**Universidade de Aveiro** Departamento de Biologia
**Ano 2013/2014**

**Sandra Catarina Oliveira Braz**

# Alternative Polyadenylation of Rho GTPases: a gene/cell specific process

# Poliadenilação alternativa das Rho GTPases: um processo específico de cada gene e de cada tipo celular

**Universidade de Aveiro** Departamento de Biologia
**Ano 2013/2014**

**Sandra Catarina Oliveira Braz**

# Alternative Polyadenylation of Rho GTPases: a gene/cell specific process

# Poliadenilação alternativa das Rho GTPases: um processo específico de cada gene e de cada tipo celular

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Andrea Patrícia Ribeiro da Cruz, Investigadora Pós-Doc no Instituto de Biologia Molecular e Celular da Universidade do Porto, da Doutora Maria Alexandra Marques Moreira Mourão do Carmo, Investigadora Principal no Instituto de Biologia Molecular e Celular da Universidade do Porto, e do Doutor Manuel António da Silva Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro

This work was supported by "NORTE-07-0124-FEDER-000003-Cell Homeostasis Tissue Organization and Organism Biology" co-funded by ON.2—O Novo Norte, under the QREN, through the FEDER and by FCT.

The work was supported by FEDER through the Operational Competitiveness Programme – COMPETE and by National Funds through FCT– Fundação para a Ciência e a Tecnologia under the projects FCOMP-01-0124-FEDER-028252 (PTDC/BEX-BCM/0468/2012) and FCOMP-01-0124-FEDER-021201 (PTDC/SAU-GMG/116621/2010).

*“Choose a job you love, and you will never have to work a day in your life.”*

*Confucius*

**O júri**

Presidente

Professora Doutora Maria de Lourdes Gomes Pereira
Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro

Professora Doutora Maria Teresa Burnay Summavielle
Investigadora Principal no Instituto de Biologia Molecular e Celular da Universidade do Porto

Doutora Andrea Patrícia Ribeiro da Cruz
Investigadora Pós-Doc no Instituto de Biologia Molecular e Celular da Universidade do Porto

## Durante a realização deste trabalho foram efectuadas as seguintes publicações científicas e comunicações:

Publicações científicas:

**Curinha A**, **Braz SO**, Pereira-Castro I, Cruz A, Moreira A (2014) *Implications of polyadenylation in health in disease*, Nucleus. Sep 5;5(6)

Andrea Cruz, Diogo Teixeira, **Sandra O. Braz**, Rui Camacho, João Relvas, Alexandra Moreira *PBS Finder, a bioinformatic tool for clustering analysis of protein binding to mRNA UTRs* Under revision in RNA

Comunicações em formato de Poster:

**Braz SO,** Cruz A, Relvas JB, Moreira A (2014) *Expression regulation of Rho GTPases family members during olygodendrocytes differentiation and myelination,* 7º Encontro Investigação Jovem da Universidade do Porto, Porto, Portugal, 12 -14 Fevereiro

**Braz SO,** Relvas JB, Moreira A, Cruz A (2014) *Alternative Polyadenylation of Rho GTPases: a gene/cell specific process,* The Complex Life of mRNA, EMBL Heidelberg, Alemanha, 5 – 8 Outubro

Cruz A, Domingues S, **Braz SO**, Moreira A, Relvas JB (2014) *Role of PABPC1 and YBX1 in oligodendrocyte differentiation,* The Complex Life of mRNA, EMBL Heidelberg, Alemanha, 5 – 8 Outubro

**S.O. Braz**, J.B. Relvas, A. Moreira, A. Cruz (2014) *Alternative polyadenylation of Rho GTPases in central nervous system cells,* $I^3S$ 4$^{th}$Annual Meeting, Póvoa de Varzim, Portugal, 30 – 31 Outubro

Andrea Cruz, Sofia Domingues, **Sandra O. Braz**, Alexandra Moreira, João B.Relvas (2014) *Role of RNA-binding proteins in oligodendrocyte differentiation*, $I^3S$ 4$^{th}$Annual Meeting, Póvoa de Varzim, Portugal, 30 – 31 Outubro

**Agradecimentos**

Agradeço em primeiro lugar à Alexandra por me ter cativado para este fabuloso mundo, e por me ter proporcionado a oportunidade de 'viver a ciência' de uma forma tão próxima e intensa e, também a oportunidade de pertencer a um grupo tão fantástico como o GR. Agradeço também o apoio e entusiasmo que sempre me dedicou.

O maior dos obrigados é necessariamente para a minha Andrea, que tão bem me acolheu e sem a qual certamente este trabalho não teria sido realizado, ou pelo menos não com o mesmo sucesso. Obrigada por toda a ajuda, por todos os ensinamentos, por toda a partilha e paciência… Obrigada por me apoiares sempre, principalmente quando as coisas foram menos fáceis, por me dares a liberdade de pensar e fazer escolhas. De certo modo fizeste-me perceber que é isto que eu quero fazer de agora em diante. Agradeço-te as discussões científicas que me fizeram evoluir e, principalmente aquelas menos científicas que temos nos nossos momentos solitários de manhã no laboratório ☺ Agora que está prestes a acabar a orientação fica o mais importante, a amizade. OBRIGADA POR TUDO…

Em segundo lugar tenho que agradecer a este GR fantástico que me acolheu, fazendo-me sentir desde o primeiro dia parte integrante. Obrigada a todos os elementos que co-habitaram comigo, contribuindo para o meu crescimento científico e pessoal.

À Dra. Isabelocas um obrigado do tamanho do mundo por toda a ajuda em todo o meu percurso, mas principalmente na minha luta com as clonagens…

Mafis, Boal & Baldi obrigada por todos os momentos de relax e perfeita parvalheira, tornaram a minha estadia aqui muito mais divertida, vou-me lembrar sempre de vocês e dos nossos momentos.

Necessariamente tenho também que agradecer ao Dr. João por todos os 'laivos de sapiência' que me dispensou e por toda a dedicação a este projecto, mas principalmente pelo constante incentivo à prática do 'thinking out of the box'. Obrigado também ao GCB, em particular à Sofia pela incansável ajuda e disponibilidade.

Aos meus preferidos: Ana, obrigada por teres sido a melhor parceira que eu podia ter, sempre comigo em todos os momentos. Obrigada por toda a ajuda, por todas as conversas até às tantas (excepto aquela sobre qPCR até à meia noite…) e por toda a amizade; Rita, obrigado por todas as vezes que ouviste os meus desabafos, e por teres sempre uma palavra certa no momento certo; Marcos, obrigado pelos momentos de pausa, tão preciosos muitas vezes. Aos três agradeço por me acolherem como membro mais recente do vosso grupo, pelos momentos de descontracção e companhia nas idas ao Mac. Sem vocês esta jornada teria sido bem mais difícil, foram (talvez) a melhor coisa que este mestrado me deu.

Um eterno obrigado aos meus Pais e Irmão e Avó, pela paciência para os meus horários loucos e pelo apoio mas sobretudo pelo exemplo, devo-vos o que sou.

Finalmente, a ti Zé agradeço-te o teres acreditado sempre mais em mim que eu mesma, sem ti dificilmente teria chegado até aqui, és a minha inspiração. Obrigado pelo apoio incondicional, pela cumplicidade e por desdramatizares a minha vida.

**Palavras-chave** *Poliadenilação alternativa, Rho GTPases, diferenciação, RNA mensageiro, região 3' não traduzida, regulação transcripcional, RNA-binding proteins*

**Resumo**

A poliadenilação alternativa (APA) é um mecanismo importante de regulação genética que ocorre em 70% dos organismos eucariotas. Este mecanismo compreende a formação de extremidades 3' alternativas por poliadenilação em diferentes locais do mRNA, de acordo com os sinais de poliadenilação (pAs). Na APA, a escolha dos pAs é um mecanismo co-transcripcional que depende de factores auxiliares *cis* e *trans* necessários para os processos de clivagem e poliadenilação de todos os pré-mRNAs. Além disso, o uso dos pAs proximais ou distais está relacionado com eventos fisiológicos gerais. Consensualmente assume-se que em estados de proliferação ocorre o encurtamento, enquanto em estados de desenvolvimento e diferenciação ocorre o alongamento das extremidades 3' não traduzidas (3'UTRs). Este padrão de APA é confirmado em tecidos cerebrais, onde a maior parte das células são diferenciadas, no entanto não existe uma alteração completa para a isoforma de mRNA longa uma vez que a isoforma curta continua a ser expressa. As Rho GTPases são 'interruptores' moleculares essenciais a vários processos celulares, incluindo a diferenciação, no entanto nada é conhecido sobre a sua regulação transcripcional. Assim, começamos a explorar se estes genes são regulados por APA. Descobrimos por análise de 3´RACE que, as Rho GTPases clássicas, expressam duas formas alternativas de mRNA. Contudo durante a diferenciação dos oligodendrócitos (OLs), eles expressam preferencialmente a isoforma mRNA mais curta, e não se observou uma alteração para a escolha da isoforma mais longa, em contraste com os dados de estudos globais do genoma em tecido cerebral. Uma vez que estas proteínas são altamente reguladas por GEFs e por GAPs, provavelmente não necessitam de regulação a nível transcripcional. As Rho GTPases atípicas, que estão constitutivamente activas, apresentam um indução global dos pAs distais, distintas das Rho GTPases clássicas. Curiosamente, este padrão sugere que APA é um mecanismo específico do gene. Como 3'UTRs mais longas providenciam mais locais de ligação para microRNA ou proteínas de ligação ao RNA (RBPs), isto sugere que as Rho GTPases atípicas requerem uma regulação mais fina ao nível co-transcriptional, por APA. Adicionalmente, mostramos que a APA é também específica de cada tipo celular, pela análise da expressão do mRNA em outras células da glia (microglia, astrócitos), e em diferentes tipos de neurónios (corticais, estriatais e hipocampais). Nós observamos o mesmo padrão de APA para as Rho GTPases selecionadas em todas as células da glia. No entanto, em neurónios corticais e do estriado, observámos a existência do alongamento do 3'UTR no mRNA da *Rac1* durante o crescimento axonal, o que resulta num aumento da quantidade total de proteína. Em resumo, estes resultados indicam, pela primeira vez, que a APA é um mecanismo específico de cada gene e de cada tipo celular. Para além disso, descobrimos uma expressão diferencial de ambas as isoformas da *Cdc42* durante a diferenciação dos OLs e do nervo ciático. Durante a diferenciação *in vitro* de OLs e *in vivo* do nervo ciático, observámos um aumento do rácio da expressão entre *Cdc42 Iso1/Cdc42 Iso2*. Mais ainda, a expressão constitutiva de *Cdc42 Iso2* em OLs induz um atraso na diferenciação, enquanto a expressão constitutiva da *Cdc42 Iso1* induz um aumento das ramificações, sugerindo uma exacerbação do fenótipo de diferenciação. Assim, estas observações sugerem um papel distinto para as diferentes isoformas de *Cdc42* durante a diferenciação de OLs. Globalmente, esta tese abre novos caminhos para explorar no futuro, que podem ter um impacto no nosso conhecimento, na regulação do processo de mielinização/remielinização.

**Keywords** *Alternative polyadenylation, Rho GTPases, messenger RNA, 3' untranslated region, transcriptional regulation, differentiation, RNA-binding proteins*

**Abstract**

Alternative polyadenylation (APA) is an important mechanism of gene regulation that occurs in 70% of eukaryotic organisms. This process comprises the formation of alternative 3' ends of an mRNA by cleavage of the pre-mRNA and polyadenylation at different sites according to the polyadenylation signals (pAs). The choice of pAs in APA is a co-transcriptional mechanism that depends on auxiliary *cis*- and *trans*-acting factors. The usage of the proximal or the distal pAs has been related to global physiologic events. It is consensually assumed that in proliferative conditions there is preferential usage of proximal pAs, while during development and in differentiated cellular states occurs lengthening of the 3'UTRs by selection of the distal pAs. This pattern is also confirmed in brain tissues, where most of the cells are differentiated, and where it was observed a lengthening of the 3' UTRs. However, there is not a complete switch for the distal pA, since the shortest mRNA is still expressed. Rho GTPases are key molecular switchers essential for several cellular processes, including differentiation, however nothing is known about transcriptional regulation in these genes. Therefore, we started to explore if Rho GTPases genes undergo APA. We found by 3'RACE analyses, that classical Rho GTPAses express two alternative mRNA isoforms. However during oligodendrocytes differentiation, they preferentially express the shortest mRNA isoform, and we did not observe a switch towards the distal pA usage, in contrast with the published genome-wide data obtained from brain tissues. Since Rho GTPases are tightly regulated at the protein level by GEFs and GAPs, they may not require this mode of co-transcriptional regulation. The atypical RhoBTB2, which is constitutively active, present a global induction of distal pA sites, distinct from the classical Rho GTPases. Interestingly, this pattern suggests that APA is a gene specific mechanism. As longer 3'UTRs contain more binding sites for miRNAs and RNA binding proteins (RBPs) this suggests that atypical Rho GTPases require a fine-tune regulation at the co-transcriptional level, by APA. Additionally, we showed that APA is also cell-specific, by analyzing the expression of the different mRNA isoforms of Rho GTPases in other glial cells (microglia, astrocytes) and different types of neurons (cortical, striatal and hippocampal). We observed the same APA profile for the selected Rho GTPases in all glial cells types. However, in cortical and striatal neurons we observed a lengthening in the 3'UTR *Rac1* mRNA during axonal growth, which results in the increase of the total protein levels. Taken together, our results indicate for the first time that APA is a gene- and cell- specific mechanism. In addition, we have found a differential expression of both *Cdc42* isoforms during OL and sciatic nerve differentiation. During *in vitro* OL and *in vivo* sciatic nerve differentiation we observed an increase in the expression ratio between *Cdc42 Iso1*/*Cdc42 Iso2*. Further, constitutive expression of *Cdc42 Iso2* in OLs induces a delay in differentiation, whereas constitutive expression of *Cdc42 Iso1* induces an increase in OL branching, suggesting an exacerbation of the differentiated phenotype. Thus, these observations suggest a distinct role for the different *Cdc42* isoforms during OL differentiation. Overall, this thesis opens new avenues to explore in the future that can impact our understanding on the regulation of the myelination/remyelination processes.

# Index

**Figures**

## Abbreviations

| | |
|---|---|
| APA | Alternative polyadenylation |
| mRNA | Messenger RNA |
| pA | polyA signal |
| Pre-mRNA | Precursor mRNA |
| 3'UTR | 3' Untranslated Region |
| Rho GTPases | Ras homologue family of small guanosine triphosphatases |
| GEFs | GTPase exchange factor |
| GAPs | GTPase-activating proteins |
| miRNAs | microRNAs |
| RBPs | RNA-binding proteins |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| Cdc42 | Cell division control protein 42 homolog |
| CNS | Central nervous system |
| E | Embryonic day |
| div | Days *in vitro* |
| OL | Oligodendrocytes |
| PNS | Peripheral Nervous System |
| SC | Schwann cells |
| OPC | Oligodendrocyte precursor cell |
| P | Post-natal day |
| PDGF-A | Platelet-derived growth factor alpha |
| FGF-2 | Fibroblast growth factor |
| GDIs | guanine nucleotide dissociation inhibitors |
| RhoA | Ras homolog gene family, member A |
| WAVE | WASP-family verprolin-homologous protein |
| mDia | Mammalian Diaphanous |
| PAK | p21-activated kinase |
| LIMK | LIM domain kinase |
| WASP | Wiskot-Aldrich syndrome protein |
| IRSp53 | Insulin-receptor insulin substrate p53 |
| a.a. | aminoacid |
| ROCKI/II | Rho-associated, coiled-coil containing protein kinase 1 |
| MLC | myosin light chain |
| Par | partitioning-defective proteins |
| PI3 | phosphatidylinositol 3–kinase |
| IQGAP | IQ motif containing GTPase activating protein |
| N-WASP | Neural- Wiskot-Aldrich syndrome protein |
| NRG1 | Neuregulin 1 |
| CA | Constitutive active |
| DN | Dominant negative |
| RhoBTB2 | Rho-related BTB domain containing 2 |
| PA | Polyadenylation |
| snRNA | Small nuclear RNA |
| polyA tail | Poly adenosine tail |
| PAP | polyA polymerase |
| nt | nucleotide |
| PAS | Polyadenylation site |
| DSE | Downstream sequence elements |
| USE | Upstream sequence elements |
| CPSF | cleavage and polyadenylation specificity factor |
| CstF | cleavage stimulation factor |
| CFI/II | cleavage factor I/II |
| PABP | PolyA binding protein |
| mRNP | messenger ribonucleoprotein particles |
| RBD | RNA-binding domains |
| PABPN1 | PolyA binding protein nuclear 1 |
| Elavl | ELAV Like RNA Binding Protein |
| Nova1 | neuro-oncological ventral antigen 1 |

**Abbreviations (continuation)**

| | |
|---|---|
| Pre-miRNA | Precursor microRNA |
| miRISC | miRNA-induced silencing complex |
| AGO | Argonaut |
| GW182 | Glycine-trytophan protein of 182kDa |
| CDS | Coding sequence |
| 3'RACE | 3' Rapid amplification of cDNA ends |
| ON | Optic nerve |
| BDNF | Brain-derived neurotrophic factor |
| GFAP | Glial Fibrillary Acidic Protein |
| Pum2 | Pumilio RNA-binding family member 2 |
| Ptbp1/2 | Polypyrimidine tract binding protein 1/2 |
| YBX1 | Y box binding protein1 |
| AREs | Au-rich elements |
| SN | Sciatic nerve |
| QK | Quanking |
| MBP | Myelin basic protein |
| hnRNP | Heterogeneous nuclear ribonucleoprotein |
| bp | Base pairs |
| RIP | RNA-binding protein Immunoprecipitation |

# INTRODUCTION

# 1. Central Nervous System Cells

## 1.1. Neurons

The nervous system of higher vertebrates, besides its role in coordination the vital functions, enables its members to perform fast and coordinated movements, respond to pain stimuli, and exert complex cognitive functions such as learning, memory and social behavior. The cellular basis of the vertebrate nervous system is built upon two major cell types: neurons and glia cells. Neurons are the main signal relaying cells and differ from other cell types present in the nervous system in their organization of fibrillar or tubular proteins that constitute the cytoskeleton.[1] During maturation, neurons undergo a transition trough several stages of differentiation until becoming fully mature, i.e. acquiring axon-dendrite polarity.[2] During this process, neurons develop lamellipodial and filopodial protrusions (at embryonic day 18 (E18)), followed by extension of multiple immature neuritis (at E18+1-2 days of *in vitro* culture (div)) which undergo axonal specification (E18+2-4div) and branching (E18+4-15div), and harbour dendritic spines and axon initiation at the fully differentiated stage (at E18+15-25div).[3] The molecular mechanisms underlying neuronal development comprise: local protein translation and degradation; a wide range of signalling pathways; transcription regulation by microRNAs[4] (miRNAs) or RNA-binding proteins (RBPs)[5]; and extracellular cues[3]. Although sharing the mechanisms involved in neuronal growth, different neurons have particular gene expression[6] and gene regulation[7] programs according to their sub type.

## 1.2. Glial Cells

Glial cells, derived from the Greek word “γλία”, meaning “glue”, provide important support to the neurons. In the CNS, there are three types of glial cells: astrocytes that provide trophic and structural support to the neurons and act in several other aspects of the neuronal homeostasis and metabolism; oligodendrocytes (OL) that produce myelin sheaths around axons in order to increase nervous conduction speed and microglia, the resident immune cells. In the peripheral nervous system (PNS) the glial population is constituted by Schwann cells (SC).[8] Glial cells, are essential for the correct development, maintenance and function of the brain network. They are the most abundant cells in the mammalian brain, and exist in a ratio of 3 glial cells for 1 neuron.[1] Although glia cells also

extend complex processes, these are less prominent and have completely different functions than those of neurons. During OL maturation, bipolar and proliferative progenitor cells (OPC) will give rise to more differentiated myelinating-competent OL containing long radial processes and branching (Figure1). Between these two developmental stages OPC undergo a complete cellular reprogramming in which they stop proliferating and initiate a differentiation program.[9, 10] This developmental program is regulated in response to extracellular cues, which are sensed by the OL. Such cues could be integrin, growth factor or chemokine receptor activation. Since OPC have a strong tendency to differentiate in absence of proliferative stimulus, this proliferation-differentiation transition needs to have fine-tune regulation. Similar to neurons, OL differentiation is regulated trough several mechanisms, such as epigenetic control[11, 12]; gene expression programs (whereas during morphological alterations, cells express a well defined panel of markers that define each stage)[10, 13]; and transcriptional and post-transcriptional regulation [14]. As OL-neuron interactions are fundamental for neurons to function, the other way around is equally vital for OL development and myelination. The OL depends on neuronal signals, such as platelet-derived growth factor alpha (PDGF-A) or fibroblast growth factor (FGF-2), to decide the time and place for differentiation[15].

