Abstract

Over the past several years, typical small Rho-GTPases cycling between a resting GDP-bound state and an active GTP-bound state, i.e. RhoA, Rac, Cdc42, have been shown to play an important role in several aspects of nervous system development, including neurite outgrowth and differentiation, axon pathfinding, dendritic spine formation and maintenance, as well as myelination (Franklin *et al*, 2008; Krause *et al*, 2008). However, the roles played and the signaling cascades modulated by atypical constitutively active Rho-GTPases (RhoD, RhoH, RhoBTB, Rnd and Miro) are far from clear (Aspertrom *et al*, 2007).

The Rnd subfamily consists of three splice variants: Rnd1, Rnd2 and Rnd3/RhoE. RhoE is ubiquitously expressed and is found in both cytosolic and membrane fractions, localizing at least partially to the Golgi complex as well as to the plasma membrane (Riento *et al*, 2005). Rnd3 was isolated through its ability to interact with p190RhoGAP, a GTPase-activating protein, acting like a RhoA antagonist (Wennerberg *et al*, 2003). Rnd3 protein activity is presumably regulated by alteration of protein expression levels and through ROCK1- and PKCα-mediated phosphorylation (Riento *et al*, 2005; Madigan *et al*, 2009). So far only two other RhoE effectors have been identified: Socius and Rapostlin (Chardin, 2006)

Up to now little is known about Rnd3-mediated functional processes and signaling mechanisms.Transient expression in 3T3 fibroblasts can induce loss of actin stress fibers, rounding of the cell body and blockage of cell cycle progression (Riento *et al*, 2005; Villalonga *et al*, 2004). In PC12 cells, Rnd3 has been recently reported to play a potential role in the regulation of neurite outgrowth (Talens-Visconti *et al*, 2010).

The aim of this study is to uncover the Rnd3/RhoE interactome and to identify new potential players in Rnd3/RhoE atypical Rho GTPase signaling in 3 different cell types: fibroblasts (NIH 3T3), neurons (PC12), and myelinating glial (MSC80) cells. This knowledge will allow follow-up analysis of different cell mechanisms (actin reorganization, cell motility/migration, neurite outgrowth, myelination). To address this question we applied Stable Isotope labeling by

Amino acids in Culture (SILAC), which is a powerful tool for interactome mapping (Mann, 2006).

Resumo

Durante os últimos anos as típicas Rho-GTPases, que transitam entre o estado inactivo de ligação ao GDP e um estado activo de ligação ao GTP i.e. RhoA, RAC e Cdc42, foram descritas como desempenhando um papel importante no sistema nervoso, nomeadamente expansão e diferenciação de neurites, definição da trajectória de axónios, formação e manutenção da espinha dendritica tal como da mielinização (Franklin *et al*, 2008; Krause *et al*, 2008).

Contudo as funções desempenhadas e a via de sinalização modulada por uma forma constitutivamente activa de RhoGTPases atípicas (RhoD, RhoH, RhoBTB, Rnd e MIRO) ainda não estão descritas (Aspenstrom *et al*, 2007).

A sub família Rnd é composta por três variantes: Rnd1, Rnd2 e Rnd3 ou RhoE. RhoE é expressa constitutivamente e está presente nas fracções menbranares e citosólica, localizando-se parcialmente no complexo de Golgi e membrana citoplasmática (Riento *et al*, 2005).

RhoE foi isolada devido à sua capacidade de interagir com uma proteína activadora de GTPase, p190RhoGAP, actuando como antagonista de RhoA (Wennerberg *et al*, 2003).

A actividade de RhoE é supostamente regulada pela alteração dos níveis de expressão e através de fosforilações por parte de ROCK-I e PKCα. Até agora apenas SOCIUS e Rapostlin foram identificados como activadores de RhoE (Chardin, 2006).

Ainda pouco é conhecido acerca dos processos funcionais e mecanismos de sinalização mediados por RhoE.

A expressão transiente, em fibroblastos células 3T3 NIH, pode induzir perdas de fibras de stress, arredondamento do corpo celular e bloquear a progressão do ciclo celular (Villalonga *et al*, 2004; Riento *et al*, 2005). Em células PC12, foi descrito recentemente que RhoE desempenha uma função importante na regulação de expansão de neurites (Talens-Visconti *et al*, 2010)

O que é proposto neste projecto é revelar os interactores de RhoE, de modo a que se possa identificar potenciais candidatos da via de sinalização de RhoE. Os principais objectivos deste trabalho são a identificação desses mediadores em três tipos de células: fibroblastos (NIH3T3), neurónios (PC12) e

células de mielinizantes (MSC80); e o estudo dos diferentes mecanismos celulares (reorganização da actina, migração celular, expansão de neurites, mielinização).

A metodologia utilizada consiste num ensaio de SILAC, Cultura celular com Amino ácidos com Isótopos marcados. Esta técnica tem vindo a ser utilizada recentemente como uma técnica fidedigna para o mapeamento de interactores (Mann, 2006).

I. Introduction

The nervous system of the vertebrates is a prerequisite for fast and efficient coordination of the various body functions, ranging from pain response and motor coordination up to higher cognitive functions such as learning, memory and social behavior. It can be subdivided into the central nervous system (CNS), consisting of brain and spinal cord, which processes information, and the peripheral nervous system (PNS), formed by the peripheral nerves that distribute the information. The major cellular components of nervous tissue are the neurons and the neuroglia. While neurons conduct electrical signals, glial cells, which are much more abundant than neurons, fulfill supportive and trophic roles critical for the normal function of the nervous tissue. There are three major types of supporting cells in the CNS: oligodendrocytes, astrocytes and microglia. In the PNS, the Schwann cell (SC) is the main neuroglial component. Glia has important developmental roles, guiding migration of neurons in early development and regulating neuronal survival and differentiation. Two types of glial cells (oligodendrocytes in the CNS and SCs in the PNS) produce myelin sheaths, which insulate nerve axons, thereby allowing long-distance fast saltatory conduction of electrical signals essential for nervous system function. Other glial cells (astrocytes) contact endothelial cells to form a selective filter around the brain capillaries, the blood-brain barrier, which prevents toxic substances and pathogens in the blood from entering the brain. Some glia promotes efficient signaling between neurons by maintaining appropriate concentrations of ions and neurotransmitters in the neuronal environment (Halassa and Haydon, 2010). Recent findings also indicate a role for glial cells in the formation, maintenance and function of synapses (Hamilton and Attwell, 2010; Pfrieger, 2010). Following injury, glial cells are also major regulators of neuronal repair (Bruce *et al*, 2010; Cafferty *et al*, 2008).

Over the past several years, it has become clear that the family of small Rho GTPases and their interacting molecules play key roles in various aspects of both CNS and PNS development (Feltri *et al*, 2008; Krause *et al*, 2008). These include: neuronal differentiation, neurite outgrowth (Talens-Visconti *et al*, 2010), axon pathfinding, dendritic spine formation and maintenance, and

myelination (Feltri *et al*, 2008). Given the importance of small Rho GTPases in several CNS and PNS functions, it is not surprising that mutations in genes encoding a number of Rho GTPase regulators and effectors have been associated with different human neurological diseases (Govek *et al*, 2005).

1. The family of small Rho GTPases

Small Rho GTPases represent a distinct family within the superfamily of Ras-related small GTPases and are broadly expressed in all eukaryotic cells. Up to now twenty-two mammalian genes encoding small Rho GTPases have been described (Jaffe and Hall, 2005). The Ras proto-oncogene product was discovered more than 20 years ago (Aspertrom *et al*, 2007). The revelation that oncogenic mutations in the *Ras* genes occur frequently in human carcinomas triggered the search for Ras-like genes and Rho became one of the first gene products to be identified in this effort. Although Rho signaling probably does play a role in tumor growth/metastasis (Ridley, 2001), so far no mutations in Rho genes have been found in human tumors.

According to their sequence homology small Rho GTPases can be divided into eight subfamilies **(Figure 1):** Cdc42 [Cdc42, TC10, TCL (TC10-like), Chp, Wrch-1], Rac (Rac1, Rac2, Rac3, RhoG), Rho (RhoA, RhoB, RhoC), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoD (RhoD and Rif), RhoH/TTF, RhoBTB (RhoBTB1 and RhoBTB 2) and Miro (Miro-1, Miro-2).

The small Rho GTPases can also be divided in typical and atypical depending on their ability (or lack of it) to cycle between an active –GTP bound and an inactive –GDP bound state.

