C in a rocking platform. The blot was washed 3 times in TBS-Tween before blocking again and proceeding with antibody incubation as previously indicated. The quantification of the bands was done with Odyssey v3.0 software.

2.4 Immunoprecipitation (IP)

In this work two types of immunoprecipitations (IP) have been done.

2.4.1 Classic IP

Cells were lysed for 30 minutes in RIPA lysis buffer. For classic IP, specific antibodies were incubated with whole cell lysates for 4 hours or overnight at 4 °C on a rotating wheel. In parallel Protein G Sepharose 4 Fast Flow (GE Healthcare™) was blocked with 100 µl of IgG free BSA (10 mg/ml). Then, protein G was washed three times in RIPA, added to cell lysate and incubated for 1.5 h at 4 °C. The beads were then washed four times in RIPA and analyzed by immunoblotting.

2.4.2 IP anti-HA agarose beads

For anti-HA immunoprecipitation, cells were lysed for 30 min in RIPA lysis buffer with or without AlF_4^- (5 mM NaCl, 3 mM $AlCl_3$). First, the lysates were pre-cleared with 10 µl of Protein G and incubated for 1 hour at 4 °C on a rotating wheel. The beads were discarded and 25 µl of monoclonal anti-HA agarose beads (Sigma Aldrich) were incubated with the lysate for 4 hour at 4 °C on a rotating wheel. Then, the beads were washed five times in RIPA with or without AlF_4^- and analyzed by immunoblotting.

2.5. Analysis of activated GEFs in cell lysates

The glutathione S-transferase (GST)–RhoAG17A pull-down assay was used to detect activated GEFs in cell lysates (García-Mata *et al.*, 2006). This mutant of RhoA cannot bind nucleotide and therefore has high affinity for GEFs.

2.5.1 Expression and purification of GST-RhoAG17A

BL21 competent cells were transformed with the pGEX-4T-GST-RhoAG17A plasmid by heat shock and plated on LB-agar plates containing ampicillin. Next day

one colony was picked from the LB-agar plate, and a 100 ml overnight culture in LB-ampicillin was made while shaking at 37 °C at about 230 rpm. Then the overnight culture was diluted 1:10 in LB-ampicillin and incubated at 37 °C with shaking for 30 minutes. Bacteria were induced to produce RhoA protein by addition of IPTG (final concentration of 100 μM) while shaking overnight at room temperature. The bacteria were collected by centrifugation at 4000 × g, 15 minutes at 4 °C and resuspended in a total of 10 ml cold RhoAG17A lysis buffer. All the purification steps were carried out at 4 °C. Then the bacteria were sonicated on ice for 6-8 times, 15 seconds each time and the lysates were clarified by centrifugation at 20000 × g, 15 minutes at 4 °C. Then the supernatant were mixed with 150 μl Glutathione Sepharose 4B beads (GE Healthcare™), and incubated at 4 °C for 1 hour with continuous rotation. Next, beads were washed two times with 10 ml of RhoAG17A lysis buffer and two more times with 10 ml of HBS supplemented with 5 mM $MgCl_2$ and 1 mM DTT. Beads were resuspended in 2/3 HBS, 5 mM $MgCl_2$, 1 mM DTT, and 1/3 glycerol, aliquoted and stored at -80 °C.

2.5.2 Pull-down of active GEFs

Cells were seeded in p60 plates the day before transfection with plasmids encoding for HA-GEF-H1 and $G\alpha_{12}$ proteins. Twenty-four hours after transfection cells were serum deprived for another 24 hours. Cells were washed twice with ice-cold HBS and harvested in 250 μl of ice-cold RhoAG17A lysis buffer using a cell scraper. Lysates were centrifuged at 16000x g for 1 minute at 4 °C and the supernatant was transferred to a fresh 1.5 ml tube. The protein concentration from total cell lysate fraction was measured using the BioRad Protein Assay, and the same amount of protein was used in each condition. Lysates were pre-cleared by rotating it with 50 μl of GST bound to Glutathione Sepharose (1 mg/ml) for 10 minutes at 4 °C. GST beads were spun down and lysates were transferred to a new tube. An aliquot of 25 μl from each sample was saved in SDS buffer for further analysis. The GST-RhoAG17A beads (10 μg) were added to each lysate, rotated for 45-60 minutes at 4 °C, and washed three times with GSTRhoAG17A lysis buffer. Protein complexes were dissolved by adding 5 μl of 5X SDS-PAGE sample buffer and were resolved by western blot analysis.

2.6 SRE-Luciferase assays

The best-characterized effector of activated RhoA is the Rho kinase, ROCK, which controls actin polymerization and leads to serum response factor (SRF) activation. For the serum response factor (SRF) activation assay the reporter plasmids firefly luciferase Serum Response Element (pSRE.L) and the Renilla luciferase Thymidine Kinase (pRL-TK) were used. The pSRE.L luciferase reporter plasmid encodes for the firefly luciferase positioned downstream of a mutant serum response element (SRE) which contains SRF binding sites but eliminates the ternary complex factor binding site. The pRL-TK plasmid encodes Renilla luciferase positioned downstream of a thymidine kinase promoter. Thus, co-transfection of pSRE.L and pRL-TK allow the production of Renilla luciferase and firefly luciferase that can be measured by use to the Dual-luciferase® reporter assay system (Promega). HEK-293 cells were seeded at a density of ~250.000 cells in 6-well plates and allowed to grow for 24 hours. Cells were co-transfected with 0.1 μg of SRE.L luciferase reporter plasmid, 0.01 μg of pRL-TK plasmid, plus the indicated cDNAs. Total amounts of transfected DNA were kept constant among wells by supplementing the empty vector DNA. The cells were cultured in the presence of 10% FBS for 6 hours and then serum starved for 24 hours. Cells were washed with PBS and lysed in 200 μl of 1X Passive Lysis Buffer (Promega) for 20 minutes in rotation at 4 °C. Lysates were clarified by centrifugation at 13000 rpm for 15 minutes. A volume of 20 μl of cell extracts were mixed with 25 μl of LAR II and assayed by luminometry for firefly luciferase activity and next, 25 μl of Stop&Glo reagent was added and firefly Renilla activity was determined. Firefly luciferase activity measurements were normalized for the corresponding Renilla luciferase values. Measurements were performed using a TD-20/20 luminometer (Sirirus®).

2.7 Fluorescence immunocytochemistry

Cells were plated on the top of cover glasses (Thermo Scientific) of 18 mm diameter and a width of 0.16 mm contained in 12-wells plates. Cells were washed twice in PBS before they were fixed with formaldehyde 3.7% in PBS for 15 minutes

at room temperature or with ice-cold methanol at -20 °C for 10 minutes. The fixation solution was removed and cells were washed three times with PBS. Afterwards, cells were permeabilized and blocked by incubation with blocking solution (0.5% NP-40, 1% BSA in PBS) for 10 minutes at room temperature. Then, the coverslips were transferred to a wet chamber and incubated with primary antibodies in blocking solution for 1 hour at room temperature, washed several times with PBS and incubated with Alexa Fluor® (Invitrogen) secondary antibodies in blocking solution for 1 hour at room temperature. When double labeling of primary antibodies from the same host was needed monovalent Fab fragments of affinity-purified (Jackson) were used. For that, an additional incubation with 20 µg/ml of Fab fragment was carried out in blocking solution for 1 hour at room temperature after the first primary antibody incubation.

Cells were washed several times with PBS and mounted with Pro Long® Gold with DAPI (Invitrogen) for fluorescence microscopic observation. Images were captured with a confocal laser scanner microscope (Leica SP5) and some results were analyzed by the upright fluorescence microscope E600 (Nikon). All the confocal images showed in this work were obtained by a SP5 confocal microscope from Leica Microsystems installed in the CSIC-IBMB-PCB Advanced Fluorescence Microscopy Unit and directed by Elena Rebollo.

2.8 Live cell imaging

Live cell imaging of diving cells was performed in a sealed chamber at 37 °C on a Leica AF7000 inverted microscope with 40x1.25 oil objective and differential interference contrast (DIC) optics. Multidimensional acquisition was controlled by Leica LAS AF software. Cells were imaged every 30 seconds for a period of 10hours.

2.9 Wound-healing assay

Cell motility was measured by the wound-healing migration assay. HeLa cells infected with a scrambled shRNA (shScr1) or a shRNA specifically targeting GEF-H1 protein (shGEF-H1) were grown to a confluent monolayer in a cell culture

dishes. After wounding detached cells or cell debris were removed by washing with PBS. Fresh medium was added immediately. Images of the wound closure were taken by phase contrast microscopy at initial time (0 h) and after 24 hours.

2.10 Microtubule co-sedimentation assay

Twenty-four hours after transfection with the plasmids encoding HA-GEF-H1 and $G\alpha_{12}Q231L$ cells were lysed in GTB buffer supplemented with 1% NP-40, by passing them through a 25-gauge syringe needle. Afterwards, cells were incubated on ice for 30 minutes, to depolymerize the microtubules, and then the lysate was centrifuged at 20,000x g for 80 minutes at 4 °C to remove cellular debris. The supernatant was diluted with GTB supplemented with 0.1% NP-40 and 20 μM Paclitaxel (Taxol) (Sigma Aldrich) was added to each sample. After incubation for 30 minutes at 37 °C, the reaction mixture was centrifuged at 20,000x g for 40 minutes at room temperature. The resultant pellets were resuspended in lysis buffer, separated by SDS-PAGE and subjected to western blotting analysis with whole lysates or supernatants.

2.11 Focus forming assay

NIH3T3 cells infected with a scrambled shRNA (shScr1) or a shRNA specifically targeting GEF-H1 protein (shGEF-H1) were transfected with pcDNA3.1-$G\alpha_{12}Q231L$ or empty vector (pcDNA3.1). Medium was changed every 3-4 days and after three weeks cells were fixed with ice-cold methanol at -20ºC for 10 minutes. Then, cells were stained with 2 ml of 1% methylene blue at room temperature for 2 minutes. Cells were washed several times with water and formed foci were counted.

2.12 Analysis of cell cycle by flow cytometry

Cells were plated in 10 cm dishes and twenty-four hours after, cells were trypsinized with 1 ml of trypsin and resuspended in 9 ml of fresh media. Then, 10^6-10^7cells were centrifuged at 1200 rpm for 3 minutes at room temperature and

pellet was washed with PBS. The suspension was centrifugated again, and the recovered cells were resuspended in 500 µl of PBS with a Pasteur pipette. Afterwards, 4.5 ml of 70% ethanol was mixed to the suspension to fix the cells for a minimum of 2 hours. At this point cells can be stored between 0 to -40 °C for several months. To stain the cells with propidium iodide, cells were centrifugated and washed in PBS for 60 seconds. The pellet was resuspended in 1 ml of 0.1% Triton X-100, 0.2 mg/ml DNase-free RNase A and 20 µg/ml of propidium iodide for 30 minutes at room temperature. After that, cells are ready for the analysis by flow cytometry.

Flow cytometric experiments were carried out using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Florida) in Centres Científics i Tecnològics de la UB-Flow Cytometry Unit and directed by Dr. Jaume Comas. The instrument was set up with the standard configuration: excitation of the sample was done using a standard 488nm air-cooled argon-ion laser at 15mW power. Forward scatter (FSC), side scatter (SSC) and red (620 nm) fluorescence for PI were acquired. Optical alignment was based on optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division). Time was used as a control of the stability of the instrument. Red fluorescence was projected on a 1024 monoparametrical histogram. Aggregates were excluded gating single cells by their area vs. peak fluorescence signal. DNA analysis (Ploidy analysis) on single fluorescence histograms was done using Multicycle software (Phoenix Flow Systems, San Diego, CA).

2.13 Statistical analysis

In all cases, experiments were analyzed for statistical significance by t-test. Asterisks are indicative of the following significances: * =$p<0.05$, **=$p<0.005$, ***=$p<0.001$.

RESULTS

1. $G\alpha_{12}$ regulates mitosis

G12 proteins are implicated in a myriad of cellular processes such as growth and proliferation, cytoskeleton rearrangement or migration and invasion (Kelly *et al.*, 2007). For many years it was known that $G\alpha_{12}$ was involved in cell division (Suzuki, Hajicek, *et al.*, 2009). As such, previous results from our group (Aragay *et al.*, 1995) demonstrated that antibodies against $G\alpha_{12}$ microinjected in the cells blocked cell division induced by thrombin in 1321N1 human astrocytoma cells. Also just after the discovery of $G\alpha_{12/13}$ proteins, the Simon's group (Jiang *et al.*, 1993) demonstrated that $G\alpha_{12}$ overexpression induce focus forming formation, suggesting that $G\alpha_{12}$ affects cell division and proliferation. After that and for many years the emphasis on $G\alpha_{12/13}$ signaling has focused on the Rho-actin polymerization axis and, in spite of the wealth of information accumulated (see Introduction), not much is known on the role of these proteins in cell division. Recently, a new concept has emerge suggesting that heterotrimeric G proteins do not only function and reside at the plasma membrane close to GPCRs and effectors but that they can be located at the cytoplasmic structures and in different organelles and endomembranes. This concept has open new ways of thinking about G protein signaling. Hence, our goal in this work has been to investigate the location and role of $G\alpha_{12}$ during mitosis.

1.1 Mitotic distribution of $G\alpha_{12}$

Before starting with the analysis of $G\alpha_{12}$ in cell division the expression of $G\alpha_{12}$ was analyzed in different cell lines (Figure R1). Cells growing in normal conditions were fixed and stained with an anti-$G\alpha_{12}$ specific antibody. $G\alpha_{12}$ was found along the cytoplasm with some staining in the membrane and in areas around the nuclei in cells in interphase (Figure R1A). Interestingly, $G\alpha_{12}$ was accumulated in the area linking adjacent cells in what it seemed cells in division (Figure R1B). The same $G\alpha_{12}$ staining pattern was present in HeLa, MDCK and COS-7 cells. Again, the majority of the cells in cytokinesis presented the strong staining in the cellular bridge. Some cells showed staining in structures connecting the two cells in regions were DNA was stained with DAPI (Figure R1C). When a cell is

dividing the telomeres of sisters chromatids fuse together and segregate in two resultant cells. Sometimes the segregation fails and a chromatin bridge is observed between the daughter cells. Some of the cells observed seemed to have this alteration with chromatin bridge. Curiously, $G\alpha_{12}$ was present in the chromatin bridge. But no nucleus staining was observed with this antibody, indicating that it was not an unspecific DNA staining of the antibody used. Moreover no staining was observed in absence of primary antibody.

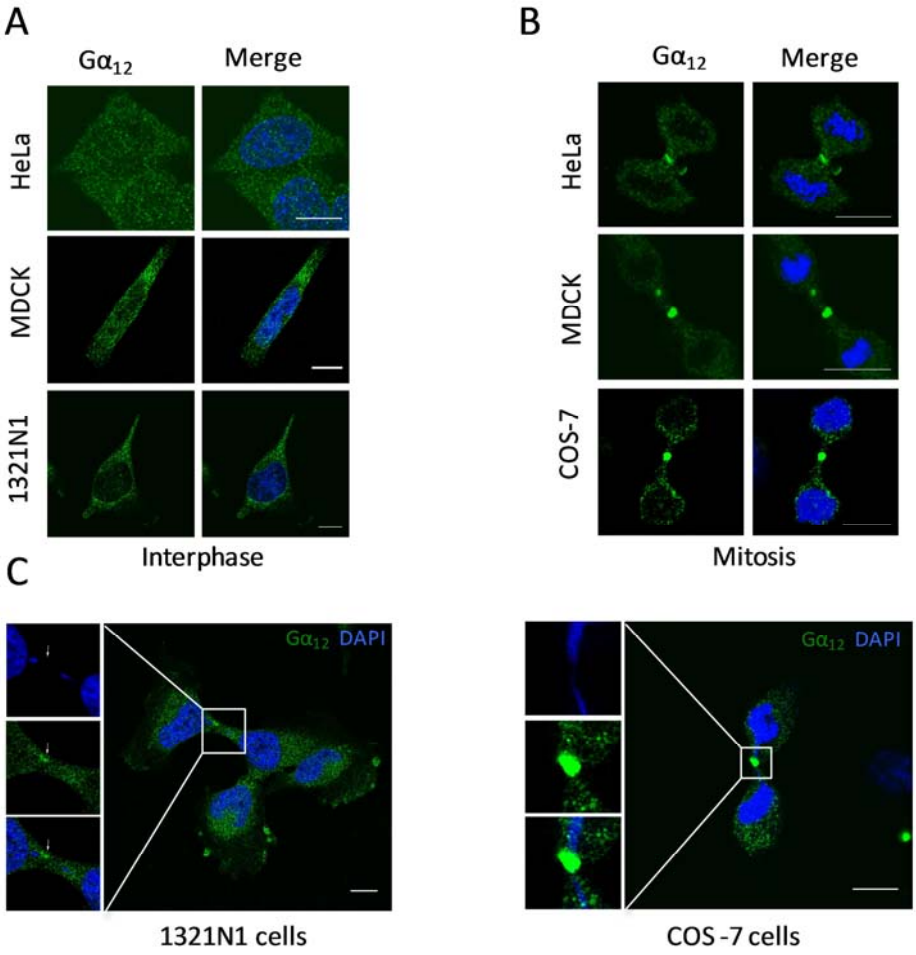


Figure R1. Particular staining pattern of $G\alpha_{12}$ during cytokinesis. **(A)** Asynchronous cells were fixed and probed with antibody against endogenous $G\alpha_{12}$ (Abcam) in HeLa, MDCK and 1321N1 cells**. (B)** Synchronic HeLa cells were fixed and immunolabeled against endogenous $G\alpha_{12}$ with specific antibody from Abcam in HeLa, MDCK and COS-7 cells. **(C)** Asynchronous cells were fixed and probed with antibody against endogenous $G\alpha_{12}$(Abcam) in 1321N1 and COS-7 cells. Cells were visualized by confocal microscopy. The image corresponds to one layer of Z-stack. Images are representative of the majority of the cells present on the plate from two independent experiments. Scale bars: 10 µm.

The $G\alpha_{12}$-pattern observed was obtained using a polyclonal anti-$G\alpha_{12}$ antibody against the C-terminal amino acids 370-379 of rat $G\alpha_{12}$ protein (Abcam). The antibody could recognize specifically a band of 44 kDa of endogenous and transfected $G\alpha_{12}$ in HEK-293 cells in western blot analysis (Figure R2A). It was important to verify further the specificity of the antibody; therefore experiments were designed utilizing another anti-$G\alpha_{12}$ antibody from Santa Cruz Biotechnology made against the N-terminus region of mouse $G\alpha_{12}$. The results obtained resembled the staining at the midbody observed with the previous Abcam antibody (Figure R2B). So, two different antibodies against $G\alpha_{12}$ resulted in the same mitotic pattern, which suggest that the staining of $G\alpha_{12}$ is specific.

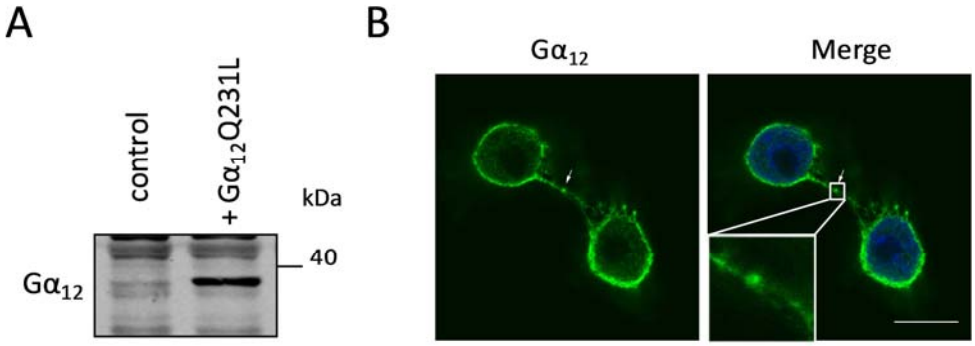


Figure R2. (A) HEK-293 cells were transfected or not with pcDNA3-$G\alpha_{12}$ Q231L. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-$G\alpha_{12}$ from Abcam. **(B)** $G\alpha_{12}$ pattern staining in dividing MEF cells with anti-$G\alpha_{12}$ antibody from Santa Cruz. Asynchronous cells were fixed and immunostained with antibody against endogenous $G\alpha_{12}$. The image corresponds to one layer of Z-stack. Cells were visualized by confocal microscopy. Scale bars: 10 μm.

To further verify the specificity, we decided to knockdown the expression of $G\alpha_{12}$ in HeLa cells utilizing two different shRNA sequences targeted against the coding region of the human GNA12 gene (see Material and Methods). A scrambled shRNA available in Mission shRNA system (Sigma Aldrich) was used as control. Cell lysates were analyzed by western blot with anti-$G\alpha_{12}$ antibody (Figure R3A). The cells infected with sh-1 were not capable of expanding after viral infection. So, it was not used for further experiments. sh-2 infected cells showed reduction of the endogenous expression of $G\alpha_{12}$. The shRNA cells were also tested by immunofluorescence (Figure R3B) and with the same confocal microscopy set up only a faint background was observed in sh$G\alpha_{12}$ treated HeLa cells compared to

control cells that presented the expected pattern. More important shGα_{12} HeLa cells had no staining of the midzone in dividing cells, indicating that the pattern observed was specific.

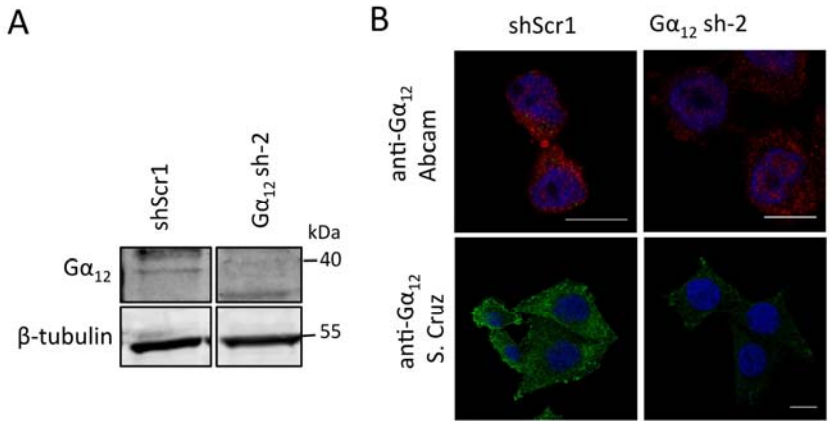


Figure R3. Gα_{12} knockdown in HeLa cell line. HeLa cells were infected by lentivirus with one sequence of shRNA against Gα_{12}(sh-2) and a control shRNA and selected with puromycine. **(A)** Lysates from control shRNA (shScr1) or Gα_{12} shRNA-expressing HeLa cells were evaluated by immunoblotting with β-tubulin expression used as a control. **(B)** Fluorescent confocal micrographs of HeLa control cells (shScr1) or Gα_{12} knockdown cells were fixed and probed against endogenous Gα_{12} with two different specific antibodies, one from Abcam (red) and the other from Santa Cruz (green). Scale bars: 10 μm.

Another approach taken to investigate whether the pattern observed corresponded to Gα_{12} was to use of a chimeric Gα_{12} protein fused to GFP. A previous work from our lab (M. Masià thesis, 2013) obtained a functional chimeric GFP tagged Gα_{12} protein. The construction of this functional chimeric protein was a real challenge since Gα subunits cannot be modified in their N- and C-terminal sequences due to their interaction with their effectors and receptors through both, N-terminal and C-terminal domains. Therefore, GFP had to be inserted within the internal Gα sequence (Hughes *et al.*, 2001; Yu, 2002). An additional problem was encounter for the G$\alpha_{12/13}$ subfamily since the insertion of GFP in internal sequences, which function for Gα_q or Gα_s, blocked the activity of Gα_{12}. Finally, a construct with GFP located between the residues proline 139 and valine 140 was obtained that showed functionality by *in vitro* pull-down experiments with RH-RGS

domains and in cell rounding experiments (M. Masià thesis, 2013). Here, we were able to demonstrate that the chimeric protein was also present in cells (Figure R4). It is worth noticing that high expression levels of $G\alpha_{12}$-GTP were toxic for the cells, as it is expression of wild type $G\alpha_{12}$. Cells with lower expression levels, present $G\alpha_{12}$ mainly located homogeneously in the entire cell. Searching for dividing cells it was possible to observe an increase of $G\alpha_{12}$ expression at the midbody in a cell during mitosis, which corroborate our results with different antibodies (Figure R4, inset). Overall these results indicated that $G\alpha_{12}$ localized at specific areas of the cell during mitosis and suggested a possible role for the protein in during the cytokinesis process.

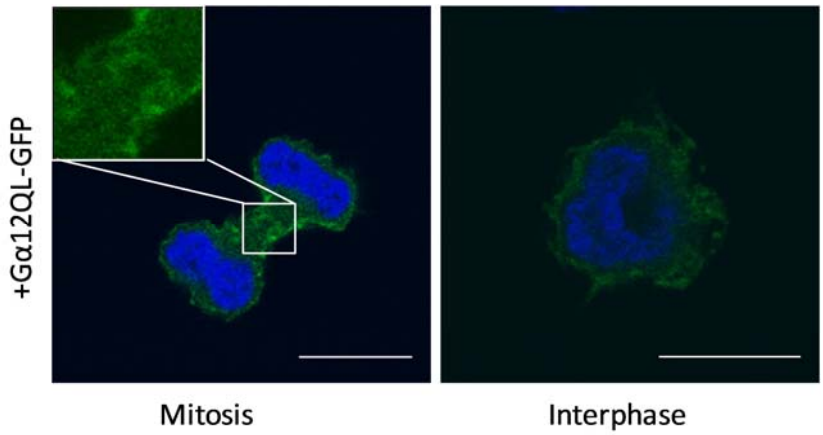


Figure R4. $G\alpha_{12}$-GFP is accumulated at the cleavage furrow region during the late stages of mitosis. Fluorescent confocal micrographs of HeLa cells transfected with pcDNA3.1-$G\alpha_{12}$Q229L-GFP. Twenty-four hours after transfection cells were fixed and mounted with ProlongGold with DAPI. The picture corresponds to one layer of Z-stack from confocal SP5 microscope. Scale bars: 10 μm.

Since the preliminary data indicated that $G\alpha_{12}$ could be positioned in the midzone in dividing cells, we aimed to identify the subcellular localization of the G protein in each stage of mitosis. For that purpose HeLa cells were synchronized with nocodazole treatment and, 2 hours later, stained with anti-$G\alpha_{12}$ antibodies to detect endogenous $G\alpha_{12}$ (Figure R5). Immunofluorescence staining identified $G\alpha_{12}$ throughout the cell cycle. In the early mitosis, during metaphase $G\alpha_{12}$ was distributed predominantly throughout the cytoplasm in a dotty pattern. Once the cell has entered in anaphase, $G\alpha_{12}$ is localized in rows that could correspond to the spindle apparatus along the microtubule tracks. In telophase the intensity of $G\alpha_{12}$

staining diminished and $G\alpha_{12}$ was mostly localized to the spindle midzone. Also a pattern of lines was observed. Finally, in cytokinesis $G\alpha_{12}$ was condensed within the intercellular bridge in a structure that seems to be the midbody, or Flemming body. The staining in cytokinesis was always very intense. We could not identify any cell in prophase.

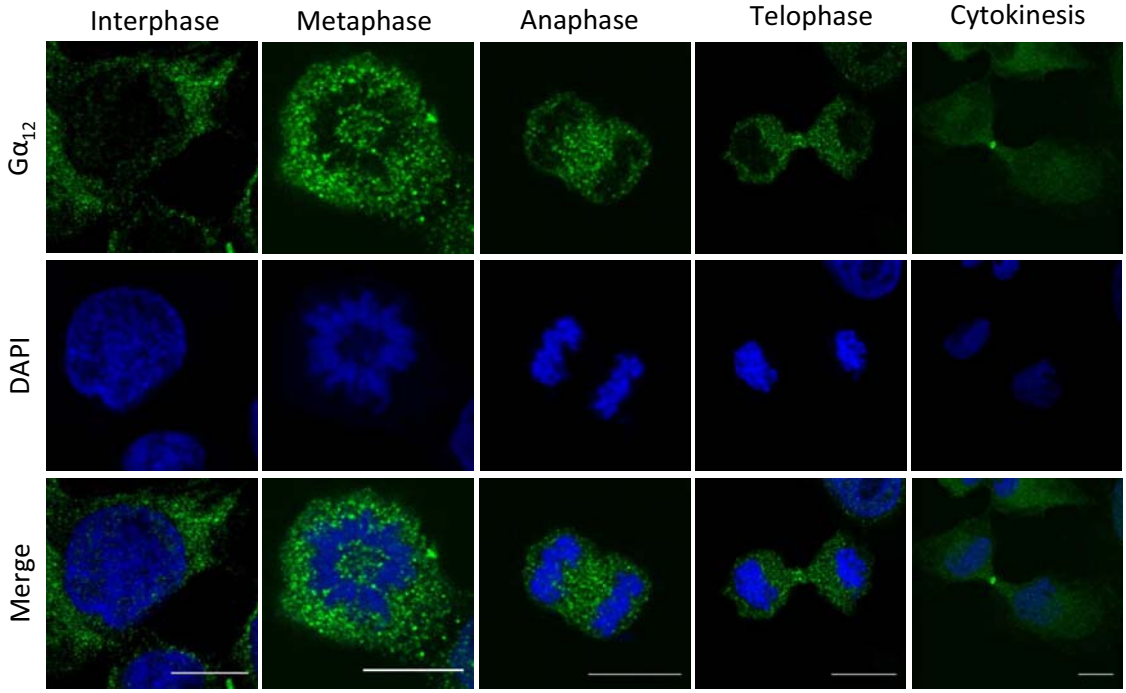


Figure R5. Mitotic distribution of $G\alpha_{12}$. HeLa cells were synchronized with nocodazole, fixed and probed against endogenous $G\alpha_{12}$ with a specific antibody purchased from Abcam. One layer of Z-stack is shown. Images were obtained by using a Leica SP5 confocal microscopy and are representative of the majority of cells present in the plate. Scale bars: 10 μm.

As mentioned before both antibodies seem to stain similar structures in the cells. Nonetheless, in order to further confirm the mitotic pattern, cells were synchronized and stained with the Santa Cruz antibody. Mouse embryonic fibroblast (MEF) cells were stained against endogenous $G\alpha_{12}$ protein (Fig. R6). In early telophase $G\alpha_{12}$ was located in the spindle apparatus, and when mitosis progresses $G\alpha_{12}$ was concentrated in a dense structure at spindle midzone. Again the dotted pattern follows linear structures that resembled the microtubules. In later stages $G\alpha_{12}$ was more prominent at the midbody. The pattern observed suggest that $G\alpha_{12}$ might hold functional significance at different stages during

mitosis and predominantly interesting seemed the localization at later stages of mitosis.