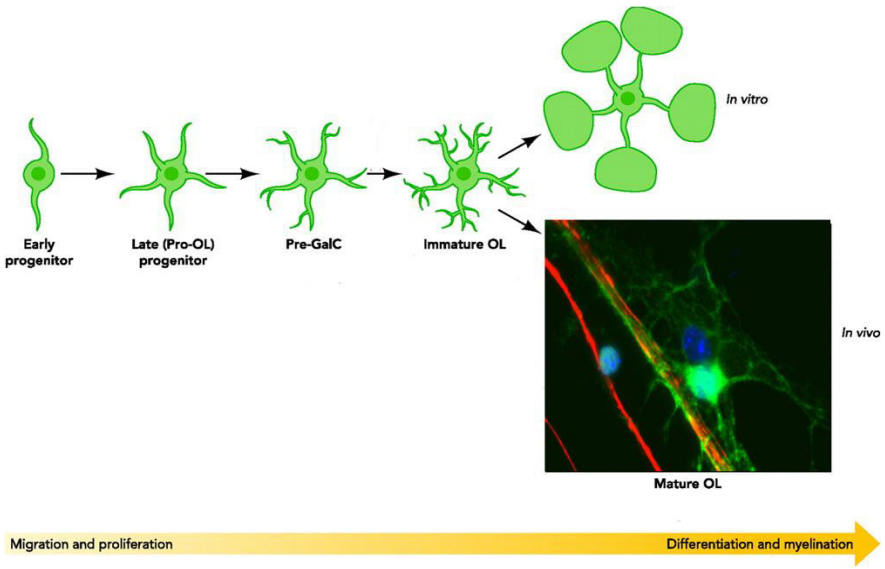



**Figure 1. Oligodendrocyte development and differentiation.** Representative scheme of the morphology alteration of oligodendrocytes during development and differentiation and an *in vivo* image of a mature oligodendrocyte (in green) myelinating an axon (in red). (Adapted from Jackman N. *et al.* 2009, Physiology)

## 2. Rho GTPases

Rho (Ras homologue) family of small guanosine triphosphatases (Rho GTPases) includes a large subgroup of the Ras superfamily of 20-30kDa GTP-binding proteins.[16] They act as molecular switches integrating signals from the environment to intracellular signal transduction pathways, thereby controlling a wide range of essential biochemical responses in eukaryotic cells.[17] The network involving Rho GTPases is thought to be very complex since approximately one percent of the human genome encodes proteins that either regulate or are regulated by direct interaction with members of the Rho family.[18] Rho GTPases are ubiquitously expressed from yeast to mammalians, and within these they share over 50% of sequence identity.[19] They are divided in 6 subfamilies, according to their sequence similarity and/or functionality: the classical RhoA (Ras homolog gene family, member A)-, Rac1 (Ras-related C3 botulinum toxin substrate 1)- and Cdc42 (Cell division control protein 42 homolog)-related subfamilies; and the atypical Rnd and RhoBTB (Rho-related BTB (Broad Complex/Tramtrack/Bric-a-brac) domain) subfamilies (Figure 2).[20, 21] As molecular switches, typical Rho GTPases cycle between two conformatial states: inactive, in GDP-bound state or active, when binding to GTP. This alternation is controlated by three classes of regulatores: GTPase exchange factors (GEFs), which promotes the dissociation of GDP to allow the binding of GTP; GTPase-activating proteins (GAPs), which increase the activity of Rho GTPases by promoting the

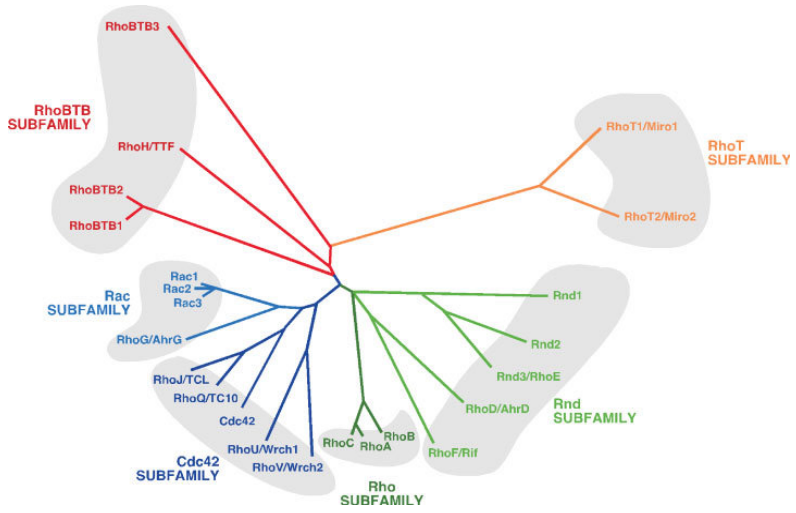



**Figure 2. Dendrogram showing the classification of Rho/Rac subfamily members according to structural similarity criteria.** Members of each subfamily are highlighted using the same color code and grouped by shaded areas. The first symbol used for each GTPase corresponds to that approved by the Human Genome Organization Gene Nomenclature Committee. (Bustelo X. *et al.* 2007, Bioessays)

return to the inactive state; and guanine nucleotide dissociation inhibitors (GDIs), that stabilize the GDP-bound form and inhibit the association of GTPases to the membrane (Figure 3).[22] In addition to this specfic regulation, Rho GTPases are also tightly regulated at expression level.[21] When activated, these GTPases interact with their downstream effectors, which in turn regulate a wide range of mechanisms such as microtubles dynamics, transcription activation or membrane trafficking. These interactions, as well the resulting functions, are likely to be specific for each cell-type and physiologic condition. Rho GTPases are therefore fundamental for diverse cellular processes including cell growth, cytokinesis, cell motility, cell adhesion, cell transformation, invasion and neuronal development. Our knowledge about Rho GTPases arises mostly from the best studied proteins Rac1, Cdc42 and RhoA.[17, 23-26]

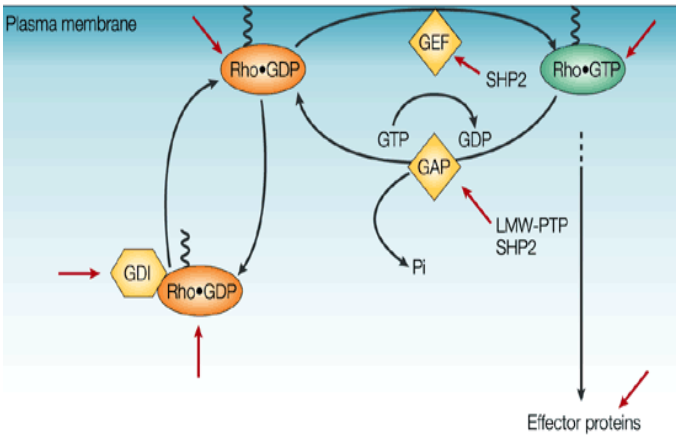



**Figure 3. GEFs, GAPs and GDIs regulating Rho GTPases.** The activity of the Rho GTPases is modulated by Rho regulatory proteins of the following classes: GEFs, which activate Rho; GAPs, that facilitate inactivation of GTP-bound Rho by increasing GTPase activity; and GDIs that prevent dissociation of GDP and inhibit activation. Phosphatases could potentially regulate the Rho GTPases at many levels, as shown by the red arrows. (Adapted from Larsen M. *et al.* 2003 Nature)

## 2.1. 'Classically activated' Rho GTPases (Rac1, Cdc42 and RhoA)

Rac1 is known to regulate signaling pathways for cell proliferation, apoptosis and activation of immune cells. However, its major function is the organization of the actin cytoskeleton (adhesion and differentiation) and the lamellipodium.[18, 27, 28] Activated Rac1 triggers a broad list of downstream effectors including proteins from the Wiskot-Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE), mammalian Diaphanous (mDia) and p21-activated kinase (PAK), which are implicated in the major

functions described below (Figure 4). The fact that the *Rac1*-knockout is embryonic lethal and the *Rac2*-, *Rac3*- and *RhoG*-knockout are viable and do not have developmental defects demonstrates the importance of *Rac1* relatively to the other members of the Rac subfamily.[28]

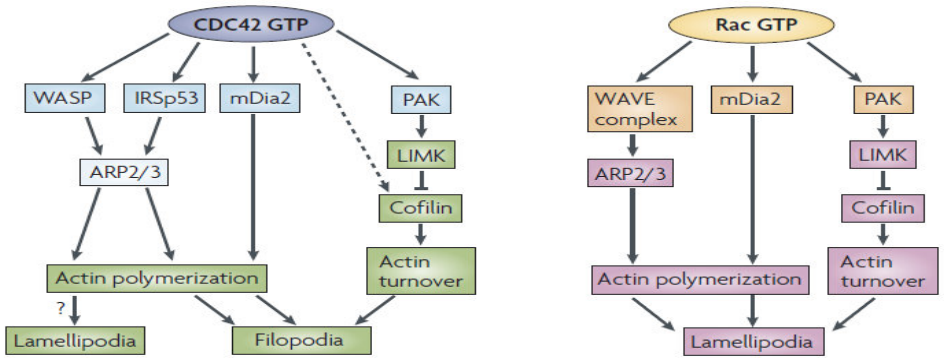



**Figure 4. Cdc42 and Rac1 effectors in lamelipodia and filopodia pathways.** Cdc42 induces actin polymerization by binding to WASP, or through IRSp53 Tyr kinase to induce branched actin filaments using the ARP2/3 complex. Rac activates the ARP2/3 complex through the WAVE complex. Cdc42 and Rac also induce actin polymerization by activation of mDia2. Rac- or Cdc42- mediated activation of the Ser/Thr kinase PAK phosphorylates, LIM kinase (LIMK), which phosphorylates and inhibits cofilin, thereby regulating actin-filament turnover. In the neuronal growth cone, Cdc42 might result in reduced cofilin phosphorylation by an unknown mechanism (dotted line), thereby stimulating actin polymerization and filopodium formation. (Adapted from Heasman and Ridley 2008, Nature)

Cdc42 function (as the other Rho GTPases) is related with receptor-mediated signal transduction leading to induction of gene transcription, cell cycle progression, apoptosis, migration, chemotaxis, and cell fate determination. [18, 28, 29] However, the primordial function of the Cdc42 is the regulation of cell polarity and actin cytoskeleton resulting in the filipodium formation. When activated, Cdc42 signals through WASP, mDia, the insulin-receptor substrate p53 (IRSp53) and PAK to perform its functions in actin polymerization (Figure 4).[28, 29] Its function is thought to be essential during embryonic development as Cdc42-knockout mice that die at embryonic day 7.5.[30] Recently, two spliced isoforms were described for Cdc42: the canonical isoform that is ubiquitously expressed and the brain specific isoform (further referred as Cdc42 Isoform 1 and Isoform 2 respectively).[31] These isoforms differ only in their C-terminal exon, a region where they have a few amino acidic (a.a.) changes resulting in different lipidations of the proteins.[32] Thus, both Cdc42 Isoforms have a prenylation in that region, however Isoform 2 has an additional palmitoylation.[31, 32] The palmitoylation of the Cdc42 Isoform 2 is essential for its membrane localization and in neurons plays a role in dendritic protrusion and dendritic spine formation.[32] Additionally, the dual lipidation increases the affinity of Isoform 2 for the

plasma membrane.[33] Although its biologic function is poorly known, this specific feature of Cdc42 represents an additional mechanism of regulation, highlighting the fine-tuned activities of Rho GTPases.

RhoA was the first identified member of Rho GTPases. Similar to Rac1 and Cdc42, RhoA transduces extracellular signals and, through it effectors, regulate cell migration, adhesion, survival, cell division, gene expression and vesicle trafficking in a cell-type dependent manner.[18, 34] Rho has been postulated to be a major regulator of actomyosin and focal adhesion dynamics, but in several cell types, these effects are only seen in cooperation either with the other members of the Rho subfamily (RhoB and RhoC) or with its effectors (e.g. ROCKI/II (Rho-associated, coiled-coil containing protein kinase 1/2) or mDia) or with other Rho GTPases (Figure 5).[34] In the past few years, several papers highlighted the important functions of Rho GTPases in regulating both the actin and microtubule cytoskeletons and the machinery involved in establishing polarity, during CNS and PNS development.

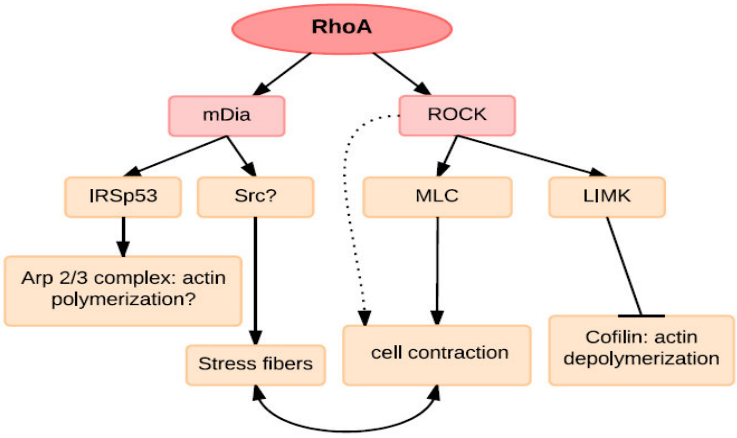



**Figure 5. RhoA effectors linked to actin reorganization.** Rho via ROCK can stimulate myosin light chain (MLC) phosphorylation and together with mDia induces stress fibre formation. mDia is dependent on Src for its contribution to stress fibres and has also been reported to interact with IRSp53, which can mediate Arp2/3-complex-induced actin polymerization. ROCK can also phosphorylate a number of other target proteins that may contribute to actin reorganization, including LIMK, which inhibits cofilin-mediated actin depolymerization. (Adapted from Ridley A.J. 2001, Journal of Cell Science)

In the CNS neuronal development requires Rac1 and Cdc42 activity for neurite formation and outgrowth, as well as for axonal guidance.[23, 26, 35] During axon polarization Cdc42 activates Rac1 via the Par3/Par6 complex and Rac1 GEFs (Tiam1 or Tiam2/STEF), which are known to be essential to neuronal polarization and extension. This mechanism is a positive feedback loop between Rac1 and Cdc42, whereas the former could activate the latter through activation of PI3-kinase (phosphatidylinositol 3–kinase). In addition, Rac1 is

activated by GEF DOCK7 to allow the microtubule growth in the nascent axon. Neuronal differentiation progresses with axon extension and, for this is required an additional effector of Rac1 and Cdc42, the IQGAP3 (IQ motif containing GTPase activating protein) and, N-WASP (Neuronal- WASP) that is targeted only by Cdc42.[23, 36] Nevertheless, Rac1 functions are not restricted to the axon, and they also affect branching of the dendrites.[35, 37] Regarding axon guidance, Rac1 and Cdc42 are associated with attractive cues and forward protrusion of growth-cone, while RhoA is associated with repulsive cues and growth-cone collapse. The RhoA and Rac1/Cdc42 are also thought to have antagonistic roles during axon formation and outgrowth.[23, 34, 36] The functions of these Rho GTPases are not restricted to neurons, and it are equally important for the appropriate differentiation of OL and SC, even though through different mechanisms.[24] Rac1-null SC lead to hypomyelination of the sciatic nerves and defects in radial sorting (similar to those seen on laminin or β1 integrin mutants). Rac1 activity, which is regulated by β1 integrin, is necessary for process extension and stabilization, and consequently for efficient radial sorting of axon bundles in a β1 integrin-dependent way.[38] Recently two downstream effectors of Rac1 were described that directly influence myelination: NF2/merlin and cAMP.[39] In the other hand, Cdc42, which activity is regulated by NRG1 affects SC proliferation and consequently radial sorting of axons (Figure 6).[38] In OL, differentiation and the proper axon ensheatment and myelination depend on Rac1 and Cdc42 activity, and on RhoA inactivity.

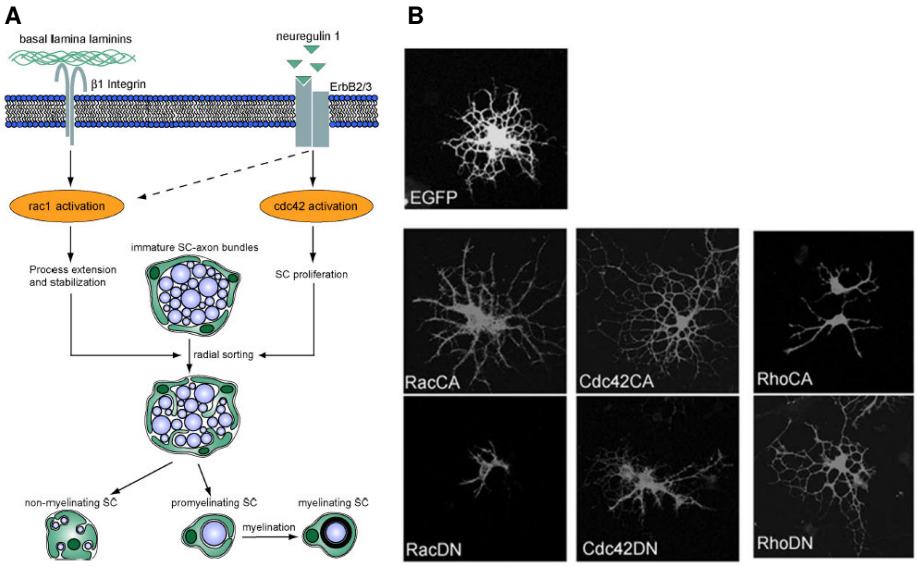



**Figure 6. Regulation of SC and OL development by Rho GTPases.** **A)** Proposed mechanism for regulation of SC by Cdc42 and Rac1, which plays different but essential roles during SC development. Both Rac1 and Cdc42 are required for efficient radial sorting of axon bundles, a prerequisite for subsequent axon

myelination. **B)** Effects on OL differentiation upon expression of constitutive active (CA) or dominant negative (DN) Rho GTPases. Constitutively active Cdc42 and Rac1 and dominant negative RhoA induce outgrowth of OL, whereas dominant-negative Cdc42 and Rac1 or constitutive active RhoA inhibit OPC differentiation, when compared with the empty vector (EGFP). (Adapted from Benninger Y. *et al.* 2007, The Journal of Cell Biology and Liang X. *et al.* 2004, The Journal of Neuroscience)

Similar to neurons, Rac1 and Cdc42 act synergistically in process extension and branching during the OL differentiation and myelination.[38, 40] These two proteins are both necessary for proper axon ensheathing and their loss leads to the accumulation of a large amount of cytoplasm in the inner tongue of the process resulting in the formation of myelin outfoldings.[40, 41] The Rho GTPase effectors participating in myelination are poorly known, however a few studies have shown that the Rac1 effector WAVE1[42] or the Fyn phosphatase, which affects RhoGAP p190 activity [41]. This is in contrast with RhoA, which has a negative role in the OL differentiation inhibiting process extension. Although the molecular effectors of this regulation are not completely known, ROCK is thought to be one of them[41].

### 2.2. Atypical Rho GTPase RhoBTB2

Rho BTBs are a group of atypical Rho GTPases that differ from the classical in their size; in their regulation by gene expression or protein stability and in the constitutive GTP-bound state. They are much larger than classical Rho GTPases and have additional BTB domains, which are important for protein:protein interactions. Although the inactivation of *RhoBTB* genes did not reveal any associated phenotype, they are reported, in particular *RhoBTB2*, as tumour suppressor genes.[21] As classical Rho GTPases, *RhoBTBs* are ubiquitously expressed in human, mouse and rat tissues. *RhoBTB1* and *3* have higher expression levels then *RhoBTB2*. Albeit having lower mRNA levels, *RhoBTB2* expression is significantly increased in the brain tissues, suggesting a tissue specific role.[43]

## 3. Gene expression regulation

The control of gene expression is a biological process essential to all organisms. Thus, gene expression requires an accurate regulation of several events during the development, division and differentiation of the cell. This regulation can be done at the transcriptional and co-transcriptional level or at the post-transcriptional levels. Transcriptional regulation occurs through the binding of regulatory proteins with specific DNA motifs, chromatin remodelling or by the interaction with the transcription machinery.