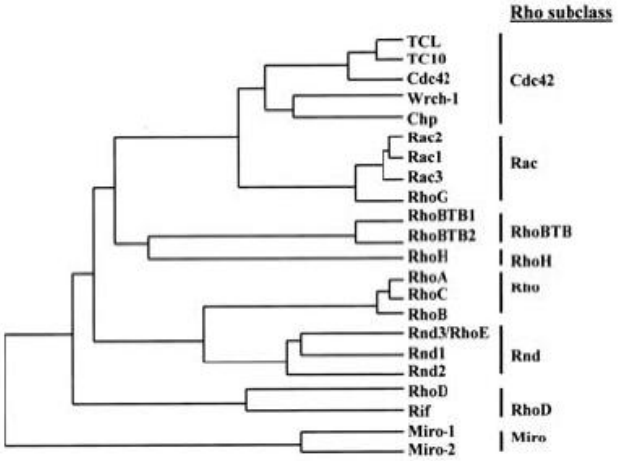

Figure 1. Family of the small Rho GTPases. This representation is based on aligments of the Rho GTPase domains employing the Clustal W algorithm. (Adapted from Aspenstrom, 2004)

1.1 Typical RhoGTPases

Typical Rho GTPases subfamilies include: Cdc42, Rac, and Rho. Their functions have been widely studied. They are key regulators of cytoskeletal dynamics and affect many cellular functions, including cell polarity, migration, vesicle trafficking and cytokinesis **(Figure 2 A)**. They are binary molecular switches, which cycle between a resting GDP-bound state and an active GTP-bound state. Their activation is controlled by guanine nucleotide-exchange factors (GEFs) that stimulate the release of GDP, allowing its replacement by GTP. Activated Rho GTPases bind to their effector proteins and activate them (Bishop and Hall, 2000). Once the active GTP-bound form has interacted with effectors, inactivation can be triggered by GTPase activating proteins (GAPs), which promote GTP hydrolysis to GDP, thereby returning the protein to its resting state. For most Rho-family proteins, this GDP-bound form is predominant at the resting state and interacts with a guanine dissociation inhibitor (GDI) protein that covers the C-terminal geranylgeranyl moiety and stabilizes it in a cytosolic Rho–GDI complex (Jaffe and Hall, 2005) **(Figure 2 B).**

The subcellular localization and thus biological activity of Rho-GTPases can be also regulated by C-terminal modification, i. e. prenylation and palmitoylanition as suggested for the Rho subfamily members RhoA, RhoB, and RhoC (Bustelo *et al*, 2007). These proteins share 85% sequence identity, differing mainly in their C-terminus. Therefore, it is likely that their specificity

would be at least partly due to differences in their sub-cellular localization (Wheeler and Ridley, 2004).

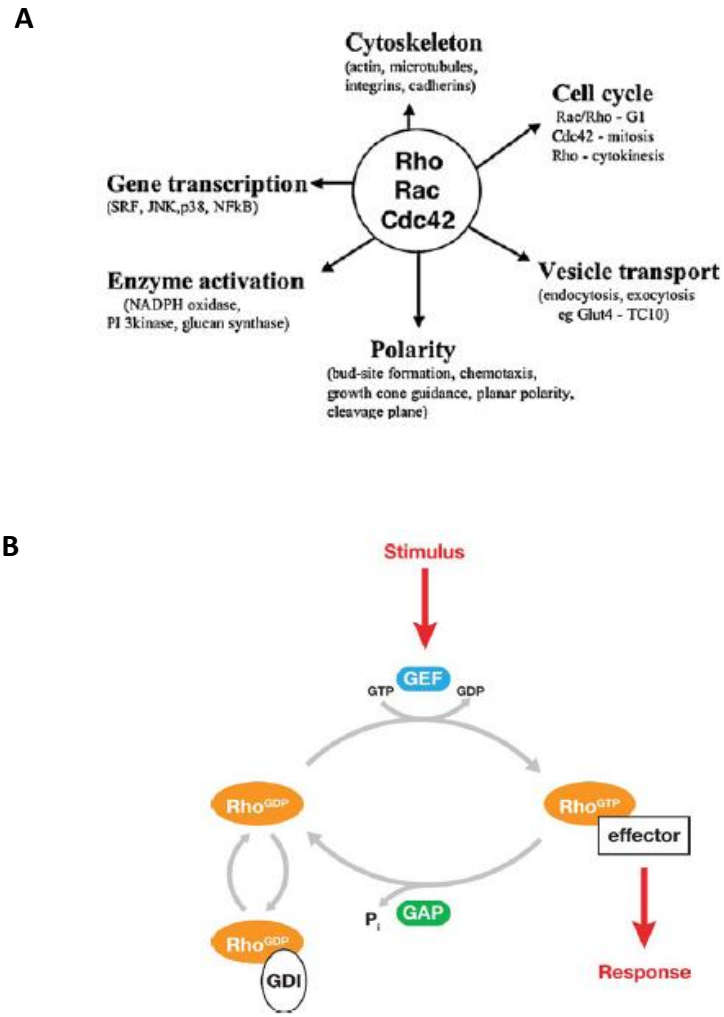

Figure 2. Rho GTPases signaling. **A**) Cellular functions in which typical Rho GTPases have been shown to be involved (Adapted from Hall, 2005). **B**) The GTPase cycle. Typical small Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound form. In mammalian cells, their activity is regulated by a large family of 85 GEFs, an equally large family of 80 GAPs, and 3 GDIs. Active GTPases interact with effector proteins to mediate a response (Adapted from Jaffe and Hall, 2005).

Additionally, typical Rho-GTPases can also be regulated by direct phosphorylation, ubiquitination, protease cleavage, or internalization (Bustelo *et al*, 2007).

1.2 Atypical RhoGTPases: The Rnd Subfamily

Atypical Rho GTPases - Rnd (Rnd1, Rnd2, Rnd3 /RhoE), RhoD (RhoD and Rif), RhoH/TTF, RhoBTB (RhoBTB1 and RhoBTB 2) and Miro (Miro-1, Miro-2) – have not been as widely studied as typical Rho GTPases. However, they might play important roles in the homeostasis of different cells and in cancer (Aspertrom *et al*, 2007).

Atypical Rho GTPases rarely follow the simple scheme depicted above for typical Rho GTPases and there have been very few indications for the existence of GEFs or GAPs able to bind to them. Instead, they are often positively regulated at the level of expression and negatively regulated by targeted degradation, namely proteosomal (Aspenström *et al*, 2004; Wennerberg *et al*, 2004; Rossman *et al*, 2005). Atypical Rho GTPase function is also likely to be regulated by protein-protein interactions, involving types of domains that are not found in classical Rho proteins (Chardin, 2003).

The Rnd subfamily represents a sub-group of the Rho family of small GTP binding proteins which in mammals consists of three proteins: Rnd1/Rho6, Rnd2/Rho7 and Rnd3/Rho8/RhoE. Rnd proteins, although more closely related to Rho proteins than to Rac, Cdc42 or other atypical members, have some unusual properties compared with other Rho-family proteins. Rnd1, Rnd2 and Rnd3 are always bound to GTP and are not regulated by the same kind of effectors. Studies in neurons have provided important insights into the mechanisms controlling their activity and revealed that it is dependent on expression levels, localization and phosphorylation, rather than the GDP/GTP switch (Chardin, 2006).

1.2.1 Rnd3/RhoE: an introduction

The predicted molecular size of RhoE is approximately 26 kDa. RhoE has amino- and carboxy- terminal domains similar to the above described Rho proteins. The amino terminus contains an additional 5 amino acids, and the carboxyl terminus contains an additional 65 amino acids (**Figure 3 A**). In addition, the presence of a carboxy-terminal methionine, which is also seen in K- and N-Ras, suggests that unlike other Rho proteins that are geranylgeranylated, RhoE might be modified by farnesylation.

RhoE was the first member of the family to be identified, and was isolated through its ability to interact with p190RhoGAP, a GAP for RhoA. However, unlike RhoA, RhoE does not bind to the GAP domain of p190RhoGAP, but to the central region of the protein (Riento *et al*, 2005).

Unlike other small G proteins, RhoE, along with two other proteins Rnd1/Rho6 and Rnd2/RhoN, does not hydrolyze GTP. The main reason for this is the presence of serines in the positions equivalent to Ala59 and Gln61 in Ras (**Figure 3 B**). By analogy to previously characterized GTP-binding proteins, it would be expected for RhoE GTP-bound state to be constitutively active. If this is the case, RhoE activity is likely to be regulated by means other than nucleotide cycling, such as, expression level, subcellular localization, or phosphorylation (Foster *et al*, 1995). Recent studies have shown that the phosphorylation state of Rnd3 has direct consequences on its cellular localization (Madigan *et al*, 2009). In the cell, it can be found both in membrane and cytosolic fractions (Guash *et al*, 1998) but its phosphorylation causes loss of plasma membrane localization and translocation to the cytosol (Madigan *et al*, 2009)

RhoE is widely expressed (Nobes *et al*, 1998) in tissues and its expression is particularly high during the development of the nervous system. In the adult nervous system, its expression levels drop significantly suggesting a more important role for this protein during embryogenesis or early postnatal development. In fact, there is increasing evidence indicating crucial roles for

Rnd proteins during brain development. The time course of RhoE expression during postnatal development is comparable to that of Rnd1, whose expression levels are high in the early postnatal rat brain, peaking at P14 and then decreasing in adulthood (Ishikawa *et al*, 2006). However, the expression pattern of Rnd1 and RhoE partially differ: for example while Rnd1 is expressed in the granule cells of the cerebellum and in the dentate gyrus (Nobes *et al*, 1998) RhoE is not (Ballester-Lurbe *et al*, 2009).