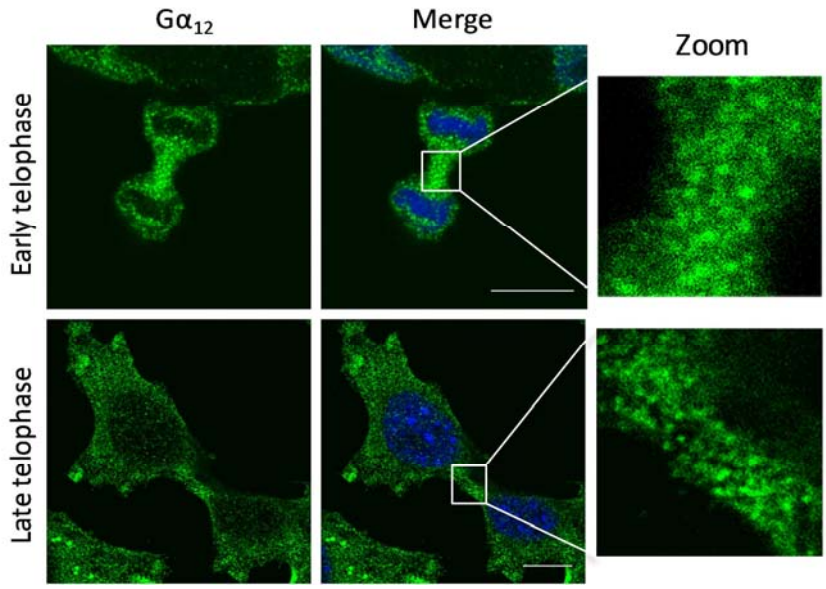


Figure R6. $G\alpha_{12}$ pattern staining in dividing MEF cells with anti-$G\alpha_{12}$ antibody from Santa Cruz. Asynchronous cells were fixed and immunostained with antibody against endogenous $G\alpha_{12}$. The image corresponds to one layer of Z-stack. Cells were visualized by confocal microscopy. Scale bars: 10 μm.

1.2 $G\alpha_{12}$ localizes at the midbody structure

As explained in the introduction, following chromosome segregation in mitosis, a process called cytokinesis separates the daughter cells (Figure R7A). In the first step of cytokinesis, the contractile ring (a structure composed of filamentous actin (F-actin) and the motor protein myosin-2, among others structural and regulatory proteins) promotes ingression of the cleavage furrow. Actin polymerization at the contractile ring requires activation of the small GTPase RhoA and it is assembled during anaphase generating the required force to constrict the cell membrane to form a cleavage furrow. While furrowing largely partitions the cytoplasm into two domains, the sister cells still remain connected by an intercellular bridge. The intercellular bridge contains dense bundles of antiparallel microtubules, which overlap at the midbody region. At later stages, cytokinesis completes by a process termed abscission, which leads to irreversible fission of the plasma membrane between dividing daughter cells (Guizetti and Gerlich, 2010). Given the pattern observed for $G\alpha_{12}$, it was possible that $G\alpha_{12}$

localized either at the contracting ring where actin polymerization is needed or at the cleavage furrow. Therefore, synchronic HeLa cells were stained against endogenous $G\alpha_{12}$ and F-actin with phalloidin toxin (Figure R7B), which binds specifically to filamentous actin. Interestingly, phalloidin labeled the contractile ring of actin (Figure R7B, see arrows), although $G\alpha_{12}$ was located in an area more inside the cell. A perfectly defined ring of $G\alpha_{12}$ was observed in the midzone in later stages and there was no colocalization with F-actin. Thereby we ruled out the possibility that $G\alpha_{12}$ was a member of the contractile ring at the cleavage furrow.

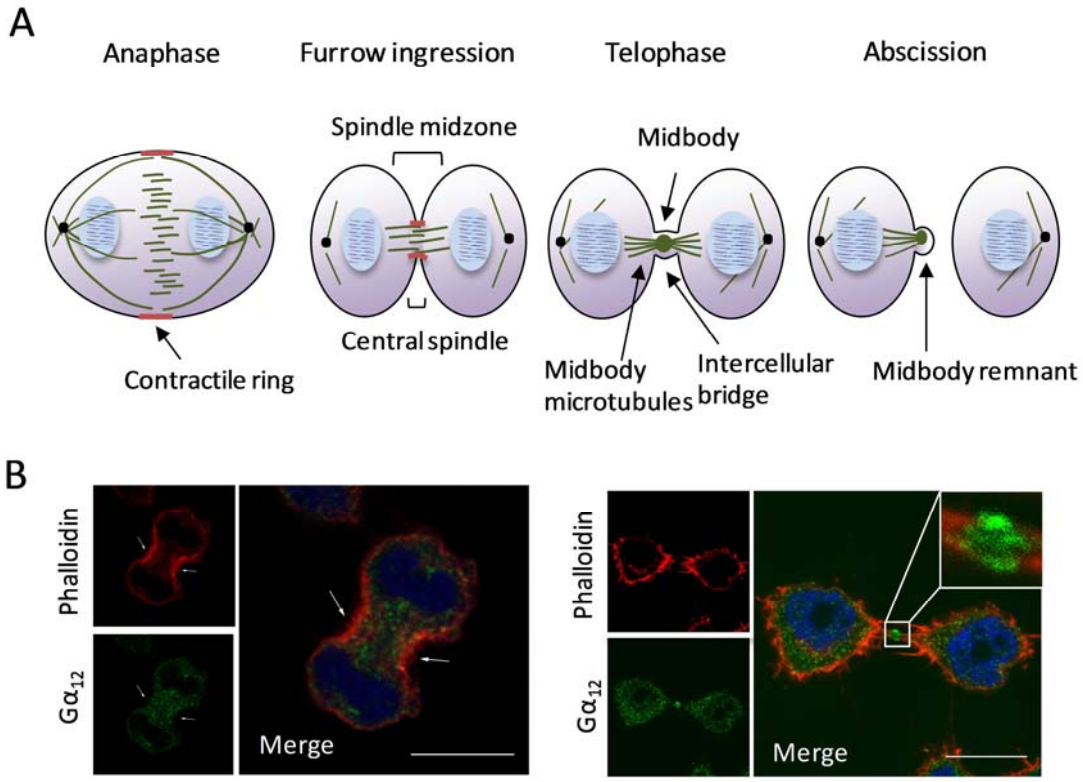
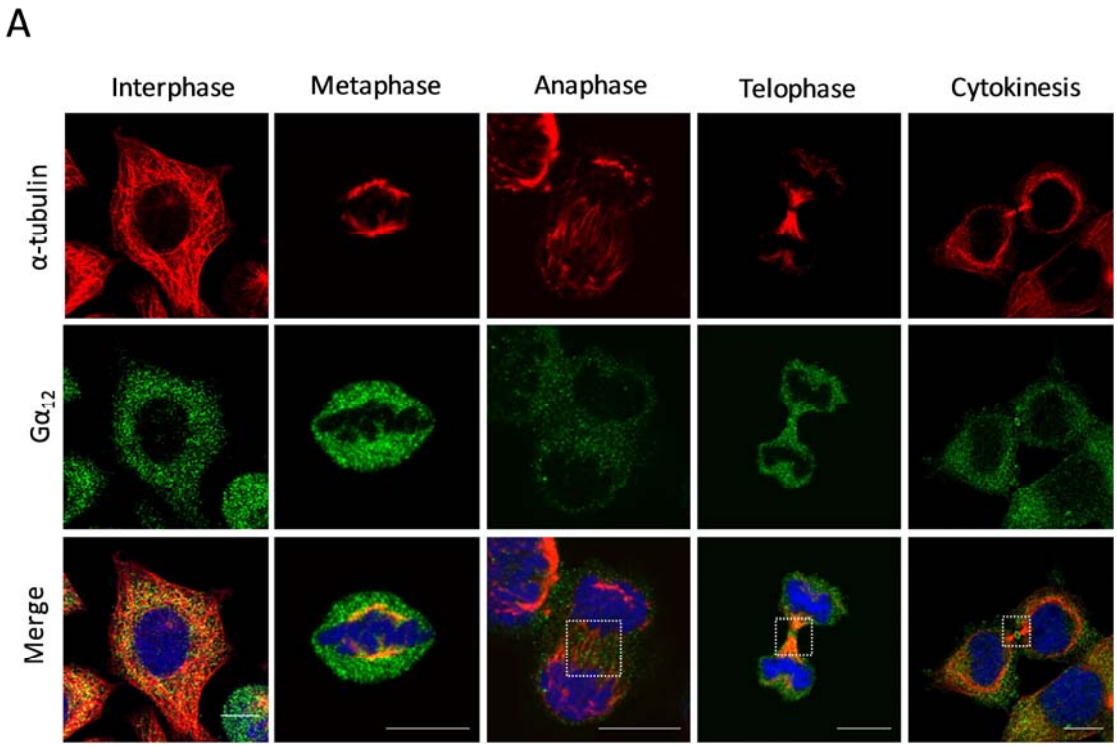


Figure R7. During mitosis $G\alpha_{12}$ does not co-localize with actin ring. **(A)** Schematic diagram of the different stages of cytokinesis (microtubules, green; chromatin, blue; plasma membrane, black; contractile actin ring, red; centrosomes, black). Adapted from (Guizetti and Gerlich, 2010) **(B)** Confocal microscopy imaging of synchronous HeLa cells. Detection of actin was carried out using Alexa555-phalloidin (red) and anti-$G\alpha_{12}$(green) (Abcam). The white box represents the zoom area and white arrows showed contractile ring of actin. The picture corresponds on single layer of Z-stack. Images are representative of the majority of the cells present on the plate. Scale bars: 10 µm.

After the ingression in cytokinesis, cells are partitioned into two daughters but remain connected for some time by a narrow intercellular bridge known as the midbody. As commented before, midbody is composed by antiparallel microtubule bundle and its core is a matrix of material with high electronic density, whose composition is still unknown. To ascertain if the $G\alpha_{12}$ immunoreactivity observed during cytokinesis of synchronic HeLa cells was the midbody; cells were stained against endogenous $G\alpha_{12}$ and endogenous α-tubulin (Figure R8). In line with what we had seen before, confocal micrographs showed dotted $G\alpha_{12}$ along spindle microtubules during anaphase prior to furrow ingression. As the contractile ring compresses the microtubules of the spindle apparatus during telophase, $G\alpha_{12}$ staining appeared along the microtubules and, also, as a concentrated ring-structure encircling the region of the former midzone spindle. Later in cytokinesis, $G\alpha_{12}$ became compacted around the dense matrix of the midbody. Thus, these data demonstrated that $G\alpha_{12}$ accumulates on the mitotic spindle during mitosis and later at the midbody.



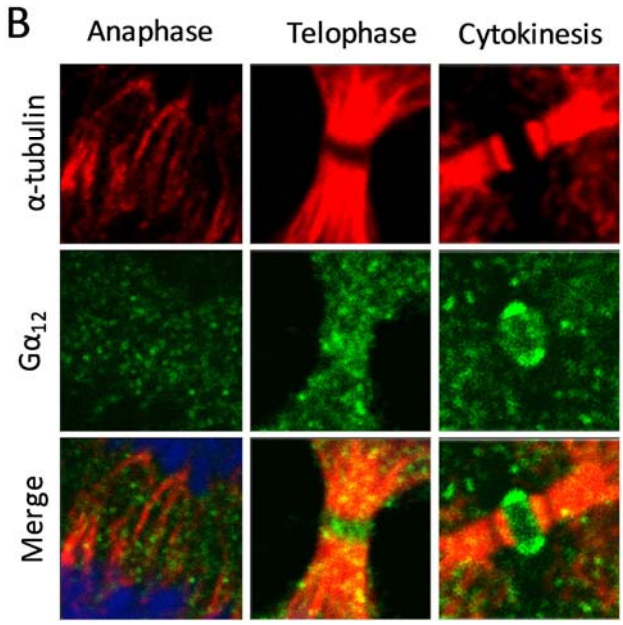


Figure R8. $G\alpha_{12}$ is associated with mitotic microtubules throughout mitosis. **(A)** Confocal microscopy imaging of synchronous HeLa cells. Detection of endogenous proteins was carried out with anti-$G\alpha_{12}$ (green) (Abcam) and anti-α-tubulin (red). White boxes shown zoom area represented in B. **(B)** Fluorescent confocal micrographs of zoom area in A. The images correspond to one layer of Z-stack and represent the majority of cells in the sample of three independent experiments. Scale bars: 10 μm.

1.3 $G\alpha_{12}$ localizes at the centrosomes

When we stained MEF cells with anti-$G\alpha_{12}$ we noticed that the antibodies were staining a structure located around the nucleus that reminded the centrosome (Figure R9A). The centrosome is an organelle composed of centrioles surrounded by the pericentriolar material that contains protein complexes and serves as the main microtubule organizing center (MTOC) (Delaval and Doxsey, 2010). The centrosomes are implicated in important cellular processes such as cell cycle regulation and microtubule organization. Pericentrin was the first component of the pericentriolar material described (Doxsey *et al.*, 1994) and is involved in the microtubule polymerization in MTOCs. To ascertain if the dotted pattern of $G\alpha_{12}$ could correspond to the centrosomes, HEK-293 cells were transfected with $G\alpha_{12}$Q229L-GFP protein and endogenous pericentrin was stained with a specific antibody. Indeed, mitotic cells that expressed fluorescent $G\alpha_{12}$Q229L protein (Figure R9B) presented the same perinuclear dotted structure as seen with anti-$G\alpha_{12}$, and colocalized with pericentrin. The localization coincided with the position of the centrosomes at opposite sides of the nuclei from the dividing furrow. Interestingly, another Gα subunit, $G\alpha_i$, was shown before to be

present in the centrosomes and at the midbody (Cho H et al, 2007). $G\alpha_{12}$ is present at the centrosomes and therefore might have a role in the stabilization of microtubules. Recent evidence has shown that $G\alpha_{12/13}$ are necessary for MTOC polarity and microtubule dynamics in cell polarization (Goulimari *et al.*, 2008). Taking together these data suggest that $G\alpha_{12}$ is associated to MTOC regulating microtubule dynamics during cell division. Our results that $G\alpha_{12}$ could be found in centrosomes, along the microtubules tracks and in the last step of cytokinesis condensed in the midbody ring, suggest that $G\alpha_{12}$ could be a key regulator of mitosis.

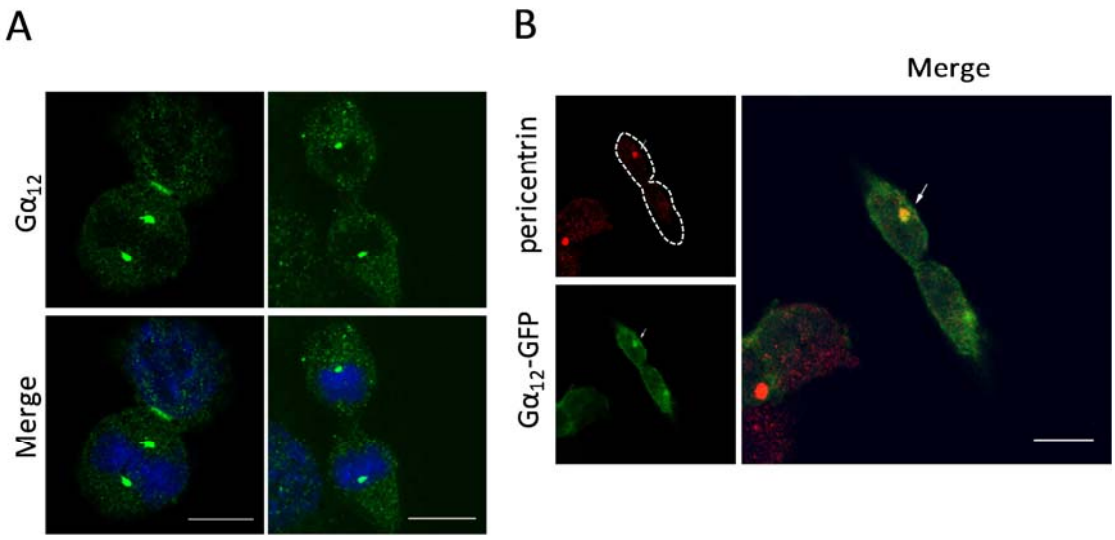


Figure R9. $G\alpha_{12}$ colocalizes with pericentrin. **(A)** Asynchronous MEF cells were fixed and probed for endogenous $G\alpha_{12}$ with a specific antibody from Abcam. **(B)** Fluorescent micrographs showed GFP-tagged $G\alpha_{12}$Q229L expressed transiently in HEK-293 cells Detection of pericentrin was carried out by using a specific antibody (red). White arrows indicate the colocalization. Images were taken by confocal microscopy. One layer of Z-stack is shown. Images are representative of the majority of the cells present on the plate. Scale bars: 10 μm.

1.4 Depletion of $G\alpha_{12}$ causes defects in cell division

To investigate whether $G\alpha_{12}$ is an important regulator of mitosis, particularly in the late stage of mitosis, we used MEF cells depleted of $G\alpha_{12}$ and $G\alpha_{13}$ and compared them with wild type MEF cell searching for failed cytokinesis by immunofluorescence. In fact we hypothesized that perturbation of endogenous $G\alpha_{12}$ function might increase the extent of multinucleation. Cells were stained

against three different membrane markers: N-cadherin, β-catenin or phalloidin and DAPI for DNA staining. Observation under confocal microscopy showed some cells in knockout $G\alpha_{12/13}$ with multiple nuclei (Figure R10A) that were no present in wild type cells. Knockout cells showed more multinucleated cells compared with wild type cells, with most cells containing two nuclei marked with an asterisk.

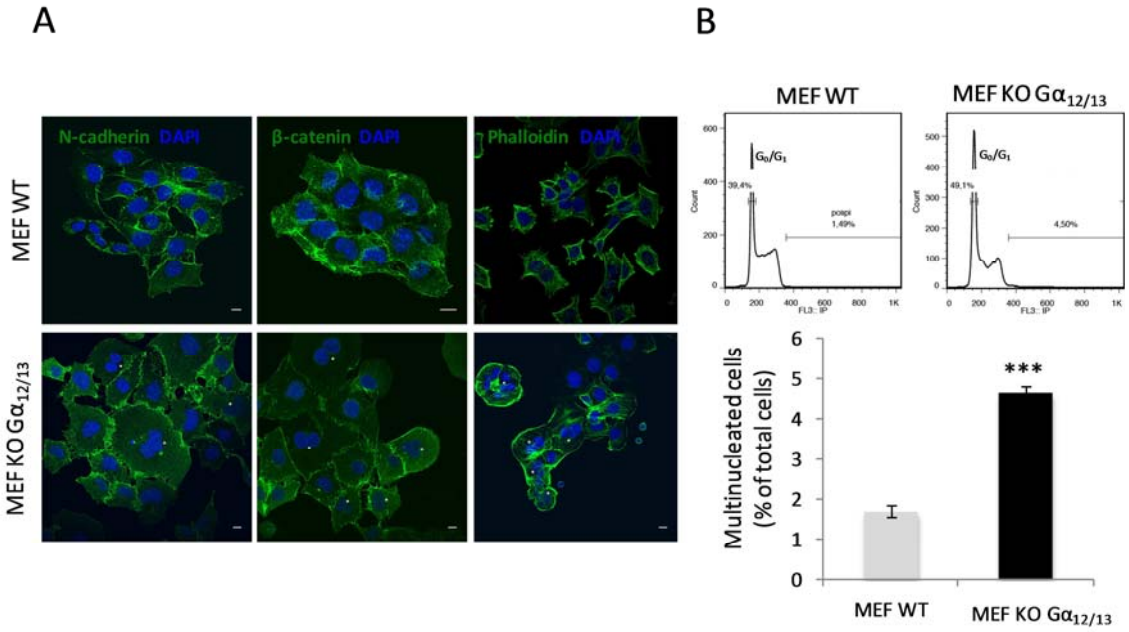


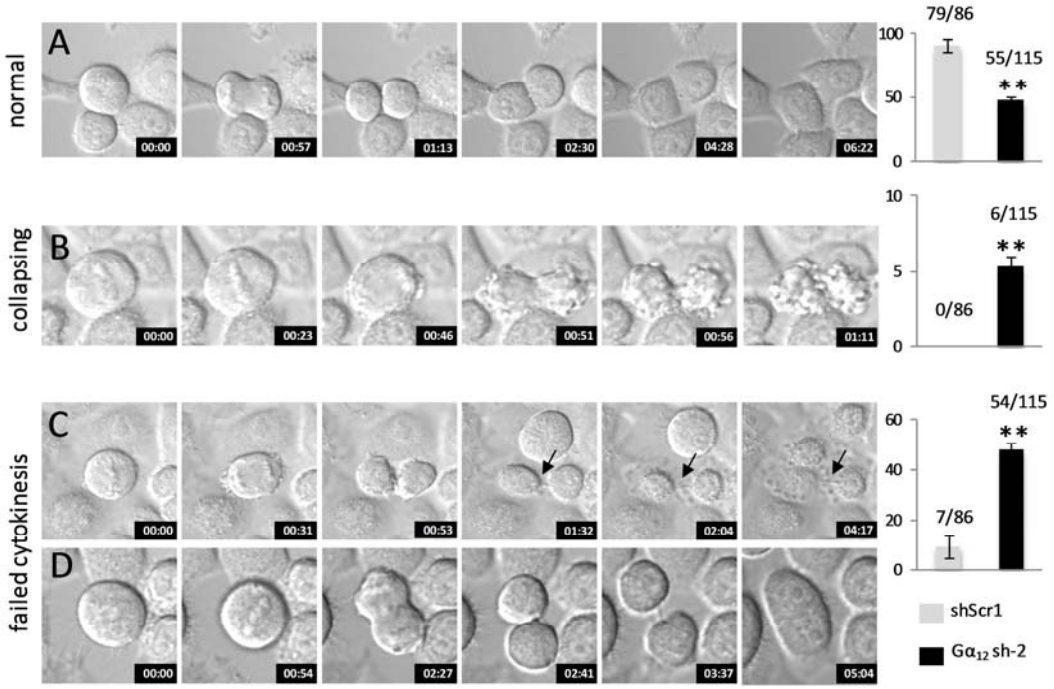
Figure R10. Multinucleation in MEF $G\alpha_{12/13}$ (-/-) KO cells. **(A)** Asynchronous MEF wild type and MEF $G\alpha_{12/13}$ KO cells were stained with the indicated antibodies and visualized with confocal microscopy. The image correspond one single layer of Z-stack. Cells harboring more than one nucleus are indicated by an asterisk. Images are representative of the majority of the cells present on the plate. Scale bars: 10 μm. **(B)** Quantification of multinucleated cells using flow cytometry. MEF wild type and $G\alpha_{12/13}$ KO cells were stained with propidium iodide (IP) and analyzed by flow cytometry. Values shown are the mean of three independent experiments and error bars indicate ± SEM. For statistical analysis, data were evaluated by two-tailed Student's t test. Asterisk indicates $p<0.001$.

To corroborate this preliminary observation cell cycle analysis were performed using flow cytometry (Figure R10B). Knockout cells showed an increase in multinucleation (4.58% ± 0.36%) compared with wild type cells (1.44% ± 0.2%), indicating that the depletion of $G\alpha_{12/13}$ was affecting to cell division.

Parallel to these analyses we analyzed $G\alpha_{12}$-depleted HeLa cells with shRNA. In fact, we observed that sh$G\alpha_{12}$-depleted (sh-2) cells presented aberrant mitosis under confocal microscopy compared to control cells (shScr1) (Figure

R11). The majority of cells progressed through mitosis, and chromosome alignment, segregation, and furrow ingression appeared normal in control cells (shScr1). In contrast, $G\alpha_{12}$-depleted cells showed different phenotypes of mitotic aberrations. We observed that some of the sh$G\alpha_{12}$ cells undergoing cytokinesis presented cortical aberrations that lead them to collapse and further go to apoptosis (Figure R11B). As the furrow started to constrict, about the half of the progressing $G\alpha_{12}$-depleted cells (i.e., 47.82% of all cells) exhibited membrane aberrations, including excessive cortical blebbing and the incapacity to resolve cytokinesis with the persistence of the intercellular bridge (Figure R11C, arrows). In addition, other cells followed cytokinesis with normality but they presented problems in furrowing, resulting in polinucleated cells (Figure R11D).

As mentioned, the vast majority of the control cells did not exhibit membrane instabilities, and passed normally through cell division. Then, we measured the time needed for cells from anaphase to starting of telophase. Both control and sh$G\alpha_{12}$ cells took an average of 23 min, showing no clear differences (Figure R11E) between control (shScr1) and $G\alpha_{12}$-depleted cells (sh-2). These results indicate that the early steps of mitosis seem do not be affected by $G\alpha_{12}$. After telophase started, then sh$G\alpha_{12}$ cells could be retained for long periods before excision, which generally resulted in a failed cytokinesis. Overall, these results indicate that $G\alpha_{12}$ appears to be important for the coordination of the cortical activities during cytokinesis.



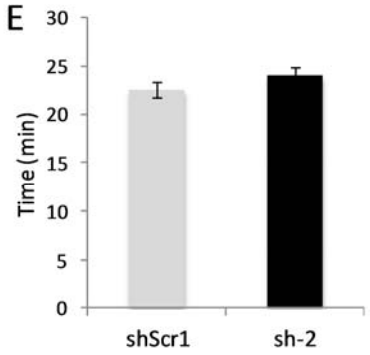


Figure R11. Downnregulation of $G\alpha_{12}$ causes mitotic aberrations. Asynchronous HeLa cells were infected by lentivirus containing a sequence of shRNA against $G\alpha_{12}$ (sh-2) and a sequence of scrambled control shRNA (shScr1). Distribution of mitotic phenotypes in HeLa $G\alpha_{12}$ sh-2 or control shScr1 cells. Classification of phenotypes; **(A)** regular cytokinesis; **(B)** collapsing: cortical hyperactivity during metaphase, finally collapsing; and **(C) (D)** failed cytokinesis: membranous aberrations during cytokinesis that are not compensated and gave rise to cytokinesis failure. Values are given as percentage of total cells, with error bars indicating ±SEM. Actual numbers are indicated. In 3 independent experiments, a total of 115 $G\alpha_{12}$-depleted cells and 86-control-depleted cells were scored (**p≤0.005). **(E)** Quantification of division time from anaphase to telophase. Error bars indicate ± SEM, n=30.

We then performed cell cycle analysis of shGα_{12} and control HeLa cells by flow cytometry (Figure R12). A high population of Gα_{12}-depleted cells (Gα_{12} sh-2) was found in G_0/G_1 phase (71.07% ± 0.59%), probably arrested, compared to control cells (shScr1) (58.37% ± 1.22%). Cells can enter into the G_0 phase due to a lack of nutrient or growing factors, and during this period cellular machinery is dismantled. Thereby, the depletion of Gα_{12} may be altering the mitotic machinery and cells enter the G_0 phase to prevent mitotic aberrations. The differences observed with the knockout cells may be due to the adaptation process that knockout cells underwent compared to shRNA treated cells that are depleted only a week before the analysis. These functional data of cell cycle are in line with our previous observations suggesting that Gα_{12} has an important role in cell division and indicating that the loss of Gα_{12} affects the cellular regulation of cell division.

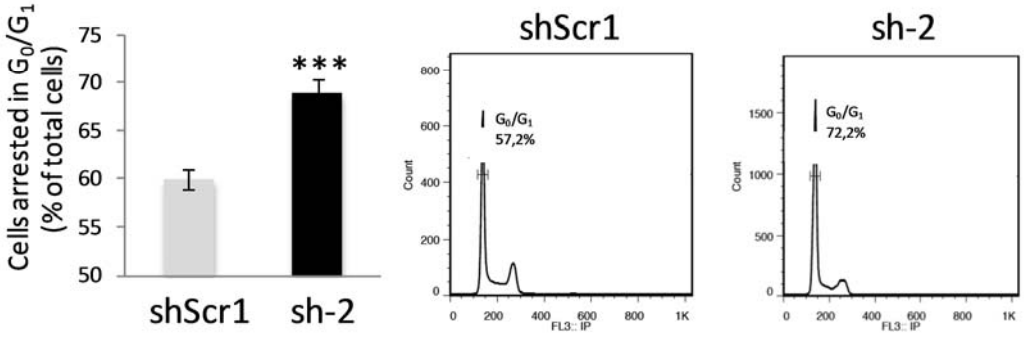


Figure R12. Accumulation of cells in G_0/G_1 phase caused by Gα_{12} perturbation. Asynchronous HeLa cells were infected by lentivirus containing a sequence of shRNA against Gα_{12} (sh-2) and a sequence of scrambled control shRNA (shScr1). Cells were fixed, stained with propidium iodide (IP) and analyzed by flow cytometry. HeLa shGα_{12} cells show and increase of arrested cells at G_0/G_1 phase compared with control HeLa shScr1 cells. Values shown are the mean of two independent experiments and error bars indicate ± SEM. For statistical analysis, data were evaluated by two-tailed Student's t test. Asterisk indicates p≤0.0001.

2. Identification of GEF-H1 as a novel effector for $G\alpha_{12}$

2.1 $G\alpha_{12}$ protein interacts with GEF-H1

Once established that $G\alpha_{12}$ is essential for cellular cytokinesis, we aimed to identify the downstream effectors of $G\alpha_{12}$ implicated in the cytokinesis process. A critical component of the spatial-temporal control of cytokinesis involves the small GTPase RhoA whose localization and activation is maintained by Rho guanine nucleotide exchange factors (RhoGEFs) and GTPase activating proteins (RhoGAPs). It is through the regulation in the interplay between RhoGEFs and RhoGAPs that RhoA translocates to the equatorial cortex and initiates the assembly and ingression of the contractile ring (Bement *et al.*, 2005; Yüce *et al.*, 2005; Loria *et al.*, 2012). Different RhoGEFs have been described to be involved during mitosis to ensure the proper assembly and ingression of the cytokinetic furrow. Thus, MyoGEF, ECT2, GEF-H1, and LARG exhibit temporally distinct functions for the proper completion of mitosis (see Figure I13). To date, GEF-H1 is thought to be implicated in telophase and early cytokinesis (Birkenfeld *et al.*, 2007). LARG was shown to be required for the abscission, the last step of cytokinesis (Martz *et al.*, 2013). LARG is a RH-RhoGEF activated by $G\alpha_{12}$ upon phosphorylation (Suzuki *et al.*, 2003). Our working hypothesis is that $G\alpha_{12}$ could be activating both RhoGEFs, GEF-H1 at early steps of cytokinesis and LARG in the final cleavage. Whereas the mechanism of activation of LARG by $G\alpha_{12/13}$ proteins it is well established, nothing is known about the activation of GEF-H1 by $G\alpha_{12}$. As a consequence, we decide to investigate if GEF-H1 could be a downstream effector for $G\alpha_{12}$.