Regulation at the co-transcriptional level includes pre-mRNA processing: capping, splicing and polyadenylation. Post-transcriptional regulation comprises mRNA stability, transport, and translation mechanisms. Some of the key events that are regulated at the co-transcriptional level are alternative splicing, polyadenylation (PA) and alternative polyadenylation (APA) and include several players *cis*- and *trans*-regulatory elements, RBP and small RNAs such as miRNAs. All of these mechanisms are emerging as key components in this regulatory process.[44-46]

## 3.1. Co-transcriptional regulation

Pre-mRNA (precursor mRNA) processing is a key step in mRNA maturation and in the specificity of several cellular programs such as tissue-specific gene expression, apoptosis, sex determination or development, implying a tight regulation of the mechanisms involved. The production of a mature and functional mRNA requires three well defined processing steps: (1) capping, that consists in the addition of a cap structure at the 5'end of the nascent transcript and prevents it from degradation; (2) splicing, involving the assemblage of small nuclear RNAs (snRNAs) with proteins in the small nuclear ribonucleic particles (snRNPs) to form the spliceossome, in order to remove the introns from the (pre-mRNA); (3) 3'end formation, that comprises the cleavage of pre-mRNA followed by the polymerization of an adenosine tail (polyA tail).[47, 48] Through alternative splicing or PA, one single transcriptional unit can produce several mRNA molecules that could lead to different proteins or mRNAs with different 3' UTR lengths. These fundamental processes occur co-transcriptionaly and, although being independent mechanisms, are in close crosstalk and interact with the transcription machinery.[48, 49]

## 3.2. Polyadenylation

The maturation of eukaryotic mRNAs requires a precise 3'end formation. As referred above this process comprises two steps, one specific endonucleolytic cleavage and the polymerization of the polyA tail by the polyA polymerase (PAP) (histone genes are the exception). This reaction is called polyadenylation (PA) and is driven by *cis*-elements present in pre-mRNA and *trans*-acting factors. The length of the polyA tail was recently described as organism-, development stage-, and tissue-specific and could vary between

30 to 100 nucleotides[50, 51], in contrast with what was consensually accepted before (polyA tails with approximately 200 adenosines)[44-46]. The PA occurs in almost fully processed eukaryotic mRNAs[48], and influence the stability, translation and transport of mRNA to the cytoplasm[44, 46]. However recent studies have, in part, challenged this view showing that the effect of the polyA tail on mRNA stability is restricted to the early developmental stages.[50, 51] The consensus and stronger signal for PA (pAs) is the *cis* sequence AAUAAA (though the signal might be a weaker variant) that is recognized by the 3'end processing machinery, and normally is localized 10-35 nucleotides (nt) upstream of the cleavage and polyadenylation site (PAS).[52] The cleavage and polyA tail addition occurs between this hexamer and an U/GU-rich downstream sequence element (DSE), which affects PA efficiency. Upstream U-rich sequence elements (USE) that present before the pAs could also affect the efficiency, providing binding sites for several protein factors.[44-46] The core machinery responsible for cleavage and polyadenylation comprises four main protein complexes: cleavage and polyadenylation specificity factor (CPSF), that identify pAs and catalyse the cleavage reaction; cleavage stimulation factor (CstF), that recognize the DSE; and cleavage factor I (CF $I_m$) and II (CF $II_m$), which are important for the assembly of the other members of the protein complex. For correct 3'end processing the core machinery have to engage 25 protein factors and around 60 individual proteins such as symplekin, PAP or polyA binding protein (PABP) (Figure 7).[53, 54] This assemblage requires a fine-tune regulation to allow the accurate mRNA maturation.[48, 53] The impairment of one of these factors or functions leads to a PA defect, related with a wide range of diseases or cellular conditions.[55]

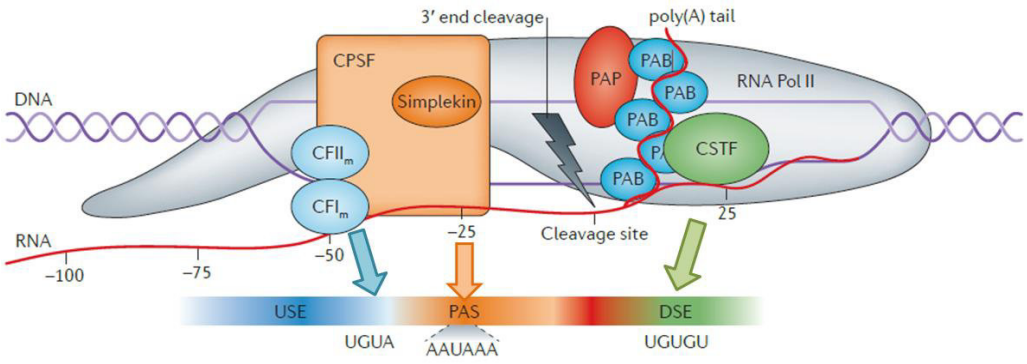



**Figure 7. Core pre-mRNA 3'end processing machinery involved in cleavage and polyadenylation.** The cleavage factors CF $I_m$, CF $II_m$ bind to their cis regulatory elements, USE, and CPSF binds to the pAs, whereas CstF binds to the U/GU-rich region, DSE. PAP polymerizes the polyA tail and PABP binds along the polyA tail. (Adapted from Elkon R. *et al.* 2013, Nature)

## 3.3. Alternative polyadenylation

In the human genome 70% of the genes have multiple pAs in their 3' untranslated regions (3'UTRs), and in mammalian, 80% of the genes undergo APA.[56, 57] APA controls gene expression by production of alternative mRNA isoforms due to the usage of different pAs present in one gene. Usage of one PAS over another is often attributed to the relative strength of the *cis* and *trans*-acting elements and to a close relation with transcription as mentioned above. The different types of APA differ in the localization of pA signal (intronic or exonic), which generates either different mRNA coding sequences (resulting in different proteins produced), or mRNAs with 3'UTRs of different lengths (Figure 8).[44, 46, 58]

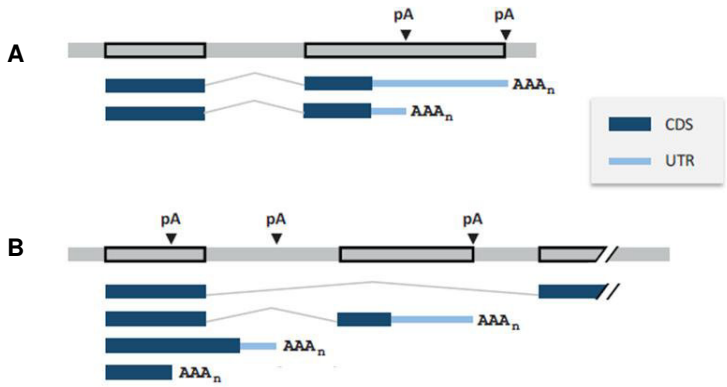



**Figure 8. Alternative cleavage and polyadenylation sites (PAS). A)** Alternative polyadenylation (APA) in the 3' most exon. A hypothetical gene is shown, with two pAs located in the 3'UTR originating two mRNAs with different 3'UTR lengths. **B)** APA in upstream regions producing mRNAs containing different coding regions, and consequently, different proteins (Adapted from Tian B. and Manley J.L. 2013, Trends in Biochemical Sciences )

In the past few years, with the increase usage of genome wide methodologies, APA has been related with global physiologic events. Recent studies associate the usage of the shortest 3'UTRs with proliferation and transformation of the cells. The usage of proximal pAs is observed during the activation of T lymphocytes, cell proliferation and in cancer cell lines. Usage of proximal pAs has been related to the production of higher amounts of protein, due to the loss of regulatory elements, present in the longest 3'UTRs, such as target sites for miRNAs.[59, 60] On the other hand the longest 3'UTRs are present preferentially in differentiation and development programs.

Several studies have shown that different cell types were reprogrammed to generate induced pluripotent stem cells through APA modulation[61], additionally 3'UTR lengthening was seen in the mouse embryonic development studies[62]. In addition, various works using RNA sequencing (RNA-seq) have shown that in brain tissues, both from *Drosophila* and

mammalian models, there is a diffuse and extensive lengthening of the 3'UTRs. Curiously was found that brain 3'UTR extensions are significantly longer than previously annotated.[63, 64] Alternative mRNA isoforms (resulting from APA) with longer 3'UTRs have more *cis*-regulatory elements in pre-mRNA such as binding sites for RBPs (involved in mRNA stability, localization and translation), miRNAs (involved in mRNA translation inhibition, cleavage/degradation), and regions with AU-rich content (important for PA site definition). Consequently, the differential signals usage implies that these RNA sequence elements will be differential used, affecting the transcript produced and ultimately modulating gene expression, both in a negative or positive manner. Therefore the differential selection of pAs in APA is a co-transcriptional mechanism that allows the mature mRNA present alternative places for regulation (Figure 9). [44, 46, 58 65]

## 3.4. RNA Binding Proteins

The RBPs are key regulators of gene expression both at co- and post-transcriptional levels. The pre-mRNA processing is intrinsically related with the binding of RBPs to the specific sequences present in the mRNA resulting in the formation of messenger ribonucleoprotein particles (mRNP) complex. This interaction occurs trough the specific RNA-binding domains (RBD) in the RBP, which could be repeats of the same type or a conjugation of different domains. Together, the high variability of these RBD, the alternative splicing of RBPs pre-mRNA, the auxiliary functional domains, and the high diversity of RNA-binding sequences leads to a variety of mechanisms of regulation by RBPs in one cell.[65] The importance of the RBPs is denoted by the high evolutionary conservation of their binding sequences.[66] In short, cells are able to generate diverse mRNPs specific for each mRNA. Several aspects of the RNA biology such as transcription, pre-mRNA splicing, PA/APA, modification, export, localization, translation and turnover can be affected by RBPs. An efficient PA is dependent on specific RBPs, while one of the major players, the CPSF, binds to the pre-mRNA by its RNA-binding subunits. Poly(A) binding protein, nuclear 1 (PABPN1) is another important RBP for PA, that additional to CPSF, its binding promote the activity of PAP. [65] The impairment of RBPs biology, as mutations or alterations in their relative concentrations in the cell can lead to severe disorders.[55] In other way, APA *per se* is able to escape or induce the regulation through RBPs according to the production of the shortest or longest mRNA isoforms respectively. Therefore, cells can use this mechanism, in addition to the

expression of cell-specific RBPs to achieve their cellular fate.[62, 67] The usage of this type of regulation is most evident and known in neuronal development and specification, where the couple between the 3' lengthening[64, 68] and the expression of brain-specific RBPs such as ELAVl/Hu (ELAV Like RNA Binding Protein) proteins or NOVA (neuro-oncological ventral antigen)[69] occurs. Another important feature of the neuronal cells involving RNPs is the mRNA transport and the local translation. As described above neurons extend long axons that need the transport of mRNAs to the apical zone for local translation of proteins in a rapid response manner.[69, 70] The deregulation of these processes is one of the major causes of neuronal diseases.[55] Although less is known, some studies have demonstrated the fundamental role of RBPs in OL differentiation and myelination. The quaking (QK) family of RBPs has identified in glial progenitors and OLs, where the differential expression of spliced isoforms coincides with the development of OLs and the onset of myelination. Mutation on *qk* gene leads to a failure in the development of mature myelinating OLs.[71] Another important family of RBPs is the Musashi (Msi) family, which is preferential expressed in CNS stem cells, and its downregulation is required to the differentiation of OLs.[72] Similar to neurons, in OLs the transport and local translation of specific mRNAs is also observed. The best studied is the myelin basic protein (*Mbp*) mRNA, which requires the formation of a RNP complex, where different family members of the RBPs hnRNP (heterogeneous nuclear ribonucleoprotein) play different functions through binding to the 3'UTR.[73-75] This co-transcriptional regulation by RBPs is tightly associated with those of miRNAs, indeed recent studies have confirmed either competition or collaboration between them.[76, 77]

## 3.5. MicroRNAs

miRNAs are a class of nonconding RNAs with approximately 21 nucleotides in length that are able to post-transcriptionally silence mRNAs by binding to them through complementary or semi-complementary sequences. In mammals, the activity of approximately 50% of protein-coding genes is controlled by miRNAs. Although a single miRNA is often predicted to target several individual transcripts, their expression can impact the definition of the cell-specific protein profile, since miRNAs are expressed in a tissue-specific and/or developmental-stage-specific manner. The mRNA target sequences for miRNAs could be also binding sites for other miRNAs as well for RBPs, which give a multiple combinations for regulatory effects.[76] In short, the biogenesis of miRNAs, initiate

with the formation of the pre-miRNA (precursor miRNA), which start by the transcription of an independent gene or an intron of one protein-coding gene by RNA polymerase II. This pre-miRNA is processed first by Drosha (in the nucleus) and by Dicer (in the cytoplasm) enzymes resulting in a ~20bp miRNA/miRNA duplex. This duplex is then separated and one of the strands becomes the mature miRNA that is incorporated into miRNA-induced silencing complex (miRISC). In miRISC, miRNAs will induce the translational repression or degradation, through the action of Argonaute (AGO) proteins and glycine-tryptophan protein of 182kDa (GW182), of the target mRNA.[78, 79] The major determinant of the AGO action is a 6-8 nucleotidic domain at the 5' end of the miRNA (this domain is called seed region). The seed region binds to the complementary region ('seed matches') on target mRNA and causes a decrease in its expression. The repressive effect is highest when the seed matches occur in the 3'UTR.[80]

Regulation by miRNA activity has some of the most interesting examples in neurons, where miRNAs have a role in the regulation of localized protein expression. Thereby, the mRNAs can be inactivated by miRNAs to reach the dendritic spines, where it becomes accessible to the translation machinery, and also they can be downregulated in the dendritic spines by the local expression of specific miRNAs.[78] In addition, neuronal determination and development are also dependent on miRNA activity, either through promoting the differentiation of the neural stem cells and progenitors (*e.g.* miR-124 and miR-9) or inducing their proliferation (*e.g.* miR-134, miR-25 or miR-137), according to the cluster of genes that are targeted.[4] Another important role of the miRNA function in the CNS is the control of the production, differentiation and health of myelinating OLs. Specific miRNAs act in OPCs and in OLs through: (1) promoting OPC expansion; (2) suppressing OPC-expressed genes to promote differentiation; (3) the overall suppressing of inappropriate non-OL lineage gene expression in OPCs and OLs; and (4) by suppressing transiently genes required at high levels during myelin sheath formation. Several members of the miR-17-92 cluster have been described as necessary and sufficient for OPC proliferation.[81] The two most highly induced miRNAs in differentiating OLs are miR-219 and miR-338; they promote normal OPC differentiation into OLs.[82] Recently, in one study it was shown that myelination of the CNS is promoted by the expression of miR-23a.[83] Thus several steps of development and differentiation of CNS can be regulated either through inducing or repressing miRNAs. The impairment of these functions by the loss of miRNA target sequences can lead to several disorders or cellular conditions[55].

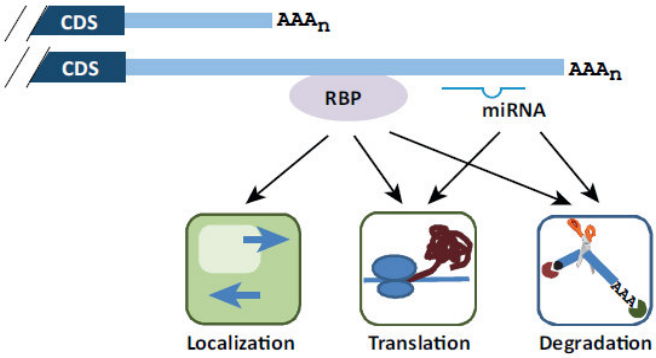



**Figure 9. Regulation by *cis*- and *trans*-acting elements in the 3' UTR originated by APA.** Two mRNA isoforms are shown containing different RBPs binding sites and miRNA target sites. Their impact on mRNA localization, translation and degradation are indicated. CDS, coding sequence. (Tian B. and Manley J.L. 2013, Trends in Biochemical Sciences)

# Aims

In this thesis we addressed three main aims:

Part I

- Characterization of Rho GTPases APA during OL differentiation.
- Understand if the mechanism of Rho GTPases APA is cell type specific.

Part II

- Study the function of Cdc42 Iso1 and Cdc42 Iso2 in OL differentiation.

# MATERIAL & METHODS

## *In silico* analysis

The UCSC genome browser was accessed to check for APA sites of mRNAs. The nucleotide sequences were obtained from NCBI database. Conservation of nucleotide sequences among different species was performed using the Geneious v4.8 software[84]. To predict putative binding sites of RBPs, the PBSFinder[85] (tool developed by us), RBPDB[86] and SFmap databases[87] were used; while for microRNAs predition, the microRNA.org[88], mirWalk 2.0 database[89] and Targetscan.org were used.

## Cell Culture

**Primary mixed glial cultures**. Primary mixed glial cultures (composed of OPC, microglia and astrocytes) were harvested from post-natal day (P) 1 to P2 neonatal Wistar rat cortex following a standard protocol[90] with minor modifications[91]. In brief, dissociated rat neonatal cortices were cultured at 37 °C in 7.5 % CO2 in DMEM with 10 % FCS and penicillin/streptomycin for 10 days *in vitro*.

**Microglia.** To remove the microglia on the top of the mixed glial cultures, the flasks were shaken for 1 h at 200 rpm on an orbital shaker. Microglia were plated at 1 x $10^6$ cells per ml into 10 cm plate in DMEM/F12 supplemented with 1 ng/ml GM-CSF and 10% FBS media. The cells were left 24 h for recover and adhesion, and were used directly for total RNA extraction.

**Oligodendrocytes.** After shaker to remove microglia, mixed glial cultures were shaken at 240 rpm overnight to dislodge the loosely attached OPC. These OPC were further purified from contaminating microglia by a differential adhesion step. Purified OPC were plated at a density of 20,000 cells per 0.8 $cm^2$ well in proliferation media (SATO media [L-glutamine (4 mM), putrescine (16 µg/ml), T4 (400 µg/ml), T3 (400 µg/ml), progesterone (6.2 ng/ml), sodium selenite (5 ng/ml), BSA V (100 µg/ml), insulin (5 µg/ml), holo-transferrin (50 µg/ml)] supplemented with PDGF-A (10 ng/ml), FGF-2 (10 ng/ml), 1 % penicillin/ streptomycin). After 2 days the cells are harvested, for day 0 time point, or maintained for induction of differentiation. The proliferation media was replaced with differentiation media (SATO media supplemented with1 % penicillin/ streptomycin and 0.5 % FCS). Cells are collected at 3, 5 or 7 days of differentiation. All cells were cultured in Poly-D-Lysin and Laminin coated plates. All mixed glial cultures were maintained until a total of three shakes were performed.

**Astrocytes.** Upon the three shakers describe above, the mixed glial cultures were subject to trypsinization and plated in new flask for further astrocyte purification. A total of three steps of purification were performed. After obtaining a pure astrocytes culture, the cells were used directly for total RNA extraction.

**Neurons.** High density cultures of rat cortex, hippocampal and striatal neurons were prepared from E18–E19 Wistar rat embryos as previously described[92], and were obtained from Teresa Summavielle's laboratory. Briefly, neuronal cultures were maintained in serum-free Neurobasal medium (Gibco Invitrogen), supplemented with B27 (GibcoInvitrogen), glutamate (25 μM), glutamine (0.5 mM) and gentamycin (0.12 mg/ml). Cells were cultured at a density of $9 \times 10^4$ cells/cm$^2$ on poly-D-lysine coated 6-well microplates, and kept at 37 °C in a humidified incubator with 5% CO2/95% air, for 24 h, 7 or 14 days in vitro (DIV), and were used directly for total RNA extraction.

**Optic and sciatic nerves.** Optic and sciatic nerves were harvested from P2, P14 and P30 Wistar rats followed by a rapid freeze at -80ºC. The tissues were homogenized in TRIzol (Invitrogen) reagent before proceeding to the total RNA extraction.

## RNA extraction

**Total RNA** was extracted with TRIzol (Invitrogen) reagent following the standard protocol. The cells were incubated 5min at RT with 1 mL of TRIzol and then overnight at -80ºC. To separate the RNA fraction chloroform was added to the lysates, mixed and centrifuge for 15 min at 11400 rpm at 4ºC. The aqueous phase was transferred to a fresh tube, where Glicoblue (15 mg/mL, Ambion Life Technologies) and the same volume as the aqueous phase of Isopropanol was added and mixed. The samples were frozen overnight at -80ºC and after, centrifuged twice, 20 min and 10 min at 11400 rpm at 4ºC, with a washing step with 75% cold ethanol between centrifugations. The pellet was air dried and resuspended in Nuclease-free water (Thermo Scientific). RNA quantification was performed in a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and the RNA stored at -80ºC.

**The extraction of RNA enriched with small RNAs** was performed using mirVana™ isolation kit (Ambion, Life Technologies) and following the manufacturer's protocol. Briefly, the cells were counted and $10^2$ to $10^7$ cells were pelleted and washed in cold PBS (1x). Cells were lysed in the lysis/binding buffer and incubated with miRNA Homogenate Additive provided in the kit. For RNA fraction extraction were added one volume of Acid-Phenol:Chloroform and centrifuged. The aqueous phase was recovered into a fresh tube and 1.25 volumes of 100% ethanol were added and mixed. This mixture was passed

through a filter cartridge before a wash step with miRNA Wash Solution 1. At this point was preformed the DNAse (DNase I recombinant, RNase free, 10 U/µL, Roche) treatment on column for 15 min. Samples were washed again with miRNA Wash Solution 1 and twice with mRNA Wash Solution 2/3. After discard the flow-through, RNA was eluted in 95ºC pre-heated Elution Solution. RNA quantification and storage was performed as described previously.

## Reverse transcription

**DNase treatment.** To avoid DNA contamination, the total RNA was treated with DNase (DNase I recombinant, RNase free, 10 U/µL, Roche) in DNase I Incubation Buffer and Nuclease-free water (Thermo Scientific), for 1h-1,5h at 37ºC. The incubation was followed for 10 min at 75ºCfor enzyme denaturation.

**Reverse transcription reaction.** cDNA was prepared using SuperScript III™ Reverse Transcriptase enzyme (Invitrogen, Life Technologies) and 500ng of total RNA following the manufacture's guidelines. Briefly, the RNA was denatured for 5 min at 65ºC with dNTPs (10 mM), Random Hexamers (50 mM) and Nuclease-free water (Thermo Scientific), followed for 5 min at 4ºC. The mixture for reverse transcription was prepared with the cDNA synthesis buffer (5X), DTT (0,1 M), RiboLock RNase Inhibitor (Thermo scientific) and SuperScript III RT (200 units/µL, Invitrogen) reverse transcriptase enzyme, added to the denaturated RNA samples and gently mixed. The samples were then incubated for 10 min at 25ºC, 60 min at 50ºC and 10 min at 70ºC to inactivate the enzyme. For discard genomic DNA contaminations, negative reactions were performed without SuperScript III.

## RACE (Rapid Amplification of cDNA Ends)

**Reverse transcription reaction.** The cDNA synthesis was performed using the SMARTer™ RACE cDNA Amplification kit (Clontech) following the manufacturer's protocol, shortly described here. Initially, the RNA and 3'-CDS Primer A were added to sterile water. The samples were incubated at 72ºC for 3 min, cooled at 42ºC for 2 min and then briefly centrifuged. During the incubation step were prepared the Master Mix with Buffer Mix for 3'RACE-Ready cDNA synthesis (5x First-Strand Buffer, DTT (20mM) and dNTP Mix (10 mM)), RNases Inhibitor (40 U/µL) and SMARTSribe™ Reverse Transcriptase (100 U). This Master Mix were added to the denaturated RNA samples and

incubated at 42ºC for 90 min and at 70ºC for 10 min. Finally the first-strand reaction products were diluted in 20-250 µL of Tricine-EDTA Buffer and stored at -20ºC.