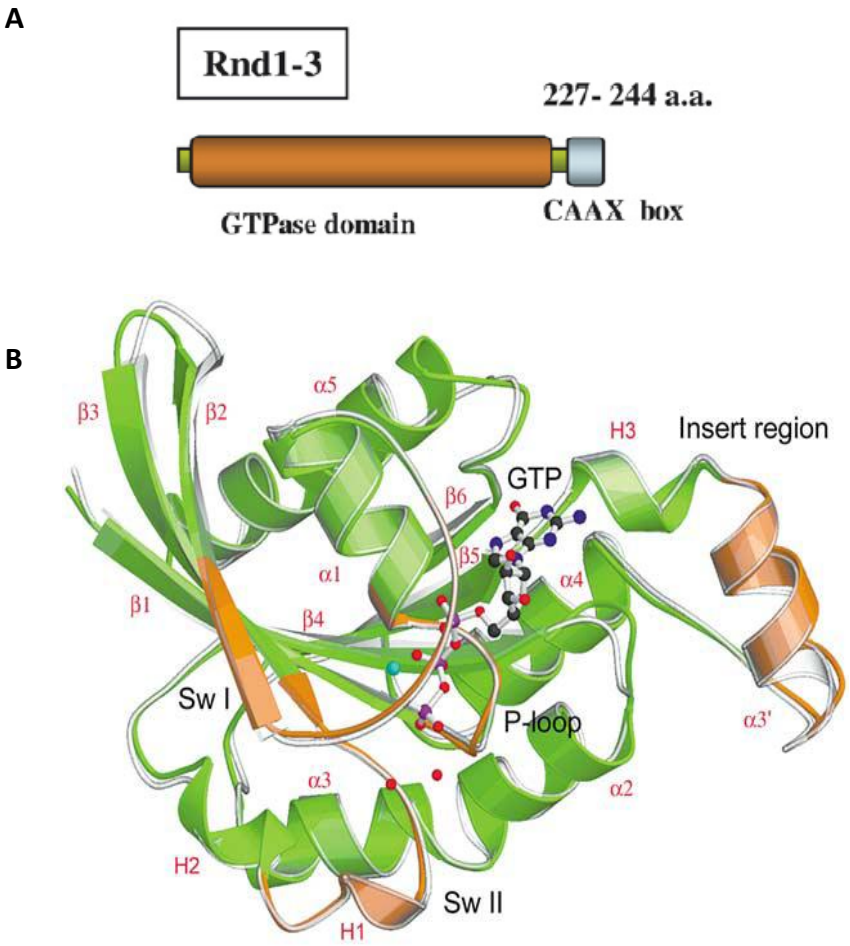

Figure 3. **A**) Schematic representation of the atypical Rho GTPases. CAAX (Cystein–Aliphatic–Aliphatic–Any amino acid) box is a membrane targeting signal (Adapted from Aspenstrom, 2007). **B**) Rnd3 Structure. Ribbon representation of Rnd3WGTP (green) compared with RhoA(G12V)WGTPQS(grey) were analyzed by the program DSSP and drawn using the program Bobscript. Structural comparison of Rnd3 and RhoA was carried out with the least squares option of the program O. P-loop, switch I, switch II and insert-helix are highlighted in orange (Adapted from Fiegen *et al*, 2002).

RhoE is widely expressed in the CNS, especially in some highly plastic areas - like the olfactory bulb, hippocampal or cortical neurons - and in

neurones producing long axons - like the motoneurones. In addition, RhoE is a marker for both the rostral migratory stream and external germinal layer of the cerebellum, cell populations that display intense migratory behaviour during development. This further suggests a potential role for RhoE in neuronal development, as shown for other Rho GTPases (Ballester-Lurbe *et al*, 2009)

RhoE signaling pathways have only been partially revealed. Acting like a RhoA antagonist (Wennenberg *et al*, 2003), RhoE exerts its inhibitory effects on ROCK-I by binding to its effector region. It has been proposed that ROCK-I mediated RhoE-phosphorylation forms part of a feedback loop regulating RhoA signaling (Komander *et al*, 2008). RhoE phosphorylation can also be mediated by PKCα, which increases RhoE stability and activity (Riento *et al*, 2005, Ballester-Lurbe *et al*, 2009) and, as a consequence, RhoA-antagonizing function resulting in altered actin cytoskeleton organization and cell motility (Guasch *et al*, 1998). In addition to its role in actin dynamics, RhoE has been reported to be involved in the control of cell cycle and survival in a few cell lines (Villalonga *et al*, 2004; Bektic *et al*, 2005; Poch *et al*, 2007). So far, in addition to ROCK, only two other RhoE effectors have been identified: Socius and Rapostlin (Chardin, 2006).

Little is known about Rnd3-mediated functional processes, however several data point to a potential role in cytoskeleton remodeling. Transient expression of RhoE in 3T3 fibroblasts can induce loss of actin stress fibers, rounding of the cell body and blockage of the cell cycle progression (Riento *et al*, 2005; Villalonga *et al*, 2004). In PC12 cells, Rnd3 has been recently reported to play a potential role in the regulation of neurite outgrowth (Talens-Visconti *et al*, 2010). Unpublished data from our group support a role for Rnd3 in regulating process extension in Schwann cells (Gonçalves *et al*, unpublished).

1.2.2 RhoE: a role in cell cycle and cancer

An elegant study made by Priam Villalonga and collaborators states that RhoE has a novel function in regulating cell cycle progression, independent of

its ability to inhibit ROCK I. In this study increased RhoE expression in fibroblasts inhibits cell proliferation and prevents serum-starved cells from entering the cell cycle in response to growth factor stimulation (Riento *et al*, 2005). Furthermore RhoE does not prevent many early signaling responses to growth factors, including activation of ERKs (extracellular signal- regulated kinases) and PKB (protein kinase B)/Akt, RhoA and Rac1. It was observed that RhoE prevent accumulation of cyclin D1, which normally occurs between 4 and 6 h after stimulation. Since cyclin D1 is important for cell cycle progression, this could explain the action of RhoE. Cyclin D1 protein levels are regulated by transcription, translation and degradation. RhoE does not affect cyclin D1 mRNA levels but predominantly affects its translation. Interestingly, cyclinD1 expression is unable to rescue the growth arrest induced by RhoE, suggesting that RhoE may affect the translation of other mRNAs. However, expression of the viral oncogenes adenoviral E1A and papilloma viral E7 does rescue cells from RhoE-induced growth arrest, indicating that the effects of RhoE are reversible (Riento *et al*, 2005).

RhoE overexpression has been reported in several types of cancer and has always been related with increased invasiveness of tumoral cells. This could be related to the fact that Rnd proteins control rearrangements of the actin cytoskeleton and changes in cell adhesion (Nobes *et al*, 1998). In particular, increasing evidences suggest that RhoE could play an important role in carcinogenesis and tumor progression. In non-small cell lung cancer RhoE expression has been proposed as an unfavorable prognostic factor for patients (Zhang *et al*, 2007). In melanoma, RhoE depletion inhibited collective and border cell movement out from spheroids in a ROCK1/2-dependent manner, implicating RhoE in the acquisition of an invasive melanoma phenotype (Klein *et al*, 2009). Contradictory results have been reported for prostate cancer, where RhoE appears to be downregulated and where its re-expression resulted in cell cycle arrest at the G_2/M phase and induced apoptosis (Bectic *et al*, 2005). It appears that RhoE cannot be only considered as an oncogene or tumor-supressor gene. Its function shows to vary depending on the organs and cells involved, the stimulating signals, or the way the cancer develops.

RhoE is also upregulated following vincristin treatment, resulting in inhibited anti-tumor-drug-induced apoptosis due to the downregulation of the proapoptotic protein Bax (Li *et al*, 2009).

1.3. Aim of the project

Despite of the key role played by RhoGTPases in a wide variety of cellular functions, atypical RhoGTPases remain largely unstudied.

Our project aims to broaden the knowledge concerning atypical RhoGTPases through the study of the interactions and signaling pathways modulated by one member of the Rnd family of atypical RhoGTPases: Rnd3/RhoE. In order to achieve this goal we planned to apply a multidisciplinary approach combining molecular biology, cell biology and proteomics.

In this study, the role of RhoE was addressed in three different cell populations: PC12 (neuronal-like cells), MSC80 (Schwann myelinating glia cells) and 3T3 NIH (fibroblasts). The effect of RhoE overexpression and depletion by shRNA-mediated knock-down was studied *in-vitro*. An expression vector containing a RhoE-myc fusion protein was produced subsequent immunoprecipitation studies. This vector will allow us to perform a proteomic (SILAC) experiment with the goal to identify RhoE interacting proteins.

Due to the fact that RhoE exists in a constitutively active GTP-bound state, it has been postulated that it would need to be regulated through different mechanisms then the GDP/GTP cycling, which regulates typical RhoGTPases (RhoA, Cdc42 and Rac1). These mechanisms could include regulation of expression levels, post-translational modifications - like phosphorylation or farnesylation. However, modulation of RhoE dependent signaling could also arise from the differential expression of unknown interactors in different cell populations.

The discovery of not yet identified interactors will be a key step for a deeper understanding of atypical RhoGTPases signaling. The availability of new

potential candidate interactors could potentially also allow the identification of new cellular functions for atypical RhoGTPases and, in this particular case for RhoE.