2.1.1 $G\alpha_{12}$ co-immunoprecipitates with GEF-H1

To evaluate whether GEF-H1 could be a downstream effector of $G\alpha_{12}$, immunoprecipitation experiments were done with the two members of the subfamily of G12 proteins, $G\alpha_{12}$ and $G\alpha_{13}$, and full length GEF-H1. The hemagglutinin(HA)-tagged GEF-H1 was co-expressed with the empty vector, or the constitutively active forms of $G\alpha_{12}$ or $G\alpha_{13}$ in HEK-293 cells. Results showed that $G\alpha_{12}$Q229L co-immunoprecipitated with HA-tagged GEF-H1; whereas $G\alpha_{13}$Q226L co-immunoprecipitated at levels similar to the background of the control line

(Figure R13A). In order to corroborate these results, a reciprocal co-immunoprecipitation assay was performed immunoprecipitating Myc-tagged $G\alpha_{12}Q229L$ in cells co-expressing HA-tagged GEF-H1. In line with our previous observations, HA-GEF-H1 co-immunoprecipitated with Myc-$G\alpha_{12}Q229L$ (Figure R13B). It was important to assess the co-immunoprecipitation with endogenous proteins due to the low levels of $G\alpha_{12}$ in the majority of cells, co-immunoprecipitation was carried out transfecting $G\alpha_{12}Q231L$ and immunoprecipitating endogenous GEF-H1 in HEK-293 cells. As can be seen in Figure R13C, $G\alpha_{12}Q231L$ co-immunoprecipitates with endogenous GEF-H1.

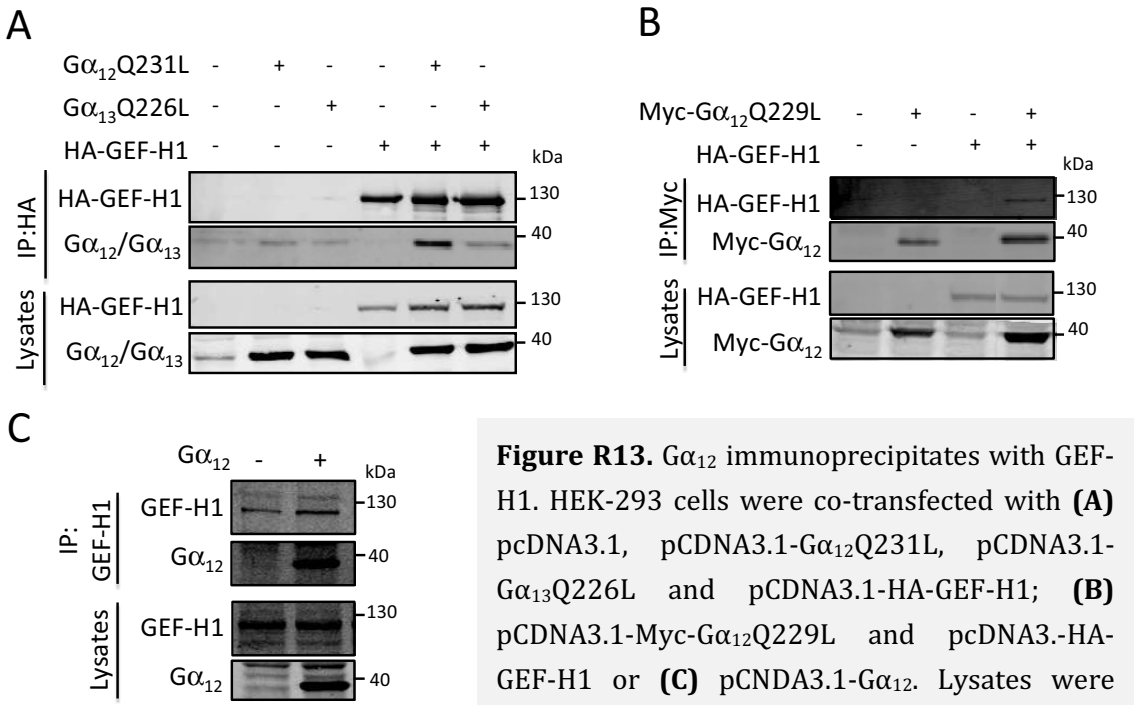


Figure R13. $G\alpha_{12}$ immunoprecipitates with GEF-H1. HEK-293 cells were co-transfected with **(A)** pcDNA3.1, pCDNA3.1-$G\alpha_{12}Q231L$, pCDNA3.1-$G\alpha_{13}Q226L$ and pCDNA3.1-HA-GEF-H1; **(B)** pCDNA3.1-Myc-$G\alpha_{12}Q229L$ and pcDNA3.-HA-GEF-H1 or **(C)** pCNDA3.1-$G\alpha_{12}$. Lysates were subjected to immunoprecipitation 24h after transfection with **(A)** anti-HA fused to agarose beads anti-Myc antibody or **(C)** anti-GEF-H1 for endogenous immunoprecipitation of GEF-H1 and analyzed by anti-HA, anti-$G\alpha_{12}$, anti-$G\alpha_{13}$, anti-Myc or anti-GEF-H1 immunoblotting. Data are illustrative of at least three independent experiments with similar results.

To analyze the domains of GEF-H1 involved in the interaction with $G\alpha_{12}$ protein, we engineered expression plasmids with HA-tagged forms of the N- and C-terminal truncated domains of GEF-H1 into the pcDNA3.1. The N-terminal fragment comprises from amino acid 1 to 571 and contains the DH-PH domains. The C-terminal domain was subcloned from amino acid 572 to 986. In both cases,

the HA coding sequence was cloned in-frame immediately upstream of the coding sequence of the protein, thus generating an amino-terminal HA-tagged GEF-H1 N- and C-terminal truncated forms. Figure R14A shows the cartoons illustrating these constructs. The full length and truncated forms were detected with an anti-HA specific antibody when expressed in HEK-293 cells (Figure R14B). Despite of transfecting equal amounts of all GEF-H1 expressing vectors or reducing the amount of the C-terminal containing plasmid, the total protein level of the C-terminal peptide was always higher.

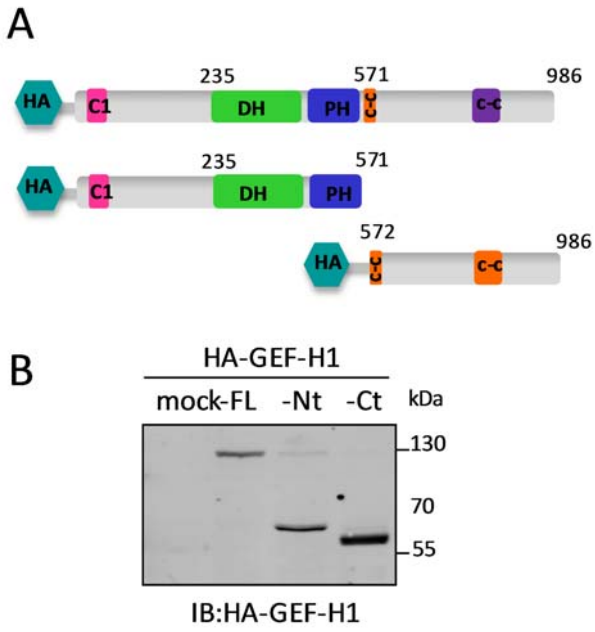


Figure R14. Expression of epitope-tagged full length, N- and C-terminal truncated forms of GEF-H1. **(A)** Cartoon of the different forms of GEF-H1: full length (1-986 aas), N-terminal (1-571 aas), and C-terminal (572 to 986 aas). **(B)** Lysates from control HEK-293 cells and cells expressing the differents constructs of GEF-H1 were subjected to western blot analysis with anti-HA antibody. Experiments were repeated at least three times with similar results.

2.1.2 GEF-H1 interacts with $G\alpha_{12}$ through the DH-PH domain

As mentioned in the introduction GEF-H1 is a member of the Dbl family that lacks the RH domain. So, in principle there is no assumption of which domain could be implicated in G protein binding. Interestingly, GEF-H1 has, in its C-terminal region, a coiled-coil motif that structurally shares common treats with this RGS-like domain of the RH-RhoGEFs (Figure R15, obtained from M. Masià thesis, 2013). Thus, taking in account the sequence similarity of the coiled-coil domain between the members of GEF-H1 subfamily we first hypothesized that this region would be a "RH-like" domain through which the RhoGEFs of this subfamily would interact with G proteins.

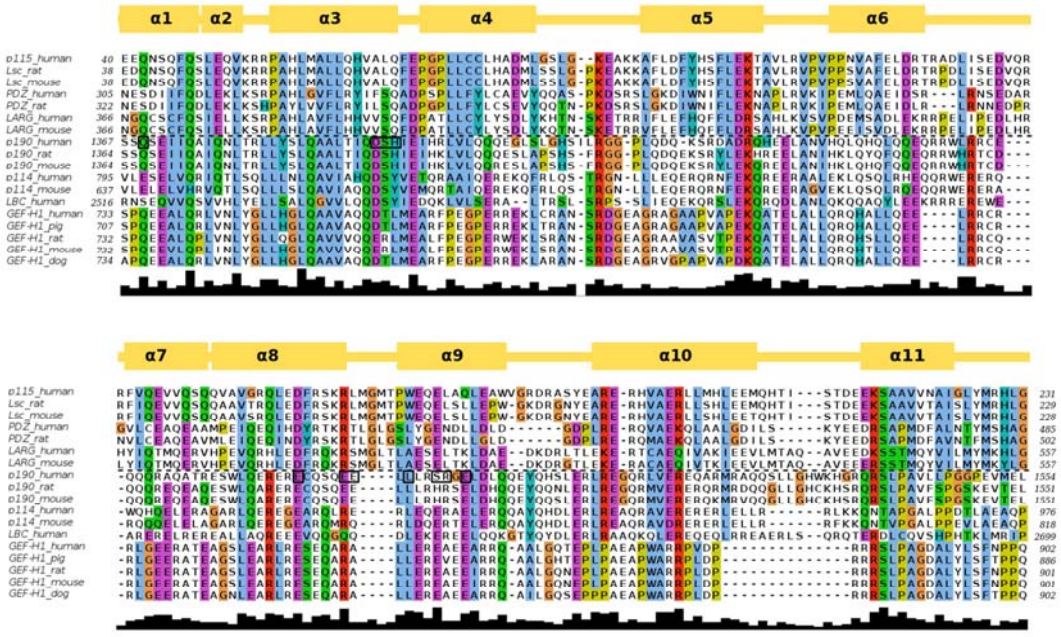


Figure R15. Sequence alignment between the RH domains of RH-RhoGEFs proteins family and members of the GEF-H1 family. Amino acids are colored based on conservation according to the Clustal scheme. Gold boxes over the alignment indicate the localization of helices on the alignment based on crystal structures. Black bars under the alignment represent the degree of conservation at each specific position. Numbers indicate the start and end of the RH-like domain in the sequence. Dotted lines separate p115-like from GEF-H1-like sequences. Obtained from thesis of M.Masià.

The ability of the HA-GEF-H1 truncated forms (Figure R14) to bind $G\alpha_{12}$ was investigated by co-immunoprecipitation experiments. HA-tagged GEF-H1 full length (FL), -Nt, and -Ct truncated forms were transiently transfected alone or together with $G\alpha_{12}Q231L$, and immunoprecipitated using a monoclonal anti-HA bead-fused antibody. The results showed that $G\alpha_{12}$ co-immunoprecipitated with the amino terminal form of GEF-H1, whereas no co-immunoprecipitation was observed with the C-terminal form (Figure R16). The N-terminal truncated form contains the DH-PH domains, which confer its catalytic activity towards RhoA. Binding of $G\alpha_{12}$ to regions outside of the RH domain of RH-RhoGEFs has been recently suggested for other RhoGEFs (Chen *et al.*, 2005; Suzuki, Tsumoto, *et al.*, 2009).

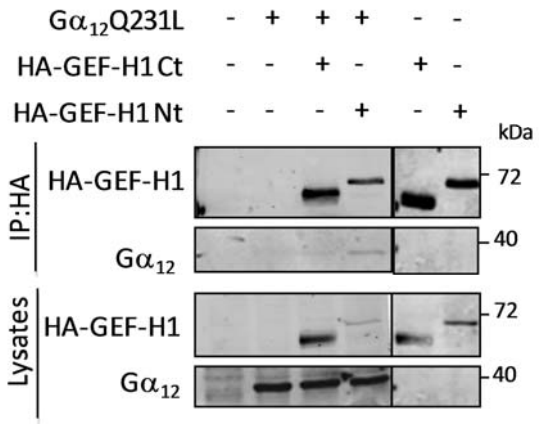


Figure R16. $G\alpha_{12}$ immunoprecipitates only with the N-terminus of HA-GEF-H1 containing the DH and PH domains. HEK-293 cells were transfected with different combination of mtDSRed (control vector) or plasmids encoding $G\alpha_{12}$Q231L, HA-GEF-H1-Ct and HA-GEF-H1-Nt. Twenty-four hours after transfection, cells were lysed and immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analyzed by western blot with specific antibodies. Blots shown are representative of three independent experiments.

Hence, we next investigated the ability of the DH-PH domains of GEF-H1 to interact with $G\alpha_{12}$. For that purpose another truncated mutant was obtained utilizing the same cloning strategy as described before. Thus, a DH-PH HA-tagged construct of human GEF-H1 from amino acid 235 to 571 and a point mutant of the full length HA-GEF-H1C53R were used to carry out co-immunoprecipitation assays in presence of constitutively active $G\alpha_{12}$ protein in HEK-293 cells (Figure R17). The GEF-H1C53R mutant cannot bind to microtubules and was shown before to be enzymatically more active (Birkenfeld *et al.*, 2007). The figure R17 showed that $G\alpha_{12}$ co-immunoprecipitated with HA-GEF-H1(DH-PH) and in fact, this truncated form showed increased immunoprecipitation than the full length protein. On the other hand, HA- GEF-H1C53R also co-immunoprecipitated more efficiently with $G\alpha_{12}$ than the wild type form (Figure R17C). It is possible that GEF-H1 has autoinhibitory domains in full length protein, which are absent in the DH-PH construct. On the other hand the association to microtubules will be inhibitory. Overall, immunoprecipitation assays showed that the DH-PH domains of GEF-H1 are sufficient for the binding with $G\alpha_{12}$.

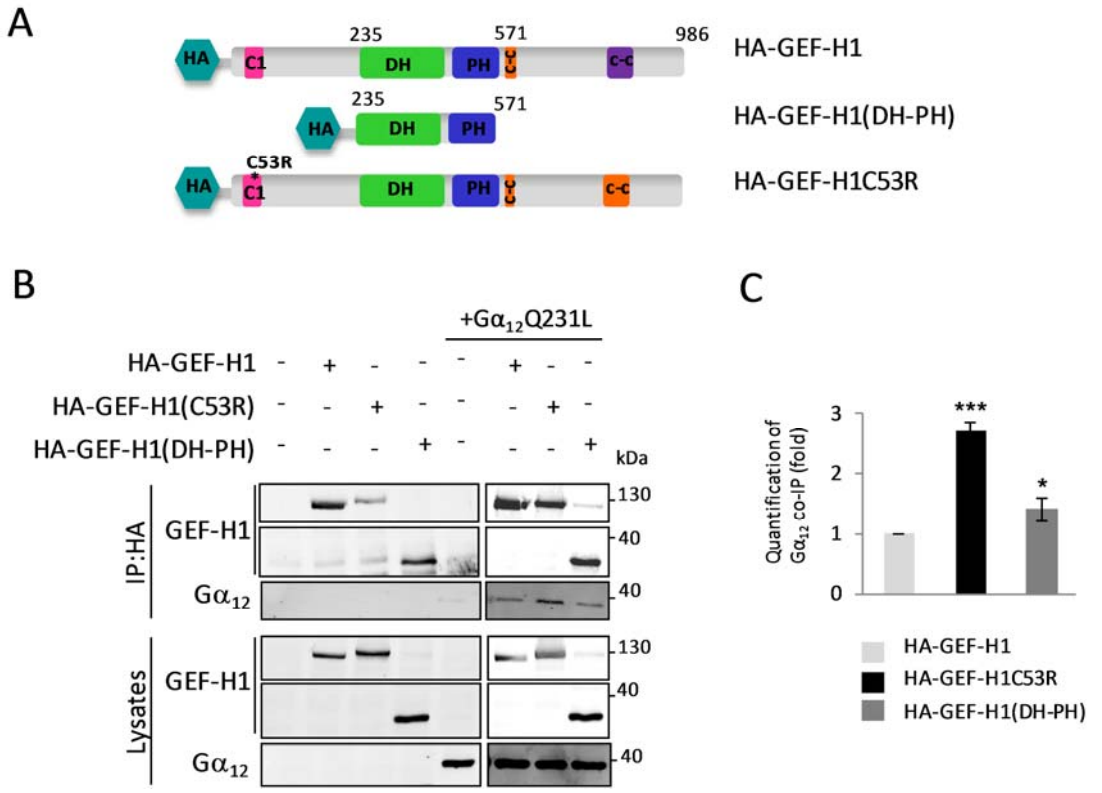


Figure R17. The DH-PH region of GEF-H1 is sufficient to co-IP with $G\alpha_{12}$. **(A)** HEK-293 cells were transfected with different combinations of plasmids encoding $G\alpha_{12}$Q231L, HA-GEF-H1, HA-GEF-H1-HAC53R and HA-GEF-H1(DH-PH). Twenty-four hours after transfection, cells were lysed and the different constructs of HA-GEF-H1 were immunoprecipitated with an anti-HA agarose-conjugated antibody. Immunoprecipitates and total lysates were analyzed by western blot with specific antibodies. Control lane shows transfection with empty pCDNA3.1 vector. **(B)** Representative cartoon of the different constructs used. **(C)** The blots were quantified by Odissey, and results were expressed as a ratio relative to the value obtained for full-length GEF-H1. The graph shows means ± S.E.M of 3 independents experiments. Values significantly different from full-length GEF-H1 are marked with one ($p \leq 0.05$) or three asterisks ($p \leq 0.005$).

2.1.3 The interaction between GEF-H1 and $G\alpha_{12}$ is enhanced upon $G\alpha_{12}$ activation

The $G\alpha_{12}$ protein binds to other RhoGEFs upon receptor activation in its GTP form. The effect of G protein activation on GEF-H1 interaction was analyzed immunoprecipitating HA-GEF-H1 in presence or in absence of AlF_4^- (Figure R18A).

AlF_4^- mimics the γ–phosphate of GTP, falsely activating the Gα protein signal transduction system. As can be observed, there was an increase of the $G\alpha_{12}$ bound to GEF-H1 in presence of AlF_4^- (Figure R18B).

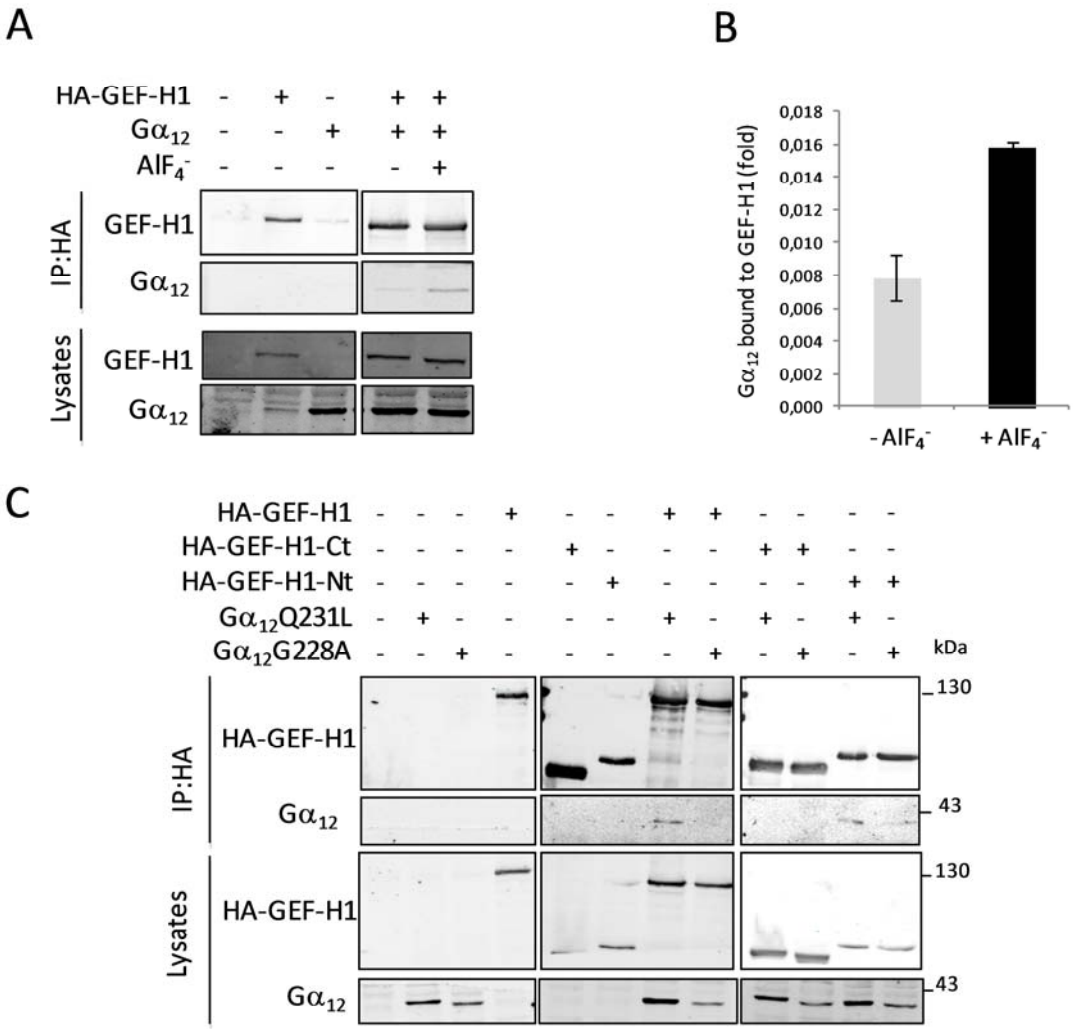


Figure R18. Co-IP of $G\alpha_{12}$ with GEF-H1-HA is enhanced upon activation. **(A)** $G\alpha_{12}$ and GEF-H1-HA expressing HEK-293 cells were immunoprecipitated with anti-HA-agarose in presence or in absence of 5 mM NaCl and 3 mM $AlCl_3$. Immunoprecipitates or whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-GEF-H1 and anti-$G\alpha_{12}$ antibodies. Control lane shows transfection with mtDSRed vector. The blots were quantified by Odissey, and results were expressed as a ratio of co-immunoprecipitated $G\alpha_{12}$ relative to immunoprecipitated GEF-H1. **(B)** Quantification of $G\alpha_{12}$ bound to GEF-H1. The graph shows means ± S.E.M of three independents experiments. **(C)** HA-GEF-H1, HA-GEF-H1-Ct, HA-GEF-H1-Nt, $G\alpha_{12}$Q231L and $G\alpha_{12}$G228A expressing HEK293 cells were lysed, subjected to immunoprecipitation using anti-HA agarose beads and analyzed by SDS-PAGE. Proteins were detected with the indicated. Data are illustrative of three independent experiments with similar results.

Moreover, we investigated the interaction with the constitutively inactive GDP-bound $G\alpha_{12}$G228A mutant. Full length, C-terminal and N-terminal forms of HA-GEF-H1 were immunoprecipitated in cells expressing $G\alpha_{12}$Q231L or $G\alpha_{12}$G228A mutants (Figure R18C). The results showed that $G\alpha_{12}$Q231L co-immunoprecipitated with full length and N-terminal construct of GEF-H1, as we had seen before, whereas $G\alpha_{12}$G228A was unable to co-immunoprecipitate with the different constructs of GEF-H1. Collectively, data in figure R18 indicate that the activation state of $G\alpha_{12}$ is important for the interaction with GEF-H1.

Recently, it has been described RhoGEF-uncoupled $G\alpha_{12}$ mutants (Myc-$G\alpha_{12}$Q229L(EEE) and Myc-$G\alpha_{12}$Q229L(MM)) that failed to interact with the well known $G\alpha_{12/13}$-activated RhoGEF LARG and p115RhoGEF (Ritchie *et al.*, 2013). Though GEF-H1 does not have a RH domain it still can be possible that GEF-H1 binds to $G\alpha_{12}$ by a similar mechanism as the RH-RhoGEFs. HEK-293 cells were transfected with Myc-$G\alpha_{12}$Q229L, Myc-$G\alpha_{12}$Q229L(EEE), Myc-$G\alpha_{12}$Q229L(MM) and the different constructs of HA-GEF-H1 were immunoprecipitated (Figure R19). As can be observed in the figure both mutants of $G\alpha_{12}$ co-immunoprecipitated with full length and N-terminal truncated form of GEF-H1 indicating that the mutations of $G\alpha_{12}$ that affected RH-RhoGEF binding are not sufficient to lose the interaction with GEF-H1. Thus, these data indicate that the mechanism of interaction between GEF-H1 and $G\alpha_{12}$ is different from the established mechanism with the known RH-RhoGEFs LARG and p115RhoGEF proteins.

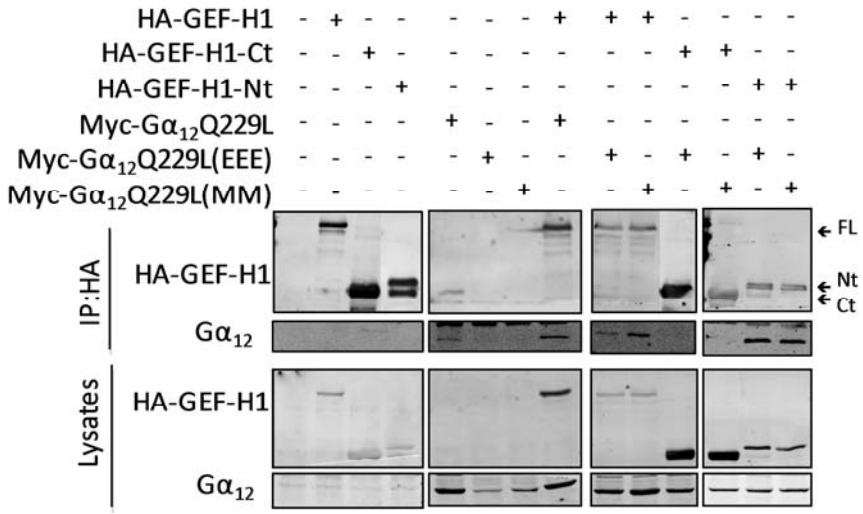


Figure R19. Mutants of $G\alpha_{12}$ immunoprecipitate with full length and the N-terminus of GEF-H1-HA. HEK-293 cells were transfected with different combination of mtDSRed (control vector) or plasmids encoding Myc-$G\alpha_{12}$Q229L, Myc-$G\alpha_{12}$Q229L(EEE), Myc-$G\alpha_{12}$Q229L(MM), HA-GEF-H1, HA-GEF-H1-Ct and HA-GEF-H1-Nt. Twenty-four hours after transfection, cells were lysed and the different constructs of HA-GEF-H1 were immunoprecipitated with anti-HA-agarose. Immunoprecipitates and total lysates were analyzed by western blot with specific antibodies. Blots shown are representative of three independent experiments. Black arrows indicate full length (FL), C-terminal (Ct) and N-terminal (Nt) forms of HA-GEF-H1.

2.2 $G\alpha_{12}$ mediates GEF-H1 activation

Taken together, the results obtained till now indicate that $G\alpha_{12}$ protein co-immunoprecipitates specifically with GEF-H1, which could be an indication of a possible direct interaction. We also determined that the domain important for this assembly is located at the N-terminal region where the DH-PH domain is found, and seems that the DH-PH domain could be sufficient for the interaction. It is well established the direct role of G12 subfamily in the stimulation of the intrinsic GEF activity of the other RH-RhoGEFs proteins, leading to RhoA activation, and in turn, promoting the activation of the RhoGTPase activity towards the Gα subunits. Thus, we aimed to explore into the biochemical and functional characterization that $G\alpha_{12}$ exerts over GEF-H1 upon their interaction.

2.2.1 $G\alpha_{12}$ enhances GEF activity of GEF-H1

The direct role of $G\alpha_{12}$ in promoting intrinsic GEF activation of GEF-H1 was assessed by the ability of GEF-H1 to bind RhoA by an affinity precipitation assay, as described previously (García-Mata *et al.*, 2006). This assay takes advantage of a "nucleotide free" mutant RhoAG17A, which has high affinity for active GEFs. The mutation (G17A) renders the protein unable to bind GDP or GTP and this state mimics the intermediate state that is bound to the GEFs. A GST-tagged version of this mutant protein was expressed and purified from *E. coli*, bound to glutathione-Sepharose beads and used to pull-down active GEFs from cell lysates. HEK-293 cells were transfected with HA-tagged GEF-H1 in presence or absence of constitutively active $G\alpha_{12}$ and whole cell lysates were subjected to pull-down assay. Pull-down with GST-RhoAG17A beads containing no RhoGEF expression resulted in no GEF-H1 precipitation (Figure R20). The GST-RhoAG17A protein captured some GEF-H1 from the control cell lysates, which could be due to a GEF-H1 basal activity. Notably, the amount of active GEF-H1 was increased in cells co-transfected with the active $G\alpha_{12}$ subunit. The total cell lysates showed similar amounts of GEF-H1 in the control and the co-transfected samples, suggesting that the expression of $G\alpha_{12}$ protein did not alter GEF-H1 levels and the input used in the assay was equal. The levels of GEF-H1 in the pull-down and the lysates were quantified by densitometry (Odyssey system). The amount of active GEF-H1 in each sample was normalized to the corresponding total GEF-H1 present at the lysates, and the data were expressed as fold increase compared with the control taken as unity. In three independent experiments, we found a consistent increase in GEF-H1 precipitated with GST-RhoAG17A following $G\alpha_{12}$Q231L expression. These results demonstrate that constitutively active $G\alpha_{12}$ protein induced an increase of GEF-H1 capable of associating with RhoAG17A, probably reflecting activation of GEF-H1.