**3'RACE.** The PCR reactions were performed also with SMARTer™ RACE cDNA Amplification kit (Clontech) by preparing, for each PCR reaction, an Master Mix with PCR-Grade Water, 10x Advantage 2 PCR Buffer and dNTP Mix(10 mM) that were added to the 3'-RACE-Ready cDNA (previously prepared), UPM (universal primer mix) (10x) and GSP (gene specific primer) (10 mM) (Table 1). Negative control reactions were performed using only one primer of the primer pair, UPM or GSP primer. The samples were submitted to a thermal cycles starting at 98ºC for 2 min followed for 5 cycles at 98ºC for 30 sec and at 72ºC for 3 min and, 5 cycles again at 98ºC for 30 sec, 70ºC for 30 sec and 72ºC for 3 min and finally, 25-27 cycles at 98ºC for 30 sec, 68ºC for 30 sec and at 72ºC for 3 min with an final extension at 72ºC for 7 min. The 3'UTR-enriched cDNA were stored at -20ºC.

**Nested PCR.** For nested PCR were added 5x Phusion HF Buffer (Thermo Scientific), dNTPs (10 mM), GSP, NUP, Phusion High-Fidelity DNA Polymerase (2 U/µL, Thermo scientific) and Nuclease-free water (Thermo Scientific) to the 3'UTR-enriched cDNA resulting from 3'RACE. The samples undergoes thermal cycling starting at 98ºC for 1 min to activate the enzyme, followed by 25-27 cycles at 98ºC for 10 sec, 65ºC for 30 sec and 72ºC for 2 min and the final extinction at 72ºC for 7 min and cooling to 4ºC.

**Table 1. Sequence of primers used in 3'RACE reactions.**

| Target | Primer pair | Sequence 5'->3' |
|---|---|---|
| **Rat Rac1** | Rac1_4 F | CTCCCATTACCTACCCGCAGGGGCTAGC |
| **Rat RhoA** | RhoA F | GCTCTGCAAGCTAGACGCGGGAAG |
| **Rat Cdc42 Isoform1** | Cdc42_Iso1 F | GAACCGAAGAAGAGCCGCAGGTGTG |
| **Rat Cdc42 Isoform2** | Cdc42_Iso2 F | CCGTTTTCTCCTTCCCCCCTTTGCTGC |

## Cloning into TOPO® vector

DNA extracted from the gel bans (produced by the 3'RACE reaction) was prepared for TOPO® TA Clonning Reaction (Invitrogen, Life technologies). First, dATPs were added to the purified DNA in a mixture of 4 µL DNA, 0,25 µL dATPs (10 mM), 0,8 µL GoTaq Buffer (5X) (Promega), and 0,1 µL GoTaq® DNA Polymerase (Promega). This reaction was performed at 72ºC for 15 min. Cloning was done by adding 4 µL of DNA from the

previous reaction and 1 µL salt solution to 1 µL vector, and incubating these mixture for 30 min at RT.

## Transformation of competent bacteria

For transformation 6 µL of the TOPO-clonning reaction were added to 100 µL of chemical competent cells. Cells were incubated for 15min in ice following a heat shock at 42ºC for 1 min and put back 5 min on ice. 400 µL of LB medium were added and the cells incubated at 37ºC for 45-60 min in an orbital shaker. Transformed cells were than centrifuge and the resuspended pellet was plated and grown in LB plates with Ampicilin (in 1:100 ratio), overnight at 37ºC.

## Colony PCR

The colonies from de previous plates were submitted to colony PCR. The reaction consisted in 0,15 µL of GoTaq® DNA Polymerase, 2 µL Green GoTaq® Reaction Buffer (1X) (Promega), 0,75 µL $MgCl_2$ (25 mM) (Promega), 0,5 µL M13 forward (AGCATCAAATGGCGTGAGA) and reverse (AGCATCAAATGGCGTGGAGA) primers (10 mM), 0,5 µL dNTPs (10 mM) mix and ddH2O up to 10 µL. With a sterile tip, the selected colony is dipped into the PCR tube and subsequently used to inoculate new ampicilin LB plates, and incubated at 37 ºC overnight. The positive colonies are selected and inoculated in liquid LB medium. Plasmid DNA was extracted from the suspension cultures using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen), following the manufacturer's protocol, and samples were sequenced outside the lab.

## Real-Time qPCR

For mRNA expression quantification, the primer pairs efficiency was optimized initially and secondly was performed the quantification itself. Briefly, a standard curve with serial dilutions of cDNA (undiluted, 10- or 100-fold diluted) was added to each primer pair (10 pmol) (Table 3), the SYBR® Select Master Mix (Applied biosystems, Life technologies), Nuclease-free water (Thermo Scientific) to a final volume of 10 µL. Reactions were incubated in the StepOne Real-time PCR System (Applied Biosystems) thermocycler following the program recommended by the company (50ºC for 2 min, 95ºC for 2 min and, 40 cycles at 95ºC for 15 sec and 60ºC for 1 min). The annealing temperature was adjusted to 58ºC in order to obtain more efficiency for some primer pairs. For an ideal

efficiency the slope of a standard curve generated with 10-fold dilutions would be -3.32. The slope obtained is used to calculate the primer pair efficiency using the formula $E=10^{(-1/slope)}$ and the correspondent efficiency percentage was calculated by the formula % E= (E-1) x100). The primer pair was considered efficient between 90-110% of efficiency. To quantify the relative mRNA expression levels were used the guidelines described above, with minor modifications. The reactions were analysed using a method that generates the expression of the target gene (TG) relative to the reference gene (RG), the comparative CT method ($2^{-(Ct(TG)-Ct(RG))}$), assuming the maximum efficiency, since the results do not suffer significant changes when using the real efficiencies. The reference genes used were Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) that were described as good housekeeping genes for the working models[93, 94]. These expression levels were also used to generate ratios between the longer isoforms and the total mRNA levels and, to normalize the expression of a specific sample relatively to a reference sample (*e.g.* day 7 of differentiation over day 0)[95].

## MicroRNA expression quantification

**Reverse transcription.** The microRNAs experiments were performed using the TaqMan® MicroRNA Assays (Life Technologies) protocol. Shortly, the RNA enriched with small RNAs was extracted as previously described, following by a reverse transcription reaction. For this reaction were mixed 10 mM dNTPs, RiboLock RNase Inhibitor (Thermo scientific), 10x Reverse Transcription Buffer, MultiScribe™ Reverse Transcriptase (50 U/µL) (Applied biosystems, Life Technologies), and Nuclease-free water (Thermo Scientific) with 1-10 ng of RNA and 5x RT primer (Table 2). The mixture was incubate on ice for 5 min before proceed to the thermal cycling that was started at 16ºC for 30 min, heated at 42ºC for 30 min and then at 85ºC for 5 min and finally cooled at 4ºC.

**Real-time qPCR.** For expression quantification of microRNAs was prepared a master mix for each probe, containing TaqMan® Universal PCR Marter Mix II no UNG and TaqMan® Small RNA Assay (20x) (Table 2) (Applied biosystems, Life Technologies), Nuclease-free water (Thermo Scientific), and reverse transcription product from the previous reaction. This reaction was transferred to the reaction plate, which was placed in the StepOne Real-time PCR System (Applied Biosystems) thermocycler using the recommended program (2 min at 50ºC; 10 min at 95ºC, and 40 cycles of 15 sec at 95ºC and 60 sec at

60ºC). The results were analysed with the previously described CT method using as reference gene the human U6 small nuclear RNA[96].

**Table 2. References of Taqman Probes.**

| Target | Reference |
|---|---|
| **hsa-miR-195** | 000494 |
| **mmu-miR-137** | 001129 |
| **hsa-miR-92a** | 000431 |
| **U6 snRNA** | 001973 |

## Actinomycin D treatment

The transcription inhibition was performed adding an antineoplastic antibiotic, Actinomycin D (Sigma-Aldrich®) to the cultured cells. No previously studies describing the ideal concentration of Actinomycin D for OL were preformed, so we used the concentration of 0,4mg/mL (an intermediate value between several cellular types). The chemical compound was added in that concentration to the culture media and the cells were collected after 10, 30, 60 and 90 min of exposure. The total RNA were extracted and converted in cDNA and gene expression analyse through RT-qPCR. The expression levels of the Actinomycin D samples were normalized to untreated samples that represent 100% of mRNA expression in order to achieve the mRNA expression decay in treated samples. These values were compared with a previous described long non-coding RNA2700046G09Rik[83] (Table 3) as a control.

## RNA immunoprecipitation

RNA immunoprecipitation (RIP) was performed using the EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore).

**Lysate preparation**. Following the manufacturer's guidelines, 8 x $10^6$ to 11 x $10^6$ OPCs were washed twice with ice-colded PBS and centrifuged at 1500 rpm for 5 min at 4ºC. The pellet was resuspended in complete RIP Lysis Buffer (RIP Lysis Buffer, Protease Inhibitor Cocktail and RNase Inhibitorfor each used antibody) and the lysate was incubated for 5 min on ice before was stored at -80ºC.

*Preparation of magnetic beads for immunoprecipitation (IP). For each IP (protein of interest, positive and negative controls) magnetic beads were washed separately twice*

*with RIP Wash Buffer and incubated 30 min in rotation at RT with 5 µg of each antibody* diluted in RIP Wash Buffer. The beads were then centrifuged and washed again as before and placed on ice.

**IP of RNA-binding Protein-RNA complexes.** The previously prepared lysates were centrifuged at 14000 rpm for 10 min and, the supernatant was added to de magnetic beads resuspended in RIP IP Buffer (RIP Wash Buffer, 0,5M EDTA and RNase Inhibitor for each used antibody). At this time 10% of supernatant for Input (that will be used for generate the standard curve for comparison) was removed and stored at -80ºC. The samples were incubated in rotation overnight at 4ºC and briefly centrifuged then for remove the supernatant. Following 6 washes steps with RIP Wash Buffer were performed.
**Purification of RNA.** The immunoprecipitated samples and the previously separated input were mixed to the Proteinase K Buffer (RIP Wash Buffer, 10% SDS and Proteinase K) before incubation with shaking at 55ºC for 30 min. The input and the immunoprecipitate supernatant were added to RIP Wash Buffer and phenol:chloroform:isoamyl alcohol and, centrifuged at 14000 rpm for 10 min at RT. The RNA extraction was performed as described previously with minor modifications specific of the EZ-Magna RIP kit.
**Analyses of immunoprecipitatedRNA.** The RNA (up to 2 µg) was converted in cDNA and the relative occupancy of the immunoprecipitated protein at each RNA site was estimated as follows: $2^{Ct\ (input)-Ct\ (IP)}$, where Ct (input) and Ct (IP) are mean threshold cycles (Ct) of quantitative RT-qPCR done in duplicate on RNA samples from input and specific immunoprecipitations, respectively. As negative control, IgG was used.

## Cell transfection

The CG4 cell line (a cell line of rat central nervous system glial precursors) was transfected with GFP-cdc42prenyl and GFP-cdc42palm plasmids (Addgene) using Lipofectamine® 3000 Reagent (invitrogen, Life Technologies) and following the manufacturer‘s protocol. In brief, lipofectamine was diluted in Opti-MEM medium (Gilbco®, Life Technologies) and added in 1:1 ratio to a master mix containing the plasmidic DNA (0,5-5 µg/µL) and P3000 reagent (2 µL/µg DNA) also diluted in Opti-MEM. TheDNA-lipid complex were added to the cells ($0,5x10^5$ cells/well) at 70%-90% of confluence and incubatedfor 5 min at RT initially, and then for 2-4 days at 37°C before analysis by fluorescence microscopy.

## Immunofluorescence (IF)

For IF analyses, cells (plated over coverslips treated with Poly-D-Lysin and Laminin) were fixed with 4% PFA for 10 min, washed twice for 5 min with PBS (1x) and store at 4ºC. The cells were then permeabilized with 0,1% TX-100 for 5-15 min and washed twice with PBS (1x) for 5 min before the blocking step, which was performed by incubation with 10% NGS (Normal Goat Serum) in PBS (1x) for 30-60 min at RT, and probed with mouse mAb anti-α-tubulin (T5168, Sigma), overnight at 4ºC in blocking solution. After 60 min at RT, in dark, Alexa Fluor 647-conjugated goat anti-mouse IgG (A21235, Molecular Probes) in blocking solution was used as secondary antibody. The incubations were followed by 3 washes with PBS (1x) for 5 min each. 4´,6-Diamino-2-phenylindole hydrochloride (DAPI) (Molecular Probes) was used to detect nuclei. The coverslips with the cells were mounted and analysed in Leica DMI 6000B microscope.

## Western-blot

Whole-cell lysates were prepared adding RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with phosphatase and protease inhibitors, directly to the cells and stored at -80ºC. The samples were then quantified (following the BSA standard curve protocol) and, the proteins were separated by SDS-PAGE using 5% (stacking gel with $H_2O$, 30% Acrilamide Mix, 1,0 M Tris (pH 6,8), 20% SDS, 10% APS and 0,01% TEMED) and 12% (resolving gel with $H_2O$, 30% Acrilamide Mix, 1,5 M Tris (pH 8,8), 20% SDS, 10% APS and 0,004% TEMED) of Polyacrilamide Gels. The loading buffer was added to the proteins in 1:1 dilution and the mixture was run in running buffer (25 mM Tris-Cl, 192 mM glycine and 0,1% SDS) for 1h30 min ( 30 min at 80V and 1h at 100V). The samples were transferred with the iBlot® Gel Transfer stacks Nitrocellulose, Mini kit in iBlot® gel transfer device (Life Technologies) to the nitrocellulose membrane. For blocking, the membrane was incubated in 10% of powder milk/PBS-T solution (8 mM disodium phosphate, 150 mM NaCl, 2 mM monopotassium phosphate, 3 mM KCl, 0.05% Tween® 20) during 1h at room temperature. This solution was replaced by a solution with primary antibody diluted in 5% powder milk/PBS-T solution and incubated overnight at 4ºC and followed by 3 washes with PBS-T of 5 min each. Finally, the membrane was incubated 1h with the appropriate secondary antibody in 5% of powder milk/PBS-T and washed again as before. All of the incubations were done in agitating platforms. Detection was performed using enhanced

luminescence (ECL Prime; Amersham/ GE Healthcare) solution in a ratio 1:1 of A and B reagents as recommended by the manufacturers.

## Antibodies

A rabbit monoclonal Ab (mAb) anti-YBX1 (ab12141; Abcam) was used for RIP experiments. The mouse mAb anti-Rac1 (ab33186, Abcam) and mouse mAb anti-tubulin (T5168 Sigma) were use as primary antibody and goat-anti mouse peroxidase (115-035-146, Jackson Immuno Res.) was used as secondary antibody for Western blot.

**Table 3. Sequences of primers used in RT-qPCR.**

| Target | Primer pair | Sequence 5'->3' |
|---|---|---|
| **GAPDH** | GAPDH F | TGGAGTCTACTGGCGTCTT |
| | GAPDH R | TGTCATATTTCTCGTGGTTCA |
| **2700046G09Rik** | lncRNA F | CCCTAGTTCAATTCCCAGCA |
| | lncRNA R | TAACTCTTGCATGCCTGGTG |
| **Rat Rac1** | Rac1 F | GAAGCTGACTCCCATTACCTACCC |
| | Rac1 R | GGGACAGAGAACGGCTCGGATAG |
| | Rac1 pA2 F | AACTGAATAAAAGTGTCACGGGT |
| | Rac1 pA2 R | GAGACACTGATAAGATCGGC |
| **Rat RhoA** | RhoA F | GACGGGAAGCAGGTAGAGTT |
| | RhoA R | CGCCTTGTGTGCTCATCATT |
| | RhoA pA2 F | ACCCCAGACTAGATGTAGTATTTTT |
| | RhoA pA2 R | ACACTTTCTGTGGGAATGACA |
| **Rat Cdc42 Isoform1** | Cdc42_Iso1 F | GAGACTGCTGAAAAGCTGGC |
| | Cdc42_Iso1 R | TCTTCTTCGGTTCTGGAGGC |
| | Cdc42_Iso1 pA2 F | TCAGATTATTTTGTTTGCAAGTTCC |
| | Cdc42_Iso1 pA2 R | AGATCAGGACTGGGGAAGGA |
| **Rat Cdc42 Isoform2** | Cdc42_Iso2 F | GAGACTGCTGAAAAGCTGGC |
| | Cdc42_Iso2 R | CTCGAGGGCAGCTAGGATAG |
| | Cdc42_Iso2 pA2 F | AAACAAAGGAATAAAACCATCCTGT |
| | Cdc42_Iso2 pA2 R | GAGTTTGCTCTAAGGTGTTGAGT |
| **Rat RhoBTB2** | RhoBTB2 F | TCCCTCGAGACATGAAGGCT |
| | RhoBTB2 R | GCACGCTGATAGTGGTCCTC |
| | RhoBTB2 pA2 F | CATCGTCCCATGGGCTTACT |
| | RhoBTB2 pA2 R | GCCTTGATGTGGGCTCTGAA |
| **Rat Pum2** | Pum2 F | ACTACAATTCCCAACAGCAGC |
| | Pum2 R | GAGCAAGGCCTGCTGAGAA |
| **Rat Nova1** | Nova1 F | TTCCATCTCAACTTCCGCCC |
| | Nova1 R | TCAGAGAGAGGCTGGACGAA |
| **Rat Ptbp1** | Ptbp1 F | CGTCCCAGACATAGCAGTCG |
| | Ptbp1 R | ATGATGAAGGGGCCGTTGCT |
| **Rat Ptbp2** | Ptbp2 F | CACCTGTCCAACATCCCTCC |
| | Ptbp2 R | AGCTTCTTCCACTGTTGCCAT |
| **Rat Elavl1** | Elavl1 F | GCATCACCAGGCACAGAGAT |
| | Elavl1 R | CGGGGACATTGACACCAGAA |
| **Rat Elval4** | Elavl4 F | GCGTAAAGAGACTGATGTCTGGA |
| | Elavl4 R | CCGTCAATGGTGATTGGGGA |
| **Rat Ybx1** | Ybx1 F | GAAGGAGAAAAGGGTGCGGA |
| | Ybx1 R | TGGTAATTGCGTGGAGGACC |

# RESULTS & DISCUSSION

# 1. Alternative Polyadenylation in Rho GTPases

## 1.1. Rho GTPases produce two mRNA isoforms with different 3'UTR lengths due to the usage of two conserved pA sites

APA is a mechanism that generates alternative mRNA isoforms allowing a differential expression of the same gene, which has been related to different cellular programs such as differentiation. During cell differentiation significant changes in the gene expression program occur. Since Rho GTPases are critical players in OLs differentiation and undergo alterations in it expression throughout this process, they are strong candidates to undergo APA. We started by identifying *in silico* putative PAS in the 3'UTR of human *Rac1*, *Cdc42 Iso1*, *Cdc42 Iso2* and RhoA using the UCSC Genome browser database (genome.ucsc.edu/). In this bioinformatics analysis we detected more than one PAS for all of the analysed human genes (Figure 10).

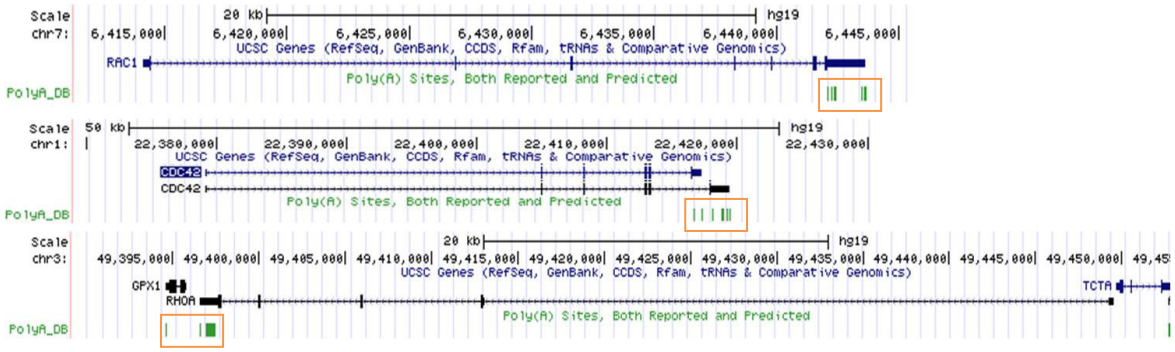



**Figure 10. Reported and predicted PAS for human Rac1, Cdc42 Iso1/2 and RhoA.** The scheme represent the genomic sequence of the genes with the black boxes (*Cdc42 Iso1* and *RhoA*) and blue boxes (*Rac1* and *Cdc42 Iso2*) showing the exons, and the narrow boxes show the 3'UTRs. Both predicted and reported PAS are surrounded by orange rectangles. (Adapted from UCSC Genome Bioinformatics).

Since in the genome browser the information about PAS for the rat genome is not available, the rat 3'UTRs were analysed to confirm the presence and the sequence of the putative pAs. In *Rac1* 3'UTR, that have 1709 base pairs (bp) in length, five putative pAs were confirmed; four of these are canonical signals and the second signal is a strong variant (ATTAAA) (Figure 12A). The 3'UTR of the *Cdc42 Iso1* have 2251bp in length and also have five predicted pAs, however none is the canonical signal, four of them are the variant ATTAAA and one is a large sequence adenosines. This adenosine sequence is present also in humans and mouse, and is described as a strong pAs (Figure 12B)[97]. In the case of the *Cdc42 Iso2* the 3'UTR is shorter (1358bp in length) than *Cdc42 Iso1* and consequently only two putative pAs were detected, the first is the canonical AATAAA and

the second is the variant ATTAAA (Figure 12C). Finally, analysis of the *RhoA* 3'UTR, that have 1073bp in length, revealed the presence of three canonical pAs (Figure 12D).