2.1 Materials

2.1.1. Reagents and cell-media

DMEM Dulbecco's Modified Eagle's Medium + Glutamax (Gibco, 31966)
DMEM-low glucose, without arginine, leucine, lysine (SIGMA, D0443)
L-Lysine monohydrochloride, sol: H_2O (SIGMA, L8662)
L-Leucine, sol: HCL (SIGMA, L8912)
L-Arginine, sol: H_2O (SIGMA, A8094)
13C6 L-Arginine (Cambridge Isotope laboratories, CLM-2265)
13C6 L-Lysine (Cambridge Isotope laboratories, CLM-2247)
Dialyzed Serum (Invitrogen, 26400–044)
Penicillin/Streptomycin (Gibco, 15140)
(PDL) Poly-D-Lysine (SIGMA, P7405)
(FBS) Fetal bovine serum (Gibco, 10270-106)
Trypsin 0.25% EDTA (SIGMA, 25200)
(NGF) Nerve growth Factor 7S, Murine, Natural (Gibco, 17504-044)
(G418) Gentamycin (SIGMA, G1397)
Puromycin, Dihydrochloride (Calbiochem, 540222)
Cell dissociation Buffer (Invitrogen, 13151-014)
Vector pcDNA 3.1/ myc-HIS (-) B (Invitrogen, V855-20 version B, production discontinued)
Vector pcDNA 3.1/ myc-HIS (-) B RhoE
pCMV-Flag-GFP RhoE 1-244 (kindly provided by Guasch R., Grupo Investigacion Biología Molecular del Cáncer)
Lipofectamine 2000 (Invitrogen, 18292-011)
Polybrene – Hexadimethrine bromide (SIGMA, 9268)
Dynabeads Co-Immunoprecipitation Kit (Invitrogen, 143-21D)
Dyna Magnet (Invitrogen, 123-21D)
Ampicillin 100mg/ml stock (1000x) (AppliChem, A0839)
DNA MidiPrep (Qiagen, 12243)

PCR Purification Kit (Qiagen, 28104)
Gel Extraction Kit (Qiagen, 28704)

2.1.2. Antibodies

Primary Antibodies

α - RockI, mouse monoclonal (BD Transduction Laboratories 611136)
α - Myc tag rabbit polyclonal (Abcam 9106)
α - UBXD5 (Socius) rabbit polyclonal (Abcam 65233)
α - His mouse monoclonal (GE Healthcare 27-4710-01)
α - PCKα mouse monoclonal antibody (Millipore 05-154)
α - RhoE mouse monoclonal antibody (Millipore 05-723)
α - glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mouse monoclonal (Hy Test 5G4)

Secondary Antibodies

Peroxidase-conjugated AffiniPure Donkey anti-rabbit IgG (Jackson ImmunoResearch 711-035-152

Peroxidase-conjugated AffiniPure Goat anti-mouse IgG (Jackson ImmunoResearch 115-035-146)

2.1.3. RhoE Primers

FW CTC TCG AG ATG AAG GAG AGA AGA GCC AGCC
RV GAG AAT TC CAT CAC AGT ACA GCT CTT CGC T

2.1.4. Restriction Enzymes:

XhoI (Fermentas)
EcoRI (Fermentas)
BamHI (Fermentas)

2.2 Solutions

LB medium

10g Bacto-tryptone

5g yeast extract

10g NaCl

1X PBS

8 g NaCl

0, 2 g KCL

1, 44 g Na_2HPO_4

0, 24g KH_2PO_4

In 800mL of destilled H_2O

Adjust the pH to 7.4 with HCl.

Add H_2O to 1 liter.

1X PBST

0.1% Tween20 in 1X PBS

Blocking buffer

5% nonfat dry milk

0.1% Tween20 in 1X PBS

SDS-PAGE running buffer

25mM Tris

200mM glycine

0.1% SDS

5x Laemmli SDS Sample Buffer

100mM Tris-HCl pH 6.8

20% Glycerol

5% SDS

200mM DTT

6mM Bromophenolblue

12% acrylamide separating gel

12% acrylamide (BIORAD 161-0156)

0.375M Tris-HCl pH 8.8

10% SDS

20% APS (BIORAD 161-0700)

0.4% TEMED (BIORAD 161-0800)

5% acrylamide stacking gel

5% acrylamide

0.125M Tris-HCl pH 6.8

10% SDS

20% APS

0.1% TEMED

Transfer Buffer

48 mM Tris

39 mM Glycine

20% Methanol

AP detection buffer

0.1M Tris-HCl pH 9.5

0.1M NaCl

TAE buffer

40 mM Tris base

20 mM Glacial acetic acid

1 mM EDTA

pH 8.0

2.3. Cell Lines

2.3.1 MSC80 cells

The MSC80 cell line was derived from purified mouse Schwann cells by Professor Anne Baron van Evercooren in France. This cell line is an aneuploid cell line with a doubling time of 17 hours and has been maintained through more than 110 passages. Most of the MSC80 cells are of bipolar or stellate (3-5 processes) shape. A few others are irregular in shape, gigantic, and multinucleated. All MSC80 cells express antigens of myelin-forming Schwann cells such as S-100, 228/58, laminin and other glycoproteins of the extracellular matrix. However, they also express the non-myelin-forming Schwann cell antigen GFAP. When induced to form aggregates in agar, they form intercellular junction and basement membrane-like structures. In addition, after transplantation in or at a distance from a lysolecithin induced lesion, MSC80 cells form myelin around the host demyelinated axons. MSC80 cells thus express, when isolated *in vitro*, some of the normal myelin-forming Schwann cell phenotype. In addition, they present the major advantage of forming myelin associated with axons *in vivo* (Evercooren, 1992)

2.3.2 NIH 3T3 cells

The NIH 3T3 cell line was obtained from Swiss albino mouse embryonic tissue. Although the morphology of the early NIH 3T3 cells, established by Todaro and Green, was virtually indistinguishable from that of normal fibroblasts, with passaging they became considerably flatter, finely granular, and more difficult to trypsinize.

2.3.3 PC12 cells

The PC12 cell line, originally derived from a transplantable rat pheochromocytoma, are small (5–10 μm), have a limited amount of cytoplasm,

and have a long doubling time (>2 days). One important feature of PC12 cells is their ability to respond to nerve growth factor (NGF, see below for more details), and therefore serve as a model system for primary neuronal cells. NGF-treated (primed) PC12 cells cease proliferation, grow long neurites, and show changes in cellular composition associated with neuronal differentiation. Once PC12 cells have become differentiated, the already low efficiency of transfection by many of the current methods such as calcium phosphate, lipofection, and electroporation, drops further (Karen Kelly-Spratt, 1998)

2.3.4 Priming PC12 cells: Nerve Growth Factor (NGF)

Nerve growth factor (NGF) is perhaps the prototypical growth factor, in that it is one of the first to be described. Stanley Cohen and Rita Levi-Montalcini discovered NGF in the 1950s while being faculty members at Washington University in St Louis. However, their discovery, along with the discovery of other neurotrophins, was not widely recognized until 1986, when they won the Nobel Prize in Physiology or Medicine.

NGF is a small secreted protein important for both regulation of neuronal development and maintenance of sympathetic and sensory neurons. Without it, these neurons undergo apoptosis. It also functions as a signaling molecule. NGF strongly promotes axonal growth. Studies have shown that it causes axonal branching and promotes elongation. NGF binds to at least two classes of receptors: the p75 LNGFR (for "low affinity nerve growth factor receptor") neurotrophin receptor (p75(NTR)) and TrkA, a transmembrane tyrosine kinase (Madduri *et al*, 2009).

NGF also possesses chemotactic properties that influence the direction of neurite growth. A number of general molecular mechanisms for the cellular response to NGF have been proposed, including involvement of phospholipid or protein methylation (Freeman *et al*, 2009; Maduri *et al*, 2009), phosphatidylinositol turnover (Levi-Montalcini, 2004). Stimulation of the Na+/K+ pump (Fahnestock *et al*, 2004), and elevation of intracellular cAMP and free CA2+ levels.

There is also evidence that NGF circulates throughout the entire body and is important for maintaining homeostasis. Interestingly, its precursor pro-

NGF, may also have important apoptotic and neurotrophic properties (Levi-Montalcini *et al*, 2004).

2.4. Methods

2.4.1. PCR reaction

One single PCR reaction consisted of the following reagents
10x PCR Buffer (Fermentas): 2,5µl
Forward primer (10µM): 1µl
Backward primer (10µM): 1µl
MgCl2 (25mM) 2µl
dNTPs (10mM) 0.5µl
Taq 5U/µl (Fermentas, EP 0403): 0.22µl
Template: 1µl DNA
H2O to 25µl

PCR Cycle:
The following PCR scheme was used for all different PCRs:
Segment 1: 95°C 5min
Segment 2: 95°C 1min
60°C 1min
72°C 1min
} 35 cycles
Segment 3: 72°C 5min
Segment 4: 4°C ∞

2.4.2. Agarose Gel Electrophoresis

15 µl of the PCR product was loaded onto a 1% agarose gel in 1X TAE buffer. The electrophoresis was carried out in 1x TAE buffer with 10V/cm gel length. Ethidium bromide - a DNA intercalating dye - was used to visualize DNA fragments under UV light. Size of the DNA fragments was compared towards a DNA ladder (GeneRuler 1Kb DNA Ladder, Fermentas, SM0311)

2.4.3. Cell Culture

PC12, MSC80 and 3T3 NIH cells were cultured in DMEM + Glutamax medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Gibco-Invitrogen). Plates were previously coated with PDL (Sigma) for at least 30 min, washed 1X with sterile H2O and then left to dry in the incubator for another 30min. Cells were cultured under humidified conditions in 5% CO_2, at 37ºC. To induce differentiation PC12 cells were first seeded at a density of 1 x 10^5 cells/mL on PDL coated plates and cultured in complete medium. Then NGF 7S (Sigma) was added to the medium at a final concentration of 100ng/mL.

2.4.4. Transient transfection of PC12, MSC80 and 3T3 NIH cells

PC12, MSC80 and 3T3 NIH cells were seeded on PDL-coated 6-well plates at a density of 2.5 x 10^5 cells/well and cultured for 15–18 h so that the cells were 90–95% confluent at the time of transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were incubated in normal growth medium without antibiotics. 2 µL of Lipofectamine 2000 were mixed with 0.8 µg of DNA of each of the plasmids of interest and incubated for 20 min at room temperature, before adding the mixture to the cellsl. Transfected cells were visualized 48 h after transfection and examined for GFP expression under a Zeiss Axiovert 200M (Carl Zeiss, Germany). Cells were transfected with the pCMV5 vector expressing cDNAs for the following proteins: green fluorescent protein (GFP) (cloned from pQBI25, Quantum Biotechnologies, Montreal, Canada) and wild-type RhoE (Guasch 2007).

2.4.5. RhoE knock-down

To suppress endogenous RhoE expression, a lentiviral vector (pLK0.1-puro) harboring the shRNA Rnd3 sequence CCGGGCGTGTGTAAATAAGACAAATCTCGAGATTTGTCTTATTTACACACG CTTTTTG) was purchased from Sigma-Aldrich.