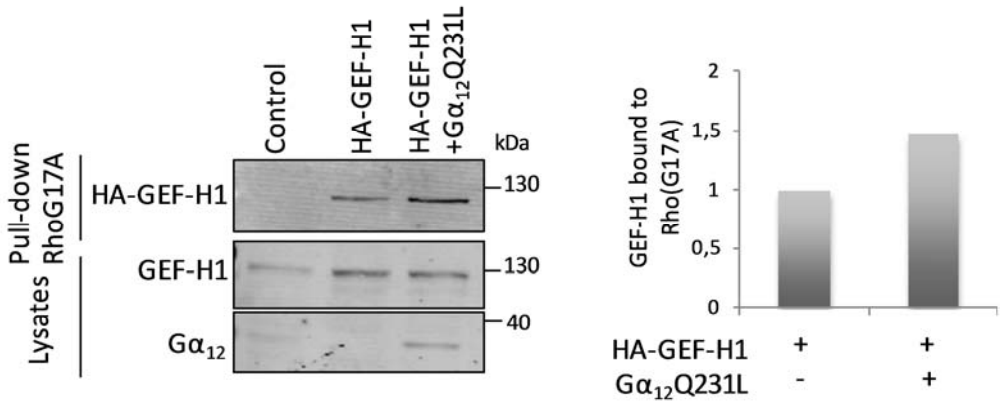


Figure R20. Pull-down of activated GEF-H1 with RhoAG17A is enhanced by $G\alpha_{12}$. Active GEF-H1 was captured from HEK-293 cells expressing GEF-H1-HA and $G\alpha_{12}$Q231L using GST-RhoAG17A fusion protein coupled to glutathione Sepharose 4B. Protein bound to the washed beads was analyzed by SDS-PAGE and immunoblotted with anti-GEF-H1 and anti-$G\alpha_{12}$ antibodies. GEF-H1 pulled-down was expressed as fold increase from GEF-H1 present at the lysates. Data shown are from one experiment representative of three independent experiments with similar results.

2.2.2 GEF-H1 mediates signals from $G\alpha_{12}$ to Rho activation

We have found that enhanced GEF-H1 activation occurs through the interaction with $G\alpha_{12}$. Thus, our next aim was to provide biochemical evidence of the functional role of GEF-H1 on $G\alpha_{12}$ signaling pathway. A method to demonstrate the activation of RhoA by heterotrimeric G proteins and RhoGEFs is the indirect measurements of the SRE-luciferase activity (Suzuki *et al.*, 2003). This method is based on the measurement of the serum response factor (SRF)-dependent transcriptional activation of a luciferase reporter gene controlled by the transcriptional regulatory element SRE.L. For that purpose, full length HA-GEF-H1 was expressed in a inducible cell line that stably expresses $G\alpha_{12}$Q231L, TREX-2T($G\alpha_{12}$Q231L) HEK-293 cells. In the T-REX™ system the expression of the gene of interest is repressed in the absence of the inductor, in this case tetracycline, and is induced when tetracycline was added to the media. So, we can express the $G\alpha_{12}$ under controlled conditions avoiding the toxic effects derived by log term expression of $G\alpha_{12}$. Cells were serum deprived overnight previous to be assayed for SRF activation in order to lower the levels of Rho activity. Expression of

$G\alpha_{12}$Q231L alone lead to approximately 2-fold increase of basal SRF activity, as we expected since $G\alpha_{12}$ was expressed in the constitutive active form of the protein (Figure R21) and expression of HA-GEF-H1 showed 2.5-fold increase. However, when GEF-H1 was co-expressed with $G\alpha_{12}$Q231L a marked enhancement of SRF activation was observed. These results suggested the involvement of GEF-H1 in the signaling pathway of $G\alpha_{12}$ to RhoA activation.

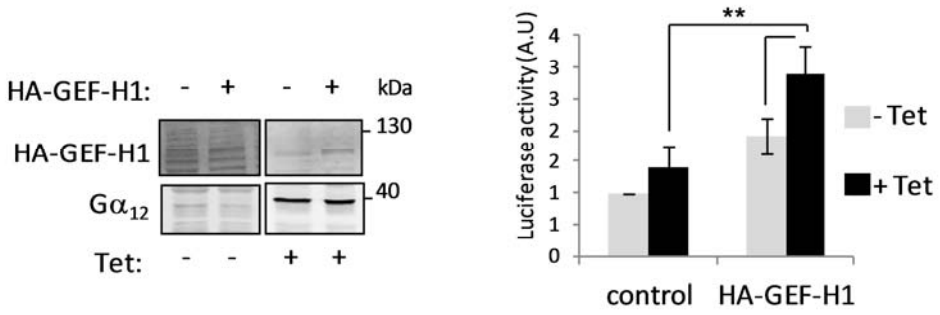


Figure R21. SRE-luciferase expression induced by GEF-H1 in TREX-2T($G\alpha_{12}$Q231L) inducible cells. A stable cell line with inducible expression of $G\alpha_{12}$ was treated in presence or in absence of tetracycline for 4 hours to $G\alpha_{12}$ expression. Cells were co-transfected with HA-GEF-H1, pSRE-L Mut and TK-Renilla. Control lane shows transfection with mtDSRed. Six hours after transfection, cells were serum-starved for 16 hours and tetracycline was added for 4 hours to $G\alpha_{12}$ overexpression. Rho activity was measured by transcription from the SRE-luciferase reporter. Data was normalized for protein expression on Renilla activity in lysates. The graph shows means ± S.E.M of 3 independents experiments. Values significantly different are marked with two asterisks ($p \leq 0.05$).

2.3 Regulation of GEF-H1

The results gathered until this point provides strong evidences for GEF-H1 as being a downstream effector of $G\alpha_{12}$ and that the DH-PH domains of GEF-H1 are sufficient for the interaction. To better understand the molecular mechanism that underlies the interaction between $G\alpha_{12}$ and GEF-H1 we focused on the effect that $G\alpha_{12}$ could exert on the regulation of GEF-H1.

2.3.1 Gα$_{12}$ promotes the release of GEF-H1 from microtubules

GEF-H1 is the unique RhoGEF that is bound and regulated by microtubules (Birkenfeld *et al.*, 2008), p190RhoGEF is also bound on microtubules but is not regulated by them. We investigated further whether Gα$_{12}$ regulates the interaction between GEF-H1 and microtubules or vice versa. For that purpose we performed microtubule co-sedimentation assay in cells expressing HA-GEF-H1 alone or with the constitutively active form of Gα$_{12}$. Co-sedimentation of GEF-H1 in the microtubule-containing pellet was intensified in the presence of paclitaxel, a microtubule-stabilizing agent. As can be observed in figure R22 the relative amount of GEF-H1 associated with microtubules was significantly decreased when Gα$_{12}$Q231L was present. In this assay, the total amount of microtubules sedimented (stabilized microtubules) was the same in all samples, indicating that the amount of GEF-H1 bound to microtubules was decreased in the presence of Gα$_{12}$Q231L. These results suggest that Gα$_{12}$ promotes GEF-H1-microtubule dissociation, and furthermore activation of the RhoGEF.

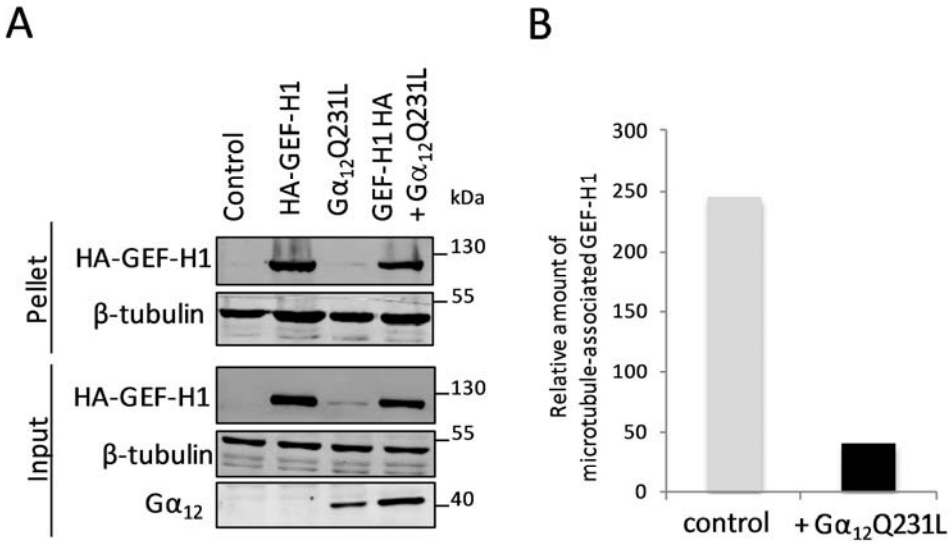


Figure R22. Effects of $G\alpha_{12}Q231L$ on the association of GEF-H1 with microtubules. **(A)** HEK-293 cells were transfected with combinations of plasmids encoding HA-GEF-H1 and $G\alpha_{12}Q231L$ and 24h after transfection cells were subjected to microtubule co-sedimentation assay. Microtubule pellets and input fractions were analyzed by western blot with specific antibodies. **(B)** The amounts of microtubule-associated GEF-H1 relative to those of β-tubulin were quantified with Odyssey's software and were normalized to GEF-H1 expression in input. Data shown is from one experiment representative of three independent experiments with similar results.

2.3.2 Homo-oligomerization of GEF-H1

Recent evidences indicate that the members of the RH-RhoGEF family can form oligomers through their C-terminal region. Furthermore, PDZRhoGEF and LARG can form heteromolecular complexes, whereas p115RhoGEF only can associates with itself. Despite of the fact that the C-terminal region has not detectable effect on the catalytic activity of the RhoGEFs, deletion of the C-terminus resulted in an increase of their GEF activity. This oligomerization takes place through the C-terminal region of the RhoGEFs and it is known to have an inhibitory role (Chikumi *et al.*, 2004; Baisamy *et al.*, 2005). Therefore we sought to address whether GEF-H1 can oligomerize and how $G\alpha_{12}$ could affect this oligomerization. To this end, cells were transfected with GEF-H1-GFP and full length, C-terminal and N-terminal truncated forms of HA-GEF-H1 in presence or absence of $G\alpha_{12}Q231L$ (Figure R23). As illustrated, co-immunoprecipitation of GEF-H1-GFP was observed with each construct of HA-GEF-H1 tested. Since C-terminal form was the highest expressed, more GEF-H1-GFP was co-immunoprecipitated. Despite the fact that the expression levels of the N-terminal form and full length protein were equal, there was a reduction on GEF-H1-GFP co-immunoprecipitated with the N-terminal construct. On the other hand, the presence of the constitutively active $G\alpha_{12}$ not seems to affect the oligomerization of the guanine exchange factor. Taken together these results suggest that GEF-H1 can form oligomers and maybe as the members of the RH-RhoGEF subfamily, through its C-terminal region where GEF-H1 has a coiled-coil motif. Further experiments should be done in order to ascertain whether this homo-oligomerization is

affecting the catalytic activity of GEF-H1. Nevertheless, $G\alpha_{12}$ does not seem to play an important role for this possible mechanism of regulation of GEF-H1.

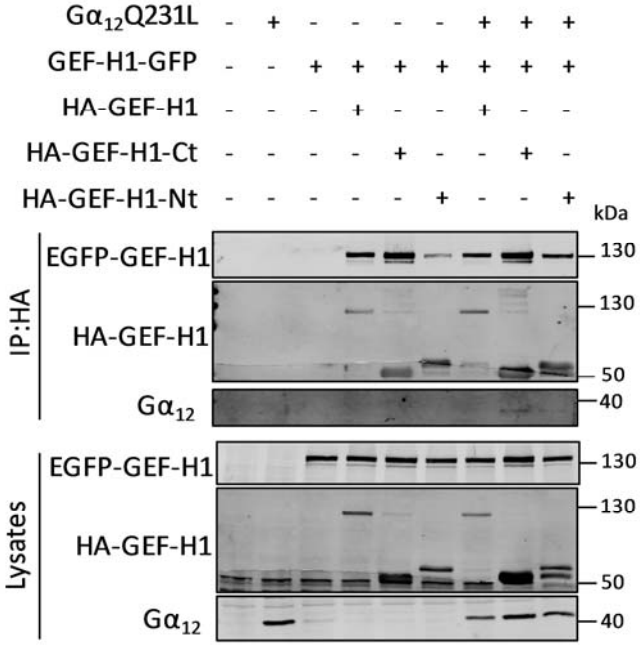


Figure R23. Dimerization of GEF-H1. HEK-293 cells were transiently transfected with different combinations of plasmids encoding $G\alpha_{12}$Q231L, EGFP-GEF-H1, HA-GEF-H1, HA-GEF-H1-HA-Ct and HA-GEF-H1-Nt. Twenty-four hours after transfection cells were lysed and the different constructs of HA-GEF-H1 immunoprecipitated with an anti-HA agarose-conjugated antibody. Immunoprecipitates and total lysates were analyzed by western blot with specific antibodies. Control lane shows transfection with mtDSRed. Data are representative of at least three independent experiments.

2.3.3 Interaction of GEF-H1 with 14-3-3

GEF-H1 has been shown to have an accurate regulation by phosphorylation (Zenke *et al.*, 2004; Callow *et al.*, 2005; Birkenfeld *et al.*, 2007; Fujishiro *et al.*, 2008; Meiri *et al.*, 2009; Yamahashi *et al.*, 2011). Several kinases, including Aurora A/B, PKA, and Pak1 have been shown to phosphorylate GEF-H1 on Ser885 creating a high affinity-binding site for 14-3-3 proteins. Since different kinases can phosphorylate the same residue it is thought that is a regulated spatial-temporal process. 14-3-3 proteins are proteins that usually act as dimers, where each monomer has a binding site for the ligand, and the interaction with 14-3-3 proteins mainly is mediated through phosphorylated serine and threonine residues

(Bridges and Moorhead, 2004). Not much is known about the significance of the interaction between GEF-H1 and 14-3-3 proteins. It has been suggested to have a negative regulatory role since its binding is associated with a decrease on GEF activity of GEF-H1. Nevertheless, the effect of kinases on GEF-H1 activity or function has been assessed only by indirect drug treatment (Krendel *et al.*, 2002; Birkenfeld *et al.*, 2007; Meiri *et al.*, 2009). Thereby we wondered whether the interaction of GEF-H1 with 14-3-3 proteins could be affected by the presence of $G\alpha_{12}$. For that purpose, cells were transfected with combinations of $G\alpha_{12}$Q231L, HA-GEF-H1, HA-GEF-H1-Ct, HA-GEF-H1-Nt, and 14-3-3 γ and the different deletion constructs and full length GEF-H1 were immunoprecipitated (Figure R24). As can be observed, 14-3-3 γ only co-immunoprecipitates with GEF-H1 in presence of $G\alpha_{12}$Q231L (Figure R24A) as well as with HA-GEF-H1-Ct, whereas no co-immunoprecipitation was observed with the N-terminal form of GEF-H1. These results are in agreement with data that shows that GEF-H1 binds to 14-3-3 through its C-terminal region (Zenke *et al.*, 2004; Meiri *et al.*, 2009). Interestingly, when the co-immunoprecipitation experiments were done in absence of phosphatase inhibitors a clear reduction of the co-immunoprecipitated 14-3-3 can be noted with the full length and the C-terminal moiety of GEF-H1 (Figure R24B).

Our results show that $G\alpha_{12}$ enhances the interaction between GEF-H1 and 14-3-3, which is inconsistent with a model that suggests a role for $G\alpha_{12}$ as a positive regulator and 14-3-3 as a negative regulator for GEF-H1. An explanation for this discrepancy could be the fact that the studies done until now do not provide enough information to clarify the regulation mechanism of GEF-H1.

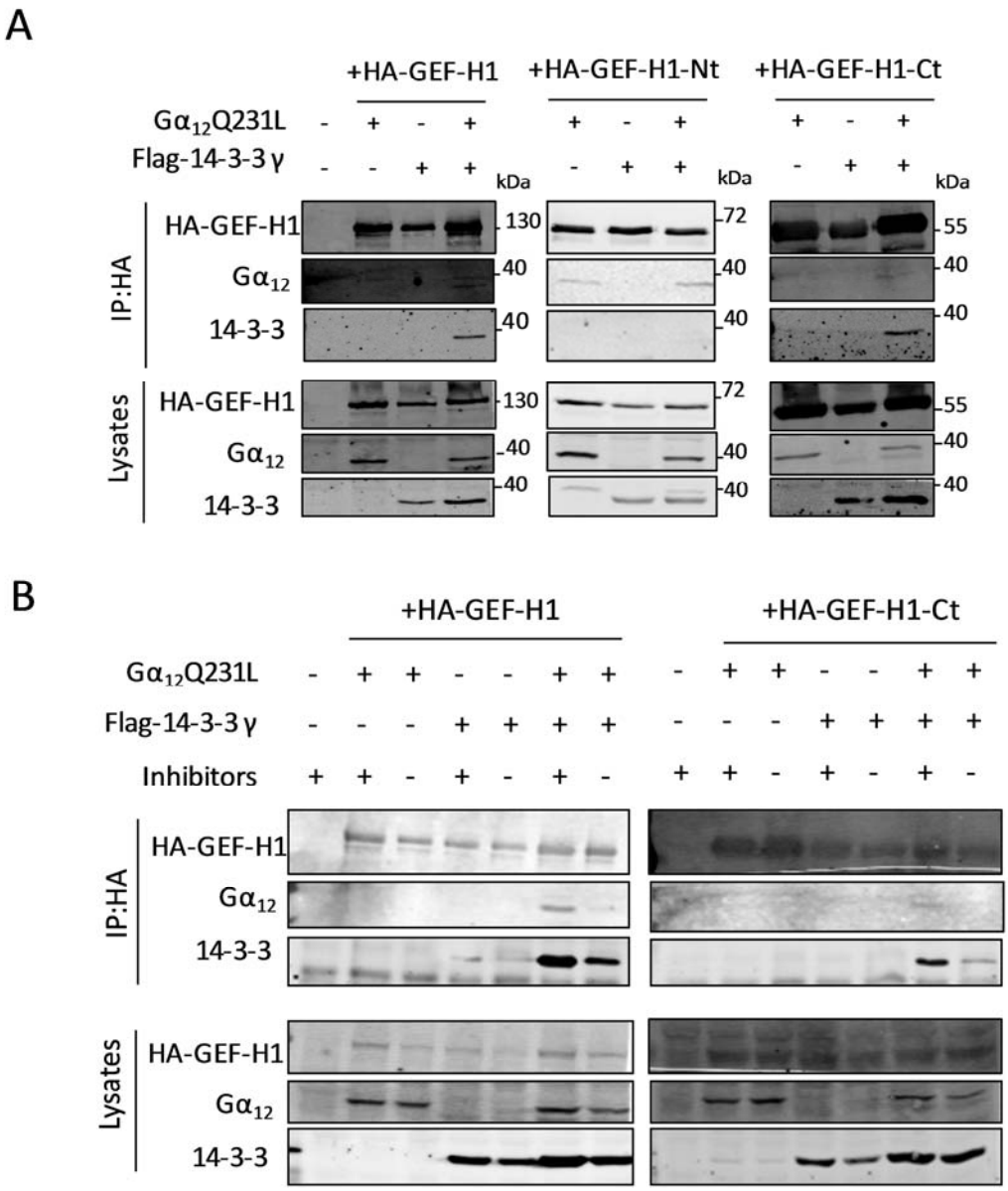


Figure R24. $G\alpha_{12}$ enhances the interaction between 14-3-3 and GEF-H1. HEK-293 cells were transfected with different combinations of mtDSRed (control vector) or plasmids encoding $G\alpha_{12}$Q231L, Flag-14-3-3 γ, HA-GEF-H1, HA-GEF-H1-Ct and HA-GEF-H1-Nt in **(A)** or $G\alpha_{12}$Q231L, Flag-14-3-3 γ, HA-GEF-H1 and HA-GEF-H1-Ct in **(B)**. Twenty-four hours after transfection, cells were lysed and immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analyzed by western blot with specific antibodies. In **(B)** cells were lysed in RIPA buffer in absence of phosphatases inhibitors (Na_3VO_4, NaF and EDTA). Blots shown are representative of three independent experiments.

2.3.4 Phosphorylation on S885 and S959 of GEF-H1 does not affect the interaction with Gα12

In order to better understand the mechanism of interaction between GEF-H1 and Gα12 we sought to determine whether phosphorylation could be important for the interaction. Thus, we took advantage of two phosphorylation mutants of GEF-H1: a phosphorylation-deficient mutant, GEF-H1S885/959A, and a phosphomimetic mutant, GEF-H1S885/959D. It has been shown that these residues are phosphorylated during mitosis. Cells were transfected with both mutants and the wild type form of GFP-tagged GEF-H1 alone or in presence of Gα12Q231L (Figure R25). Taking together all the results obtained in different experiment Gα12 co-immunoprecipitated with both mutants, GEF-H1S885/959A and GEF-H1S885/959D, with no difference compared with the wild type form of GEF-H1. Although it has been shown that these residues were dephosphorylated in order to promote GTP loading on RhoA in a mitotic context, it is not clear whether phosphorylation could inhibit GEF activity of the exchange factor. Nonetheless, the interaction of Gα12 with GEF-H1 is not affected by the phosphorylation state of the residues S885 and S959 of GEF-H1.

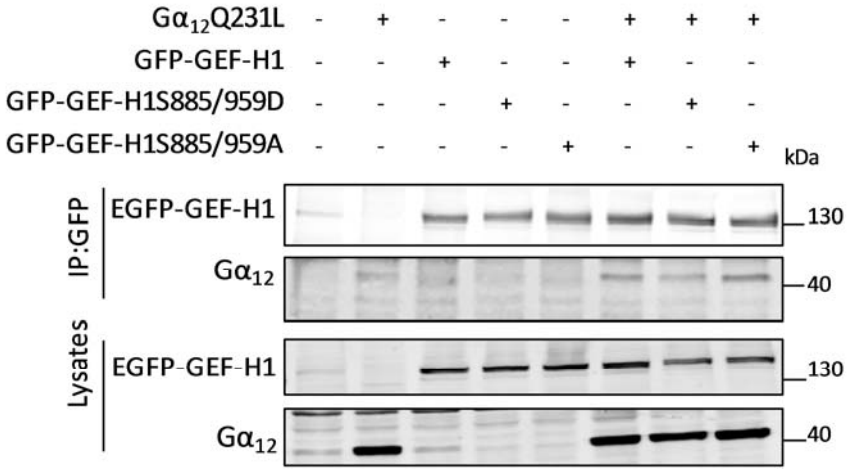


Figure R25. (A) GEF-H1 phosphorylation mutants co-immunoprecipitate with $G\alpha_{12}$. HEK-293 cells were co-transfected with pCMV5-EGFP-GEF-H1, pCMV5-EGFP-GEF-H1S885/959D, pCMV5-EGFP-GEF-H1S885/959A, pcDNA3.1-$G\alpha_{12}$Q231L or pCDNA3.1-mtDSRed. Twenty-four hours after transfection cells were lysed and subjected to immunoprecipitation using anti-GFP antibody. Immunoprecipitates and whole cell lysates were analyzed by SDS-PAGE, and immunoblotted with anti-GFP and anti-$G\alpha_{12}$ antibodies. Data are illustrative of three independent experiments with similar results.

3. Subcellular localization of GEF-H1

3.1 GEF-H1 colocalizes with the Golgi apparatus in MEF cells

In order to characterize the subcellular localization of GEF-H1, we started by visualizing endogenous GEF-H1 in MEF cells in interphase. Curiously, anti-GEF-H1 stained at the perinuclear region and a discreet vesicular staining pattern was present in both cell types (Figure R26).

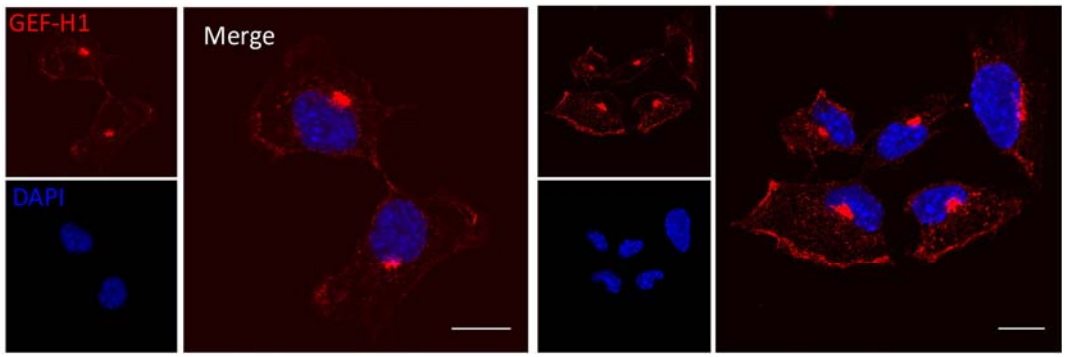


Figure R26. GEF-H1 presents a perinuclear pattern in MEF. Confocal micrographs of MEF cells immunolabeled with anti-GEF-H1 (Abcam) (red) and mounted with ProlongGold with DAPI (blue). Images shown are representative of the majority of the cells in the plate. One single confocal image layer is shown in the micrographs. Scale bars: 10 μm.

To control the specificity of the antibody, we decided to silence GEF-H1 by short hairpin-interfering RNA (shRNA). Five different shRNA sequences targeted against the coding region of the mouse ARHG2 gene (see Materials and Methods)

as well as a scrambled control (shScr1) were used. After lentivirus infection MEF cells were selected with puromycin and whole cell lysates were loaded in a SDS-PAGE gel. The western blot analysis of those lysates with an anti-GEF-H1 antibody showed that two shRNA, clone 5 and 8, decreased the protein levels (Figure R27A). Clone 6 and 7 had an intermediate silencing on GEF-H1 expression levels and clone 9 presented similar GEF-H1 expression as the control. Then, immunostaing of endogenous GEF-H1 was performed in control (shScr1) and stable GEF-H1-depleted (sh5 and sh8) MEF cells (Figure R27B). shGEF-H1-MEF cells lost the specific perinuclear staining, which probe the specificity of the antibody used. Also it demonstrates that GEF-H1 is located at the nuclear periphery as well as in the cell membrane.

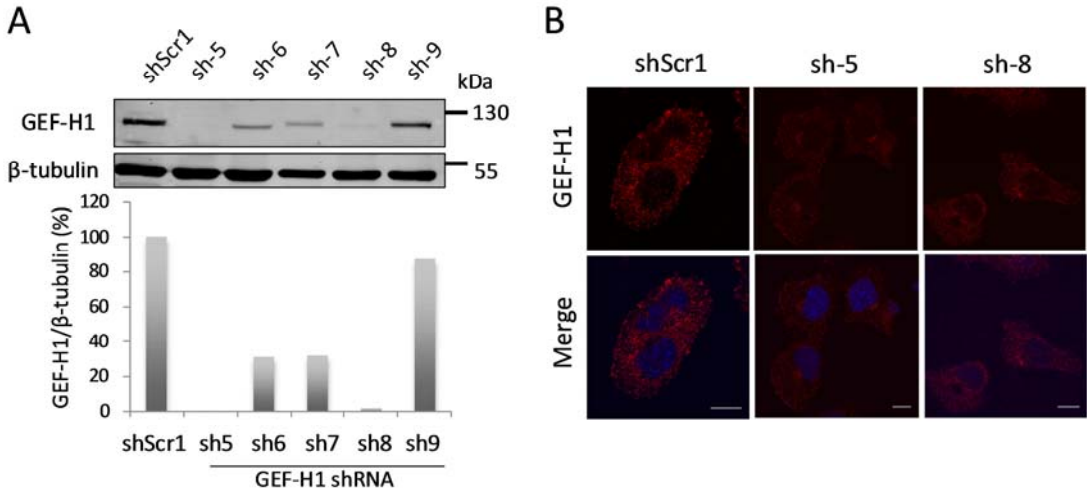


Figure R27. Transcriptional silencing of GEF-H1 by shRNA decreases protein levels with two different target sequences. MEF wild type cells were infected by lentivirus with five different sequences of shRNA (5 to 9) against GEF-H1 and a control shRNA (shScr1). **(A)** Lysates from stable scrambled (shScr1) or GEF-H1 shRNA-expressing MEF cells were evaluated by immunoblotting using an anti-GEF-H1 (Cell Signaling) and anti-β-tubulin as control (upper panel). Quantification of protein level was done by Odyssey and the level of GEF-H1 expression was normalized by β-tubulin protein level (lower panel). **(B)** The transcriptional silencing was evaluated by immunofluorescence. Confocal micrograph of stable scrambled or GEF-H1 sh-5 and sh-8 MEF fixed cells were stained for GEF-H1 (Abcam). Cells were mounted with Prolong Gold containing DAPI. Scale bars: 10 μm.

The perinucelar staining of GEF-H1 was further examined using various subcellular protein markers. Rab11 a protein involved in vesicles transport, was

used as recycling endosome marker; caveolin, a protein involved in receptor-independent endocytosis, as a caveolar marker protein; Rab1, as a marker for the intermediate compartment and endoplasmic reticulum and Mannosidase II, as a Golgi marker. MEF cells were immunostained for the above-mentioned proteins and for endogenous GEF-H1 (Figure R28). Rab11 localized in a patch in the perinuclear region that corresponds to the endocytic recycling compartment and a few small dots throughout the cytoplasm and the intercellular bridge. In this case GEF-H1 showed the same staining pattern as Rab11 with colocalization at the perinuclear region and in the dots into the intercellular bridge. Caveolin showed granular and diffuse cytoplasmatic pattern, as well as perinuclear staining, and colocalization with GEF-H1 was found in the perinuclear region. However, neither Rab1 nor Mannosidase II proteins colocalized with GEF-H1. These results indicate that GEF-H1 could also have a role in vesicle trafficking.