The presence of several putative pAs in the 3'UTRs of those genes does not mean that, all of the signals are used in a specific cell type or that they are used in the same manner or at the same time. However, as classical Rho GTPases possess diverse putative pAs, their expression during OL differentiation may be regulated by so far unexplored pre-mRNA processing events such as APA. In order to evaluate if these genes could undergo APA during OL differentiation and to map their 3' ends, 3'RACE analyses were performed (position of the RACE primers is depicted in Figure 11A). In these analyses, we used primary rat OPCs and the CG4 cell line differentiated *in vitro* for three days. The time point of three days was chosen because it is an intermediate stage where the cells are no longer proliferating but are not yet fully differentiated. Thus, probably the gene expression at this stage is a mixture of the two programs, which allow us the possibility to gain knowledge about all the mRNA isoforms produced in both conditions. The results of the 3'RACE show that two mRNAs isoforms are produced by all of the tested genes (Figure 11). The *Rac1* 3'RACE presented several bands (data not shown), but by performing a nested PCR, both the short and long mRNA isoforms of about 400bp and 1600bp respectively, were clearly visible (Figure 11B). The size of these isoforms corresponds to two of the previously described putative pAs. The usage of two pAs was also depicted in the nested PCR of *Cdc42 Iso1* 3'RACE reactions (Figure 11C). The shorter mRNA isoform have about 350bp and the longer mRNA isoform with nearly 1450bp and these sizes are in agreement with two of the predicted pAs found in *Cdc42 Iso1* 3'UTR. Interestingly, the 3'RACE for *Cdc2 Iso2* mRNA showed different results according to the cells used, indicating that mechanisms governing APA depend on the type of cell (Figure 11D). The nested PCR of the reaction in OLs samples revealed the usage of only the proximal pA, however in CG4 samples two isoforms were found, a shorter very faint band of about 200bp and an additional longer isoform, related with the distal pA, of about 800bp. Finally, the *RhoA* 3'RACE reactions revealed two close isoforms (Figure 11E) of approximately 640bp and 760 bp. The band patterns were also confirmed in CG4, OLN93, rat and mouse Schwann cells (data not shown).

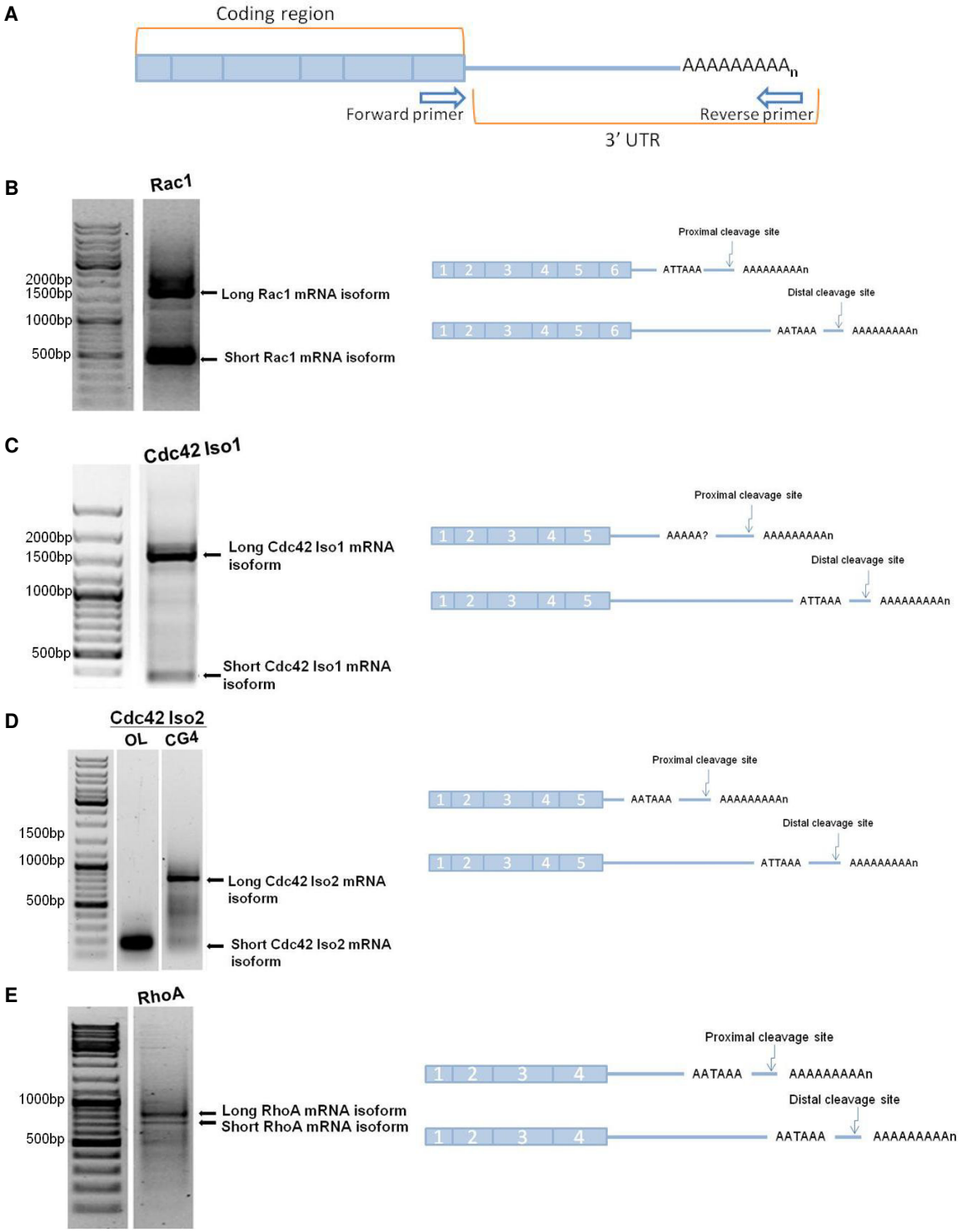



**Figure 11. 3'RACE products using rat OLs and schematic representation of mRNA isoforms of Rho GTPases. A)** Schematic representation of an mRNA showing the localization of the primers for the 3'RACE reactions. **B-E**) Representative experiment of the 3'RACE reaction. The first lane of the figures corresponds to the DNA ladder with the molecular weights in bp. The second lane shows the mRNA isoforms produced by OL at day 3 of *in vitro* differentiation. The schemes at the right represent both short and long mRNA isoforms with the indication of the pAs used. **D)** An additional lane with the results obtained using CG4 cell line is showed.

To map the pAs and the cleavage site used for each pre-mRNA, the 3'RACE products were extracted from the gel, cloned into the TOPO vector and sequenced. The results showed that production of the short *Rac1* mRNA isoform is due to the cleavage of pre-mRNA at CA (red bar) that is preceded by two pAs (Figure 12A). The pAs used is certainly the ATTAAA signal (*Rac1* pA1), because the spacing between this signal and the cleavage site, 15nt is the required for the reaction to occur, whereas the strongest canonical signal is localized too distant from the cleavage site. The longer mRNA isoform is produced by usage of the distal AATAAA signal (*Rac1* pA2) (Figure 12A). Sequencing of *Cdc42 Iso1* 3'UTR revealed cleavage at CA, producing the shorter mRNA, however the pAs that used in this case is unclear. The 3' UTR sequence have shown three noncanonical putative pAs upstream of the cleavage site, among which the adenosines stretch (*Cdc42 Iso1* pA1) seems to be the best candidate as it is the strongest signal presented. This is in agreement with other observations[97]. Interestingly, downstream of the cleavage site there is long GU/U rich sequence that usually acts as a cleavage activatory element as it binds CstF. The long *Cdc42 Iso1* mRNA isoform is produced by usage of the ATTAAA signal (*Cdc42 Iso1* pA2) and the inefficient cleavage site GA (Figure 12B). Sequencing of *Cdc42 Iso2* 3'UTR showed that the two predicted pAs were used to produce the two confirmed mRNAs. The shorter mRNA is produced through the usage of the AATAAA signal and the cleavage site CA, whereas the longer mRNA isoform use the ATTAAA signal and it is also cleaved at an inefficient GT (Figure 12 C). As the longer mRNA isoforms of both *Cdc42 Iso1* and *Cdc42 Iso2* are produced by usage of inefficient cleavage sites, it is possible that other still unidentified activatory elements and/or factors are required for the selection of the respective pAs. RhoA 3'UTR sequencing revealed the usage of two very close canonical pAs, separated by only 118nt. Within this range of spacing between, these two pAs signals are in competition[98, 99]. The cleavage of the shorter and longer isoforms occurs at CA and TT respectively (Figure 12D). Therefore, as both pAs are the canonical hexamer, both present DSEs, and the distal cleavage site is inefficient, other auxiliary elements must be involved in the distal pAs selection. Noteworthy, by visual inspection it is possible to identify a T-rich putative USE upstream the distal pA. These conserved regulatory elements activate mRNA 3' end formation[99, 100].

**A** ***Rac1*** **3'UTR**

ATGTCTGAGCCCCTCCCTCGGTCTTGGTCCCTGGAGCCTTTGTACGCTTTGCTCAGAAAATGGCGGAGTCTTCGCGTCTGATGCCAAGTTTT
TGTTACAGATTAATTTTTCCATAAAACCATTTTGAACCAATGAACCAGTCAGTAATTTTAAGGTTCTGTTTTAAATGTAAGAATTCCAACTTACA
GTCTATTAAAATTCAGCCCTAAAAC|ACAAAGCCTTCTTAAAGCCTTATTTTTCAAAATCCCCTATTCTTGCTCAGATTAAGAATTGCCAAAATA
CCTTGTGAACTAAGTTGCGTTGTGCTGAGAACATGTAAGCACTAAGCTGTTGAGAGACTTTGTCCTTAAGAAGACTGCAGCTTCTGGGCTCA
GGGGTGCAGACCCTCCCGTAGCTCCCAGACCGTGTGACACAGCACAGCCTCCTTAATGACACGCTGCCATGTAACGCACCTGTAACTTATC
AGCCCATGCTCATTACGTAACTTTGTACTGTACGTCACGATGGGTGTAACAGCTCTGCTCTTTGATTTCATAGTGAGTTCTCTAAAATACCAG
CTGACCGGCTTCTGCAGGCTTTGAACAGAACTCTGGCTCCTGTGTTGCCTCTAACGAAGTATTCTGTTCCTAGTCGTGGGTGTGCTGGGTGG
AGTGTGTGAAACACGACGTCATCAAAGGAGACAGACAGTATTTTGACTAATATGAAGTAGAGATTAATTTACACTACATTGTACATGGAGTAA
TTCAACTGAATAAAAGTGTCACGGGTAAAGCTTTTTAACGGTTAATTTCTGTCAAACAGTAGATGACAAATGGCCGATCTTATCAGTGTCTCTC
TTGAGCCCCCCTTCCCCCTGCTGTCCCTCCCCAGATGGGGCGTTGAGTCCATATTTAAACTGGCCATCCTCACAGTTGCTAACTTAGCAAGT
GCTTTTCTTTAGGACCCCCTTCTTAACGAGCAATATGTCTGACCTGTACTATAAGATCTTTCTGATAATGCATTCGGAGATTTTTTTGGTAGAT
AGTAGAAGTGCGTTCCTGTTTTCACCTTCCTTTACTCAGCTGACTAGTGCTTCCCTTCGTTTTCTAGTAACTGGGTGTAGAAATCACGTGCTG
CGGCTTTACAGTTTTTAAACTATTTTAGATATTCTGAAACATCACTGTCTTGCCAGAGTACCAACACTGTCATGTGATTGATGCCGCCCCCTCT
AGACCTCACCCACGCGGACACATGCTTCCCGTACCTTTGGGTCTGTGAGGTTCTGTCAAGCGCTAGTGCTAACGCCGTTCTGTACAACCTAA
CTCACTGGCAAGAACACAGTGTTGGGCCTTTCGACCACTAGAACAAACTTTTTTCAATTGACAGTTACAGAATTGTGGAGTGTTTTTACATTG
ATCTTTTGCTAATGCAGTTAGCAGTATGTTTTGCATGTATGACTTAATAAATCCTTGAATC|ATAAAA

**B** ***Cdc42 Iso1*** **3'UTR**

ACGCATCTCCAGAGCCCTTTCTGCACAGCTGGTGTTGTCATCATACTAAAAGCAATGTTTAAATCAAACTAAAGATTAAAAAAAATTTAAAATTC
GTTTCTGCAATAATGACAGACGACCTGCACCTACCCACATGCACTCATGTGAGACAAGGCCCATTAGTATGGCCCCCTTGCCCTTCCGGTAA
TAGTTAATTTTGAATAATTATGTATTGTCTAAAAAGCGATGAGTGCTAGTTTTGTTTCCTTGTTTAAAAAAAAAAAAAAAAAC|AACGCAAATCC
CCACAAACGTTTTGTTTTGTTTAAAAGCAAGGCATGCTTGTGCTGACTGTAACCGACTAATTCAGATCGTCAGAGCTGCTCCCTGGTCCATGT
GGAGAGTATTCTGGGGCATCTTAGTGTCTTCTGTTGTTTTGTTTTGTTTTTCTTCCACCCTTCTCTCCTTTTAGGGTGTGTATGGGGTTGTTTC
TTAGACTTGTATTTTAAAATTTGTAAACCTGAGGACAGCCTGTGGGGAGGAGGACAGATGATTTCCACATTCCACTCCCTAGGTCTAGTTTAG
CAACTGTGTCCCCACCTGGTGCTCTTAGGGAGGAGTGTGGGAATGCCTCGTTTAATAACGTACTCCTTCTTGAAAGCTGCCTTTTCTCTCCA
CCCTTAAGTGGACCCAGTCTTTGCTGAAACTCATGCAGGTGGGTGGGAGCTGTCTTGCCCCTCTCTAGGATGCACTCTACCTGTGACTGTGA
CTTTCAAGGGTATTTGTTTGCCATTTGCTGATTTGGGGGGGGGGGGATAACTTCTAACTTCTTTCATTGCTAAATGAAAAAAAGTATTGCACC
TTTTGAAATACACCAAATGGATTGAGTTCCTAATTAAAAGAGTTCTTTTGCCCCTGTCAGTCATTTTCTTATATGCTTAGCATAGATTTACAACT
CACTGTGTAGTGTTCCTAGATTCAGCTGAAGACGAGATACACAGGGTTCCTTAAGGGCTGGTGTTGGGAAACACAGCCGCACTGGTGATTG
CCATCTCCTTCAGGTGATGAGGTTACAGGCCTGCTTCATTAAGAAGCTATGAGCATGTGGGGTGGGGAGCATACTTTTAACCAACATGGGGA
CCAGTCAGGGCTGTACCCCTTTTTTTCTTCCATGCCTATGAGGAATCCTTGAACAACTGGCTGAAGCTCTCTCAAAAATGGCGAGAGATTCAT
GACTCTTTATAAGTGTTAATAGAGACTTTATCAGATTATTTTGTTTGCAAGTTCCAGTTTTTAAAAATGTTTAGGTAATCTCCTTCCCCAGTCCT
GATCTTGTAGATTCGTTAGTGCCGAACCAATGCTTTCTTGTGTCTCAACTCTTTGTATATGAATTCTTTTCAGATGTATTAAACAAACAAAACC
CTTCACAAGTG|AGCCTGAAGGTTGTTATTTTCCTCCGTCTCTTACTTTTCAGGTATTTAAAAGTGAGGATGTGACTCTTACATGACATCAACA
TCTAACAAATTTTGGTATAAAATTTTCAAGCTTTATTTTTGTCTTCTGACACTCCATATGCCTTCTCTCCTAAAATTGACAGCCAGCTCTGAAAC
TCCTCCCCTTGTATGCTGAGTAGGTCAGAGTAGTAAGTGCAGAAATGTGTGTGAAGACCTCACCTCCTGGAAGGCGGCACAAGCACACTCG
TGCAGAGCAGTGGGGCAGTAGACCACTTGCCTCAGCCTGCCTGCATTAAAG

**C** ***Cdc42 Iso2*** **3'UTR**

ACCGTTTTCTCCTTCCCCCCTTTGCTGCTGCTTCCTGTCCCACTACTGTAGAAAGATCATTTAAAACAAAGGAATAAAACCATCCTGTTTC|AA
AGCCTCTGCGTCTTCTTACTCAACACCTTAGAGCAAACTCTGTATTCATTTTTGATCATGAATGCAATTAGAATATTTTTGTGATCAGTAGTCAC
ATTAGACTTGTTTAACTTTCTGCTGCTTGAGTTGCCTGATGCTCAGAGCTTTTGGTTTGAATTATATTATTGCAAAAGGGAATGTGGTCTGGCA
TTAAGAATGATGTCTTCTTGGAGAAAATAAGAGTTTTAACACTTCTAGATGTTAGTTCTAGATGGAGAAAGTATCGCTAAACATCATTTTACTCT
TACTGATTGTTAATTGTAATTGCATGACAAACTATGAAAAGGGTTGACTTGATAGTAGAGTGTGACGGGGAAGGGAGGAGTCTCCTATGGTTT
TCCTGTGTGCAGTGAAACTTTTGTGTTGCTCTTGCTTTGGCTGTTGTGCTGTAGGTGGAGTGTTTGTCATTCTGGGATGGGGAAGGTGTCCA
TGTATCTGCTTACTGCTTTATGAGTTCATTGTTACACATTATCTCCTAATCCTAGCATCCATTTAACAATCGACTTAAGGTTTGCTGATGTTGAA
ATGTAAAGCTACTGCCTTTATATTCTCCTGCTTTCGATTCTATTGTTGTGAGGGAAACATACAGTTGTGGTTATCTTTACATTTTGAAATTAAAA
TTATACAATTGTTTG|TATAAATGCCTGGTGAAGC

**D** ***RhoA*** **3'UTR**

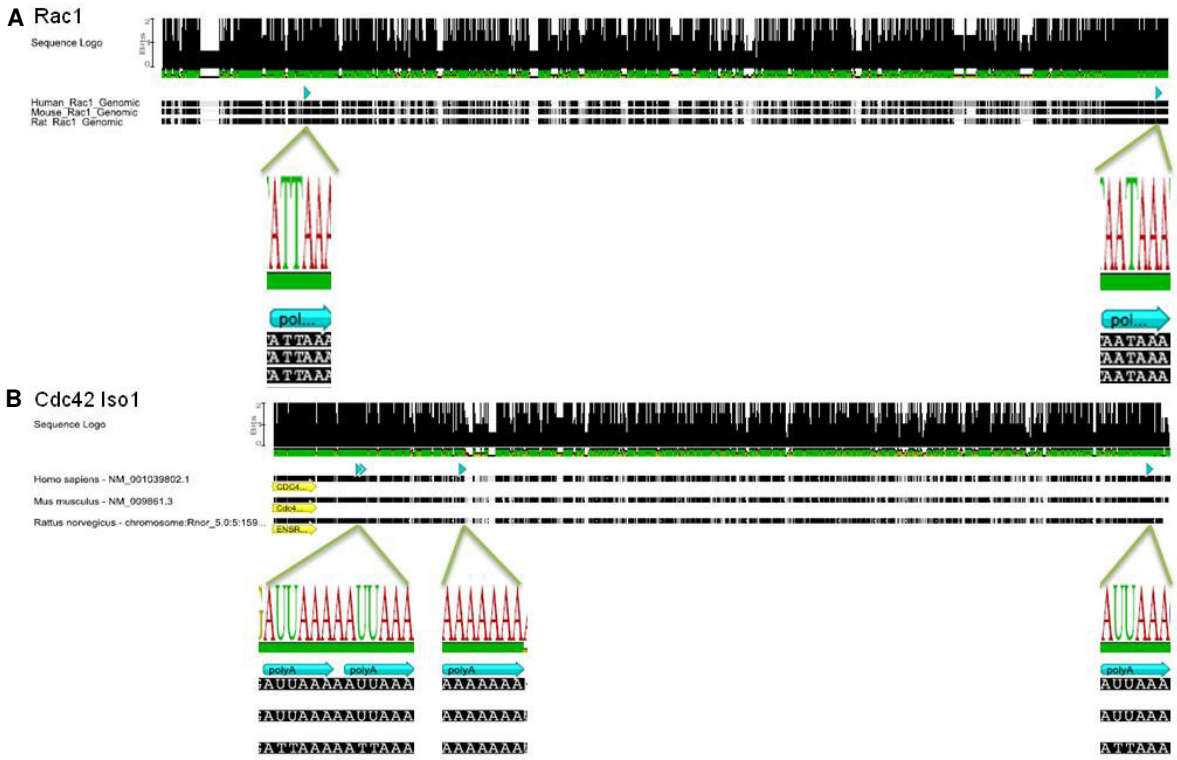
AGCCTTGTGACACGCAGCCTCATGCGGTTAATTTGAAGTGCTGTTTATTAATCTTAGTGTATGATTACTGGCCTTTTCATTTATCTATAATTTAC
CTAAGATTACAAATCAGAAGTCATCTTGCTACCAGTATTTAGAAGCCAACCATGATTATTAATGATGTCCAGCCTGTCTGACCAGCCAGGGTC
CTTCTGACACTGCTCTAACGCCCTCTCTGCACTCCACCTGACACCAGGCGCTCATTCAAAGAATTTCTTAACTTCCTGCTTCTTTCTAGAAAG
AGAAACAGTTGGTAACTTTTGTGAATTAGGCTGTAACTACTTTATAACTAACATGTCCTGCCTATTTTCTGTCAGCTGCGAGGAACTCTGGTGA
GTCACTACTTCAGAGCTTTACTCCTTAACAGATTCCATTGGCAGAGCTCTGGGGTGGGCACCCAGTGTTTGGAAACTTGATCAGCCAGAAAG
GCCCAAGTCCACGCAGCTGTGGCAGAGTTACAGTTCTGTGGTCTCATGTTAGTTACCTTATAGTTACTGTGTAATTAGTGCCACTTAATGTAT
GTTACCAAAATAAATATATCTACCCC|AGACTAGATGTAGTATTTTTTGTATAATTGGATTTCCTAATTCTGATATTTGTCATTCCCACAGAAA
GTGTATTGCTTTTTTTTTTTTTAAGAAAGTATTTGGAATAAATAAGTCAGATGGAAAAT|TCATTTTTAAAATTCCCATTTTGTCACTTTCTCTGATAA
AATATGGCCATATCTCCCCTATTTAGCCCTATATATCATTCCAGTGCCCCTTTCCAGACTGGACTGAGGAAATAGGAATTGGTTTCATGCCTG
AGGCTGTTAGACTTTGGAGGTGGCATAGCCTTTCTCACCTGGACTGCAGGGCCTGGCTCTAAGTCACAGTGCTCCTTTCTCCACACTGTTAT
CCAAGTCGCCTCCCAGAGGAGCCACCAGTTCTTATGGGTGGCAATGGGTGGGTGGGTGGGGGGTCTCCTCTCTCCAGCTGACTAAACTTTT
TTCTGTACCAGTTAATTTTTCCAACTAATAGAATAAAGG

**Figure 12. Mapping of the mRNA 3' ends of *Rac1*, *Cdc42 Iso1* and Cdc42 *Iso2* and *RhoA* isoforms. A)** *Rac1*, **B)** *Cdc42 Iso1*, **C)** *Cdc42 Iso2* and **D)** *RhoA* mRNA 3' end mapping by 3'RACE and sequencing. The pAs are shown in bold and colored in red and the cleavage sites are indicated by the red bar. Putative DSEs are underlined and putative pAs are shown in grey.