Plasmid DNA was isolated from transformed bacteria using DNA MidiPrep columns (Qiagen) following manufacturer's instructions. Virus production was performed in HEK 293 cells. Shortly, a fully confluent tissue culture 90 mm plate of HEK293 cells 24h prior to transfection (so that cells would be 90-95% confluent when transfection was performed). Cells were transfected using LIPOfectamine 2000 (Invitrogen). Thirty-six uL of LIPOfectamine was mixed with 1, 5 mL of OptiMEM, for 5min, and 6ug of lentiviral RhoE, 3ug of psPAX2 and 3ug of pLP/VSVG were mixed in 1,5mL OptiMEM. The two solutions were combined and incubated for 25min before adding, 5mL of 10%FBS medium. Cells were incubated with the resulting 8 mL of medium under standard conditions for 24 hours. Later this medium was removed and the HEK 293 cells incubated in fresh medium for 48h. Finally, the medium containing the virus was centrifuged, the supernatant collected.

To silence the endogenous RhoE protein expression, we then infect PC12, MSC80 and 3T3 cells with viruses (1,5mL of virus stock and 1,5mL DMEM complete) in the presence of polybrene (Sigma). Stable expressing cells were obtained following 3 days of selection with puromycin.

2.4.6. Immunoblotting

Protein lysates were loaded into 12% or 10% SDS-PAGE (5% stacking) polyacrylamide gels. Electrophoresis was carried out at 25mA per gel (7.3 cm x 10 cm) for 1h. The transfer PVDF-membrane (Immobilon-P, Millipore) was activated in methanol washed 1x in H2O and equilibrated in transfer buffer for 5min. Blotting was carried out at 100V for 1.5h. The membrane was blocked for 1h in blocking buffer at room temperature (RT) or at 4°C overnight. The primary antibodies were diluted in 5% non-fat dry milk in 1XPBST (anti-RhoE 1:500; anti-GAPDH 1:50000; anti UBDX5 1:500). Following 1h incubation at RT, membranes were washed and the secondary antibodies applied for 1h at RT. Prior to detection, membranes were washed 3x with 1X PBST. Detection was then carried out with the supersignal west Pico Chemiluminescent substrat

(Thermo scientific, 34080) according to manufacturer`s recommendation. Visualization was carried out using a ChemiDoc apparatus from Biorad.

3.1. Endogenous RhoE

We selected PC12 cells (Neuronal-like cells) - which have been previously reported to endogenously express RhoE (Talens-Visconti *et al*, 2010) - together with MSC80 (Schwann cells) and 3T3 NIH cells (fibroblasts), as potential cell populations to perform the screen for the function of RhoE and interactors. As we wanted to use PC12 as a model for neurons, all experiments have been performed with PC12 cells primed with NGF to allow formation of neurite-like structures **(Figure 4)**.

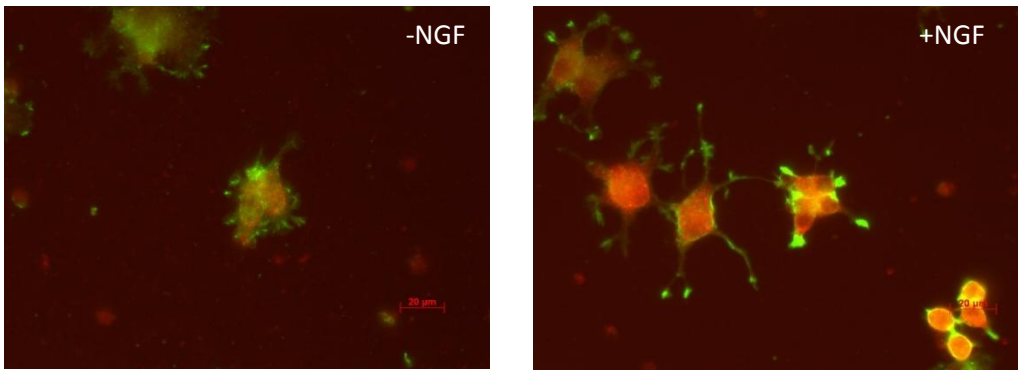

Figure 4. Priming of PC12 cells induces formation of neurite-like structures. Cytoskeleton has been visualized by immunocytochemistry staining for F-actin (phallodin-Alexa 488) and tubulin (visualized with Cy3-coupled anti-IgG secondary Ab). Representative images show the formation of neurite-like structure 12 hours following the starting of the priming.

We confirmed endogenous expression of RhoE in these 3 cell populations by western-blot. As the level of expression of this protein was similar among the three different cell populations, this suggests that RhoE is not expression level regulated **(Figure 5).**

We also examined the expression of two known RhoE interactors: Socius and PKCα. Interestingly, Socius was expressed specifically in MSC80 cells while no signal was observed in both MSC80 and 3T3 NIH cells **(Figure 5)**. As expected, PKCα is expressed in all cell populations although we observed even

higher expression levels in fibroblasts (3T3 cells) when compared to both PC12 and MSC80 cells.

The obtained results allowed us to choose PKCα as positive control for the follow-up interactome approach.

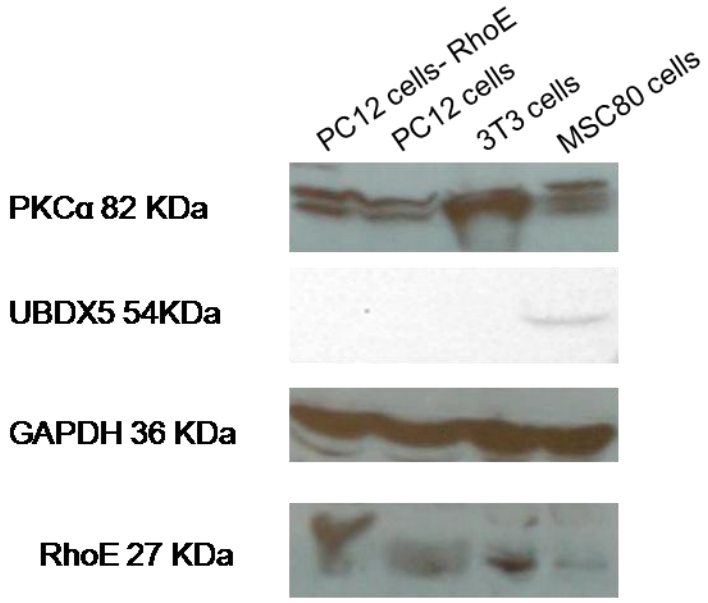

Figure 5. Endogenous expression of RhoE and its interactors Socius and PKCα in PC12, MSC80 and 3T3 NIH cells by immunoblotting. First lane shows overexpression of RhoE in PC12 cells.

3.2. Long-term RhoE overexpression

PC12, MSC80 and 3T3 NIH cells were transfected with pCMV5 FLAG-GFP-RhoE1-244 vector (**Figure 6**) in order to induce overexpression of RhoE. As in addtion to RhoE this vector also allowed for independent expression of green fluorescent protein, the transfected cells could be directly monitored under a epifluorescence microscope. Cells were kept in cultures for 96 hours before imaging with the aim of study the effects of long-term overexpression.

This is essential due to the fact that subsequent SILAC experiments will need to be performed following 5-6 doubling times (meaning between 240h and 288h *in vitro* cell culture) in order to allow near to complete incorporation of labeling aminoacids. Short-term overexpression has been previously reported to induce slight cytoskeletal changes, cell rounding and partial loss of adhesion in MDCK cells (Guash, *et al*, 1998). Long-term overexpression could therefore induce cytoskeletal changes not compatible with the experimental setup or either cell viability.

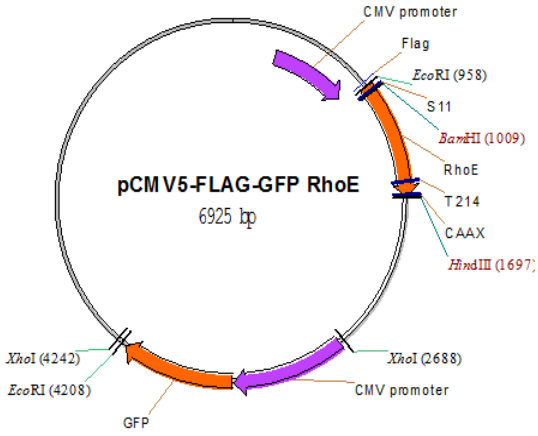

Figure 6. Vector pCMV5-FLAG-GFP RhoE 1-244 (gently provided by Guasch R).

Indeed, in our experimental setting RhoE long-term overexpression revealed a phenotype characterized by dramatic cytoskeletal changes in two out of three studied cell types. The affected cell types were both 3T3NIH and MSC80 cells. Although RhoE inactivates ROCKI, its effects are likely to be cell-dependent and in these cells we observed retraction of processes, loss of actin stress fibers (data not shown), rounding of cells and loss of adhesion already after 48 hours **(Figure 7)**. Following 72 hours of *in vitro* cell culture almost all transfected cells detached from the PDL-coated plates. This led to the progressive loss of adhesion and formation of detached apoptotic cell bodies, and as a result, cultures could not be kept in culture for long periods of time (**Figure 7**).

These results show that it is not possible to maintain MSC80 and 3T3 RhoE-overexpressing cells in culture for 5-6 passages, making it impossible to use these cells for SILAC.

These results are however extremely interesting as they further highlight the key importance of RhoE in cytoskeleton remodeling in different cell types.