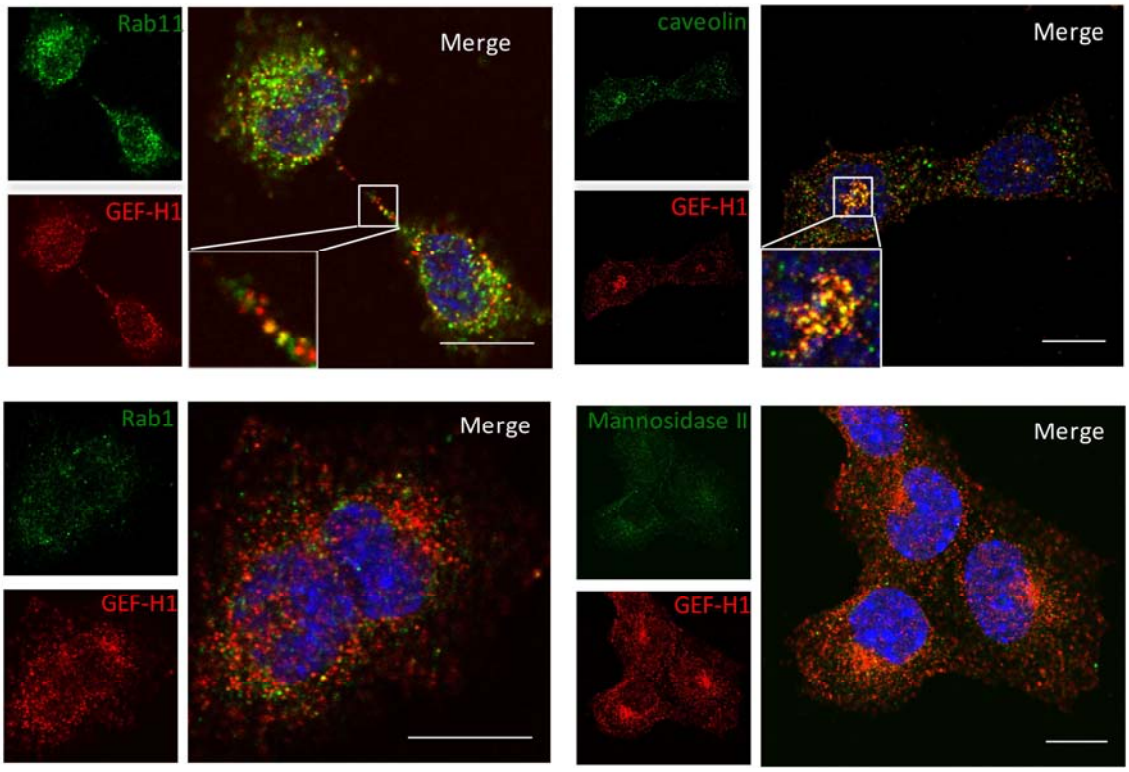


Figure R28. Subcellular distribution of GEF-H1 with Rab11, Rab1, caveolin and Mannosidase II. MEF cells were fixed and immunolabeled for endogenous GEF-H1 (Abcam) (red) and Rab11, Rab1, caveolin and Mannosidase II (green). Boxed areas are magnified images of the indicated area. The arrows in the panels denote colocalization with GEF-H1. One single confocal image layer is shown in the micrographs. Scale bars: 10 μm.

The previously mentioned Golgi marker, Mannosidase II, gave us a diffuse cytoplasmatic staining pattern despite of the perinuclear Golgi staining that we expected. For this reason and to better understand the results observed above we decided to test another Golgi marker, 58K protein (formiminotransferase cyclodeaminase) (Figure R29). Immunostaining of endogenous GEF-H1 and 58K protein showed a clear colocalization between GEF-H1 and membrane structures of the Golgi apparatus (Figure R29A).

Brefeldin A (BFA) is a fungal metabolite that causes reversible disassembly of the Golgi complex into a tubular network that is absorbed into the endoplasmic reticulum (Klausner *et al.*, 1992). BFA was used to disrupt the Golgi structure and Golgi 58K and GEF-H1 localization was examined after treatment of the cells with BFA. Cells were also treated with nocodazole, an anti-neoplasic agent, which interferes with the polymerization of microtubules. High concentrations of nocodazole induce microtubule depolymerization whereas at low concentrations nocodazole alters spindle microtubule dynamics, but microtubules do not depolymerize (Jordan *et al.*, 1992; Vasquez *et al.*, 1997; Blajeski *et al.*, 2002). Nocodazole did not alter colocalization of GEF-H1 at Golgi apparatus (Figure R29B). BFA treatment caused Golgi dissembling and, consequently, the GEF-H1 staining at the perinuclear region was lost (Figure R29B). Interestingly, during this thesis it was published that GEF-H1 is involved in vesicle trafficking through the regulation of the endocytic and exocytic pathways (Pathak *et al.*, 2012). Taking the results all together we can conclude that GEF-H1 localizes at Golgi apparatus in MEF cells and this subcellular localization would be related with this novel GEF-H1 function.

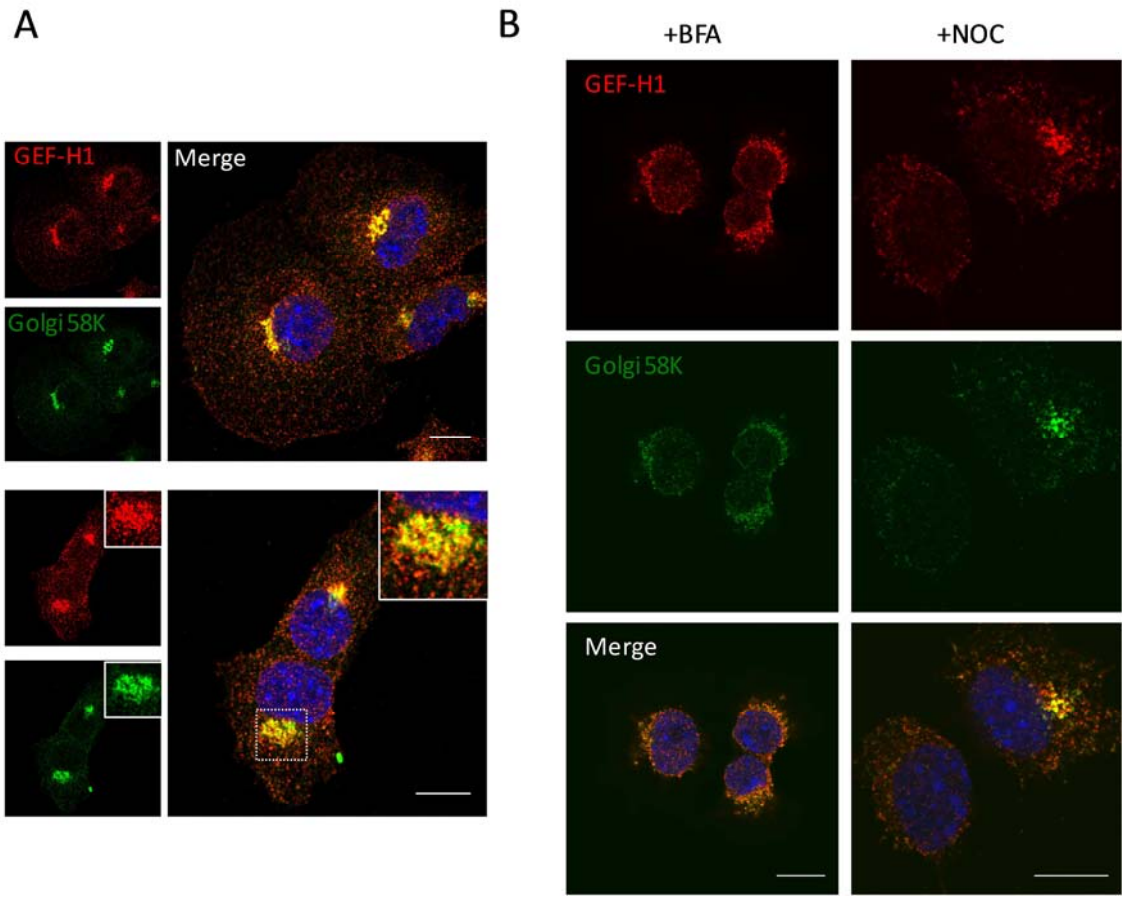


Figure R29. Subcellular distribution of endogenous GEF-H1 in different images of MEF cells shows that GEF-H1 localizes with Golgi markers. **(A)** Confocal micropraphs of MEF cells stained against endogenous GEF-H1 (Abcam) (red) and Golgi 58K protein (green). Boxed area is a magnified area of the image **(B)** MEF cells were treated with brefeldin A (5 μg/ml) or nocodazole (10 μg/ml) for 30 minutes, fixed and probed against endogenous GEF-H1 and Golgi 58K proteins. One single confocal image layer is shown in the micrographs. Scale bars: 10 μm.

3.2 Intracellular localization of GEF-H1 in HeLa cells

An earlier characterization of the intracellular distribution of full length GEF-H1 demonstrated that it is associated with microtubules (Krendel *et al.*, 2002). The expression of HA-tagged full length protein and GFP fusion protein is shown in figure R30, where it is easy to observe the characteristic microtubule localization of the protein. Though we have to mention that the antibody against GEF-H1 labels very poorly GEF-H1 in microtubules during interphase.

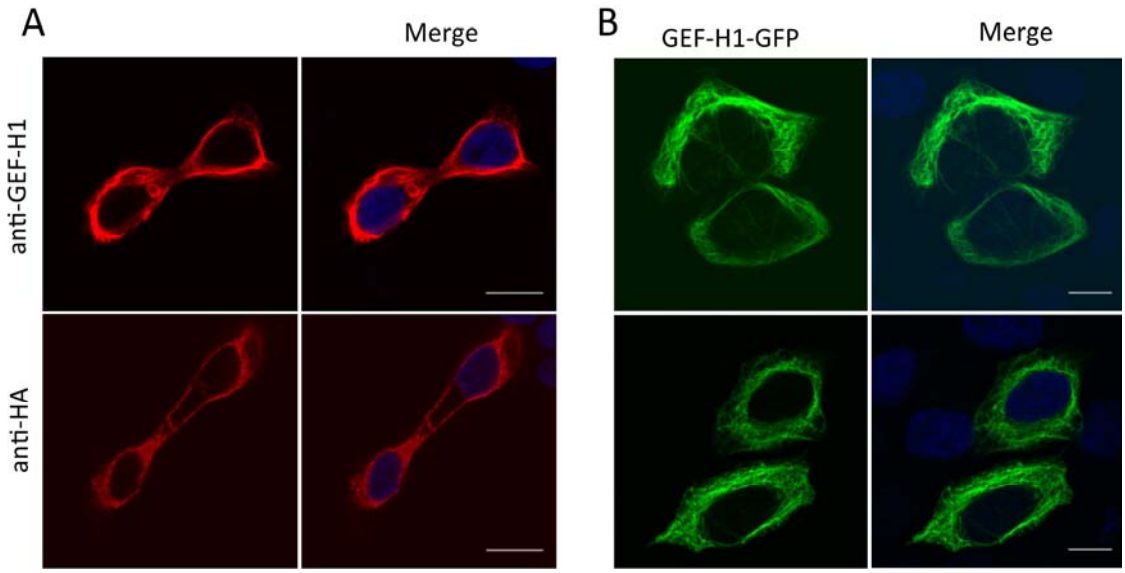


Figure R30. Subcellular distribution of either HA-GEF-H1 or EGFP-GEF-H1 shows a microtubule pattern in HEK-293 cells. **(A)** Confocal micrographs of cells transfected with pcDNA3-HA-GEF-H1. Twenty-four hours after transfection cells were fixed and stained with two different antibodies, anti-GEF-H1 (Abcam) (red) and anti-HA (red). Both antibodies show similar pattern of cell staining. **(B)** Confocal micrographs of cells transfected with pCMV5-EGFP-GEF-H1. Twenty-four hours later, cells were fixed and processed. One layer of Z-stack is shown. Images are representative of the majority of cells. Scale bars: 10 μm.

We sought to follow the localization of the endogenous GEF-H1 protein along the different stages of mitosis in HeLa cells (Figure R31). Mitotic cells with the anti-GEF-H1 antibody stained the mitotic spindle. Upon initiation of mitosis, GEF-H1 was homogeneously distributed through the cytoplasm in metaphase. Then, in anaphase GEF-H1 begin to be localized with the spindle apparatus in the equatorial plane between the daughter cells. In early telophase GEF-H1 was localized at centrosomes and midzone and finally, in late telophase, GEF-H1 labeled a ring-like structure encompassing the midzone. These data revealed that a fraction of GEF-H1 localizes to the spindle apparatus throughout all stages of mitosis and agrees with previous data (Birkenfeld *et al.*, 2007).

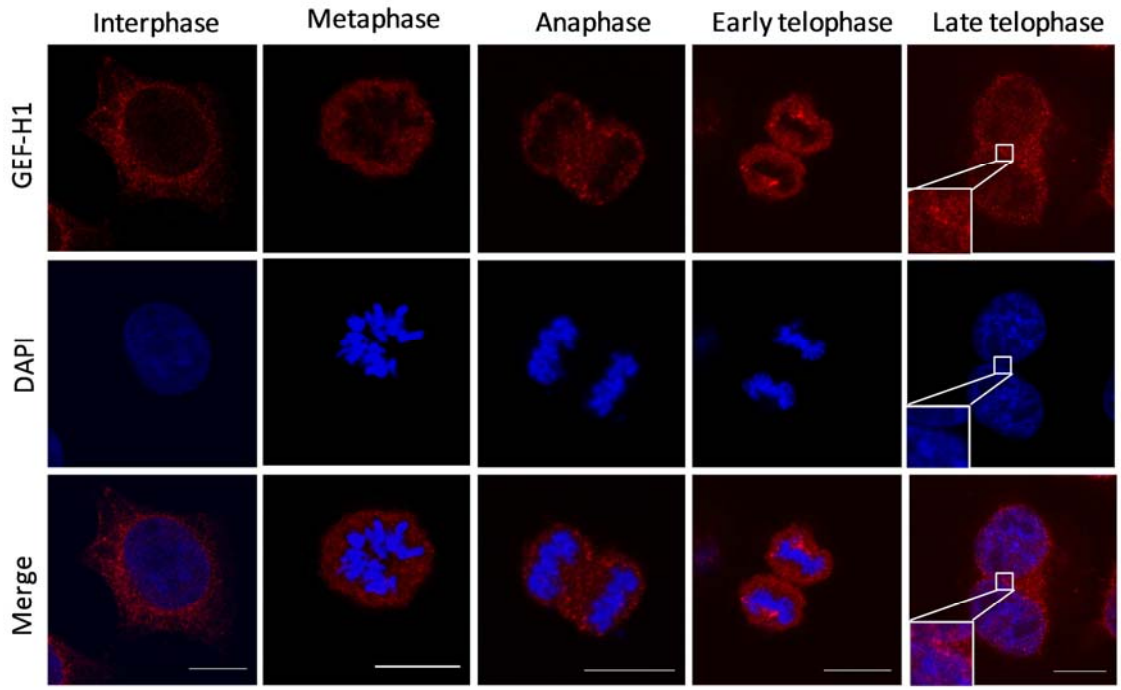


Figure R31. Distribution of GEF-H1 during mitosis in HeLa cells. Asynchronic HeLa cells were fixed and probed against endogenous GEF-H1 with anti-GEF-H1 (Santa Cruz) (red). Cells were mounted with Prolong Gold containing DAPI. Boxed areas are magnified images of the area indicated. Images were taken by confocal microscopy. The image corresponds to one single confocal layer. Scale bars: 10 μm.

3.3 GEF-H1 is located at the midbody structure

As mentioned before, GEF-H1 modulates localized RhoA activation during cytokinesis under the control of the mitotic kinases Aurora A/B and Cdk1/Cyclin B (Birkenfeld *et al.*, 2007). There is still no information on which protein activates its GEF activity in this context. The characterization of the interaction between $G\alpha_{12}$ and GEF-H1 led us to propose GEF-H1 as a novel downstream effector for $G\alpha_{12}$ signaling. Given the fact that $G\alpha_{12}$ protein is located at the midbody during cytokinesis and that GEF-H1 is coordinating cortical activities during cleavage furrow ingression we next sought to visualize GEF-H1 in cytokinesis.

Next, we examined in more detail the distribution of GEF-H1 during cytokinesis. Cells were stained against endogenous GEF-H1 (Figure R32A) and as can be observed a ring-like structure was present between two dividing cells in HeLa and 1321N1 cells. Likewise, expression of GFP-GEF-H1 fusion protein in HEK-293 cells (Figure R32B) showed that GFP-GEF-H1 was prominently localized

within the intercellular bridge at the midbody. These data confirmed that GEF-H1 is localized at the midbody region. Interestingly, this localization coincides with the localization of $G\alpha_{12}$. It remains to be established if in absence of $G\alpha_{12}$, GEF-H1 localizes at the midbody.

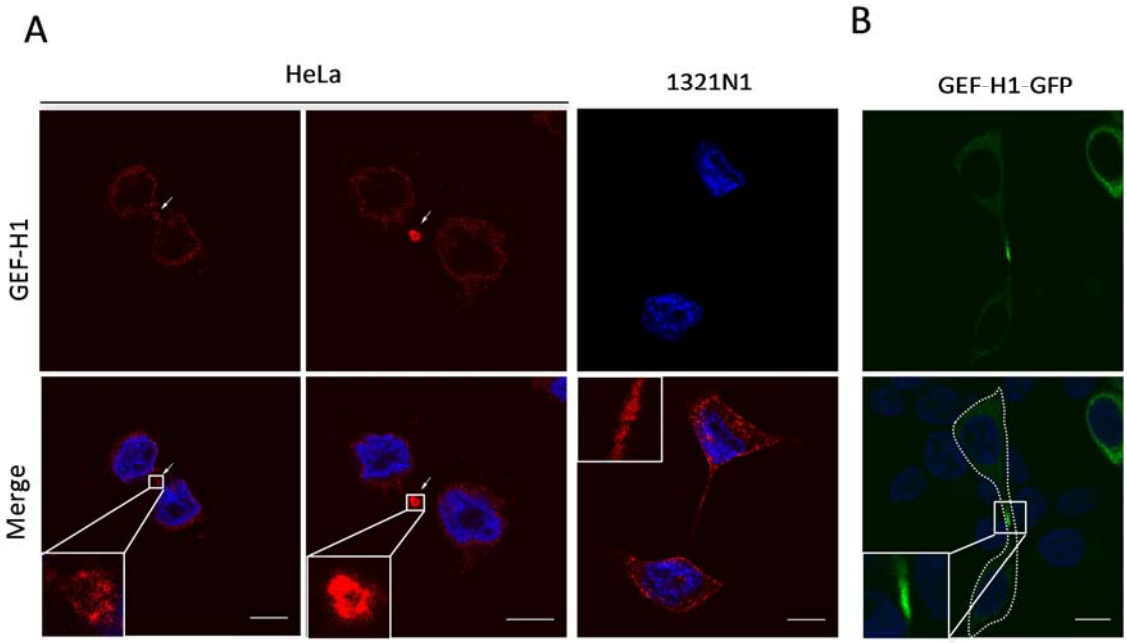


Figure R32. GEF-H1 is localized at the midbody structure during cytokinesis. **(A)** HeLa cells were pretreated with nocodazole 2h before the fixation with TCA. Asynchronous 1321N1 cells were fixed with ice-cold methanol. Confocal micrographs of cells probed against endogenous GEF-H1 (Abcam). **(B)** GEF-H1-GFP is located at midbody. Confocal micrographs of HeLa cells transfected with pCMV5-EGFP-GEF-H1, 24h after transfection cells were fixed and processed for immunofluorescence, and mounted with DAPI. Boxed areas are magnified images of the area indicated. Images show a single layer of a confocal image. Scale bars: 10 μm.

4. Involvement of GEF-H1 in $G\alpha_{12}$ signaling pathways

4.1 Colocalization of $G\alpha_{12}$ and GEF-H1 at the midbody

Given the fact that subcellular distribution analysis of $G\alpha_{12}$ and GEF-H1 through mitosis indicate that both proteins are in the same structure during cytokinesis, we performed double immunostaining of endogenous GEF-H1 and $G\alpha_{12}$ (Figure R33).

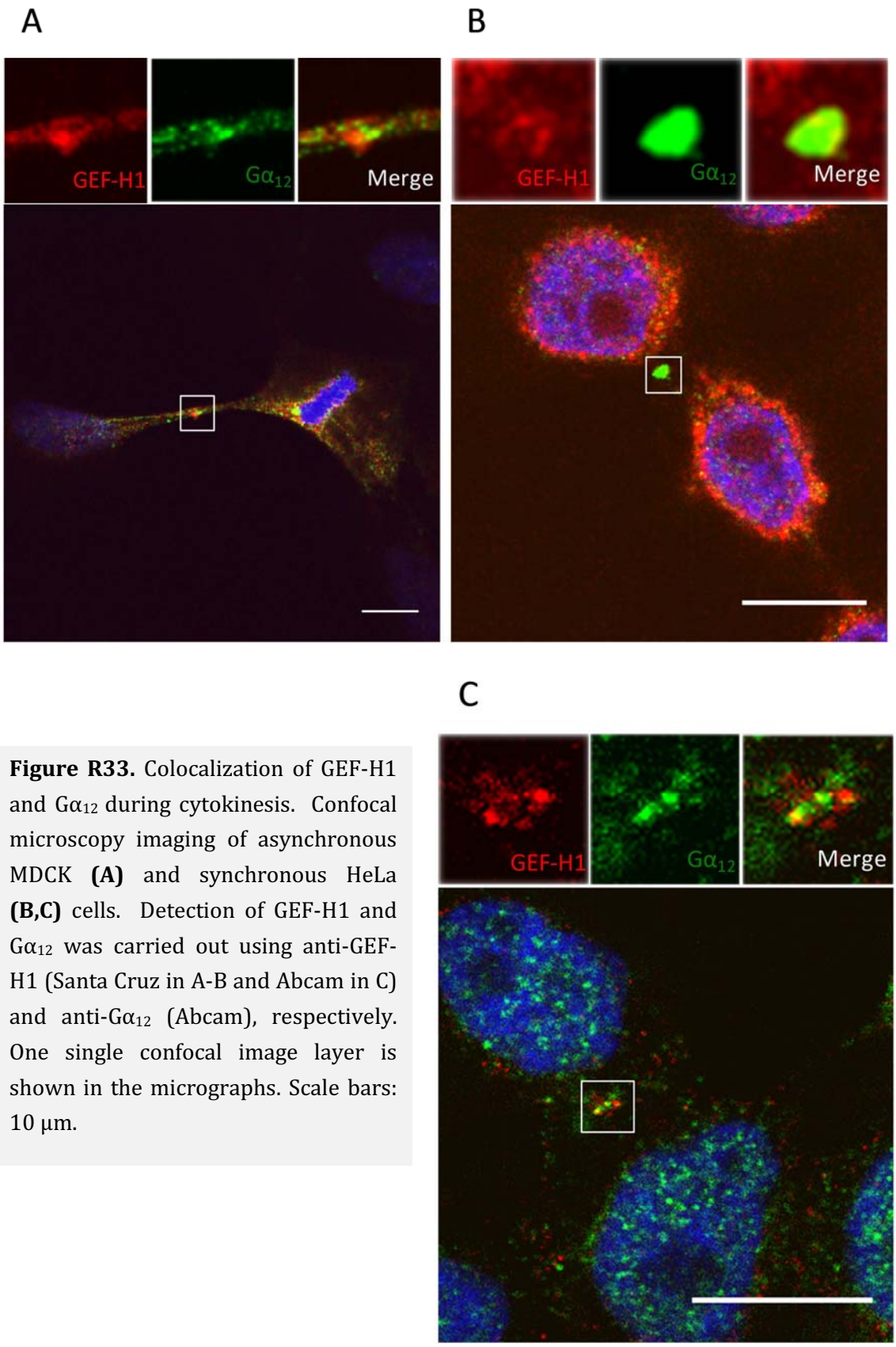


Figure R33. Colocalization of GEF-H1 and $G\alpha_{12}$ during cytokinesis. Confocal microscopy imaging of asynchronous MDCK **(A)** and synchronous HeLa **(B,C)** cells. Detection of GEF-H1 and $G\alpha_{12}$ was carried out using anti-GEF-H1 (Santa Cruz in A-B and Abcam in C) and anti-$G\alpha_{12}$ (Abcam), respectively. One single confocal image layer is shown in the micrographs. Scale bars: 10 μm.

As can be observed $G\alpha_{12}$ was prominently localized at the ring-like structure that corresponds to midbody whereas GEF-H1 staining was more discreet but also was clearly associated with midbody (Figure R33). In MDCK cells the ring of $G\alpha_{12}$ was more disperse whereas GEF-H1 labeling was more intense in the midbody (Figure R33A). Clear colocalization can be observed. On the other hand, in panel B HeLa cells showed a higher $G\alpha_{12}$ midbody staining and a GEF-H1 ring surrounding the structure of $G\alpha_{12}$. These images were taken with the Santa-Cruz antibody against GEF-H1 (Figure R33A/B). Analysis utilizing the anti-GEF-H1 antibody from Abcam also shows similar localization of GEF-H1 and $G\alpha_{12}$ in figure R33C. Taken together these data suggest that $G\alpha_{12}$ and GEF-H1 localized at the midbody during cytokinesis. Therefore, it could be possible that $G\alpha_{12}$ activates GEF-H1 during cytokinesis.

4.1.1 Phosphorylation by Aurora A kinase

As we mentioned above GEF-H1 is regulated by the mitotic kinases Aurora A/B and Cdk1/Cyclin B through mitosis by phosphorylation on Ser885 and Ser959, respectively. While GEF-H1 seemed to be dephosphorylated prior to RhoA activation, the phosphomimetic mutant GEF-H1S885/959D did not alter the enzymatic activity of GEF-H1 indicating that additional modulators are needed (Birkenfeld *et al.*, 2007). To further characterize GEF-H1 function during mitosis we followed phosphorylated GEF-H1 using a phospho-specific antibody raised against the pSer885 epitope in synchronized HeLa cells throughout the different stages of mitosis. Despite of the homogenous and slightly staining of GEF-H1 in cells in interphase, high levels of endogenous phospho-GEF-H1 during metaphase (Figure R34) were observed co-localizing with centrosomes, wherein is phosphorylated by Aurora A kinase. When cells were allowed to progress through mitosis phosphorylated GEF-H1 appear to follow microtubule tracks in anaphase, and in telophase it can be observed that GEF-H1 is located surrounding the midzone.

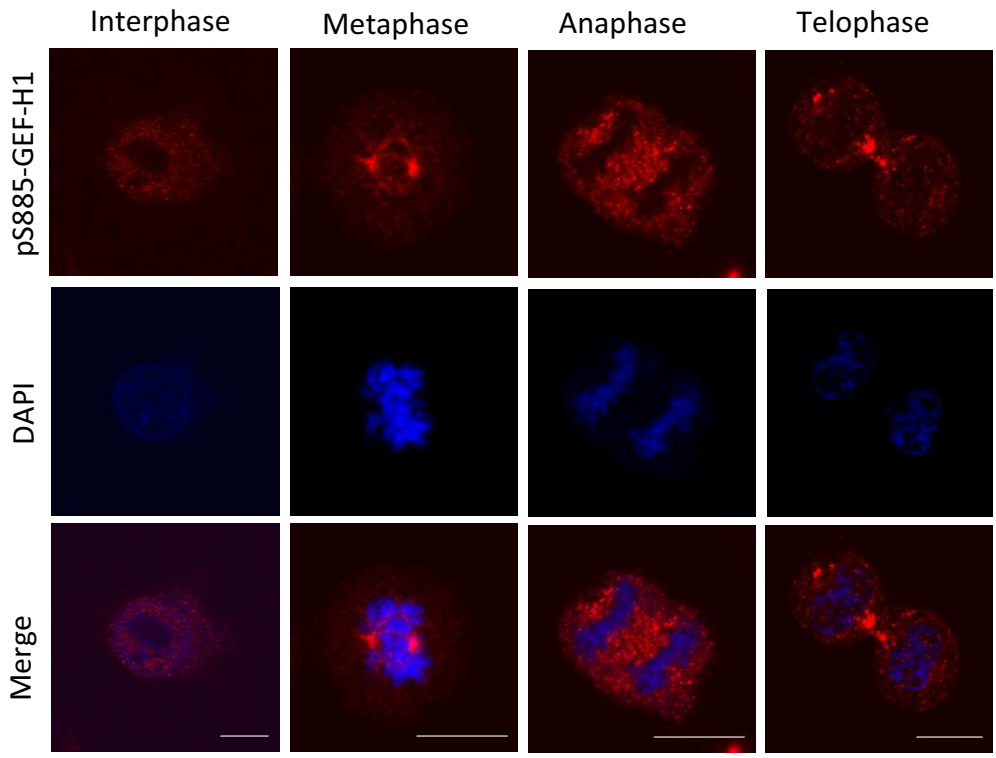


Fig. R34. Subcellular localization of phosphorylated GEF-H1. Synchronized HeLa cells were fixed and probed with anti-phospho-S885-GEF-H1. Mitotic stages were determined by labelling DNA with DAPI. The picture corresponds to one layer of Z-stack from confocal SP5 microscope. Scale bars: 10 μm.

As mentioned previously in the introduction, recent phosphoproteomic report shown that $G\alpha_{12}$ is phosphorylated during mitosis on serine 67, but nothing is known about this novel phosphorylation function as well as the kinase involved in. If $G\alpha_{12}$ is important for cytokinesis it is plausible to hypothesize that $G\alpha_{12}$ might be regulated in this process, and one mechanism of regulation could be phosphorylation. As Aurora A kinase phosphorylates GEF-H1 we considered the possibility that Aurora A kinase would be the responsible of $G\alpha_{12}$ phosphorylation. A double immunofluorescence staining was done in synchronic HeLa cells (Figure R35).

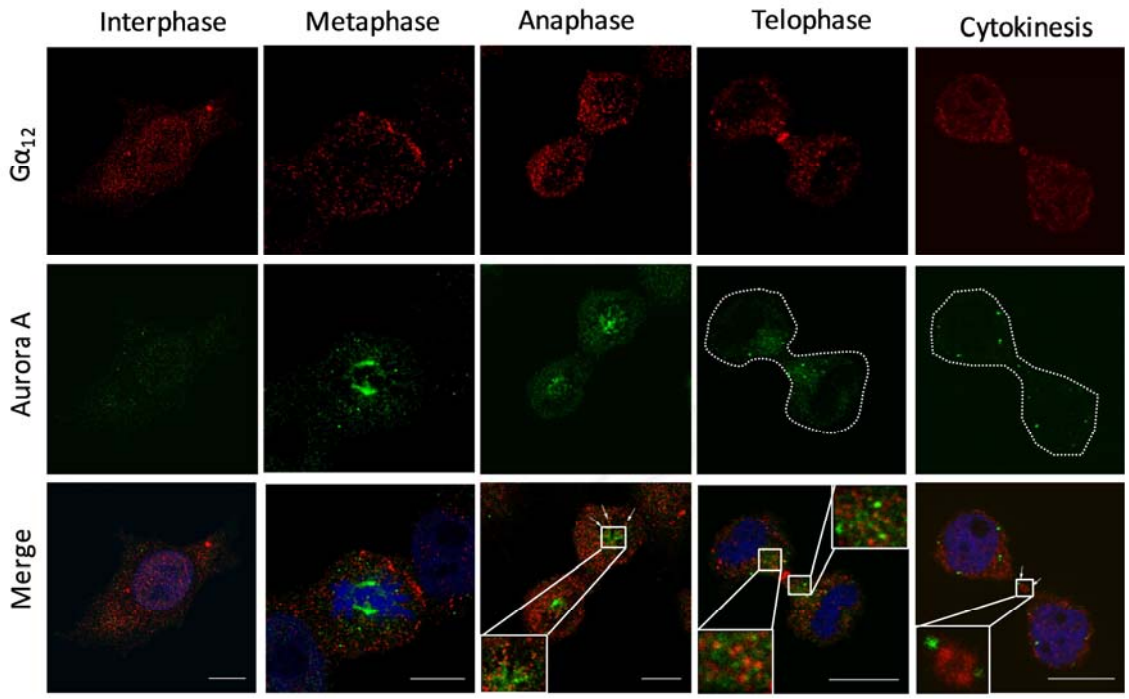


Fig. R35. Colocalization between $G\alpha_{12}$ and Aurora A kinase at different stages of mitosis. Fluorescent confocal micrographs of synchronized HeLa cells. Detection of endogenous Aurora A kinase and $G\alpha_{12}$ was carried out by using anti-Aurora A (green) and anti-$G\alpha_{12}$ (red) (Abcam) antibodies, respectively. White boxes represent the zoom area and white arrows shown co-localization structures. Images were taken by confocal microscopy. One layer of Z-stack is shown. Images are representative of the majority of the cells present on the plate. Scale bars: 10 μm.