To better characterize the pAs, the genome conservation of the 3'UTR of the selected Rho GTPases was evaluated. To that purpose, *Rac1*, *Cdc42 Iso1* and Cdc42 *Iso2*, and

*RhoA* 3' UTR sequences from rat, mouse and human were gathered from NCBI nucleotide database (ncbi.nlm.nih.gov/nuccore/) and aligned using Geneious v4.8 software. Conservation of mammalian Rho GTPases is approximately 50%[19], however the results of the conservation analysis revealed a surprisingly higher conservation of the 3'UTR between the three species, with 100% pairwise identity value for the pAs sequences (Figure 13). These results showed for the 3'UTR an identity above 80% for *Cdc42 Iso1* and *Cdc42 Iso2*, and *RhoA*, and over 76% in the case of *Rac1*.

Although these analyses were performed with only three species, the high level of conservation indicates an important role for these sequences in regulating gene expression of Rho GTPases in these organisms.



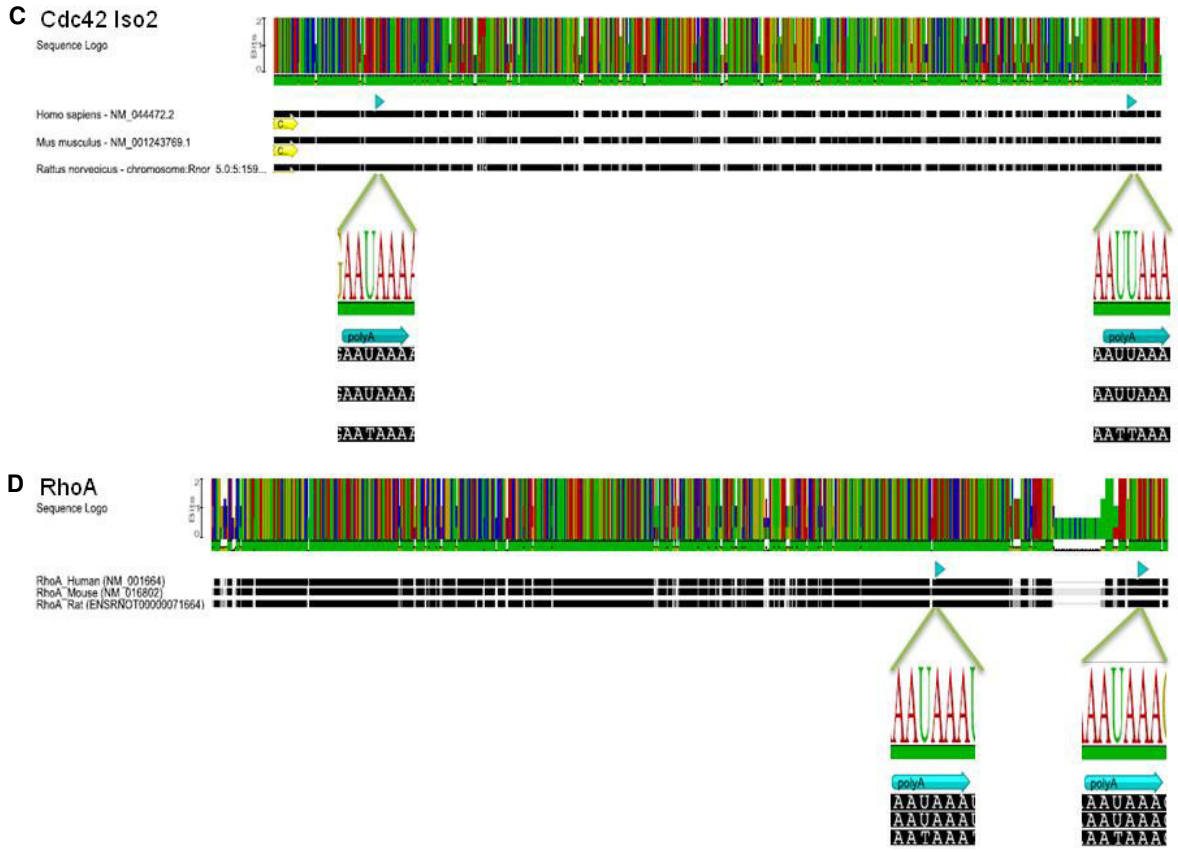



**Figure 13. Genomic alignment of Rho GTPases 3'UTR sequences (rat, mouse and human).** 3'UTRs showing the high conservation throughout all sequence (showed by the darkness of the bars), with close-up on pAs (blue arrows). The conservation of the 3'UTR for **A)** *Rac1*, **B)** *Cdc42 Iso1*, **C)** *Cdc42 Iso2* and **D)** *RhoA*. (Adapted from Geneious v4.8)

## 1.2. Classical Rho GTPases preferentially use the proximal pAs during OL differentiation

Previous genome wide studies have related the progressive lengthening of the 3'UTRs by APA in mammalian brain tissues[64], as well as in developmental processes such as differentiation[62]. Additionally, it has been described that ubiquitously expressed genes that present alternative 3'UTRs, show a change in the isoforms ratio in response to transformation or differentiation programs.[101] In order to investigate if APA have a role in Rho GTPases regulation during OL differentiation, the expression levels were analysed by RT-qPCR in cells at the OPC stage and in OLs differentiated *in vitro* for 3, 5 or 7 days. To measure the total expression of the mRNAs and the expression of the longest mRNAs isoform only, two specific primer pairs were designed as represented in Figure 14.

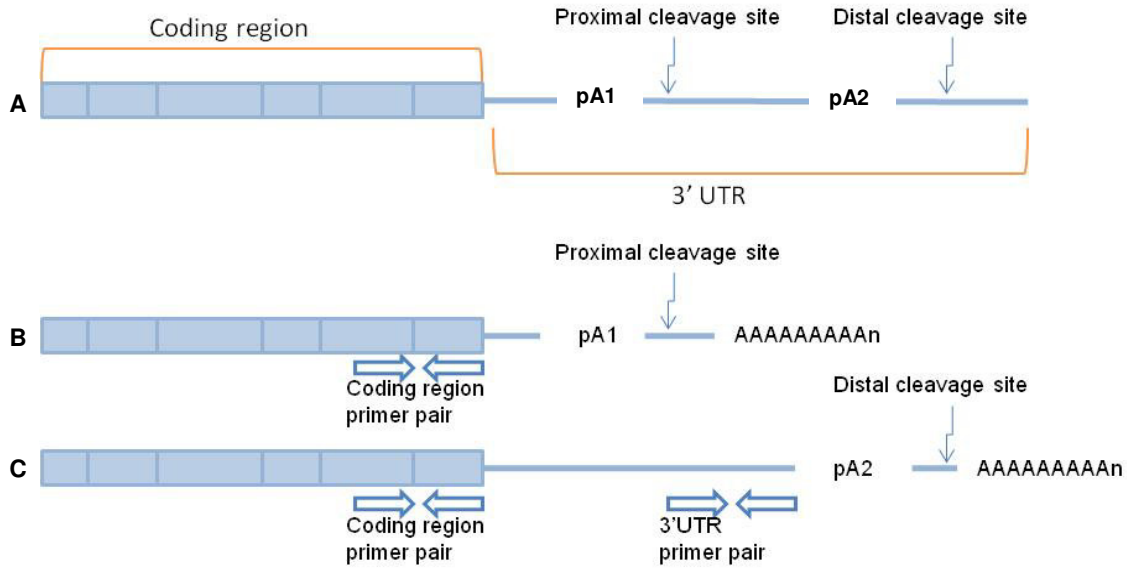



**Figure 14. Schematic representation of the primer pairs used in RT-qPCR analyses. A)** Schematic pre-mRNA sequence depicting the coding region, two pAs and respective cleavage sites at the 3'UTR. **B)** Hypothetic shorter mRNA isoform amplified by the primer pair spanning exon junctions. **C)** Hypothetic longer mRNA isoform will be amplified by the primer pair located in the coding region and also by the specific primer pair designed at the 3'UTR between the two pAs.

As expected, the RT-qPCR results have shown variable expression levels of the genes during the OL differentiation (Figure 15A). *Rac1* and *RhoA* increase its total expression during differentiation. Interestingly, the expression of *Cdc42* isoforms show an opposite profile during differentiation, there is an increase in *Cdc42 Iso1* total mRNA levels whereas *Cdc42 Iso2* present a decrease from day 0 to day 7 (although the expression levels of *Cdc42 Iso1* are 3 fold higher than those of *Cdc42 Iso2*). This suggests that

*Cdc42* isoforms could have different roles in OL differentiation, i.e. *Cdc42 Iso1* may have a particular importance in the differentiation process whereas *Cdc42 Iso2* appear to be crucial for the proliferation stage (discussed in point 2 of this section).

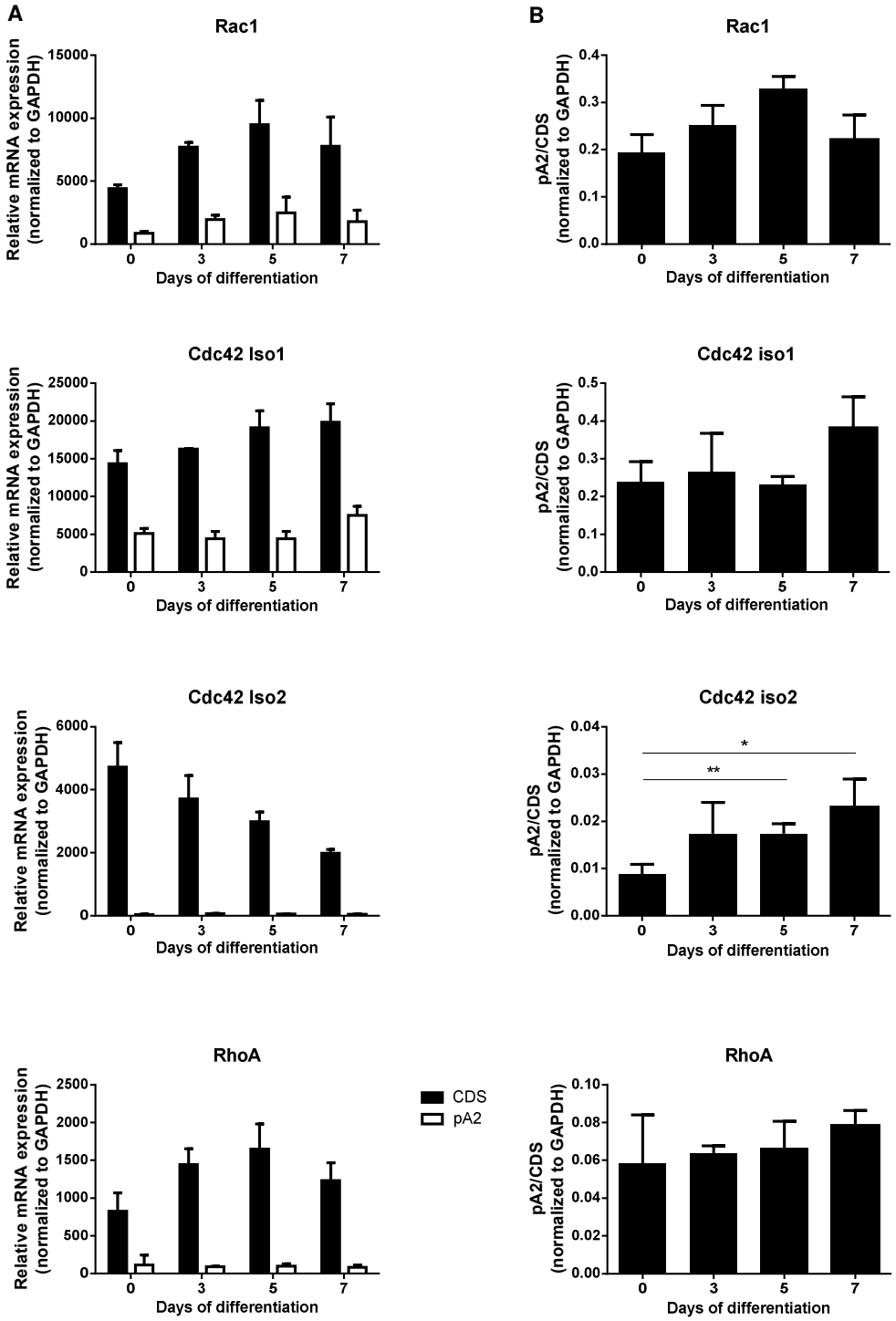



**Figure 15. Relative mRNA expression levels quantified by RT-qPCR, of the different mRNA isoforms during OL differentiation.** OLs were differentiated *in vitro* and at specific time points post differentiation, total

mRNA was collected. **A)** Total mRNA expression (CDS, colored in black), and the longest mRNA isoform expression (pA2, colored in white) was determined for each gene. **B)** The ratio of these expressions (pA2/CDS) was performed in order to obtain the variation of the pAs usage. Error bars show the standard deviation (SD) for at least three independent experiments. *p<0,05, **p<0,01.

Confirming the results obtained from 3'RACE analysis, both mRNA isoforms are expressed, however, the expression levels of the longer mRNA isoforms are considerable lower than the total mRNA expression (Figure 15A). To understand how APA varies in the genes analyzed during OL differentiation, we determined the relative usages of one pAs over another: the ratio between the levels of pA2/CDS (Figure 15B). The alterations observed in pA2/CDS for Rac1, *Cdc42 Iso1* and *RhoA* during OL differentiation were not statistically significant, and the proximal pAs is the most used for all these genes. In the case of the *Cdc42 Iso2* the results show a significant increase in the ratio pA2/CDS, from day 0 to day 7. However these alterations do not reflect a preferential usage of the distal pA but they are due to the decrease in the total mRNA (and therefore, the ratio increases). In conclusion, we observed that classical Rho GTPases do not undergo an APA switch during OL *in vitro* differentiation and the proximal pAs is the most used.

Rho GTPases act by integrating signals from the environment, therefore its expression and regulation may also be modulated by extracellular cues. Although the *in vitro* culture conditions try to reproduce as most as possible, the *in vivo* context, there are not all the signals derived from other cells, namely neurons. As our results were not in agreement with those that were previously published, which indicate a lengthening of the 3'UTR during cell differentiation and in brain tissues[61, 62, 64], we decided to confirm this observation *in vivo*. For this proposes, we used mRNA extracted from the rat optic nerves (ON), which are mainly composed by OLs[102]. ONs were collected from the animals at P2, P14 and P30, which are the *in vivo* correspondents of the three defined stages of *in vitro* differentiation (d0, d3 and d5). The results showed that the relative mRNA expression in the ON is consistent with that observed in OLs. For *Rac1* and *Cdc42 Iso1* the total mRNA increase during the differentiation without significant changes in the long mRNA isoform expression, which is less expressed (Figure 16A-B). For *Cdc42 Iso2*, we observed an increase in the total expression at P14 (Figure 16A), which differs from that observed *in vitro* OLs, suggesting that *in vivo Cdc42 Iso2* is expressed in a distinct manner. This difference could be due to the presence of signals, that not exist in culture, that regulate it expression or, less probable, by the influence of the small portion of axon present in the tissue. However, the expression of the longer mRNA isoform is in agreement with the OLs

results: its expression is almost null and the differences detected in the ratio pA2/CDS are due to the alterations in the total mRNA expression (Figure 16B).

For *RhoA* the results indicate that the total mRNA levels increase with development. However the usage of the pAs compared to the *in vitro* OLs is different. At P2 the most used signal is pA2, but throughout differentiation the increase in the expression levels is mainly given by usage of the proximal signal. Therefore, for RhoA we observe a shortening in the 3'UTR during differentiation, which contrasts with previous results in mouse embryonic development and mammalian brain[61, 62, 64].

One hypothesis for the observed differences between ON and OL regarding *RhoA* expression, can be the presence of other cell types in the ON since ubiquitously expressed genes can use APA to achieve its tissue-specificity[101]. Although their relative small number (e.g. microglia or astrocytes), *RhoA* have distinct functions in different cell types. In astrocytes, as opposed to OLs and neurons, an increase in the activation of RhoA is necessary for cell body and process expansion[103]. On the other hand, preliminary results from João B. Relvas's lab have showed a crucial function of RhoA in microglia activation (personal communication).

Taken together our *in vivo* results are in agreements with our *in vitro results* for *Rac1*, *Cdc42 Iso1* and *Cdc42 Iso2*. Furthermore, our results support the hypothesis that 'classically' activated Rho GTPases do not use APA switch as a regulatory mechanism of gene expression during OL differentiation.

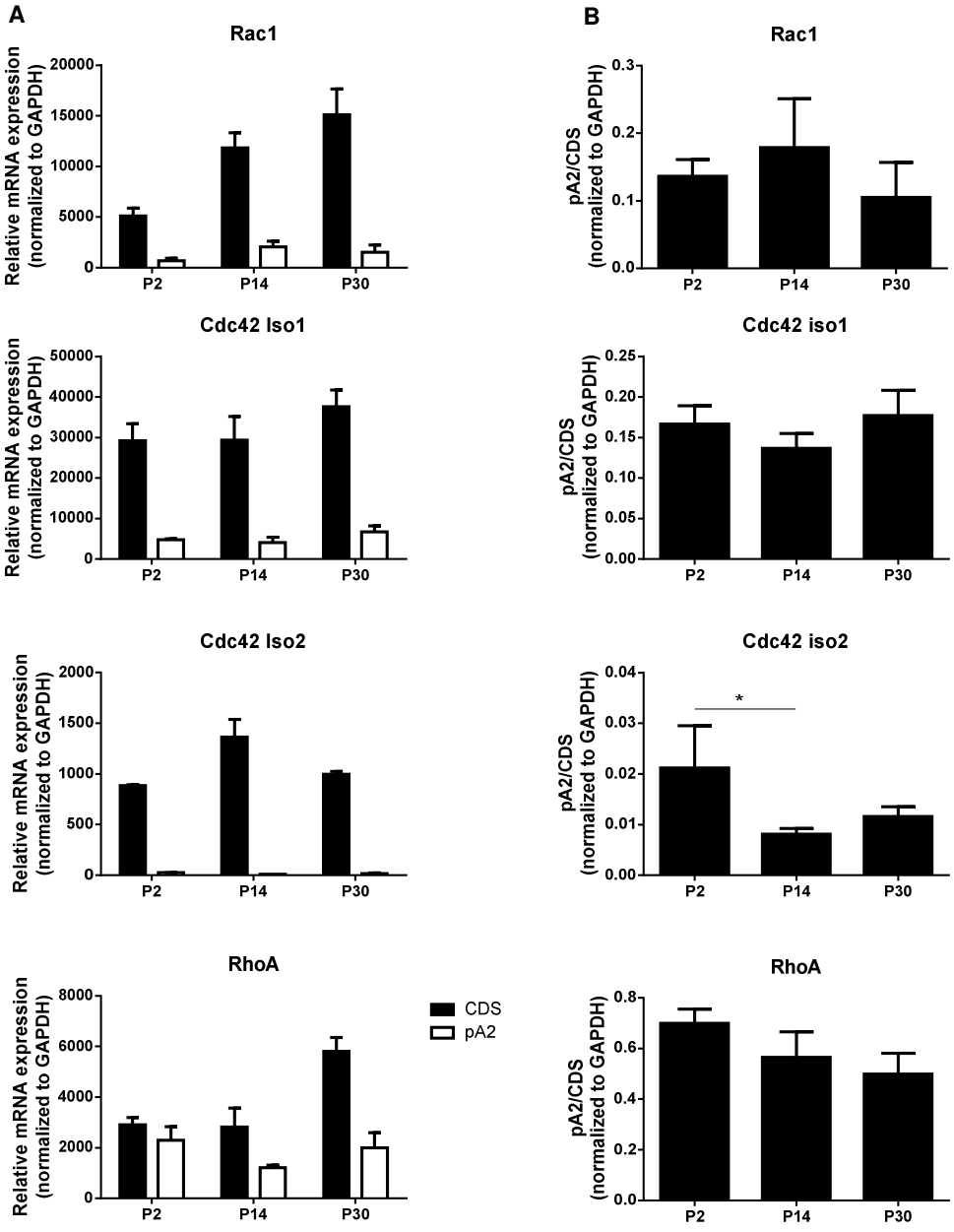
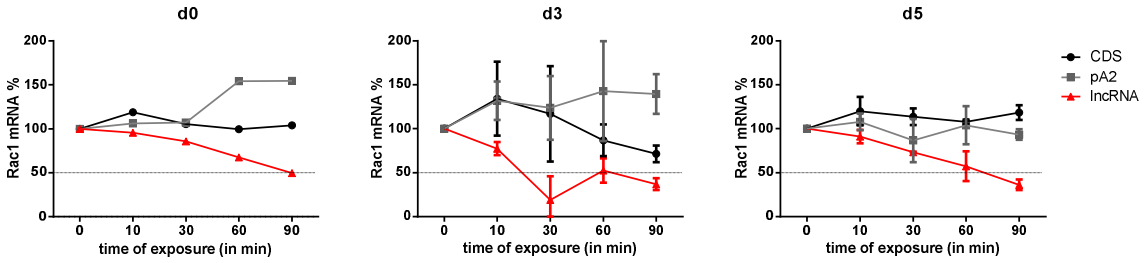



**Figure 16. Relative mRNA expression levels of Rho GTPases mRNA isoforms during ON development.** ONs from rats were collected at 2, 14 and 30 days post birth, and the total mRNA was extracted. **A)** The total and long mRNA expression was quantified for each gene by RT-qPCR.CDS (black bar), pA2 (grey bar). **B)** The expression ratio pA2/CDS was determined to obtain the variation of the pAs usage. Error bars show the standard deviation (SD) for at least three independent experiments. *p<0,05.