A role for RhoE in cytoskeleton related-signaling could also be confirmed for PC12 primed cells. Our results are in line with the previously reported data according to which RhoE stimulates neurite outgrowth in PC12 cells (Talens-Visconti *et al*, 2010) (**Figure 7**). However, neuron-like NGF-primed PC12 cells were capable to maintain adhesion to the substrate despite cytoskeletal rearrangements. Therefore, as we needed long-term overexpression for our proteomic experiments, we chose to use PC12 cells.

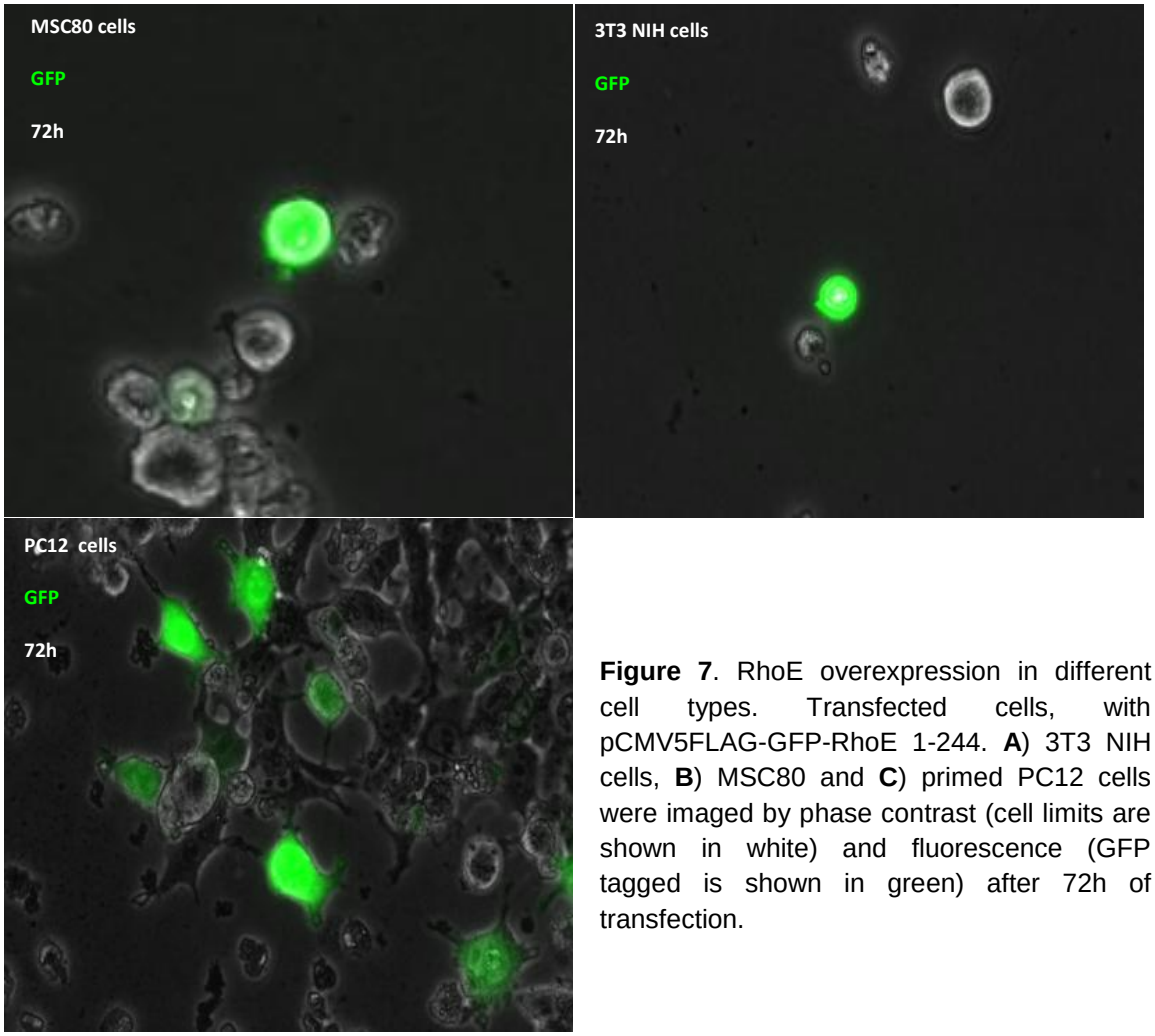

Figure 7. RhoE overexpression in different cell types. Transfected cells, with pCMV5FLAG-GFP-RhoE 1-244. **A**) 3T3 NIH cells, **B**) MSC80 and **C**) primed PC12 cells were imaged by phase contrast (cell limits are shown in white) and fluorescence (GFP tagged is shown in green) after 72h of transfection.

3.3. RhoE downregulation by shRNA

In order to further clarify the role of RhoE in the three different cell populations studied, we reduced the endogenous RhoE expression levels by lentiviral shRNA infection. Obtention of lentiviral particles and infection of cells in culture were carried out based on protocols currently available in the lab (Pereira et al 2009). The morphological phenotype of cells in culture is currently being investigated. Standard biochemical assays are also being carried out to analyze the expression of known RhoE interactors.

3.4. Vector cloning

To perform the SILAC experiments and at the same time minimizing false-positive results and the possibility of masking RhoE-binding sites, a vector overexpressing a RhoE-myc fusion protein needed to be produced.

We decided to follow in parallel two different approaches to obtain such vector:

1) Excision of GFP from the pCMV5 FLAG-GFP-RhoE1-244 vector.
2) Excision of RhoE from the pCMV5 FLAG-GFP-RhoE1-244 vector and its re-insertion into a new vector expressing Myc and His Tag, pcDNA Myc His 3.1B(-)

3.4.1. Excision of GFP from the pCMV5 FLAG-GFP-RhoE1-244 vector.

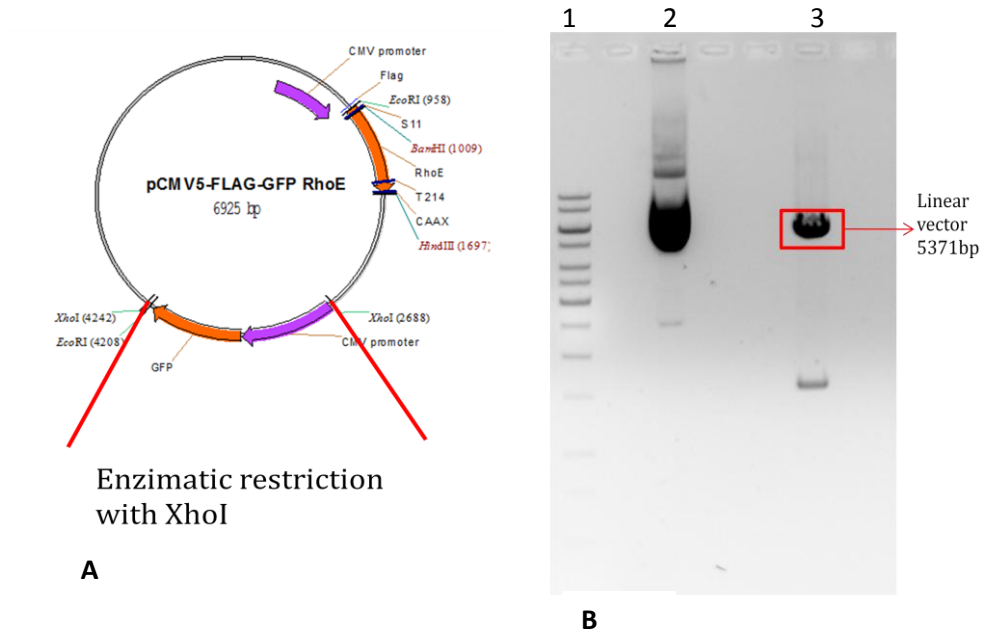

Figure 9. Map of the vector pCMV5-FLAG-GFP RhoE1-244 (**A**) and electrophoresis gel showing the vector linearized after enzymatic restriction with XhoI (**B**).

We excised GFP and the CMV promoter upstream GFP present in the vector sequence using the enzyme XhoI. However, our attempts to re-ligate the vector and restore its circularity failed, even after several trials using different protocols. It is possible that there are more than two restriction sites for XhoI, resulting in fragments not detectable in agarose gels. We will now sequence the plasmid to confirm the sequence,

3.4.2. Excision of RhoE from the pCMV5 FLAG-GFP-RhoE1-244 vector and its re-insertion into a new vector expressing Myc and His Tag, pc DNA 3.1 B (-)

To create two different restriction sequences, XhoI and EcoRI, in a PCR fragment spanning the entire RhoE cDNA fragment, we desined two primers: Fw CTC TCG AG ATG AAG GAG AGA AGA GCC AGCC and Rv GAG AAT TC

CAT CAC AGT ACA GCT CTT CGC T. The RhoE insert was obtained by PCR extension from pCMV5 FLAG-GFP-RhoE1-244 vector and was cloned in pcDNA 3.1 Myc His B (-) vector (**Figure 10, 11**).