In metaphase, Aurora A kinase was associated to centrosomes whereas $G\alpha_{12}$ was evenly distributed in the cytoplasm. Despite the clear centrosome staining of endogenous $G\alpha_{12}$ obtained in the results showed above in MEF cells (Figure R9A) it was not possible to obtain the same staining in HeLa cells. Upon initiation of mitosis and through anaphase, $G\alpha_{12}$ colocalized with Aurora A kinase at the spindle poles. During telophase there was colocalization along the microtubule tracks whereas in cytokinesis the staining of Aurora A was decreased and $G\alpha_{12}$ was located within the intercellular bridge at the midbody. There was a discreet colocalization observed in the late stages of mitosis. Further experiments will be needed to ascertain the role of the $G\alpha_{12}$ phosphorylation during mitosis.

4.2 Cell transformation induced by $G\alpha_{12}$ is altered in GEF-H1 knockdown cells

$G\alpha_{12}$ protein was shown to have transforming activity when is overexpressed as its constitutively active form in NIH3T3 cells (Jiang *et al.*, 1993). To further investigate whether GEF-H1 can be a downstream effector for $G\alpha_{12}$ protein, we transfected $G\alpha_{12}$Q231L in GEF-H1 knockdown cells (sh-5) and in control (shScr1) NIH3T3 cells. In GEF-H1 depleted NIH3T3 cells (88.5% of GEF-H1 silencing), there was a reduction on the number of foci (2.5 ± 0.19) versus control cells that show some foci (17.3 ± 1). Overexpression of $G\alpha_{12}$Q231L induces more foci (31.9 ± 0.38) than control conditions, as expected. $G\alpha_{12}$Q231L expression in cells with reduced levels of GEF-H1 formed 5.9 ± 0.22 colonies (Figure R36). Notably GEF-H1 knockdown cells showed incapacity to promote the formation of focus compare with control cells (Figure R36D). The same foci morphology was found between control and GEF-H1-depleted cells (Figure 36C). Collectively, these results suggest that GEF-H1 appears to be a $G\alpha_{12}$ downstream effector in this pathway.

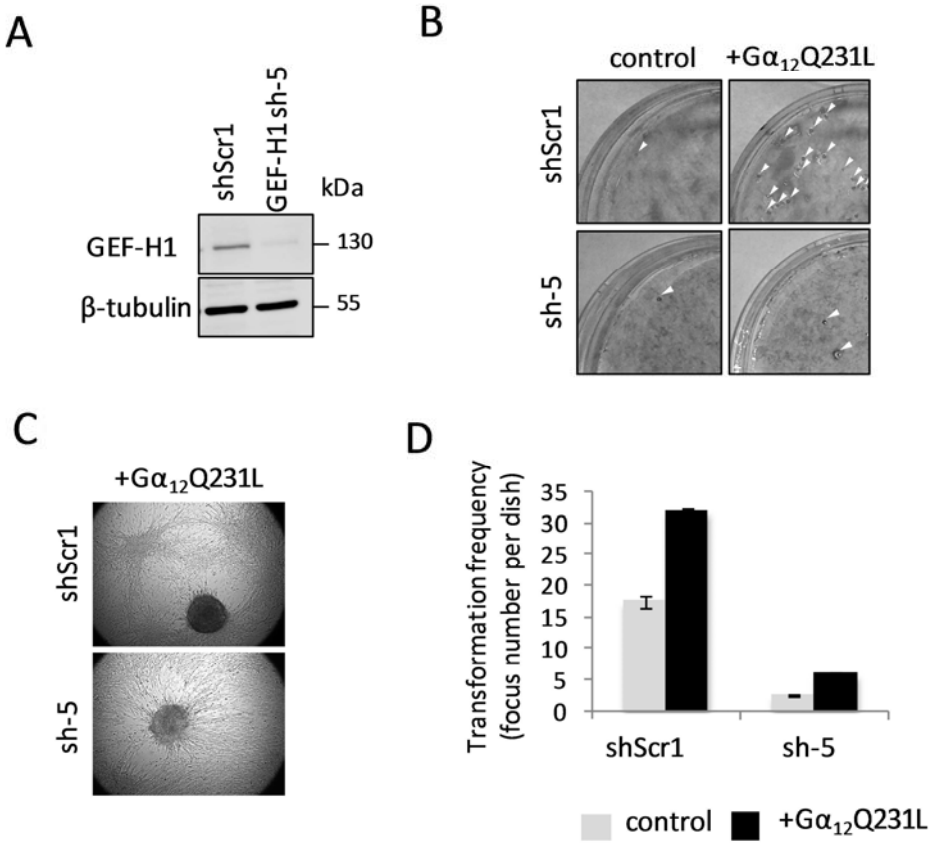


Figure R36. GEF-H1 is important to $G\alpha_{12}$-induced cell transformation. NIH3T3 cells were infected by lentivirus with one sequence of shRNA against GEF-H1, a control shRNA and cells were selected with puromycine. **(A)** Lysates from control shRNA (shScr1) or GEF-H1 shRNA-expressing NIH3T3 cells were evaluated by immunoblotting with β-tubulin expression used as a control. **(B)** Phase contrast images of NIH3T3 shScr1 or GEF-H1 knockdown cells expressing $G\alpha_{12}$Q231L or mtDSRed, used as control vector. After three weeks of culture, the dishes were fixed with ethanol and stained with methylene blue. White arrows indicate transformation focus. **(C)** Phase contrast images of the focus morphology of control (shScr1) and GEF-H1 sh-5 NIH3T3 cells transfected with $G\alpha_{12}$Q231L. **(D)** Quantification of the number of transformed foci by overexpression or not of $G\alpha_{12}$Q231L in control (shScr1) and GEF-H1-depleted cells (sh-5). Graphs show means ± S.E.M of 3 independents experiments.

4.3 GEF-H1 knockdown affects cell migration

Previous studies have shown that $G\alpha_{12/13}$ were indispensable for coordinated and directed cell migration (Goulimari *et al.*, 2005). To investigate whether GEF-H1 was downstream of $G\alpha_{12}$-coordinated cell migration we test the role of GEF-H1 in this pathway by scratch wound healing assays in GEF-H1 stable knock-down HeLa cells.

HeLa cell migration was initiated in the presence of 10% serum in a monolayer of confluent cells, and recordings were performed at 0 and 24 h after wounding. Cells migrated into the wound as sheets to reform tight monolayer within up to 24 h. The rate of wound healing was less in GEF-H1 knockdown cells compared with the shRNA control (shScr1) cells. At 24h post-scratching, the GEF-H1 knockdown cells had the wound healed at 58% compared with the wound of the control that was healed at 80% (Figure R37). These results suggest that GEF-H1 regulates cells migration after serum stimulation, a process that it is also controlled by $G\alpha_{12}$. Further studies utilizing activated $G\alpha_{12}$ and shGEF-H1 could help to answer the question if $G\alpha_{12}$ needs to activate GEF-H1 to induce cell migration.

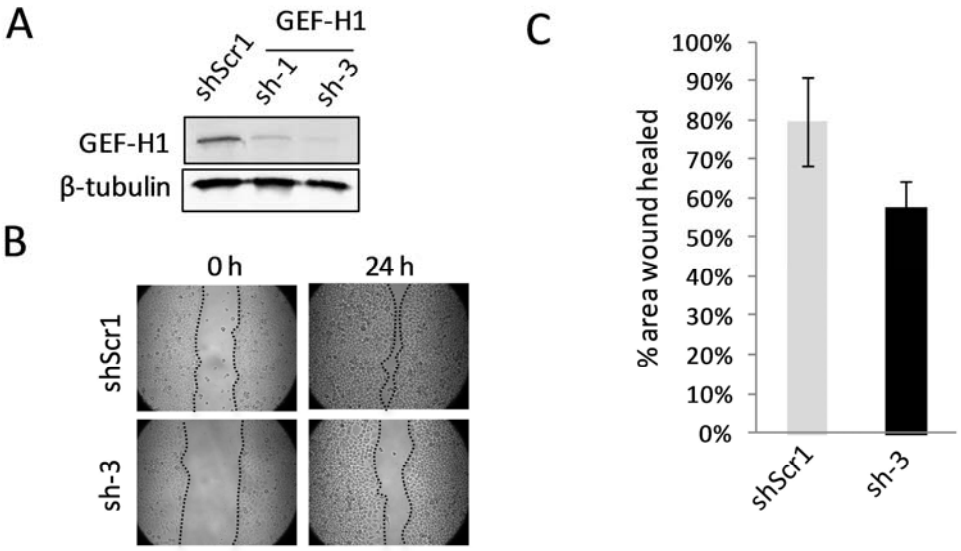


Figure R37. Knockdown of GEF-H1 prevents wound-induced cell migration. HeLa wild type cells were infected by lentivirus with two different sequences of shRNA (1 and 3) against GEF-H1 and a control shRNA (shScr1). **(A)** Lysates from stable scrambled (shScr1) or GEF-H1 shRNA-expressing HeLa cells were evaluated by immunoblotting using an anti-GEF-H1 (Cell Signaling) and anti-β-tubulin as control. **(B)** Representative phase contrast images of wounded shScr1 (upper panel) and GEF-H1 knock-down (lower panel) HeLa cells at 0 and 24 hours after wounding. Wound gap at 0 hour post-scratching was regarded as 100%. **(C)** The graph shows means ± S.E.M of 3 independents experiments.

DISCUSSION

In the present work, we have investigated the role for $G\alpha_{12}$ protein during mitosis. We have shown that $G\alpha_{12}$ is recruited at the midbody during cytokinesis and, more important, demonstrated that $G\alpha_{12}$ is essential for the process since perturbation of $G\alpha_{12}$ function resulted in mitotic defects. We have also suggested that in cytokinesis $G\alpha_{12}$ might regulate the RhoGEF protein GEF-H1. GEF-H1 is a microtubule-regulated exchange factor that couples microtubule dynamics to RhoA activation. Interestingly, the results obtained provided strong evidence of GEF-H1 as a novel $G\alpha_{12}$ downstream effector.

1. $G\alpha_{12}$ REGULATES CYTOKINESIS

Conventional theory locates G proteins to plasma membrane. Indeed, there is extensive experimental data regarding the post-translational modifications of G proteins, which are thought to facilitate their membrane localization (Sánchez-Fernández *et al.*, 2014). Despite this, there is increasing evidence that heterotrimeric G proteins are also present at cellular locations distinct from the plasma membrane including the Golgi apparatus (Stow *et al.*, 1991), endoplasmic reticulum (Audigier *et al.*, 1988), cytoskeleton (Carlson *et al.*, 1986), nucleus (Crouch, 1991), and mitochondria (Rezaul *et al.*, 2005; Andreeva *et al.*, 2008; Benincá *et al.*, 2014). Particularly, $G\alpha_{12}$ was reported to be targeted to mitochondria and to be involved in the control of mitochondrial morphology and dynamics (Andreeva *et al.*, 2008).

The studies presented herein have shown a novel and specific role for $G\alpha_{12}$ in cytokinesis. We found that $G\alpha_{12}$ was associated with the spindle apparatus in the late stages of mitosis and it was recruited at the midbody during cytokinesis. This novel localization is associated with a functional phenotype since depletion of $G\alpha_{12}$ promotes multinucleation and an increase of the cell population in G_0/G_1 phase. $G\alpha_{12/13}$ knockout MEF cells have an increase incidence of multinucleation seen under microscopic analysis and also by cell cycle analysis. $G\alpha_{12}$-depleted cells by short hairpin RNA also show multinucleated cells and failed cytokinesis when analyzed under the microscope. Curiously, flow cytometry analysis of these cells showed an accumulation in G_0/G_1 phase suggesting an arrest of the cells in this phase.

Both findings suggest that the absence of $G\alpha_{12}$ has an impact on cell cycle. Curiously, the analysis by flow cytometry of shRNA $G\alpha_{12}$-depleted cells did not show an increase in multinucleated cells. This difference with the knockout cell line could be due to experimental conditions since observation under the microscopy also showed the presence of multinucleated cells. Treatment of cells for flow cytometry analysis involved getting cells in suspension. shRNA-$G\alpha_{12}$ cells that had been grown under puromycin were very sensitive to cellular treatments. It is possible that some of these cells did not recover from the analysis. On the other hand, one has to consider that $G\alpha_{12/13}$ knockout MEF cells are a stable cell

line that came from double deficient embryos. At a stage of E8.25, those embryos have a poorly developed headfold, no somites, and unclosed and sometimes kinked neural tubes and as consequence die at embryonic day E9.5 (Gu *et al.*, 2002). The fact that the cell line is viable means that the loss of $G\alpha_{12/13}$ proteins has been compensated somehow and it is possible that the G_0/G_1 arrest has been overcome. At last, we cannot forget that knockout cells are depleted of both $G\alpha_{12}$ and $G\alpha_{13}$, instead shRNA treated cells have only diminished expression of $G\alpha_{12}$. It is important to note that the downregulation of other proteins implicated in cytokinesis promoted G_0/G_1 arrest (Gromley *et al.*, 2003; Asiedu *et al.*, 2009). Centriolin is a protein required for the later stages of cytokinesis which depletion resulted in long intercellular bridges as well as cell arrest in G_0/G_1 phase (Gromley *et al.*, 2003). Additionally, the elimination of the centrosome with a microneedle or by laser microsurgery caused defects in cytokinesis and failed to enter S phase and arrest at G_0/G_1 phase (Hinchcliffe *et al.*, 2001; Piel *et al.*, 2001). On the other hand, cell cycle analysis by flow cytometry cannot be used to definitively conclude that cells are arrested in G_0/G_1. An increase in G_0/G_1 phase could also be due to a delayed cell cycle progression. In order to confirm an arrest in G_0/G_1 phase it will be needed a time course experiment measuring the length of the cell cycle. Interestingly, and in line with these observations, we found that $G\alpha_{12}$ also colocalizes with pericentrin, which forms a complex with γ-tubulin at the centrosome, in dividing cells. Overall, both cell lines present defects in cell division, which is compatible with a role of $G\alpha_{12}$ in mitosis.

The use of time-lapse video microscopy was very informative since it allowed us to identify different mitotic phenotypes in $G\alpha_{12}$ shRNA HeLa cells, most of them resulting in defective cytokinesis. A majority of $G\alpha_{12}$-depleted cells displayed complete contractile ring ingression, but then some cells showed unresolved cytokinesis with membrane blebbing and the presence of persistent intercellular bridges. Other cells showed loss of membrane stability during late stages of mitosis, though they progress properly through mitosis they were not able to resolve abscission regressing to form binucleated cells, as mentioned before. Despite of that part of the cells presented aberrant mitosis, some cells eventually became separated which could be due to the residual expression of $G\alpha_{12}$. The mitotic phenotypes observed here are reminiscent of the depletion of

mitotic regulators, such as, the RhoA effector citron kinase or the actin bundling protein Anillin (D'Avino *et al.*, 2004; Echard *et al.*, 2004). The depletion of Anillin induced extensive membrane blebbing in the cleavage area resulting in cytokinesis failures. It has been reported that Anillin is important at several stages during post-furrowing events in cytokinesis, contributing to midbody maturation and stability. On the other hand, depletion of citron-kinase also produced multinucleate phenotype. Cells can progress normally through mitosis, central spindle assembly, and cytokinesis furrow ingression. However, they cannot terminate properly and the cells abruptly merged (Echard *et al.*, 2004). As $G\alpha_{12}$ might be important for cortical stability of the mitotic spindle, as well as for the regulation of RhoA activity in the context of cytokinesis, the question that remains to be answered is whether $G\alpha_{12}$ should be important for the formation and maturation of the midbody.

It is noteworthy that recent evidences pointed out that some members of the subfamily of $G\alpha_i$ subunits were present at the centrosomes and the midbody, playing essential roles in mammalian cell division (Cho and Kehrl, 2007). Forced expression of GTPase-deficient $G\alpha_{i1}$ resulted in defective cytokinesis whereas $G\alpha_{i3}$ wild type caused prolonged mitosis though it did not affect cytokinesis. The authors propose that the difference may arise from their differing intracellular localization. In *Drosophila*, $G\alpha_i$ is involved in directing asymmetric cell division in both neuroblasts and SOP cells (Schaefer *et al.*, 2001). It has been shown that overexpression of Gαi or depletion of heterotrimeric G protein complexes causes defects in both spindle orientation and determinant localization in both cell types, suggesting that heterotrimeric G proteins integrate two distinct pathways to orient asymmetric cell division. Also some Gα subunits ($G\alpha_{i1}$, $G\alpha_s$, $G\alpha_o$) have been shown to activate tubulin GTPase (Roychowdhury *et al.*, 1999). $G\alpha_{12/13}$ proteins are necessary for microtubule-organizing center (MTOC) polarity and microtubules dynamics (Goulimari *et al.*, 2008). $G\alpha_{12/13}$ proteins critically regulate microtubule dynamics since $G\alpha_{12/13}$ knockout MEF cells presented altered advancement rates. The authors propose a model in which $G\alpha_{12/13}$ might exert essential functions in MTOC polarization and microtubules dynamics during directional cell movement through LARG and mDia1. $G\alpha_{12/13}$ regulation of microtubules has been shown during cell migration and no information was known for other processes. Our results demonstrate that $G\alpha_{12}$ is present at the spindle apparatus through different

stages and concentrate at the late stages of mitosis. It is possible that $G\alpha_{12}$ has a role regulating the Rho function in cytokinesis but also in microtubule organization is earlier stages. Although the results obtained with video microscopy of dividing cells does not show any differences in the time frame between anaphase and telophase in presence of reduced levels of $G\alpha_{12}$. So, any functional effect of $G\alpha_{12}$ on microtubule dynamics should happen during telophase.

2. IDENTIFICATION OF GEF-H1 AS A NOVEL EFFECTOR FOR $G\alpha_{12}$

GEF-H1 is a member of the Dbl family of RhoGEFs belonging to a subfamily also composed by p114RhoGEF, p190RhoGEF, and AKAP-Lbc proteins. Recent evidences show that G proteins can stimulate these RhoGEFs although the region responsible for the interaction is still unknown (Diviani *et al.*, 2001; Niu *et al.*, 2003). As mentioned in the introduction, this subfamily of GEFs is closely related to the members of the RH subfamily: p115RhoGEF, LARG, and PDZRhoGEF. However, the main difference between the two subfamilies is the fact that they do not contain the RH domain. In order to search for a putative G protein binding domain in GEF-H1, we performed sequence comparison between the members of the GEF-H1 subfamily and the RH subfamily (Figure R15). The coiled-coil motif in the C-terminal region of the GEF-H1 subfamily shears weak homology with the RH domain (between 11 and 16%). Though this weak homology would have been insignificant it was particularly interesting that a group of residues were conserved being the ones important for the helix-helix structure of the RGS domains. In fact, the RH structure *per se* shares quite low homology with the RGS structure (Ross and Wilkie, 2000). So, it could be possible that this coiled-coil structure behave as an RGS-like domain. Previous reports showed that AKAP-Lbc could be stimulated by $G\alpha_{12}$ promoting RhoA activation although $G\alpha_{13}$ was not be able to stimulate its activity (Diviani *et al.*, 2001). In addition, the splice variant proto-Lbc was found to form complexes with $G\alpha_{12}$ coupling GPCR signaling to RhoA. The authors suggested that Lbc-RhoGEF might contain a putative RH-like domain in the C-terminal region through which Lbc-RhoGEF would interact with $G\alpha_{12}$ (Dutt *et al.*, 2004). Parallel results from our group identify p190RhoGEF as a

novel downstream effector of $G\alpha_{12/13}$ proteins; in this case $G\alpha_{13}$ interacts and activates p190RhoGEF. This work let us suggest that $G\alpha_{13}$ may be an important regulator of colon cancer metastasis ((Masià *et al.*, 2014) submitted). Interestingly, the interaction between p190RhoGEF and $G\alpha_{13}$ is through the coiled-coil domain located at the C-terminal region of the RhoGEF.

Using structural modeling approaches our group was able to suggest a model for the interaction between p190RhoGEF and $G\alpha_{13}$, wherein p190RhoGEF could interact with $G\alpha_{13}$ through residues along the α1, α3, α8 and α9 helices ((Masià *et al.*, 2014) submitted) (Figure D1A), which will be in agreement with the known interaction between RH structures with $G\alpha_{12/13}$ proteins. The crystal structure of the RH domain of p115RhoGEF in complex with the chimera of $G\alpha_{13/i\text{-}5}$ identified two distinct surfaces of RH that interact with Gα subunit (Chen *et al.*, 2005). The first involves the N-terminal βN-αN hairpin of RH (residues 17-39). The second contact surface involves several residues among the p115RhoGEF RH domain (residues 44-233: α2-α11), which contact with Switch II and the α3 helix of $G\alpha_{13/i\text{-}5}$. Specifically, α3-α4, α8-α9 and α10-α11 loops and helix α8 of RH domain are all involved in contacts with $G\alpha_{13/i\text{-}5}$. Like the RH domain of p115RhoGEF, the surface of the RH-like domain of p190RhoGEF proposed to interact with $G\alpha_{13}$ is negatively charged. It is interesting the fact that GEF-H1 is the most divergent member among the subfamily (Cook *et al.*, 2013) and it is likely that GEF-H1 evolved independently of p190RhoGEF, p114RhoGEF and AKAP-Lbc. Within the coiled-coil domain the most divergent residues lay in the α8 to α12 helices. Comparison between GEF-H1 and p190RhoGEF show two residues: Glu1484 and Arg1490, located within α8 and α9 helices that are not conserved between these two proteins (Figure D1B). Interestingly, recent evidence of our group showed that point mutants of p190RhoGEF of these charged residues, considerably reduced their binding to $G\alpha_{13}$ (Izquierdo, I). Additionally, one of the most remarkable differences is the length of helix α10 of GEF-H1, which is the shortest between the members of the subfamily (Figure D1B). Since our results show that $G\alpha_{12}$ do not bind the C-terminal region of the GEF-H1 protein, we propose that the divergent sequence found in the area of helices α8 to α12 would be the responsible of the lost of this interaction.

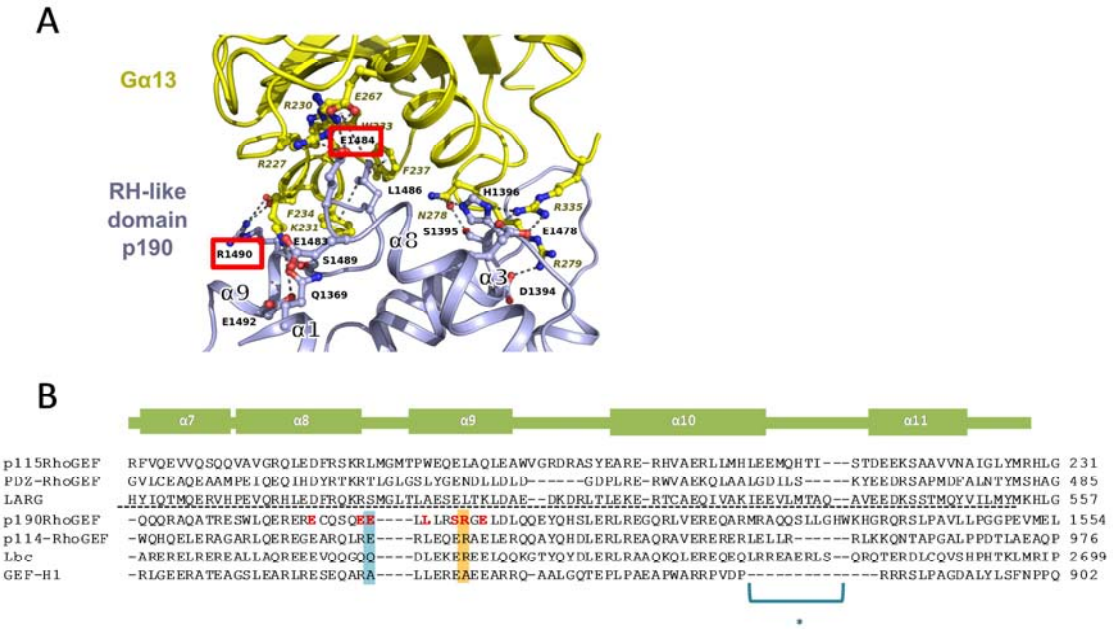


Figure D1. Computer model and sequence alignment of the coiled-coil region of GEF-H1 subfamily RhoGEFs **(A)** Computer model of the RH-like domain of p190RhoGEF in complex with $G\alpha_{13}$. RH-like domain of p190RhoGEF is shown in grey and $G\alpha_{13}$ is shown in yellow. **(B)** Sequence alignment between human RH-RhoGEFs and human members of GEF-H1 subfamily. Color boxes shown Glu1484, in blue, and Arg1490, in orange, of p190RhoGEF.

p114RhoGEF has been reported to be stimulated directly through its interaction with Gβγ (Niu *et al.*, 2003). Being a close related member of the GEF-H1 family we tested the involvement of Gβγ subunits in the activation of GEF-H1. We could not find any combination of Gβγ subunits that did co-immunoprecipitate with GEF-H1, ruling out the possibility of this activation.

As mentioned our results support the hypothesis that $G\alpha_{12}$ binds to the DH-PH domain of GEF-H1. Recent reports have implicated the DH-PH domain of other RhoGEFs in the interaction with Gα subunits. As such, $G\alpha_q$ binds to the DH-PH domain of the p63RhoGEF protein (Lutz *et al.*, 2007). The crystal structure of the $G\alpha_q$:p63-RhoGEF complex revealed that part of the α2 and α3 helices from the DH domain make contact with $G\alpha_q$ (Lutz *et al.*, 2007). On the other hand, recently reports showed that besides the interaction of p115RhoGEF with $G\alpha_{13}$ through its RH domain, p115RhoGEF can also interact through the Dbl homology domain (Chen *et al.*, 2012). The authors propose a model in which the binding of the RGS-

homology domain to $G\alpha_{13}$ facilitates the interaction with the DH domain, thereby regulating its guanine nucleotide exchange activity. However, the DH domain alone may not be sufficient to associate with the Gα subunit since the binding affinity between DH and activated $G\alpha_{13}$ is low. Other studies demonstrated that LARG could also interact through its DH-PH domain with $G\alpha_{13}$, besides the RH domain and the C-terminal region (Suzuki, Tsumoto, *et al.*, 2009). So, interactions between the DH domains of RhoGEFs and their regulatory proteins might be a common mechanism for regulation.

Many members of the Dbl family of RhoGEFs are maintained in a basal inactive conformation by intramolecular interactions involving the DH and PH domains as well as other regulatory sequences. Such interactions have been proposed to block the access of Rho GTPases to the DH domain and/or suppress the GEF activity of the exchange factor (Aghazadeh *et al.*, 2000; Bi *et al.*, 2001; Zheng, 2001). Different studies indicate that members of the RH-RhoGEFs subfamily can form homo- and hetero-oligomers by their C-terminal region in order to regulate their RhoGEF activity (Eisenhaure *et al.*, 2003; Chikumi *et al.*, 2004). Regarding to GEF-H1 regulation, it has been shown that GEF-H1 is regulated by phosphorylation but not much is known about the possible regulation by intramolecular interactions. Previous reports showed that deletion of C-terminal region of GEF-H1 dramatically increases its basal RhoGEF activity compared to its full length form (Krendel *et al.*, 2002), suggesting an inhibitory role for the C-terminal region. We found that GEF-H1 could dimerize and most likely through its C-terminus since more GEF-H1 was co-immunoprecipitated with the C-terminal truncated form of GEF-H1. Despite of the higher expression levels of the C-terminal form of GEF-H1 compared to the full length form it is important to note that similar expression levels were found with the N-terminal form however, less GEF-H1 was co-immunoprecipitated. So, these results might indicate that the dimerization takes place mainly through the C-terminal region, and most likely through the coiled-coil motif as it has seen before for the others RH-RhoGEFs. Nevertheless, we cannot discard the possibility that the N-terminal region of GEF-H1 could also be involved in intramolecular interactions of the protein because we also observed co-immunoprecipitation of GEF-H1 with this truncated form. By contrast, $G\alpha_{12}$ binding to GEF-H1 does not disrupt the dimerization and this is

consistent with previous data in which the binding of $G\alpha_{13}$ to the RH domain of p115RhoGEF, PDZ-RhoGEF and LARG does not affect their oligomerization (Chikumi *et al.*, 2004). One explanation for this result could be that the binding site for $G\alpha_{12}$ is located at the DH-PH domains of GEF-H1 and probably other modulators may be involved in the regulation mechanism of GEF-H1. Further experiments will be necessary to ascertain the possible role of the C-terminal domain on GEF-H1 activity.

It is well established the regulation of GEF-H1 through its binding to microtubules (Krendel *et al.*, 2002). The proposed model suggests that microtubule-bound GEF-H1 is inactive and its release from microtubules leads to an increase in its GEF activity towards Rho. So, the observation of a decrease in the amount of GEF-H1 bound to microtubules in the presence of the constitutively active form of $G\alpha_{12}$ supports the notion that $G\alpha_{12}$ would be promoting the release of GEF-H1 from microtubules in order to activate the protein, triggering the activation of RhoA. However, further experiments with purified proteins and microtubules would help to conclude this model.