It is possible that variations in the levels of the mRNA isoforms reflect different mRNA stabilities. Therefore, we decided to measure the mRNA half-life after actinomycin D treatment. Actinomycin D blocks transcription and therefore there is no synthesis of new mRNAs after the treatment. Thereby the mRNA decay can be accessed by quantification

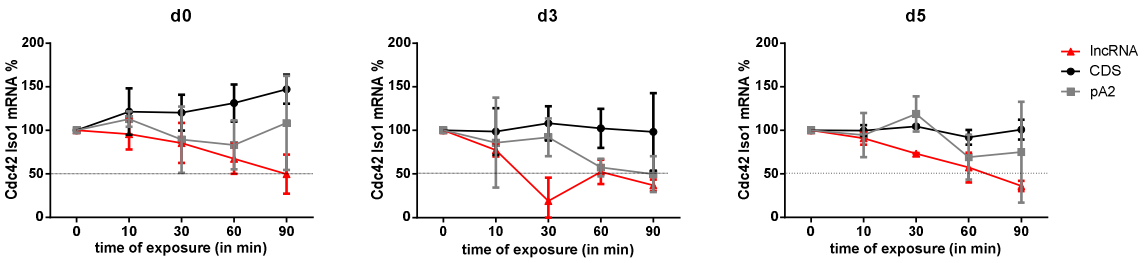
of mRNA levels before and after the exposure to the chemical compound, at different time points.

**A**



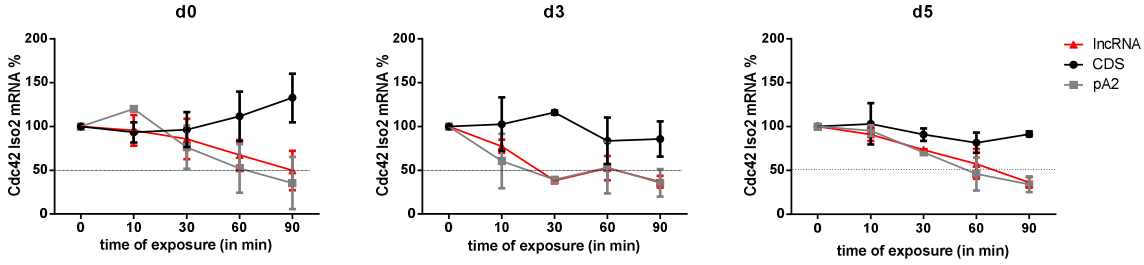

| *Rac1 mRNA $t_{1/2}$(in min)* | | | |
|---|---|---|---|
| | d0 | d3 | d5 |
| lncRNA | 90 | <30 | <90 |
| CDS | >90 | >90 | >90 |
| pA2 | >90 | >90 | >90 |

**B**



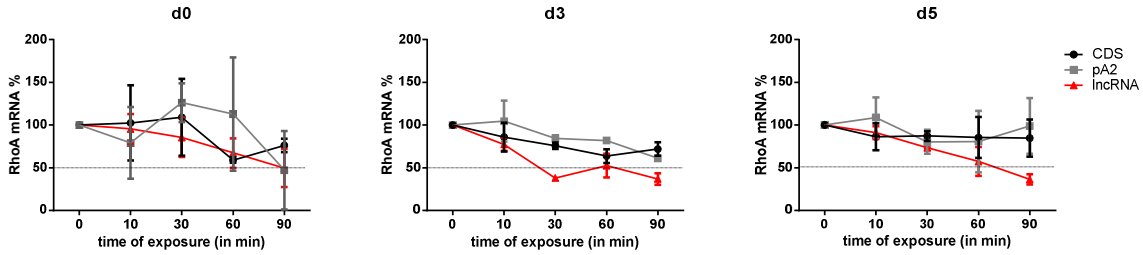

| *Cdc42 Iso1 mRNA $t_{1/2}$(in min)* | | | |
|---|---|---|---|
| | d0 | d3 | d5 |
| lncRNA | 90 | <30 | <90 |
| CDS | >90 | >90 | >90 |
| pA2 | >90 | 90 | >90 |

C




| *Cdc42 Iso2 mRNA $t_{1/2}$(in min)* | | | |
|---|---|---|---|
| | d0 | d3 | d5 |
| lncRNA | 90 | <30 | <90 |
| CDS | >90 | >90 | >90 |
| pA2 | 60 | <30 | 60 |

D




| *RhoA mRNA $t_{1/2}$(in min)* | | | |
|---|---|---|---|
| | d0 | d3 | d5 |
| lncRNA | 90 | <30 | <90 |
| CDS | >90 | >90 | >90 |
| pA2 | 90 | >90 | >90 |

**Figure 17. Stability of Rho GTPases mRNA isoforms.** Stability of **A**) *Rac1*, **B)** *Cdc42 Iso1*, **C)** *Cdc42 Iso2* and **D)** *RhoA* mRNAs in OLs differentiated *in vitro* (at day 0, day 3 and day 5) was measured by RT-qPCR after 10, 30, 60 and 90 minutes of exposure to actinomycin D (time 0 corresponds to the untreated samples). The expression levels are normalized to the untreated samples. The tables resume the half-life of the mRNA isoforms. CDS (black line), pA2 (grey line). The long non coding RNA (red line) was used as a control. Error bars show the standard deviation (SD) for at least three independent experiments. Time in minutes.

In this experiment we used OL differentiated in vitro at day 0, 3 and 5, and after Actinomycin D treatment we quantified mRNA levels at different time points: 0, 10, 30, 60,

90 minutes. As a control RNA we used lncRNA *2700046G09Rik* that has been described as stable[83]. Of note, in the culture conditions used (with laminin) the lncRNA *2700046G09Rik* showed to be unstable. Our results show a high stability for both *Rac1* mRNA isoforms (Figure 17A) and *RhoA* (Figure 17D) in all stages of OL differentiation, with approximately 100% of expression levels even after 90 min. Analysis of *Cdc42 Iso1* mRNA decay showed also a similar stability for both total mRNA and long mRNA isoform with the exception of day 3, at which occurs the progressive decrease in the levels of the longer mRNA isoform (Figure 17B). This mRNA has an half-life of 90 min of treatment (Figure 17B) suggesting a mild instability of the longer 3'UTR at day 3. Figure 17C shows a higher decay of the long *Cdc42 Iso2* mRNA isoform as opposed to total *Cdc42 Iso2* mRNA. Its half-life is reached at less than 30 min at day 3 and at 60 min of exposure in day 0 and day 5. These results are consistent with the expression levels suggesting that, in addition to the lower expression levels, the longer isoform derived from the pA2 signal usage is also more unstable.

From these results it is possible to assume that for *Rac1*, *Cdc42 Iso1* and *RhoA*, the lower expression of the pA2-derived mRNA isoforms is not due to its rapid degradation. On other hand, the lower expression of *Cdc42 Iso2* longer mRNA may be due to its instability, possibly due to instability *cis* elements or miRNA target sites localized in the longer 3'UTR

Taking all our results into account, it is reasonable to assume that the levels of the mRNAs quantified and represented in Figures 15 and 16 do not reflect instability of the longest 3'UTRs. This is in accordance with published results describing that regulation of the differential pAs usage is done through regulatory elements present in the 3'UTRs rather than through different degradation rates[63, 101].

In summary, we showed that classical Rho GTPases use the proximal pA signal during OL differentiation, in contrast with previous genome wide studies in the brain[62-64] and thus we conclude that during OL differentiation most classical Rho GTPases do not undergo APA switch towards distal pAs, *i.e.* the preferential expression of Rho GTPases shortest 3'UTRs could be related to a particular requirement for these proteins in OL differentiation. It was previously proposed for other genes that proximal pAs usage leads to an increase in the protein produced[101] and the same mechanisms may also apply to Rho GTPases: use of the proximal pA site may avoid post-transcriptional down-regulation by miRNAs, for example. Therefore, one possible explanation for our findings resides in the nature of this family of proteins. As Rho GTPases are regulated at the protein level by cycling between activated and inactivated states, through GTP binding and release respectively, to perform

their functions, they may need to be highly expressed at the mRNA level and do not require another level of regulation (by APA).

## 1.3. Atypical *RhoBTB2* mRNA expression during OLs differentiation

RhoBTB2 is an atypical Rho GTPase that stays always in the active state and is not regulated at the protein level, suggesting that it may require another level of regulation, at the mRNA level, which may implicates a better regulation at the mRNA level.

We therefore hypothesised that atypical Rho GTPases would undergo distal pAs selection during OL differentiation. Analysis of the *RhoBTB2* 3'UTR sequence have shown that it has 70,4% of pairwise identity, and three 100% conserved putative pAs (data not shown). However, by performing RT-qPCR with primer pairs for the coding region (CDS), for the region between the first and the second signal (pA2), and for the region between pA2 and pA3 (pA3), it was demonstrated that the pA3-derived isoform is not expressed (data not shown). Therefore we quantified total and pA2 *RhoBTB2* mRNA levels at 0, 3, 5, 7 days of differentiation and found that there is always a preferential usage of pA2 during OLs differentiation (Figure 18A). The selection of the pAs is not affected, as the ratio between pA2-derived and total mRNAs do not change during the process (Figure 18B). The expression of the longest 3'UTR allows the differential binding of either RBPs or miRNAs and this could affect the *RhoBTB2* expression levels, however, our results show that APA is not regulated during differentiation.

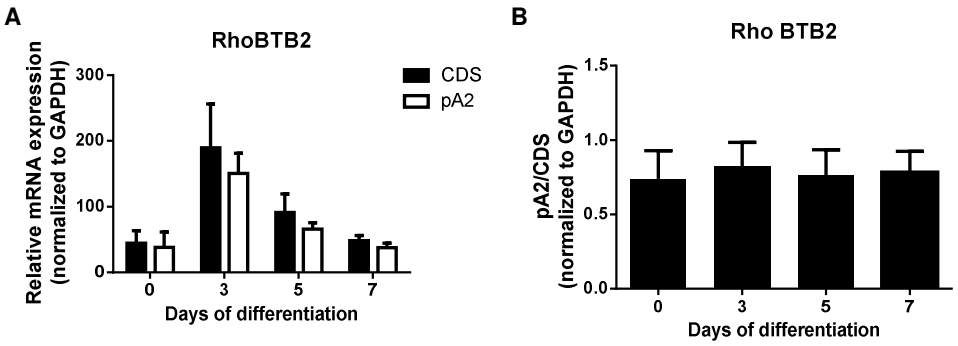



**Figure 18. Quantification of the relative mRNAs expression levels by RT-qPCR, of the different *RhoBTB2* isoforms during *in vitro* OL differentiation. A)** The mRNA expression of the two isoforms was quantified by RT-qPCR.CDS (black bar), pA2, (white bar). **B)** The ratio of mRNAs expressions (pA2/CDS) was performed. Error bars show the standard deviation (SD) for at least three independent experiments.

Using the same approach *in vivo*, in the ON we observed that both total mRNA and long *RhoBTB2* isoform are distinctly expressed compared with OLs (Figure 19). The contribution of the pA2-derived mRNA for the total expression levels is not as considerable as in OLs, however its expression is very high in comparison to classical Rho GTPases (Figure 19A). The ratio pA2/CDS significantly increases from P2 to P14

(Figure 19B), but this may be due to a decrease in the total mRNA expression (Figure 19A) rather than an increase in the levels of the longer mRNA isoform.

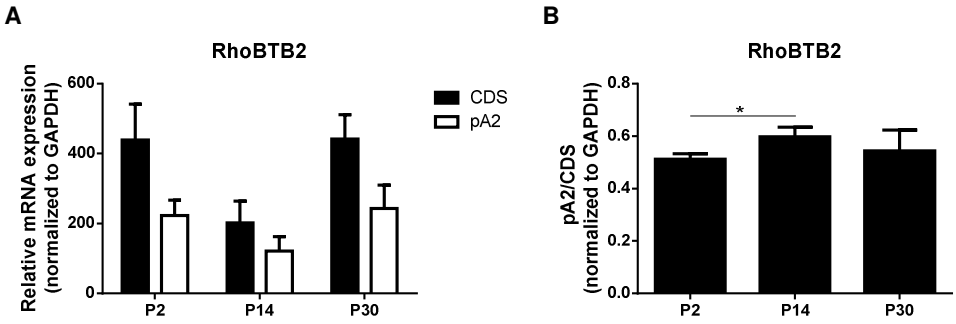
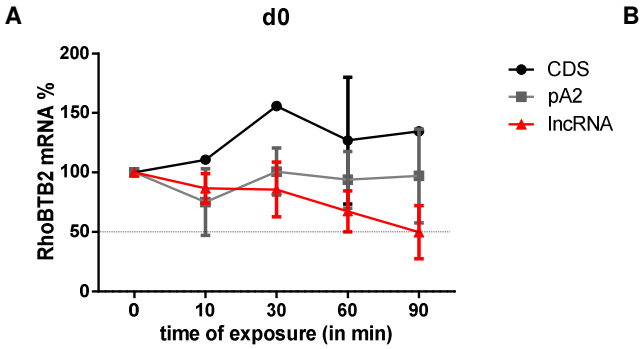



**Figure 19. Quantification of the relative mRNAs expression levels by RT-qPCR, of the different *RhoBTB2* isoforms during ON development. A)** The mRNA expression of the two isoforms was quantified by RT-qPCR.CDS (black bar), pA2, (white bar). **B)** The ratio of mRNAs expressions (pA2/CDS) was performed. Error bars show the standard deviation (SD) for at least three independent experiments. *p<0,05

To measure mRNA stability we treated OPC cells with actinomycin D. The total mRNA as well the pA2-derived isoform was quantified by RT-qPCR (Figure 20A). Both mRNAs remain in the cell for more than 90 min (Figure 20B), suggesting that the longer mRNA produced by pA2 usage is stable and not target for degradation.




| *RhoBTB2 mRNA $t_{1/2}$(in min)* | |
|---|---|
| | d0 |
| lncRNA | 90 |
| CDS | >90 |
| pA2 | >90 |

**Figure 20. Stability of *RhoBTB2* mRNA isoforms. A)** The stability of the *RhoBTB2* mRNA isoforms in OPC was measured by RT-qPCR after 10, 30, 60 and 90 minutes of exposure to actinomycin D. The mRNA levels were normalized to the untreated samples. **B)** The table resumes the half-life of the mRNA isoforms CDS (black line), pA2 (grey line); the long non coding RNA (red line) was used as control. Error bars show the standard deviation (SD) for at least three independent experiments. Time in minutes.

In brief, our results demonstrate that this atypical Rho GTPase preferentially uses distal pAs producing longer mRNAs, in contrast to the classical Rho GTPases. However,

similarly to classical Rho GTPases, atypical Rho GTPases do not undergo an APA switch during OL differentiation. Overall it is possible to infer that among Rho GTPases, APA is a mechanism that is gene specific.

## 1.4. In glial cells, Rho GTPases show the same pattern of APA, using preferentially the proximal pAs.

APA patterns have been recently described as tissue-specific[101] . Since we have observed a marked APA pattern for Rho GTPases in OLs, we decided to study this "cell type-specificity effect of" in other type of glial cells. The expression of the different Rho GTPases mRNA isoforms was therefore tested in primary microglia and astrocytes cell cultures (Figure 21), which are fully differentiated cells.

Our results show that both microglia and astrocytes appear to behave similarly to OLs, and present a "cell type-specific effect". We also show that *Rac1*, *Cdc42 Iso1* and *Cdc42 Iso2*, and *RhoA* preferentially use the proximal pAs. For *RhoBTB2* our results show that the longest isoform contributes for thereabout half of the total expression in microglia and astrocytes. In OLs, these expression levels are higher, suggesting that APA of *RhoBTB2* change in a cell-specific manner. This may explain the differences obtained between OLs in culture and ON (Figures 18 and 19). In glial cells the classical *Rac1*, both *Cdc42* isoforms and *RhoA* share the preferential usage of the proximal pAs forming the shortest mRNA isoforms whereas atypical *RhoBTB2* favors the distal pAs. Thereby, it is possible to conclude that APA of Rho GTPases is gene- and cell-type specific.

Recent work has shown a complete lengthening of the 3'UTRs in *Drosophila* CNS[63] whereas in mammalian brain this lengthening is not complete: although levels of longer mRNAs are higher, the shortest isoforms still continue to being expressed[64]. The authors have suggested that these differences were due to the presence of a higher percentage of glial cells in mammalian brain than in *Drosophila* CNS. Our results for the Rho GTPases in OLs, microglia and astrocytes support this hypothesis.

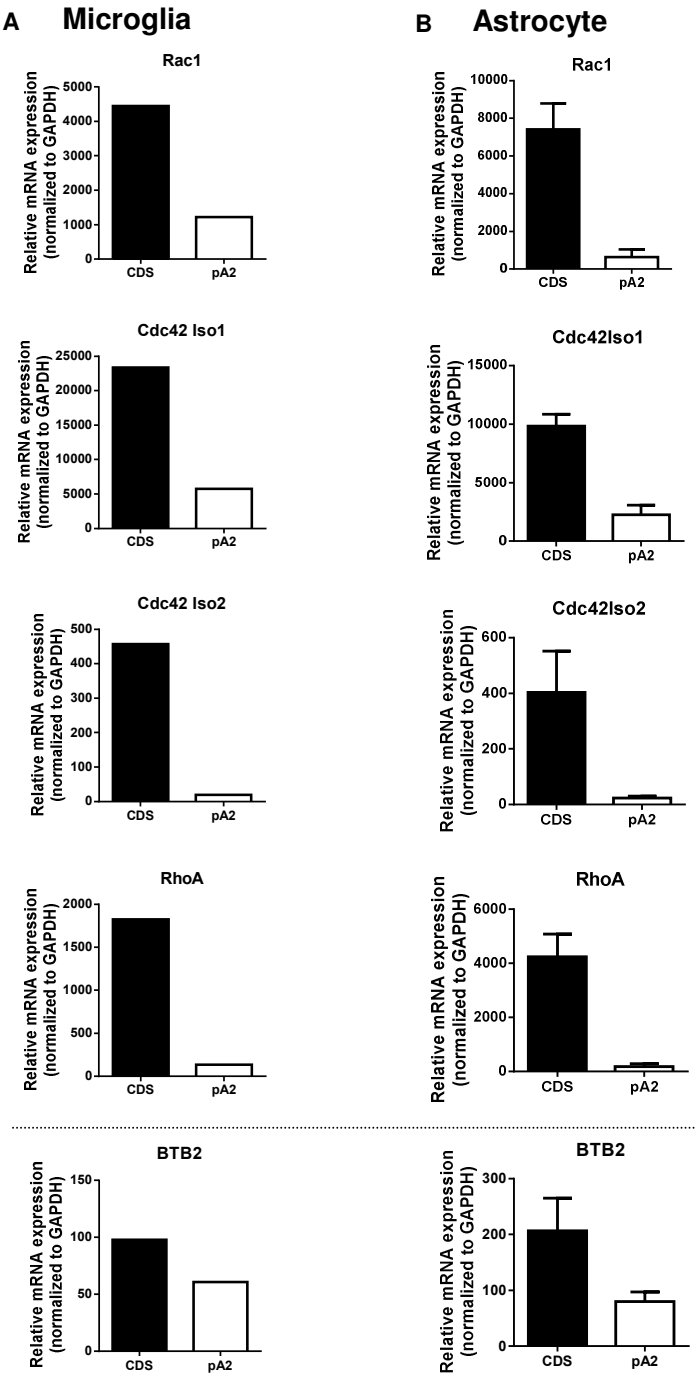



**Figure 21. Rho GTPases mRNA isoforms quantification in different cell types.** mRNA expression from the coding region (CDS, black bar), and the longest isoform produced (pA2, colored in white) was quantified by RT-qPCR. The quantification was performed either in primary **A)** microglia (only one experiment) and **B)** astrocytes. Error bars show the standard deviation (SD) for at least three independent experiments.