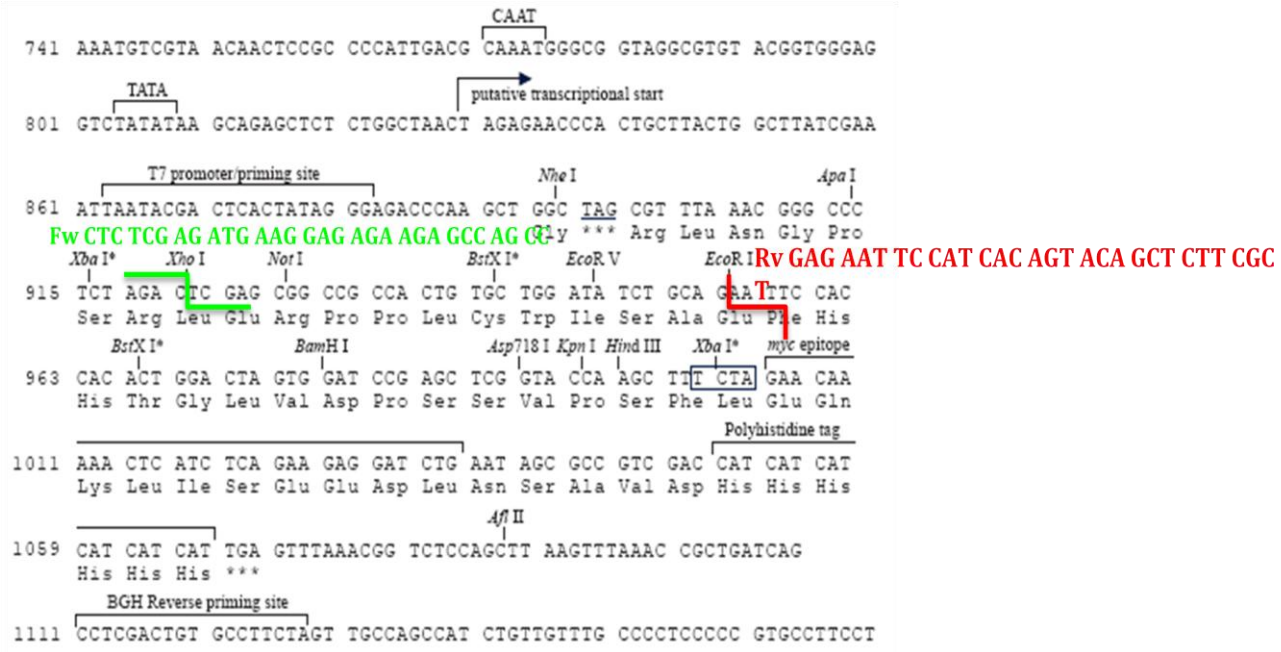

Figure 10. Multiple cloning site of version B of the pc DNA 3.1(-)/ Myc-His with the restriction enzymes that recognize the insert and the vector.

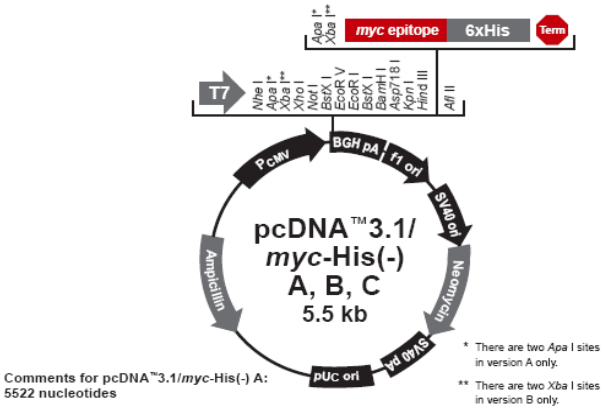

Figure 11. Vector Map of pcDNA 3.1/ myc-HIS (-) A, B and C.

To produce a C-terminus myc fusion protein, a RhoE PCR fragment cut with the restriction enzymes XhoI and EcoRI was inserted into the multiple cloning site of the version B of the pcDNA 3.1 Myc His (-) vector **(Figure 10,**

11). The integrity of the cloning vector pcDNA 3.1 Myc-HIS B (-) was confirmed by visualization in an agarose gel **(Figure 12 B)**.

The PCR amplifications were done as follows: initial DNA denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were separated on 1% agarose gels containing Orange G and visualized under a UV transilluminator **(Figure 12 A)**. PCR products were purified using PCR purification kits (Qiagen) following manufacturer instructions. The PCR products were then cut with appropriate restriction enzymes (*Xho*I and *EcoRI*) and ligated into the pcDNA 3.1 Myc His B (-) expression vector that has been cut with corresponding enzymes.

Enzymatic restriction with *EcoRI* and *XhoI* was performed for 2 h at 37ºC, and the correct size of the PCR product and linearized vector were confirmed by visualization in 1% agarose gels **(Figure 12 C)**.

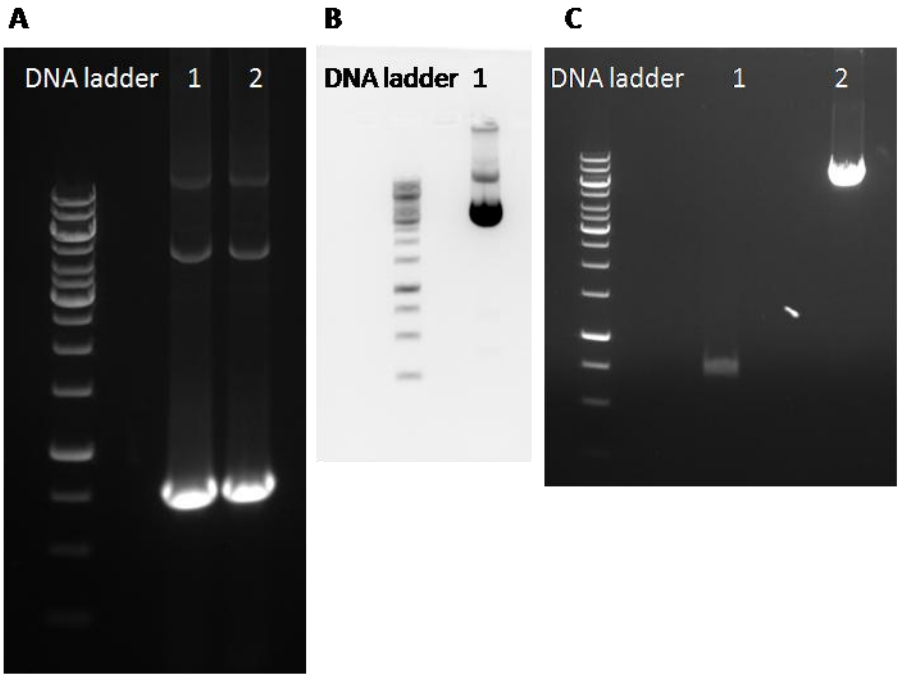

Figure 12. Results of cloning. **A**) PCR product, **B**) integrity and weight of the receptor vector (pcDNA3.1 Myc-His B (-)), **C**) restriction enzymatic reaction of the vector (lane 2) and the RhoE insert (lane 1).

The ligation reactions were transformed into *E. coli* DH5α strain, after determining the ideal ratio between insert and vector. The DNA sequence and the integrity of the constructs were determined by automated DNA sequencing.

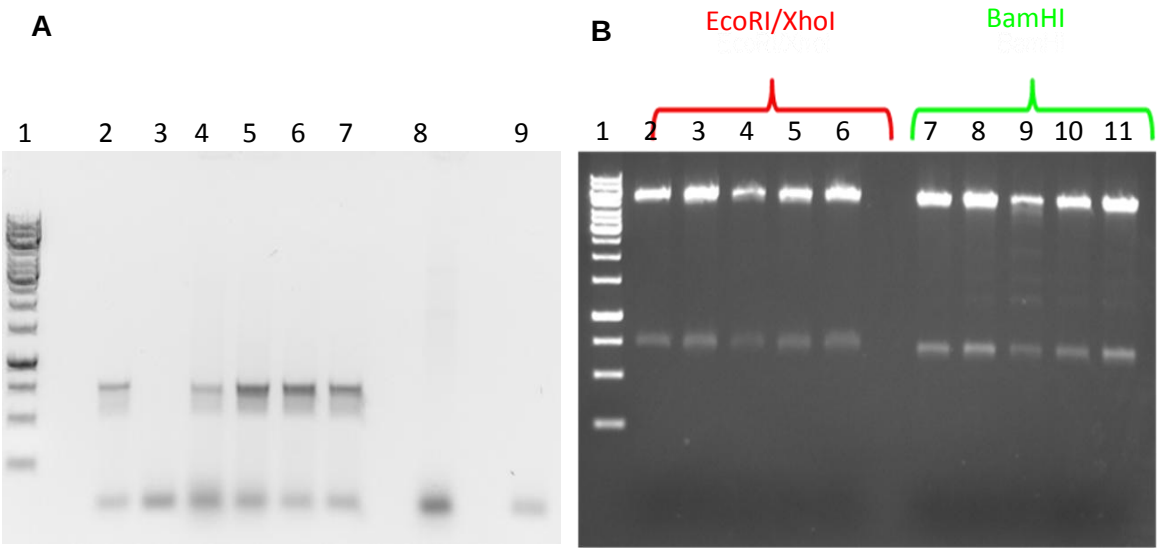

Figure 13. Cloning results of vector transformation, **A**) Correct fragment of 735 bp is present on the chosen 6/30 colonies (lanes 2-7). **B**) Restriction cuts to observe the correct orientation of insertion fragment into the vector (*EcoRI* and *XhoI* digestion lane 2-6, *BamHI* digestion lane 7-11).

We proceeded with a PCR reaction and at the same time a small scale culture in liquid LB medium was performed for each single colony. From the 30 transformed colonies obtained, we used 6 colonies to analyze the fragment extension by PCR. Only 5 showed to be positive for insert fragment presence **(Figure 13 A)**.

After this procedure in order to confirm that the insert was cloned into the right position we excise the insert from the new vector pcDNA3.1 Myc-His B (-) with XhoI and EcoRI.