As it has been mentioned before GEF-H1 could be regulated by phosphorylation. GEF-H1 undergoes phosphorylation at 38 different residues (including serine, threonine and tyrosine residues by Phosphosite (www.phosphosite.org)), indicating a complex regulation of its activity. A lot of work has been done studying the phosphorylation of serine 885. It has been reported that GEF-H1 can be phosphorylated at S885 by Aurora A/B kinases, PAK1, Par1b kinase and PKA (Zenke *et al.*, 2004; Birkenfeld *et al.*, 2007; Meiri *et al.*, 2009; Yamahashi *et al.*, 2011). For instance, during early mitosis GEF-H1 is phosphorylated by the mitotic kinases Aurora A/B and the complex Cdk1/cyclin B in order to inhibit its catalytic activity. Just prior to enter in cytokinesis, GEF-H1 is dephosphorylated allowing the activation of RhoA (Birkenfeld *et al.*, 2007). Additionally, Par1b could phosphorylate GEF-H1 to inhibit RhoA-promoted stress fibers formation (Yamahashi *et al.*, 2011). On the other hand, PAK1 phosphorylates GEF-H1 inducing 14-3-3 binding and relocation of 14-3-3 to microtubules (Zenke *et al.*, 2004). Furthermore, after PKA phosphorylation GEF-H1 also associates with 14-3-3 and its exchange activity was suppressed (Meiri *et al.*, 2009). These

observations suggest that S885 behave as an inhibitory switch of GEF-H1, which is targeted by multiple distinct kinases in a context-dependent manner.

Since phosphorylated GEF-H1 at S885 was reported to induce 14-3-3 binding that inhibits the exchange activity of GEF-H1 (Meiri *et al.*, 2009) we investigated whether $G\alpha_{12}$ could be affecting this interaction. 14-3-3 proteins are regulatory proteins that bind to a variety of cellular targets, including the proapoptotic protein Bad, Raf kinase, cell cycle-dependent phosphatase Cdc25, and others (Fu *et al.*, 2000). 14-3-3 binding regulates the subcellular localization and activity of these proteins and their ability to interact with other components of intracellular signaling pathways. Surprisingly, our results showed that only when the constitutively active form of $G\alpha_{12}$ was present we observed co-immunoprecipitation of 14-3-3 γ with GEF-H1. Our results are unexpected since our first hypothesis was that $G\alpha_{12}$ would reduce the interaction between 14-3-3 and GEF-H1 or conversely, that 14-3-3 would affect the co-immunoprecipitation of $G\alpha_{12}$ with GEF-H1. Instead, we observed an enhancing of the 14-3-3 binding to GEF-H1 when $G\alpha_{12}$ was present. Experiments were repeated several times with the same results and with different constructs of GEF-H1. Whereas the N-terminal form was not able to co-immunoprecipitate 14-3-3, the C-terminal form only co-immunoprecipitated 14-3-3 in presence of $G\alpha_{12}$. It is described that 14-3-3 binds to the C-terminal region of the protein (Zenke *et al.*, 2004; Meiri *et al.*, 2009). 14-3-3 binds mainly to phosphorylated residues of targeting proteins. We also tested whether the interaction between GEF-H1 and 14-3-3 was dependent of phosphorylation. Indeed, when we perform the co-immunoprecipitation in absence of phosphatase inhibitors a clearly decrease in the co-immunoprecipitated 14-3-3 was observed, corroborating the previous reports. Again in those conditions only 14-3-3 was co-immunoprecipitating with GEF-H1 in the presence of $G\alpha_{12}$. As consequence these results do not fit with a model wherein $G\alpha_{12}$ activates GEF-H1 and 14-3-3 might be inhibiting GEF-H1 catalytic activity. One possible explanation of the discrepancy could be that the co-immunoprecipitation assays were done with the 14-3-3 γ isoform whereas results shown 14-3-3 inactivation of GEF-H1 are done with the 14-3-3 ζ , ε and η isoforms (Zenke *et al.*, 2004; Meiri *et al.*, 2009). There are seven isoforms of 14-3-3 proteins in mammals, so it is plausible that different isoforms are implicated in different regulatory

pathways. On the other hand, it could also be possible that $G\alpha_{12}$ activates GEF-H1, therefore the protein takes an open conformation that allows its phosphorylation and later the binding to 14-3-3 which will lead to its inactivation. In fact, Meiri et al., (2012) suggest a regulatory mechanism that links GEF-H1 to microtubules through the dynein motor light chain Tctex-1 protein (Meiri *et al.*, 2012). In this report the authors demonstrate that Tctex-1 anchors GEF-H1 to microtubules through an inhibitory mechanism that involves PKA-phosphorylation of S885 of GEF-H1 and consequently 14-3-3 binding. Nevertheless, to date several reports (Zenke *et al.*, 2004; Meiri *et al.*, 2009) have been identified 14-3-3 proteins as binding partners for GEF-H1 and most likely being as a negative regulators for its catalytic activity. However, the mechanism of regulation of GEF-H1 activity seems to be more complicated and probably more players are needed in order to understand the molecular basis where $G\alpha_{12}$ was activating GEF-H1 and triggering its release from microtubules.

2.1 Proposed model

Based on all the information collected (including binding regions and activation and deactivation mechanisms), we propose a working model to account for the sequential events involved in GEF-H1 activation by $G\alpha_{12}$ (Figure D2). Initially GEF-H1 is bound to microtubules with 14-3-3 dimer that might be forcing the protein in a close conformation. In this inactive state GEF-H1 is phosphorylated in two residues: S885, the primary binding site for 14-3-3 proteins, and T114, a second ancillary site as it was described previously (Meiri *et al.*, 2009). Upon the action of some modulator protein, which we hypothesize that would be a phosphatase, GEF-H1 would be dephosphorylated promoting a conformational change (1). In an open conformation, the DH-PH domains are free to interact with active $G\alpha_{12,}$ which promotes the release of GEF-H1 from microtubules and GEF-H1 activation. Consequently, activated GEF-H1 will be able to activate Rho (2). When the cell needs to finish the signaling another regulatory protein would be acting, and we speculate that in this case would be a kinase. GEF-H1 would be phosphorylated on residue S885 in order to be inactivated (3). We speculate that this phosphorylation will promote a first 14-3-3 binding and $G\alpha_{12}$ displacement

from GEF-H1 (4). Thus, 14-3-3 binding will promote a conformational change on GEF-H1 that facilitates the $G\alpha_{12}$ release and a secondary binding of other 14-3-3 monomer, returning the protein to its inactive initial state (6). At this point we hypothesize that GEF-H1 can also undergo dimerization in order to inactivate its catalytic activity as an alternative mechanism to microtubules binding (5). And for the action of some regulatory protein GEF-H1 will dissociates and re-enters into the cycle again (7).

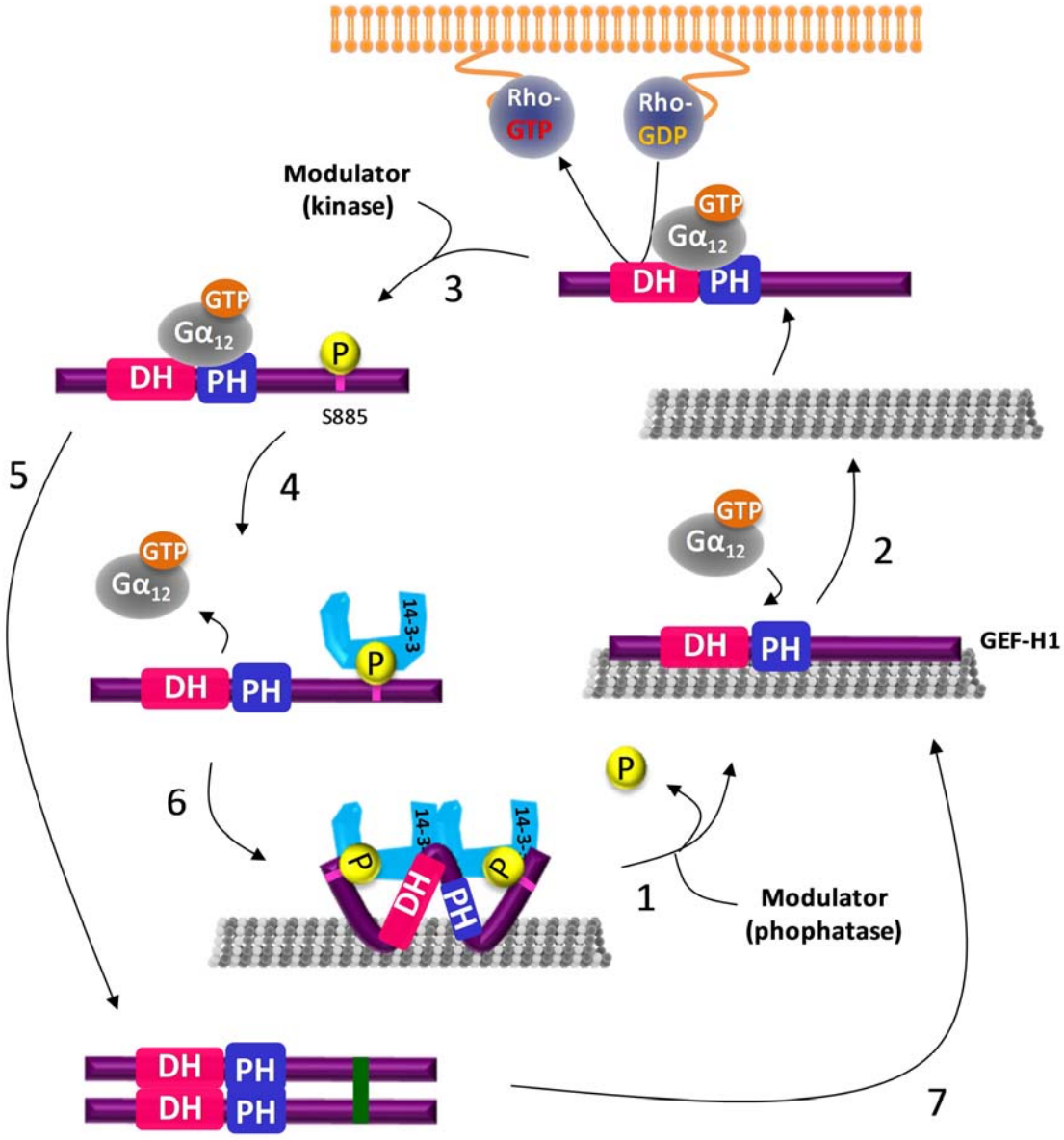


Figure D2. Proposed model of the molecular mechanism for GEF-H1 activation by $G\alpha_{12}$.

A number of questions remain to be addressed. For instance, the interplay between $G\alpha_{12}$ and 14-3-3 proteins has to be clarified. We have shown that the interaction between 14-3-3 and GEF-H1 is enhanced by the presence of $G\alpha_{12}$,

whereas it was previously reported that the binding of 14-3-3 had a inhibitory role for GEF-H1 activity (Meiri *et al.*, 2009). However, is not clear whether 14-3-3 can inhibit GEF-H1 activity. It is known that after GEF-H1 phosphorylation on S885 the protein translocates to the microtubules, and that phosphorylation promotes 14-3-3 binding. Probably 14-3-3 proteins are not able to inhibit GEF-H1 *per se* because they need a kinase for its binding to GEF-H1 but they would maintain GEF-H1 in a close conformation inhibiting its catalytic activity. 14-3-3 proteins were reported to interact with other RhoGEFs. For instance, AKAP-Lbc was shown to be inhibited by 14-3-3 binding upon PKA phosphorylation because 14-3-3 interferes with the interaction between AKAP-Lbc and RhoA (Diviani *et al.*, 2004). On the other hand, p190RhoGEF can also interact with 14-3-3 (Zhai *et al.*, 2001). However, whether this interaction affects the signaling properties of p190RhoGEF remains to be elucidated.

In this model we propose the needed of kinases and phosphatases in order to regulate the pathway. Although the specific players remain to be elucidated, $G\alpha_{12}$ would be promoting the recruitment and/or regulation of the kinases or phosphatases involved in the process. For instance, it is known that $G\alpha_{12}$ can activate the phosphatase PP2 in order to regulate the microtubule-phosphorylated protein tau (Zhu *et al.*, 2004). Interestingly, the RH-RhoGEF LARG requires Tec phosphorylation to be able to interact with $G\alpha_{12}$ (Suzuki *et al.*, 2003). The authors propose a mechanism in which activated $G\alpha_{12}$ may recruit Tec in close proximity of LARG and facilitate its phosphorylation. However, tyrosine phosphorylation of LARG by Tec does not affect its basal RhoGEF activity, but rather changes its regulation by Gα subunits.

On the other hand, it is unclear which effect would exert the dimerization on GEF-H1 activity. As it is commented before, several RhoGEFs are regulated by intramolecular interactions that dictate the accessibility of the DH domain to RhoGTPases maintaining them in an inactive conformation (Chikumi *et al.*, 2004; Baisamy *et al.*, 2005). Nevertheless, further experiments are needed in order to elucidate the entire molecular mechanism between GEF-H1 and $G\alpha_{12}$.

3. SUBCELLULAR LOCALIZATION OF GEF-H1

Recent evidences showed that GEF-H1 is implicated in the regulation of vesicles trafficking of endocytic recycling and exocytosis (Pathak *et al.*, 2012). GEF-H1 has been shown to be involved in the regulation of the exocytic complex through the activation of RhoA. Moreover, the exocyst complex is also involved to target vesicles to the midbody region during cytokinesis (He and Guo, 2009). Our results of GEF-H1 localization at Golgi apparatus would be in line with these previous observations. It is likely that the perinuclear localization of GEF-H1 in MEFs cells would be related with vesicle trafficking since the Golgi-plasma membrane trafficking is regulated by RhoA. Furthermore, colocalization experiments revealed colocalization of endogenous GEF-H1 with Rab11 and caveolin, while no colocalization was observed with Rab1 and Mannosidase II. Pathak and co-workers showed that depletion of GEF-H1 leads to an accumulation of Rab11-positive vesicles (Pathak *et al.*, 2012). Thus, Rab11, a well established recycling endosome and a post-Golgi protein marker, and GEF-H1 may be involved in the regulation of the recycling pathway. Interestingly, furrow ingression and abscission are accompanied by an increase in plasma membrane surface area by vesicle trafficking (Schweitzer *et al.*, 2005; Echard, 2008), which in fact is needed for cleavage furrow ingression (Danilchik *et al.*, 1998; Lecuit and Wieschaus, 2000) and it continues until late telophase stages (Shuster and Burgess, 2002). Two distinct populations of vesicles have been associated with completion of cytokinesis: Golgi-derived vesicles and vesicles derived from recycling endosomes (Echard, 2008). In particular, vesicles derives from recycling endosomes can be visualized by the small GTPase Rab11 (Yu *et al.*, 2007). So, we speculate that the colocalization between GEF-H1 and Rab11 would be in the recycling endosomes involved in cytokinesis. GEF-H1 also colocalized with caveolin, which is related with the Golgi apparatus as be involved in receptor-independent endocytosis. These colocalization findings may be cell-specific despite of the fact that this subcellular localization of GEF-H1 was only found in MEFs cells. To date, any evidence has been reported regarding a possible role for $G\alpha_{12}$ in vesicles trafficking.

A recent report shows how RhoA/GEF-H1 regulates the coordination of actin and microtubule cytoskeleton modulation and vesicle trafficking during migration and cell division (Pathak and Dermardirossian, 2013). Thus, it seems to be an emerging issue though the molecular mechanism underlying the process remains largely unclear. Given the fact that $G\alpha_{12}$ activates GEF-H1 during cytokinesis and GEF-H1 would be also implicated in other pathways regulated by $G\alpha_{12}$, the question that raises now is to answer whether $G\alpha_{12}$ would be upstream of GEF-H1-vesicle trafficking regulation.

4. INVOLVEMENT OF GEF-H1 IN $G\alpha_{12}$ SIGNALING PATHWAYS

Although RhoA activities have been reported to be required for cell cycle progression at different phases, most studies have concentrated on the role of RhoA in cytokinesis where the activity of RhoA is essential. RhoA organizes the assembly of the contractile ring and induces the actomyosin-driven constriction of the cleavage furrow (reviewed in (Barr and Gruneberg, 2007). RhoGEFs are critical proteins necessary for the proper assembly, constriction, and stability of the contractile ring (Tatsumoto *et al.*, 1999; Wu *et al.*, 2006; Birkenfeld *et al.*, 2007; Su *et al.*, 2011). ECT2 localizes and activates RhoA at the cleavage furrow (Nishimura and Yonemura, 2006), whereas MyoGEF act as a functional unit at the cleavage furrow to advance furrow ingression during cytokinesis (Wu *et al.*, 2006). Other reports position GEF-H1 and LARG in the late stages of cytokinesis. Being GEF-H1 in the steps before abscission mediating RhoA activation at the cell cortex (Birkenfeld *et al.*, 2007) and LARG necessary for the last step, abscission (Martz *et al.*, 2013). The fact is that both GEF-H1 and LARG are downstream effectors of $G\alpha_{12}$ ((Suzuki *et al.*, 2003) and our data presented here) and that $G\alpha_{12}$ is precisely associated with the spindle apparatus and located at the midbody during cytokinesis suggesting that most likely the activation of these proteins by $G\alpha_{12}$ takes place in a mitotic context. In fact, we could observe GEF-H1 and $G\alpha_{12}$ colocalizing in a ring-like structure that should be the midbody. Moreover, the interaction between LARG and $G\alpha_{12}$, as well as its catalytic activity, it is regulated by phosphorylation (Suzuki *et al.*, 2003) and GEF-H1 is also regulated by several

kinases. So, it is plausible that the coordination between the regulation of GEF-H1 and LARG by $G\alpha_{12}$ would be a key process in the control of cytokinesis (Figure D3). It will be very challenging to demonstrate the precise requirement of $G\alpha_{12}$ and the GEFs since depletion of either of proteins give rise to failed cytokinesis (Martz *et al.*, 2013).

For the future, it will be interesting to analyze how different RhoA GEFs are coordinated through microtubules, mitotic kinases, and associated signaling factors to modulate and fine tune different aspects of mammalian cell cytokinesis and abscission.

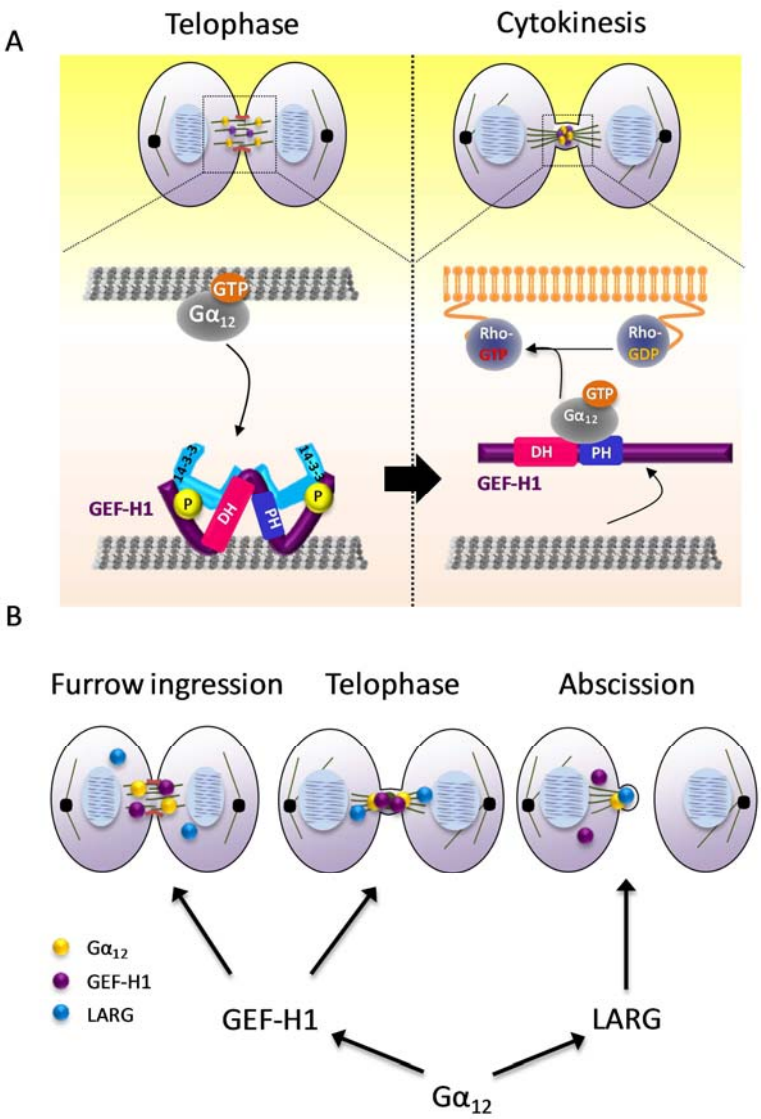


Figure D3. Putative models in mitosis **(A)** Model of the putative mechanism of interaction between GEF-H1 and $G\alpha_{12}$ in mitosis**. (B)** Model of the regulation by $G\alpha_{12}$ during mitosis.

$G\alpha_{12}$ has been shown to stimulate proliferation in many different cell lines. Several studies have identified that the mitogenic signaling by $G\alpha_{12}$ involves inputs from multiple signaling pathways emanating from the Ras as well as Rho family of GTPases, JNK, COX2, and β-catenin (Chan *et al.*, 1993; Dhanasekaran and Dermott, 1996; Meigs *et al.*, 2001). While these studies have suggested that $G\alpha_{12}$ is critically involved in cell growth regulation, the molecular mechanisms involved in this process of transformation or proliferation remain unclear. It is well known the transforming potential of $G\alpha_{12/13}$ proteins in NIH3T3 cells (Jiang *et al.*, 1993). Regarding the underlying transformation mechanism, Rac seems to play a greater role in the transforming activity of $G\alpha_{12}$ than RhoA or Cdc42 in NIH3T3 cells (Tolkacheva *et al.*, 1997). Interestingly, our results showed that GEF-H1-depleted NIH3T3 cells lost the transforming activity when the constitutive active form of $G\alpha_{12}$ was present, suggesting a putative role for GEF-H1 in this $G\alpha_{12}$-induced signaling pathway. Although GEF-H1 was originally described as a nucleotide exchange factor for Rac and RhoA (Ren, 1998), later studies suggest to be RhoA-specific (Krendel *et al.*, 2002). However, recent evidences link again GEF-H1 to Rac activation (Callow *et al.*, 2005). The authors proposed a model in which through PAK4 phosphorylation GEF-H1 would negatively regulate Rho and activate Rac. So, it would be possible that GEF-H1 will activate Rac after $G\alpha_{12}$-stimulation to promote cell transformation. Nevertheless, these are ongoing studies and more experiments are needed in order to investigate the molecular mechanism and the players involved in this pathway.

Directional cell migration is a fundamental mechanism in wound repair and embryogenesis and it is well known that many of these cytoskeletal changes are brought by Rho-family GTPases. It has been reported that $G\alpha_{12/13}$ proteins are absolutely essential for directional cell migration and wound healing involving Rho activity at the leading edge, which may control the formation of stable microtubules via Dia1 (Goulimari et al., 2005). HeLa knockdown cells for GEF-H1 showed a decrease in cell migration efficiency compared to control cells. These results would indicate that GEF-H1 is involved in cell motility. Although the implication of GEF-H1 in cell migration was already published (Nalbant *et al.*, 2009), further experiments should be done in order to ascertain whether GEF-H1 could be downstream of $G\alpha_{12}$-induced cell migration.

CONCLUSIONS

1. $G\alpha_{12}$ regulates mitosis:

1. $G\alpha_{12}$ is localized with the spindle apparatus in the late stages of mitosis, mainly in anaphase and telophase as well as in the midbody during cytokinesis.

2. Utilizing $G\alpha_{12}$-GFP we demonstrated that $G\alpha_{12}$ colocalizes with pericentrin in centrosomes structures during mitosis in HEK-293 cells. Endogenous $G\alpha_{12}$ is also found with pericentrin in murine embryonic fibroblasts.

3. Perturbation of $G\alpha_{12/13}$ function results in an increase of polinucleated cells in $G\alpha_{12/13}$ knockdown murine embryonic fibroblasts.

4. Loss of function of $G\alpha_{12}$ protein utilizing shRNA promotes aberrant mitosis, with cells undergoing completely collapse during cytokinesis, apoptosis or binucleation in HeLa cells.

5. shRNA-$G\alpha_{12}$-depleted cells present an increase in cells in G_0/G_1 phase of cell division analyzed by flow cytometry.

2. Identification of GEF-H1 as a novel effector for $G\alpha_{12}$:

6. $G\alpha_{12}$ but not $G\alpha_{13}$ co-immunoprecipitates with GEF-H1 in HEK-293 cells.

7. GEF-H1 co-immunoprecipitates with $G\alpha_{12}$ through its DH-PH domains.

8. $G\alpha_{12}$ activation enhances the binding to GEF-H1, proven by treating cells with AlF_4^-; expressing the constitutive active mutant of $G\alpha_{12}$ ($G\alpha_{12}$ Q231L) and the dominant inactive mutant $G\alpha_{12}$G228A.

9. $G\alpha_{12}$ promotes activation of GEF-H1, reflected by the induction of GEF-H1 binding to the inactive form of RhoA (RhoAG17A).

10. GEF-H1 mediates $G\alpha_{12}$-Rho activity assessed by measuring the SRE promoter luciferase activity in HEK-293 cells.

11. $G\alpha_{12}$ promotes the release of GEF-H1 from microtubules.

12. $G\alpha_{12}$ enhances the binding between GEF-H1 and 14-3-3 proteins.

3. Subcellular localization of GEF-H1:

13. GEF-H1 is localized at the Golgi, and colocalized with Rab11 and caveolin in MEF cells.

4. Involvement of GEF-H1 in $G\alpha_{12}$ signaling pathways:

14. GEF-H1 colocalizes with $G\alpha_{12}$ along the microtubule in central spindle in telophase and in the midbody during cytokinesis.

15. GEF-H1 is necessary for $G\alpha_{12}$-induced cell transformation in NIH3T3 cells.

16. GEF-H1 is involved in cell migration analyzed by the wound-healing method.

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RESUM EN CATALÀ

INTRODUCCIÓ

Les proteïnes G representen una gran família de proteïnes que s'encarreguen de la homeòstasi de les cèl·lules i de coordinar la senyalització entre els receptors acoblats a proteïnes G, que es troben a la membrana plasmàtica, amb els seus efectors intracel·lulars. Els receptors acoblats a proteïnes G són unes proteïnes composades per set dominis transmembrana que uneixen una gran diversitat de lligands i molècules petites. Representen una de les famílies més grans, amb més de 800 membres identificats al genoma humà. Quan una senyal extracel·lular s'uneix al receptor provoca un canvi conformacional que promou l'activació de les proteïnes G heterotrimèriques, les quals estan associades a la membrana plasmàtica i al receptor. Hi ha diferents famílies de proteïnes G dependent del receptor i de la via de senyalització.

Les proteïnes G heterotrimèriques estan formades per tres subunitats: Gα, Gβ i Gγ. Un cop el receptor ha sigut activat, es produeix un canvi conformacional en la subunitat Gα que facilita la seva activació intercanviant el GDP per GTP. A la vegada l'activació provoca la dissociació de la subunitat Gα del dímer Gβγ, i això la conseqüent activació dels seus efectors. El cicle de senyalització acaba un cop la subunitat Gα hidrolitza el GTP a GDP i es torna a associar amb el dímer Gβγ. Les subunitats Gα tenen capacitat intrínseca d'hidrolitzar el GTP, però moltes vegades aquest procés està catalitzat per proteïnes reguladores.

Les subunitats Gα de les proteïnes G es divideixen en quatre famílies: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ i $G\alpha_{12}$. La subfamília G12 està formada pels membres $G\alpha_{12}$ i $G\alpha_{13}$. Aquesta subfamília està implicada en diversos processos fisiològics com per exemple en el desenvolupament embrionari, el creixement cel·lular, la migració cel·lular, els, processos, d'angiogènesi i l'apoptosi. Les proteïnes $G\alpha_{12}$ i $G\alpha_{13}$ poden interaccionar i transmetre els senyals a un ampli ventall de proteïnes efectores. Les proteïnes que poden unir-se directament a les proteïnes $G\alpha_{12/13}$ es poden classificar en tres grans grups: proteïnes efectores, proteïnes adaptadores i proteïnes reguladores. Per tal de posar un exemple de cadascuna, dintre de les proteïnes efectores es trobarien les RhoGEFs o les cadherines, entre d'altres. Com a proteïnes adaptadores es trobarien la xaperona Hsp90 o la quinasa Tec, com a exemple. I per últim serien proteïnes reguladores les fosfatases PP2 i PP5 així com les proteïnes

RGS. De totes maneres, aquesta és una llista que va en augment amb el pas del temps degut al descobriment de noves tècniques experimentals que faciliten la identificació de noves proteïnes que s'uneixen a $G\alpha_{12/13}$.

Una de les vies efectores millor estudiada és la controlada per membres de la família de les RhoGTPases. La família de les RhoGTPases forma part de la súper família Ras, totes elles poden transitar entre un estat actiu, unides a GTP, i un estat inactiu, unides a GDP. Aquestes RhoGTPases estan implicades en una gran diversitat de funcions, com per exemple, el desenvolupament, la polaritat, la migració, el desenvolupament neuronal, la divisió cel·lular i l'adhesió. Aquesta gran família està subdividida en les subfamílies Rho, Rac i Cdc42. El cicle GDP/GTP està controlat per proteïnes reguladores. Per una banda, les responsables de l'activació són les anomenades proteïnes bescanviadores de nucleòtids de guanina, les RhoGEFs. Les RhoGAPs són les encarregades de catalitzar la hidròlisi del GTP a GDP, retornant les proteïnes Rho al seu estat inicial inactiu. També hi han les RhoGDI les quals s'encarreguen de la inhibició de les proteïnes Rho ja que, impedeixen la seva activació.

Una de les subfamílies millor és la família anomenada Dbl-RhoGEFs. Tots els membres d'aquesta família comparteixen un domini d'homologia a les proteïnes Dbl, anomenat DH i un domini d'homologia al domini plecstrina, anomenat PH. S'han descrit quatre membres d'aquesta família que estan regulats per les proteïnes G12. La p115RhoGEF, la PDZ-RhoGEF, la LARG i la AKAP-Lbc. Els tres primers formen una subfamília coneguda com RH-RhoGEFs, ja que al seu domini N-terminal comparteixen una seqüència amb certa homologia al domini RGS contingut a les proteïnes "reguladores de la senyalització per proteïnes G", i és a través d'ell que interaccionen amb les proteïnes G12. L'AKAP-Lbc pertany a una altra subfamília de RhoGEFs on també s'inclouen la p114-RhoGEF, la p190RhoGEF i la GEF-H1. S'ha vist que la p114-RhoGEF també pot ser estimulada pel dímer $G\beta\gamma$, i resultats recents del nostre grup han demostrat que la p190RhoGEF és una nova proteïna efectora per a la senyalització de la $G\alpha_{13}$. La proteïna GEF-H1, per la seva banda, no ha sigut vinculada a l'estimulació a través de proteïnes G.

La GEF-H1 és va descobrir al 1998 com una proteïna associada als microtúbuls. La seva unió a microtúbuls sembla que tindria un paper inhibitori sobre l'activitat catalítica de la proteïna, mentre que la despolimerització dels

microtúbuls per algun estímul extracel·lular provocaria un alliberament de la GEF-H1 dels microtúbuls, i conseqüentment, una activació de Rho. S'ha descrit que pot tenir activitat tant per Rho com per Rac. A part de la regulació per unió a microtúbuls s'ha descrit que té una regulació bastant complexa per fosforilació. Diferents quinases la poden fosforilar en els mateixos residus, però diferent context cel·lular. Per exemple, s'ha vist que les quinases mitòtiques Aurora A/B i el complex Cdk1/ciclina B poden fosforilar la GEF-H1 durant els primers estadis de la mitosi per tal d'inhibir la proteïna. La posterior defosforilació de la GEF-H1 durant la citocinesi li permetrà activar Rho, ja que es tracta d'un procés molt controlat d'una manera espai-temporal. La GEF-H1 s'ha considerat un membre clau en la interconnexió entre el citoesquelet d'actina i la dinàmica dels microtúbuls. També està involucrada en la regulació del cicle cel·lular, així com en la regulació de la polaritat, motilitat i morfologia cel·lular.

El cicle cel·lular es pot dividir en quatre fases: S, M, $G_{0/1}$ i G_2. Durant la fase S té lloc la replicació del DNA i la fase M seria la culminació del cicle cel·lular on les cèl·lules filles es separen. Les altres dos fases, la $G_{0/1}$ i la G_2, aporten temps extra a la cèl·lula per créixer. La fase M està formada per la mitosi i la citocinesi, que és quan té lloc la separació física entre les cèl·lules filles. Dintre de la citocinesi també es poden distingir diferents processos. Primer de tot s'ha d'assemblar l'anell contràctil d'actina (composat per actina, miosina i altres proteïnes) al inici de l'anafase. Durant la progressió cap a la telofase l'anell contràctil servirà de força motora per tal de separar les cèl·lules filles. Un cop les cèl·lules es comencen a separar es forma el pont intercel·lular format principalment per un fus de microtúbuls i que al seu centre té el cos intermedi o "Flemming body". El cos intermedi està format per microtúbuls i per una densa àrea proteica de la qual es desconeix la seva composició. Finalment, el cos intermedi serà l'encarregat de marcar el lloc de l'abscissió i conseqüentment, la separació física de les cèl·lules en divisió.

RESULTATS I DISCUSSIÓ

1. La proteïna $G\alpha_{12}$ regula la mitosi

Resultats anteriors del grup vam mostrar que la proteïna $G\alpha_{12}$ podria tenir un paper rellevant durant la divisió cel·lular. Es va veure que quan microinjectaven anticossos contra la proteïna $G\alpha_{12}$ en cèl·lules d'astrocitoma humanes, la divisió induïda per trombina estava bloquejada. Llavors es va decidir estudiar quin paper podria tenir la $G\alpha_{12}$ durant la mitosi.

Primer de tot es va determinar quin patró de localització subcel·lular mostrava la proteïna $G\alpha_{12}$. En cèl·lules en interfase la tinció de la $G\alpha_{12}$ va mostrar que es trobava distribuïda homogèniament pel citoplasma. Però de manera interessant, es va trobar que la $G\alpha_{12}$ es condensava en una regió molt particular entre dues cèl·lules que s'estaven dividint, al mig del pont intercel·lular que es forma. Aquesta tinció es va observar en diferents línies cel·lulars i també amb la proteïna quimèrica $G\alpha_{12}$-GFP. Quan es va seguir la tinció de la $G\alpha_{12}$ a través de les diferents etapes de la mitosi sincronitzant les cèl·lules amb nocodazol es va veure que la proteïna $G\alpha_{12}$ donava un patró que recordava al dels microtúbuls durant l'anafase i la telofase, i que després es concentrava al cos intermedi. Per tal de caracteritzar a quina estructura subcel·lular es trobava la $G\alpha_{12}$ es va procedir a fer assajos de colocalització amb diferents proteïnes implicades en la mitosi i citocinesi. Una estructura molt important que es forma a les etapes finals de la mitosi és l'anell contràctil d'actina. Aquest anell proporciona la força motora per començar a separar físicament les cèl·lules filles. Però, quan es va fer l'assaig de colocalització amb la fal·loïdina, una droga que s'uneix a la F-actina, no es va veure colocalització. Per tant, es va descartar la idea de què la $G\alpha_{12}$ fos un membre de l'anell contràctil d'actina. A continuació es va avaluar la colocalització amb la α-tubulina. Tant el pont intercel·lular com el cos intermedi estan formats per microtúbuls, addicionalment el cos intermedi conté altres proteïnes però encara es desconeix la gran majoria. Es va trobar que la $G\alpha_{12}$ estava associada als microtúbuls, durant l'anafase i la telofase. En canvi, durant la citocinesi la $G\alpha_{12}$ es concentrava en el cos intermedi. Paral·lelament, es va veure que la $G\alpha_{12}$ colocalitzava també amb la pericentrina, un component dels centrosomes. Per tant,

els nostres resultats indicaven que la $G\alpha_{12}$ estava localitzada al fus mitòtic a les etapes finals de la mitosi i què durant la citocinesi es localitzava principalment al cos intermedi. Degut a la localització amb els microtúbuls es pot pensar que la $G\alpha_{12}$ podria tenir un paper regulador en la seva organització tal com s'ha vist amb anterioritat. Es va descriure que les proteïnes $G\alpha_{12/13}$ eren necessàries per a la polarització del MTOC, centre organitzador de microtúbuls, durant la migració cel·lular.

Si la $G\alpha_{12}$ té un paper important durant la mitosi, la seva falta provocarà defectes en la divisió cel·lular. Per tal de comprovar aquesta hipòtesi es van utilitzar unes cèl·lules deficients en $G\alpha_{12/13}$, la línia *knockout* $G\alpha_{12/13}$ provinent de cèl·lules fibroblàstiques embrionàries de ratolí. Mitjançant microscòpia confocal i amb diferents marcadors de membrana plasmàtica es va observar que comparades amb la línia salvatge, les cèl·lules *knockout* de $G\alpha_{12/13}$ mostraven més cèl·lules polinucleades. Per tal de confirmar aquestes observacions es va dur a terme una anàlisi del cicle cel·lular mitjançant citometria de flux. D'aquesta manera, es va veure que a les cèl·lules *knockout*, efectivament, hi havia un augment de cèl·lules polinucleades comparades amb la línia salvatge.

Addicionalment, es va utilitzar una línia de cèl·lules HeLa a les quals es va silenciar l'expressió de la proteïna $G\alpha_{12}$ mitjançant la infecció amb lentivirus. Sorprenentment, quan es van analitzar les divisions d'aquestes cèl·lules es va veure que presentaven problemes per dividir-se, observant-ne diferents fenotips. Algunes cèl·lules mostraven col·lapse cel·lular, és a dir, començaven a dividir-se amb normalitat però de seguida la membrana cel·lular començava a presentar diferents protuberàncies i inestabilitat de la membrana cel·lular fins que la cèl·lula acabava fent apoptosi. Unes altres cèl·lules, la fracció majoritària del total, presentaven citocinesis fallides que no acabaven de resoldre's. Per exemple, algunes cèl·lules mostraven incapacitat per terminar la divisió amb la presència d'un pont intercel·lular persistent, el inici de la mitosi era normal però en el punt final quan les cèl·lules intenten dividir-se no poden i es queden connectades pel pont intercel·lular. Unes altres cèl·lules acabaven sent polinucleades pel fet de què al intentar separar-se com no podien, es tornaven a fusionar. Per tant, aquests resultats semblen indicar que la falta de la proteïna $G\alpha_{12}$ provoca defectes en la citocinesi. Per altra banda, es va analitzar el cicle cel·lular d'aquestes cèl·lules

knockdown de la proteïna $G\alpha_{12}$ es va veure que hi havia un augment en la població de cèl·lules arrestades en G_0/G_1. Tot i què, moltes cèl·lules sh$G\alpha_{12}$ presentaven polinuleació els resultats obtinguts amb el citòmetre de flux no van coincidir amb els observats prèviament amb les cèl·lules *knockout* $G\alpha_{12/13}$. Això pot ser degut a dues explicacions. Per una banda, una explicació experimental ja què les cèl·lules sh$G\alpha_{12}$ han sigut infectades i seleccionades amb puromicina i part d'aquestes cèl·lules es podrien haver perdut durant el procés. L'altra explicació seria a què degut a que la línia de MEF *knockout* $G\alpha_{12/13}$ és una línia estable i viable, que ha estat mantinguda durant anys, potser les cèl·lules han desenvolupat un mecanisme alternatiu per tal de compensar la pèrdua de les proteïnes $G\alpha_{12/13}$. En canvi, a les cèl·lules sh$G\alpha_{12}$ l'experiment té lloc immediatament després d'infectar i per això les cèl·lules pot ser que entrin en G_0/G_1.

Llavors els resultats obtinguts indiquen que la proteïna $G\alpha_{12}$ tindria una funció en la regulació de la citocinesi.

2. Identificació de la proteïna GEF-H1 com un nou efector per a la proteïna $G\alpha_{12}$

En aquest punt es van voler trobar els possibles efectors de la proteïna $G\alpha_{12}$ durant la citocinesi. La citocinesi és un procés regulat per la proteïna Rho, la qual està regulada per les proteïnes RhoGEFs. La GEF-H1 és una RhoGEF implicada en la citocinesi de la qual no es té gaire informació sobre la seva activació. Es va començar a investigar la possible interacció entre ambdues proteïnes mitjançant assajos de co-immunoprecipitació. Es va trobar que la proteïna $G\alpha_{12}$ era capaç de co-immunoprecipitar amb la proteïna GEF-H1. Per tal d'esbrinar quina podia ser la regió implicada en la unió a $G\alpha_{12}$, es van fer unes anàlisis *in silico* de les seqüències dels membres de la subfamília de la GEF-H1 i de les RH-RhoGEFs a on es va veure que contenien en la seva part C-terminal una regió súper hèlix amb certa homologia al domini RH de les RH-RhoGEFs. Llavors, la primera hipòtesi que es va formular va ser que la proteïna GEF-H1, així com els altres membres de la seva família, interaccionava amb la proteïna $G\alpha_{12}$ a través d'aquest motiu. Es van generar dos formes truncades de la GEF-H1, una forma N-terminal que contenia els dominis DH-PH i una C-terminal amb el motiu súper hèlix. Sorprenentment, només

la part N-terminal era capaç de co-immunoprecipitar $G\alpha_{12}$. Per tant, la nostra hipòtesi era errònia. Per tal d'acotar la regió d'interacció es va generar un altra forma truncada que només contenia els dominis DH-PH i certament, aquesta forma encara conservava la capacitat d'interaccionar amb la $G\alpha_{12}$. Tot i així, els resultats obtinguts no són tan sorprenents degut a què alguns membres de la família de les proteïnes RH-RhoGEF, la p115RhoGEF i la LARG, també poden interaccionar amb les proteïnes $G\alpha_{12/13}$ a través del domini DH. Tot i què en aquest cas la regió que s'uneix principalment a les proteïnes $G\alpha_{12/13}$ és el domini RH i a conseqüència d'aquest primer contacte, les RhoGEFs estableixen el segon punt d'unió amb les proteïnes $G\alpha_{12/13}$ a través del domini DH. El fet de què la GEF-H1 s'uneixi a la proteïna $G\alpha_{12}$ a través del dominis DH-PH es pot explicar degut a què entre els membres de la subfamília de la GEF-H1, la GEF-H1 és la que té el domini súper hèlix més divergent entre tots els membres. Pot ser que la GEF-H1 hagi evolucionat d'una manera independent dels altres membres de la subfamília ja que la p190RhoGEF sí que s'uneix a través del domini súper hèlix a la proteïna $G\alpha_{13}$.

Addicionalment, es va estudiar quin efecte podria tenir l'activació de la $G\alpha_{12}$ en la seva unió a la proteïna GEF-H1. Es va observar que quan la proteïna $G\alpha_{12}$ estava activada hi havia més unió a la proteïna GEF-H1, indicant que l'activació de la $G\alpha_{12}$ era important per interaccionar amb la GEF-H1.

La GEF-H1 està regulada per la seva unió a microtúbuls. Llavors es va avaluar quin efecte podia tenir la $G\alpha_{12}$ sobre la localització de la GEF-H1 als microtúbuls, ja que és una conseqüència directa de la seva inactivació. Mitjançant una anàlisi de co-sedimentació dels microtúbuls es va veure que la presència de la $G\alpha_{12}$ produïa un augment en l'alliberament de la GEF-H1 dels microtúbuls i conseqüentment, un augment en la seva activació.

Un cop vist que la $G\alpha_{12}$ podia interaccionar amb la proteïna GEF-H1, ja sigui directa o indirectament, es va voler investigar si la proteïna $G\alpha_{12}$ era capaç d'estimular l'activitat catalítica de la GEF-H1 sobre Rho. Es va fer servir un assaig de *pull-down* d'un mutant de la proteïna Rho que no pot unir nucleòtids i presenta una afinitat molt alta per les proteïnes RhoGEFs actives (RhoAG17A). Mitjançant aquest assaig es va veure que en presència de la proteïna $G\alpha_{12}$ la quantitat de GEF-H1 unida al mutant de la proteïna RhoAG17A augmentava, indicant una activació per part de la proteïna $G\alpha_{12}$. Una altra aproximació que es va prendre va ser fer

servir l'assaig de l'activitat del gen reporter de la luciferasa per tal d'avaluar l'activitat de RhoA. Es va utilitzar una línia cel·lular induïble per a l'expressió de la proteïna $G\alpha_{12}$, degut a què nivells d'expressió elevats de la proteïna $G\alpha_{12}$ provoca apoptosi, i què l'assaig de luciferasa és un assaig molt sensible. Es tracta del sistema T-REX® que consisteix en la co-transfecció de dos plasmidis. Un d'ells conté la proteïna d'interès, sota el control d'un promotor que està reprimit per l'acció d'un repressor contingut en un segon plasmidi. Només quan l'inductor, en aquest cas la tetraciclina, és present en el medi la proteïna d'interès s'expressarà. Llavors es va generar al laboratori el clonatge del plasmidi amb seqüència de la proteïna $G\alpha_{12}$Q231L i es cotransfectar amb el plasmidi del repressor en cèl·lules HEK293. L'expressió de la $G\alpha_{12}$ es va induir per un temps aproximat de 4 hores i es va procedir a fer l'assaig de l'activitat del gen reporter de la luciferasa. En aquest cas també es va observar un increment en l'activitat catalítica de la GEF-H1 quan la $G\alpha_{12}$ era present. Per tant, es resultats obtinguts fins el moment suggereixen que la proteïna GEF-H1 interacciona i és activada per la proteïna $G\alpha_{12}$.

La regulació de la GEF-H1 està controlada per la unió a microtúbuls i per fosforilació. Les proteïnes 14-3-3 són unes proteïnes que uneixen un gran ventall de proteïnes a través, principalment, de residus de serina o treonina fosforilats en les proteïnes que uneixen. La fosforilació de la serina 885 per diferents quinases condueix a la seva unió a 14-3-3 i es coneix que aquest complex es trobava localitzat als microtúbuls. Tot i què, encara no es coneix si el que provoca la translocació de la GEF-H1 als microtúbuls és la fosforilació o la unió de 14-3-3. Llavors per tal d'estudiar l'efecte de la $G\alpha_{12}$ sobre aquesta unió es van fer servir tècniques de co-immunoprecipitació. Inicialment es va hipotetitzar que si la $G\alpha_{12}$ tenia un paper en l'activació de la proteïna GEF-H1, i la 14-3-3 s'associava a un paper inhibitori, principalment, la presència de $G\alpha_{12}$ hauria de disminuir la interacció entre GEF-H1 i 14-3-3. Però, en canvi, es va observar un augment de la interacció entre la 14-3-3 i la GEF-H1 en presència de $G\alpha_{12}$. La co-immunoprecipitació en presència i absència d'inhibidors de fosfatases va demostrar que la falta d'inhibidors provocava una disminució en la unió entre la 14-3-3 i la proteïna GEF-H1. Per tant, tot i què els resultats no eren els esperats els experiments van ser repetits varies vegades i amb diferents construccions de la GEF-H1, i el resultat era sempre el mateix. Els resultats obtinguts es poden explicar

de diferents maneres. Per una banda, hi ha set isoformes de les proteïnes 14-3-3 en mamífers. La isoforma utilitzada és l'anomenada 14-3-3 γ, mentre que els treballs anteriors es van fer amb les subunitats 14-3-3 ζ, η i ε. No seria estrany que diferents isoformes poguessin fer funcions diferents. Per altra banda, es coneix molt poc sobre del mecanisme de regulació de la GEF-H1. Tot i què hi ha diversos treballs a on es demostra la interacció entre la 14-3-3 i la GEF-H1, hi ha poques evidències què demostrin la inhibició directa per la 14-3-3. Sembla més aviat que degut a la fosforilació sobre la serina 885, que és la que indueix la unió de 14-3-3, la GEF-H1 es transloqui als microtúbuls i conseqüentment, s'inhibeixi la seva activitat catalítica cap a Rho. Per tant, sembla que el mecanisme d'activació de la $G\alpha_{12}$ necessita de la participació de proteïnes reguladores fins ara desconegudes.

Un altre mecanisme de regulació de les proteïnes RhoGEFs és a través d'unions intramoleculars que impedeixen l'activació de Rho. També tenen l'habilitat d'interaccionar amb si mateixes o amb altres RhoGEFs, és a dir, fent homodímers o heterodímers. Es va estudiar si la proteïna GEF-H1 tenia aquesta habilitat i certament, es va trobar que podia dimeritzar. Es va avaluar la capacitat d'unió de la forma completa de la GEF-H1 amb les dos formes truncades, la N-terminal i la C-terminal. Les RH-RhoGEFs poden formar homodímers i heterodímers a través d'un domini super hèlix localitzat a la seva regió C-terminal. Els resultats van mostrar que la GEF-H1 era capaç de formar homodímers tant amb la part N-terminal com amb la part C-terminal, tot i què, sembla que la part C-terminal era capaç de co-immunoprecipitar més quantitat de GEF-H1 que la part N-terminal. Això es pot explicar pel fet de què la GEF-H1 té a la seva regió C-terminal un domini super hèlix estructuralment similar al domini super hèlix de les RH-RhoGEFs. Que la regió N-terminal tingui capacitat d'interaccionar potser indica que tot i què el lloc principal d'unió es troba a C-terminal, pot haver-hi un segon lloc de contacte de menys afinitat.

3. Localització subcel·lular de la proteïna GEF-H1

En aquest punt es va decidir caracteritzar la localització cel·lular de la GEF-H1. És una proteïna associada als microtúbuls. Es creu que d'alguna manera estabilitza l'estructura dels microtúbuls ja que la despolimerització dels

microtúbuls mitjançant drogues, és més difícil quan la GEF-H1 està sobreexpressada. Quan es va seguir la proteïna endògena per les diferents etapes de la mitosi en cèl·lules HeLa la GEF-H1 presentava un patró més intens a les etapes finals. Durant l'anafase semblava associada al fus mitòtic i durant la telofase associada als centrosomes així com localitzada a la zona de separació entre les cèl·lules. També es va observar, tal com s'havia descrit, que la GEF-H1 es localitzava al cos intermedi durant la citocinesi.

Per altra banda, es va analitzar la localització cel·lular de la GEF-H1 en cèl·lules MEFs i es va veure que mostrava un patró de tinció diferent al prèviament observat. En aquesta línia cel·lular es va veure que la GEF-H1 estava localitzada principalment en una regió perinuclear. Quan es van fer assajos de colocalització per tal d'esbrinar a quin compartiment subcel·lular estava, o amb quines proteïnes podia estar associada, es va trobar que la GEF-H1 colocalitzava amb l'aparell de Golgi, Rab11 i caveolina. Tot i què aquest patró de marcatge no es va trobar en altres línies cel·lulars testades això podria tenir relació amb una de les funcions de la proteïna GEF-H1. Fa relativament poc temps es va publicar que la GEF-H1 regulava el complex exocític i endocític a través de la interacció directa amb un dels components del complex exocític, amb Sec5. El transport de vesícules fins a la membrana plasmàtica necessita l'acció de Rho, i la GEF-H1 és l'encarregada d'activar-la. Per altra banda, cal destacar la importància del transport de vesícules per tal de completar la citocinesi, on Rab11 és una de les proteïnes implicades en aquest procés així com vesícules derivades de l'aparell de Golgi. Per tant, tot i què caldrien més experiments per esbrinar quina és la importància d'aquests resultats, semblaria que aquest patró de la GEF-H1 estaria relacionat amb el transport de vesícules i romandria saber si la $G\alpha_{12}$ podria estar implicada en aquest procés regulant l'activitat de la proteïna GEF-H1.

4. Implicació de la proteïna GEF-H1 en processos de senyalització regulats per la proteïna $G\alpha_{12}$

Un cop vist que la GEF-H1 pot ser estimulada per la $G\alpha_{12}$ i que totes dues proteïnes estan localitzades al cos intermedi, el següent que es va analitzar era la colocalització durant la citocinesi. Certament, es va trobar la colocalització entre la

proteïna $G\alpha_{12}$ i la GEF-H1 en diferents tipus cel·lulars durant la citocinesi, indicant una possible interacció de les dues proteïnes en el context mitòtic.

La GEF-H1 es fosforila a la serina 885 per les quinasa mitòtica Aurora A durant la mitosi per tal d'inhibir la seva activitat catalítica. Just abans de la citocinesi la GEF-H1 es desfosforila i és llavors quan ja pot activar a Rho. Per altra banda, un estudi fosfoproteòmic va mostrar que la proteïna $G\alpha_{12}$ estava fosforilada durant la mitosi en el residu de la serina 67. Però es desconeix quina és la quinasa implicada i quin efecte podria representar sobre la seva regulació. Llavors es va especular si la $G\alpha_{12}$ podria estar fosforilada per la Aurora A ja que, com la GEF-H1 estava regulada per l'acció de la Aurora A i les dues proteïnes interaccionen i aparentment regulen juntes la citocinesi. La GEF-H1 es fosforila durant la metafase, però la $G\alpha_{12}$ no va donar cap tipus de colocalització amb la Aurora A que pogués indicar que era la quinasa responsable. Com s'ha dit, tampoc es coneix quin efecte podria tenir la fosforilació sobre la regulació de la $G\alpha_{12}$, és a dir, si es tracta d'una fosforilació que activa o bé que inhibeix la seva activitat. Per tant és un tema que queda obert i el qual s'ha d'estudiar amb més profunditat.

Per tal d'aprofundir en altres vies de senyalització on la GEF-H1 podria participar juntament amb la $G\alpha_{12}$ es van estudiar dues vies regulades per la $G\alpha_{12}$. La primera es la transformació de cèl·lules induïda per la sobreexpressió de la $G\alpha_{12}$ en cèl·lules NIH3T3. La $G\alpha_{12}$ indueix la formació d'agregats cel·lulars, anomenats foci, quan és expressada en la seva forma constitutivament activada en cèl·lules NIH3T3. Llavors utilitzant cèl·lules que tenien silenciada l'expressió de la proteïna GEF-H1 es va procedir a fer l'assaig de transformació. Certament, es va observar que quan la $G\alpha_{12}$ era expressada la formació d'agregats cel·lulars augmentava de manera considerable. En canvi, en les cèl·lules deficients en la proteïna GEF-H1 es reduïa la formació dels agregats en presencia de l'expressió de la $G\alpha_{12}$ activa. A més a més, es coneix que és a través de la proteïna Rac, no Rho, que té lloc la formació dels foci. Casualment, la GEF-H1 també és capaç d'activar Rac. Aquest resultats suggereixen que la GEF-H1 pot ser una proteïna important en aquesta via de senyalització.

Per altra banda, es va estudiar la influència de la GEF-H1 en la migració cel·lular. Les proteïnes $G\alpha_{12/13}$ estan implicades en la migració cel·lular a través de l'activació de Rho, necessària per els canvis que es produeixen en el citoesquelet

durant la motilitat cel·lular. Fent servir la mateixa estratègia que abans, es va estudiar la migració cel·lular en unes cèl·lules que tenien silenciada l'expressió de la proteïna GEF-H1. Es va fer un assaig de tancament de ferida sobre cèl·lules control i cèl·lules que no expressaven la proteïna GEF-H1. El resultat va ser que a les cèl·lules que tenien silenciada l'expressió de la proteïna GEF-H1 el tancament de la ferida estava retardat respecte a les cèl·lules control, indicant una migració més lenta. Tot i què es va veure després que aquest resultat ja estava publicat, és a dir, que la GEF-H1 està implicada en la migració cel·lular, resta per veure com la $G\alpha_{12}$ i la GEF-H1 poden estar interaccionant per tal de promoure la migració cel·lular.

Aquestes noves aproximacions són experiments que s'estan duent a terme al laboratori i, per tant, moltes preguntes encara estan pendents de ser contestades per tal d'esbrinar i entendre millor la importància d'aquesta nova interacció entre la GEF-H1 i la $G\alpha_{12}$.

CONCLUSIONS

1. La proteïna $G\alpha_{12}$ es localitza al fus mitòtic durant les últimes etapes de la mitosis i al cos intermedi durant la citocinesi.
2. La $G\alpha_{12}$ colocalitza amb la pericentrina durant la divisió.
3. La pèrdua de la funció de la proteïna $G\alpha_{12}$ provoca defectes en la mitosi, concretament durant la citocinesi.
4. El silenciament de la proteïna $G\alpha_{12}$ amb la tècnica de shRNA provoca un augment de la població de cèl.lules en la fase G_0/G_1.
5. La proteïna $G\alpha_{12}$ co-immunoprecipita amb la proteïna GEF-H1 a través del seu domini DH-PH.
6. L'activació de la proteïna $G\alpha_{12}$ augmenta la seva unió a la proteïna GEF-H1.
7. La proteïna $G\alpha_{12}$ promou l'activació de la proteïna GEF-H1, reflectida a través de la inducció de la unió de la GEF-H1 a la forma lliure de nucleòtids de Rho (RhoG17A).
8. GEF-H1 participa en l'activació de RhoA induïda per $G\alpha_{12}$ en cèl·lules HEK293.
9. La proteïna $G\alpha_{12}$ promou l'alliberament de la GEF-H1 dels microtúbuls.
10. La GEF-H1 té la capacitat de dimeritzar, probablement a través del seu domini súper hèlix localitzat a la regió C-terminal.
11. La $G\alpha_{12}$ afavoreix la interacció entre la proteïna 14-3-3 i la GEF-H1.
12. La GEF-H1 es localitza al Golgi en les cèl·lules MEFs.
13. La GEF-H1 colocalitza amb Rab11 i caveolina en les cèl·lules MEFs.
14. La GEF-H1 i la $G\alpha_{12}$ colocalitzen al cos intermedi durant la citocinesi.
15. La GEF-H1 posseeix activitat de transformació en les cèl·lules NIH3T3.
16. La GEF-H1 afecta a la migració cel·lular.

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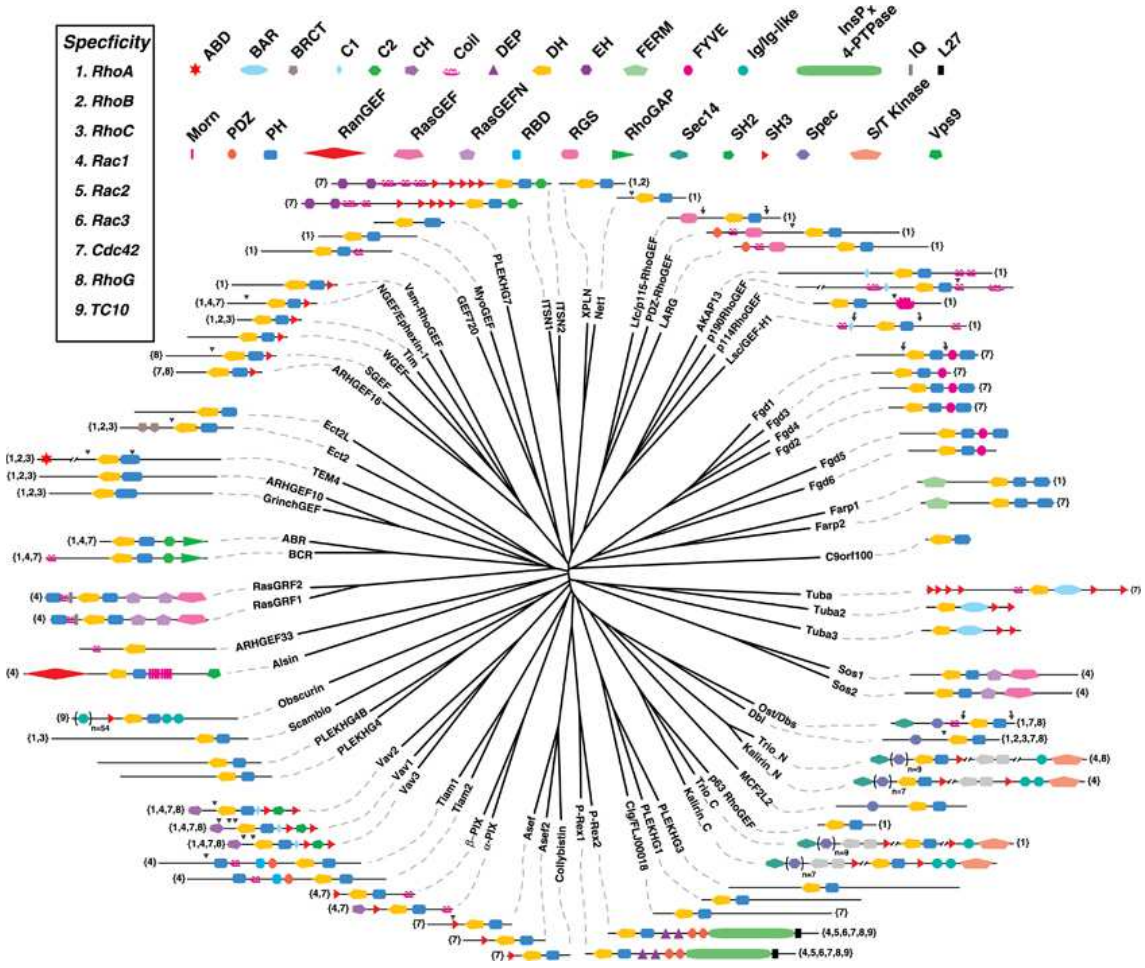
APPENDIX

APPENDIX I: Members of the human Dbl RhoGEF family



Annex I: Members of the human Dbl RhoGEF family. Black arrowheads indicate sites of genetic truncation known to activate RhoGEF function, whereas bracketed arrowheads designate biologically or catalytically active fragments of a GEF. Numbers within braces next to a Dbl protein indicate the reported Rho GTPase specificity of the DH domain. (Cook *et al.*, 2013)