To assess whether the cloned RhoE insert was correctly orientated, we also cut the positive clones with BamHI, a restriction enzyme predicted to cut once the insert and the vector. BamHI was expected to cut the RhoE insert 45bp, after the ATG start codon, and the vector just after the cloned insert

(Figure 13 B, **Figure 14)**. The analysis of the resulting DNA fragments by agarose gel electophoresis show that the RhoE insert was correctly orientated.

Figure 14. Scheme of restrictions cuts of interest.

IV. Discussion and Future Perspectives

Previous work in our lab has shown that RhoE is modulated by ECM/integrin signaling (Pereira et al 2009). In the absence of B1 integrin or integrin-linked-kinase rhoE is dramatically downregulated at both mRNA and protein level (our own unpublished observations), and its loss is associated with increased rho/ROCK activation. In relation to Schwann cell development active inhibition of ROCK is essential for the onset of radial sorting of axons, a crucial step during peripheral nervous system myelination (Pereira et la., 2009 and our own unpublished observations). Furthermore, genetic ablation of RhoE in mice (R. Guasch, and our unpublished observations) results in deficient axon fasciculation, defects in muscle innervation and increased mortality. The elucidation of how RhoE is regulated and the identification of its interacting partners is of crucial importance to understand rhoE signaling and function and is a long-term goal for this project.

In order to carry out SILAC, we need to maintain RhoE over-expressing cells for at least 5 passages in culture. It has already been described that in MDCK cells and 3T3 cells transient expression of Rnd proteins results in loss of actin stress fibers and focal adhesions (Guash *et al*, 1998, Nobes *et al*, 1998). Furthermore, transient expression of Rnd proteins in fibroblasts leads to cell rounding, hence the name Rnd (Nobes *et al*, 1998). Interestingly, the effects of Rnd proteins on the actin cytoskeleton and focal adhesions can be counteracted by an excess of activated RhoA. How Rnd3/RhoE antagonizes the signaling from Rho (Guasch et al., 1998; Nobes et al., 1998) and more specific from RhoA has been alredy described (Wennenberg, 2003) and it is depicted in Figure 15.

To test the viability of RhoE-overexpressing NIH 3T3 (fibroblasts), MSC80 (myelinating glia) and PC12 (neurons) over several passages in culture, we transfected them with an expression vector, the pCMV5 FLAG-GFP RhoE 1.244 vector (kindly provided by Guash R. and collaborators), carrying both a cDNA for RhoE and a cDNA for green fluorescent protein (GFP). By monitoring

the fate of GFP expressing cells over several weeks, we determined the viability of the transfected cells.

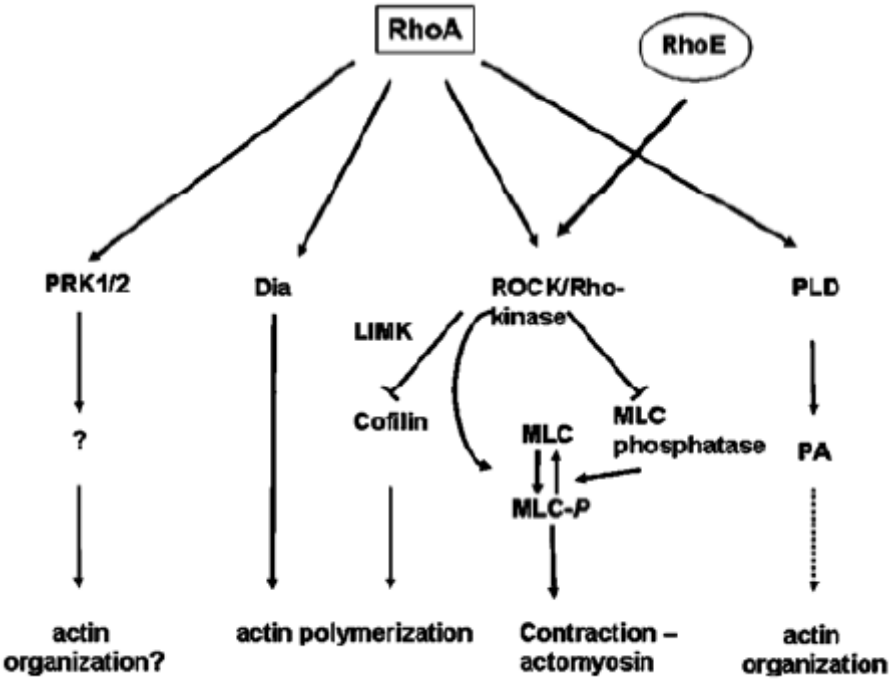

Figure 15. RhoE binds ROCK I. Targets for RhoA that are involved in RhoA-induced actin reorganization (Adapted from Riento, 2005).

Our results shown that long term overexpression of RhoE, in 3T3 NIH (fibroblasts) and MSC80 (myelinating cells) led to retraction of processes, loss of actin stress fibers and rounding of the cell body, already after 48 hours, resulting ultimately in loss of adhesion and cell death. PC12 cells were the only cells who could be maintained for long periods of time in culture, a result that is in line with a previous report by Talens-Visconti and colleagues (2010) in which transient expression of RhoE was shown to induce neurite-outgrowth. Although in general RhoE counteracts ROCK1 activity, its effects in cytoskeletal organization and process extension appear to be cell-specific.

We also analyzed the expression of some known RhoE interactores in these cells by western blot analysis. Interesting was the observation that SOCIUS is only expressed in MSC80 cells, and that PKCα expression was

higher in 3T3 cells. The physiological relevance of this result is still unclear and no functional data are available about SOCIUS in the context of myelinating glia. This will be investigated in future experiments.

Based on these preliminary data we chose to carry out our SILAC experiments using PC12 cells. PKCα will serve as a positive control for the interactome studies.

SILAC experiments require the overexpression of the studied protein, and tagged-fusion proteins allow high-yeld protein purification and are ideal for immunoprecipitation and interactome determination studies. Therefore, after several attempts, we sub-cloned the RhoE cDNA into the pcDNA3.1 Myc His B (-) to produce a myc/his tagged fusion protein. Positive clones were confirmed by enzyme restriction analysis and DNA nucleotide sequencing. Currently, we are expanding transfected PC12 cells to perform the SILAC experiment following the protocol discussed below.

SILAC Approach

The SILAC technology is a powerful tool for quantitative analyses of post-translational modifications, low abundance proteins, phosphoproteins, and membrane proteins using mammalian cells. The SILAC is based on the metabolic labeling technology using isotopic amino acids in cell culture media and, in combination with comparative MS (Mass Spectrometry) analyses, provides a useful tool to identify and quantify complex proteins samples.

In SILAC experiments, two cell populations are grown in identical cell culture media deficient in some essential amino acids. One cell population is grown in medium with heavy (isotopic) amino acid while the other cell population is grown in medium with light (normal) amino acids. The natural metabolic machinery of the cells is used to label all cellular proteins with the heavy amino acid.

SILAC Procedure

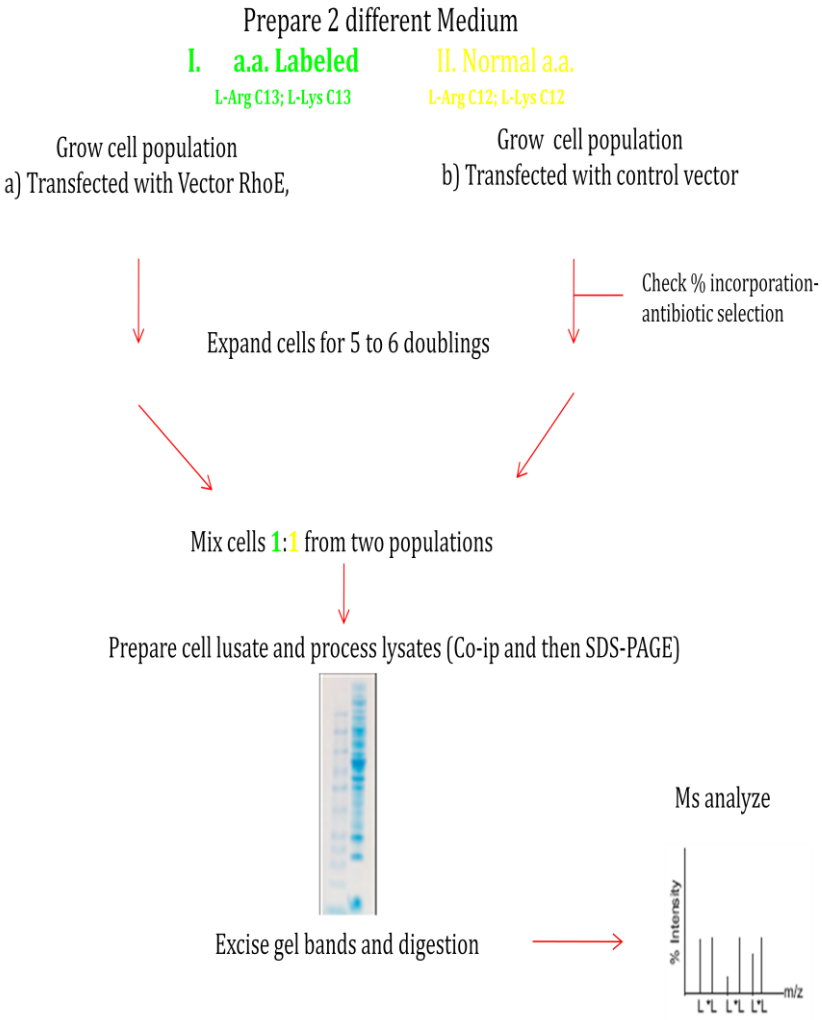

Figure 15. Representative scheme of SILAC procedure.

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