## 1.5. *Rac1* mRNA undergoes 3'UTR lengthening during axonal growth of the cortical neurons, concomitant with an increase in Rac1 protein levels

To further explore the cellular specificity of APA, and taking into account the differences observed between *Drosophila*[63] and mammalian[64] CNS, the pAs usage of Rho GTPases was accessed in rat cortical neurons. To unravel if the observed pattern of APA is characteristic of these genes and transversal to the cell types or if it is regulated in a cell-specific manner, and follow the described widespread usage of the distal pAs, the total mRNA and the long mRNA isoform were quantified by RT-qPCR.

Strikingly, in cortical neurons, we observe an increase in the long *Rac1* mRNA expression during axonal growth (Figure 22A and B). This finding demonstrates an APA switch in *Rac1* mRNA in cortical neurons during axonal growth, resulting in a complete lengthening of the *Rac1* 3'UTR due to the usage of the distal pAs. This switch in the pAs usage provides an additional possibility of post-transcriptional regulation, either by miRNA/RBP-targeted degradation or by RBP-based stabilization, and suggests that in neurons, APA is used to control the axonal growth. In addition, this difference could mean that in neurons, Rac1 may have other cellular functions that not occur in glia. Although the described roles of Rac1 in both cell types are parallel and are related to the process/dendrite extension, Rac1 is also important for the axonal maintenance. Since the axon is a particularly long structure, *Rac1* may need a differential regulation for the proper outgrowth and maintenance of this structure. Another indication that the differential expression of *Rac1* isoforms could be related to the axonal biology is the fact that the major differences are seen in day 14 of axonal growth, whereas at day 0 the expression is mostly given by the usage of the proximal signal. Similarly to what was seen for *BDNF* (brain-derived neurotrophic factor) long mRNA isoform that localizes at the dendrites and affect it normal development and functions[104], it is also possible that the long *Rac1* mRNA isoform is involved in *Rac1* mRNA cellular localization. Supporting this hypothesis, the long 3'UTR of *Rac1* contain several putative binding sites for RBP of the hnRNP family (Table 4), known to be required for mRNA export[105].

The APA pattern for *Cdc42 Iso1*, *Cdc42 Iso2*, and *RhoA* is similar to what was observed in glial cells, *i.e.* preferential proximal pAs selection without significant changes in the isoforms ratio during axonal growth (Figure 22A-B). Regarding the total expression of *Cdc42 Iso1* and *Cdc42 Iso2* mRNAs in neurons (Figure 22A) and in contrast to OLs (Figure 15A), we observed a drastic increase in the relative mRNA

expression of the *Cdc42 Iso2* and a decrease in *Cdc42 Iso1* mRNA expression. This indicates that the described brain-specific *Cdc42 Iso2* [31, 32] is instead neuron-specific, since its expression in the glia is very low. In addition this result suggests the existence of distinct roles for both Cdc42 protein isoforms in the CNS.

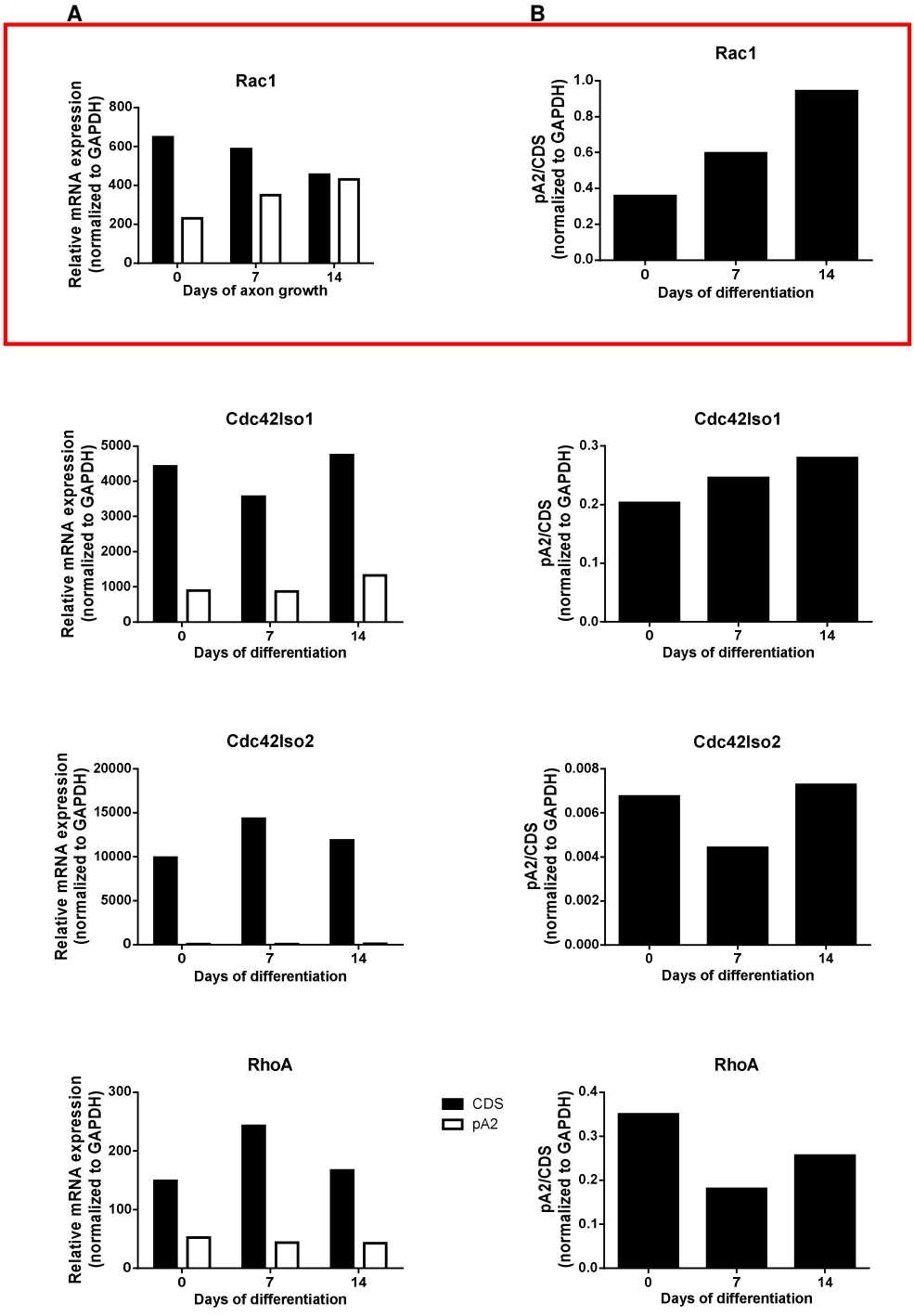



**Figure 22. Rho GTPases mRNA isoforms quantification during axonal growth of cortical neurons.** **A)** The expression of the total (CDS, colored in black) and the longest (pA2, colored in white) mRNAs was quantified by RT-qPCR. **B)** The ratio of these mRNA expressions (pA2/CDS) is presented. Results are from one experiment representative of three independent experiments.

Next, we questioned if the different *Rac1* 3'UTRs would impact protein production and therefore is physiologically relevant. For that, we quantified total Rac1 by western blot at the three time points of the neuronal growth (Figure 23).

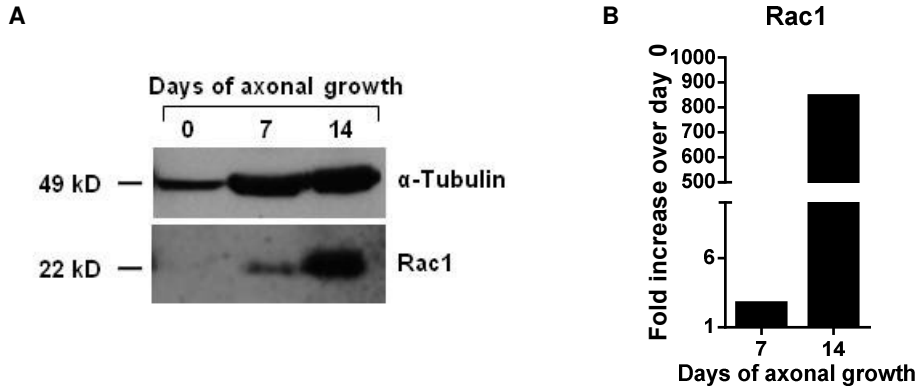



**Figure 23. Quantification of Rac1 protein levels during the axonal growth of cortical neurons. A)** Total Rac1 Protein expression at day 0, 7 and 14 of axonal growth (western blot). **B)** Quantification of the protein levels. α-Tubulin was used as a control. Results are from one experiment representative of two independent experiments.

Although the α-Tubulin protein was proved not to be the best control, since it varies from day 0 to day 7, it is possible to see a drastic increase in total Rac1 protein levels from day 0 to day 14. This result indicates that the longest *Rac1* 3'UTR leads a higher protein production and suggest that the longer 3' UTR is not target for downregulation. This possible function for the longer 3'UTR is in accordance to previous results from our lab, in which the longer p*olo* mRNA in *Drosophila* is required for efficient translation[95].

Altogether our results highlight the relevance of APA in achievement a cell-specific gene expression. However, the mechanisms that confer this fine-tune regulation as well as their functional relevance remains unknown.

### 1.5.1. Rac1 appears to be differentially regulated in different types of neurons.

The neuron-specific alternative splicing mechanisms has been largely explored in the last few years. Recently it was demonstrated that two conserved RBPs, UNC-75/CELF and EXC-7/Hu/ELAV present alternative spliced isoforms according the functional type of the neuron where they are expressed[7]. The coupling between alternative splicing and APA has been already well described for some mammalian genes[106], *e.g.* the Glial Fibrillary Acidic Protein (*GFAP*) in glial cells[107]. Although this close relationship, this has not been explored in different types of neurons. In order to access if the APA pattern of Rac1 is neuron-type-specific, we quantified the expression of the mRNA isoforms in hippocampal and striatal neurons (Figure 24). These two types of neurons differ in their morphology and functionality. Our preliminary results indicate that there are some differences in expression of both mRNA isoforms between the two-neuron types.

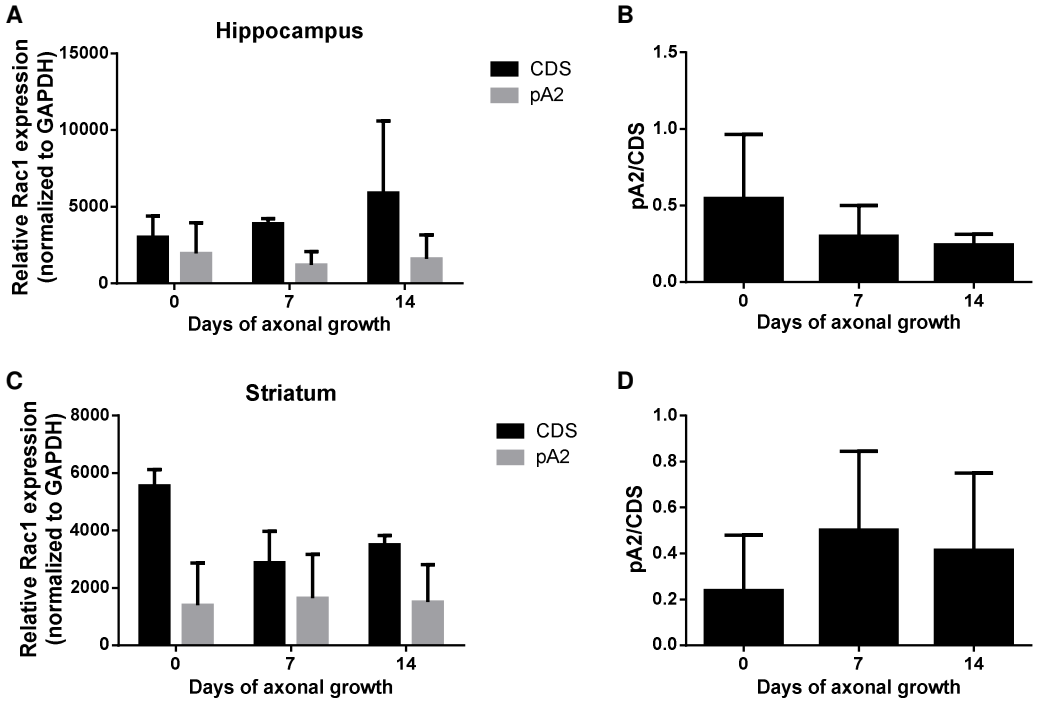



**Figure 24. Rac1 mRNA isoforms quantification during axonal growth of hippocampal and striatal neurons.** The expression of the total (CDS, colored in black) and the longest (pA2, colored in white) mRNAs was quantified by RT-qPCR using cultured **(A,B)** hippocampal and **(C,D)** striatal neurons. **B)** and **D)** represent the ratio of them RNA expression (pA2/CDS) showing the variation of the pAs usage. Error bar represent the SD of two independent experiments.

Although these differences are not statistically significant and more experiments needs to be done, it is possible to observe that during the axonal growth in hippocampal neurons the total mRNA expression of *Rac1* remains stable, being accompanied by a small decrease in the levels of the longer *Rac1* mRNA isoform (Figure 24A), whereas in striatal neurons the total *Rac1* expression decrease with no changes in the expression of the longest isoform (Figure 24C). In addition, we observe an opposed ratio of the pAs usage during the axonal growth (Figure 24B and 24D). In the hippocampus, neurons decrease the usage of the distal signal while in striatum this usage is increased. Comparing these results with those of cortical neurons it is visible that the pattern of the cortical neurons is more related to that presented by the striatal neurons (Figure 25).

There are several different types of neurons in the brain. In the cortex there is a mixture of gabbaergic and glutamatergic pyramidal neurons, in the hippocampus glutamatergic pyramidal neurons are predominant, and striatum mostly contain gabbaergic interneurons. Therefore, we expected that *Rac1* expression in cortical neurons would reflect the mixture of both types of neurons, or additionally would be similar to the expression observed in the hippocampus, given the morphological similarities and the importance of *Rac1* for that morphology [23, 36], what it has not observed.

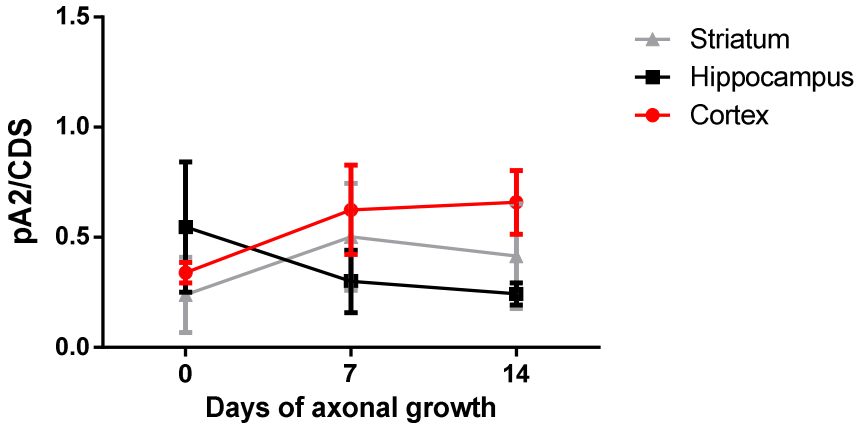



**Figure 25. Comparison of the Rac1 mRNA isoform expression ratio (pA2/CDS) in different types of neurons during axonal growth.** Error bars represent the SEM of the three (for cortex) or two (for hippocampus and striatum) independent experiments.

The activity of Rho GTPases, in particular Rac1, is regulated by the neuronal activity through the activation of glutamate receptors [108, 109], thus we hypothesise that APA is regulated by the same signal.

Although preliminary these results indicate that APA plays a role in fine-tuning gene expression, which is specific of the cell type. Furthermore, this data suggests a connection between the usage of alternative 3'UTRs and the function of the gene in each cell.

## 1.6. Candidate RBPs to regulate *Rac1* 3'UTR.

In the brain, there are two key players in the regulation of the gene expression: the longest 3'UTRs of the mRNAs and the expression of neuron-specific RBPs[110]. RBPs may regulate APA co-transcriptionally, and/or may bind to the 3'UTR of a particular mRNA regulating its fate. Since our results demonstrated a distinct Rac1 APA pattern among the different brain cells, we hypothesized the existence of a coupling between different expressed 3'UTRs, and a differential expression of specific RBPs, implying possible role in regulating Rac1 expression. Therefore, we performed an *in silico* analysis to identify the RBPs that potentially bind *Rac1* 3'UTR. Table 4 depicts RBPs (with a score over 70%) that have putative binding sites in both *Rac1* 3'UTRs (light green), only in the shortest (mild green) or in the longest (dark green) isoforms.

**Table 4. RBPs with putative binding sites in *Rac1* 3'UTR.**

| Rac1 pAs | KHSRP | YTHDC1 | RBMX | KHDRBS3 | PUM2 | SFRS1 | MBNL1 | ELAVL1 | QKI | ACO1 |
|---|---|---|---|---|---|---|---|---|---|---|
| pA1 | | | | | | | | | | |
| pA2 | | | | | | | | | | |
| | PTB | Nova1 | hnRNPF | ELAVL2 | SFRS2 | SNRPA | PABPC1 | FUS | SFRS9 | EIF4B |
| pA1 | | | | | | | | | | |
| pA2 | | | | | | | | | | |
| | VTS1 | SFRS13A | YBX2-A | SAP-49 | YBX1 | RBM4 | hnRNPU | hnRNPH1 | hnRNPA2B1 | hnRNPA1 |
| pA1 | | | | | | | | | | |
| pA2 | | | | | | | | | | |

**TAA**ATGTCTGAGCCCCTCCCTCGGTCTTGGTCCCTGGAGCCTTTGTACGCTTTGCTCAGAAAATGGCGGAGTC
TTCGCGTCTGATGCCAAGTTTTTGTTACAGATTAATTTTTCCATAAAACCATTTTGAACCAATGAACCAGTCAGTA
ATTTTAAGGTTCTGTTTTAAATGTAAGAATTCCAACTTACAGTCTATTAAAATTCAGCCCTAAAATGAC|AAAGCC
TTCTTAAAGCCTTATTTTTCAAAATCCCCTATTCTTGCTCAGATTAAGAATTGCCAAAATACCTTGTGAACTAAGT
TGCGTTGTGCTGAGAACATGTAAGCACTAAGCTGTTGAGAGACTTTGTCCTTAAGAAGACTGCAGCTTCTGGGC
TCAGGGGTGCAGACCCTCCCGTAGCTCCCAGACCGTGTGACACAGCACAGCCTCCTTAATGACACGCTGCCA
TGTAACGCACCTGTAACTTATCAGCCCATGCTCATTACGTAACTTTGTACTGTACGTCACGATGGGTGTAACAG
CTCTGCTCTTTGATTTCATAGTGAGTTCTCTAAAATACCAGCTGACCGGCTTCTGCAGGCTTTGAACAGAACTCT
GGCTCCTGTGTTGCCTCTAACGAAGTATTCTGTTCCTAGTCGTGGGTGTGCTGGGTGGAGTGTGTGAAACACG
ACGTCATCAAAGGAGACAGACAGTATTTTGACTAATATGAAGTAGAGATTAATTTACACTACATTGTACATGGAG
TAATTCAACTGAATAAAAGTGTCACGGGTAAAGCTTTTTAACGGTTAATTTCTGTCAAACAGTAGATGACAAATG
GCCGATCTTATCAGTGTCTCTCTTGAGCCCCCCTTCCCCCTGCTGTCCCTCCCCAGATGGGGCGTTGAGTCCA
TATTTAAACTGGCCATCCTCACAGTTGCTAACTTAGCAAGTGCTTTTCTTTAGGACCCCCTTCTTAACGAGCAAT
ATGTCTGACCTGTACTATAAGATCTTTCTGATAATGCATTCGGAGATTTTTTTGGTAGATAGTAGAAGTGCGTTC
CTGTTTTCACCTTCCTTTACTCAGCTGACTAGTGCTTCCCTTCGTTTTCTAGTAACTGGGTGTAGAAATCACGTG
CTGCGGCTTTACAGTTTTTAAACTATTTTAGATATTCTGAAAACATCACTGTCTTGCCAGAGTACCAACACTGTCA
TGTGATTGATGCCGCCCCCTCTAGACCTCACCCACGCGGACACATGCTTCCCGTACCTTTGGGTCTGTGAGGT
TCTGTCAAGCGCTAGTGCTAACGCCGTTCTGTACAACCTAACTCACTGGCAAGAACACAGTGTTGGGCCTTTC
GACCACTAGAACAAACTTTTTTCAATTGACAGTTACAGAATTGTGGAGTGTTTTTACATTGATCTTTTGCTAATGC
AGTTAGCAGTATGTTTTGCATTGTATGACTTAATAAATCCTTGAATC|

Pum2 Nova1 Ptbp Elavl Ybx1

**Figure 26. mRNA sequence of the *Rac1* 3´UTR. The putative binding sites for several RBPs are highlighted.**

We next quantify the expression of RBPs that have putative binding sites in both mRNA isoforms, and also, specific RBPs that bind only to one isoform. We quantified the expression of *Nova1* (Neuro-oncological ventral antigen 1), *Ptbp1* (polypyrimidine tract binding protein 1) and *Ptbp2* (polypyrimidine tract binding protein 2), all with

putative binding sites for both *Rac1* mRNA isoforms (Figure 26), in OLs during differentiation and in neurons during axonal growth (Figure 27). Although these RBPs are mostly known for their role in alternative splicing, their important functions in mRNA 3' end processing have already been well established[98, 111, 112]. In OLs, *Nova1* mRNA expression levels showed a 2-fold increase at day 3 and a gradual decrease up to day 7 (Figure 27A). In contrast, neurons present nearly a 2-fold increase at day 14, without any changes in the two first time points (Figure 27B). The increase in *Nova1* mRNA expression corresponds to the time point, which in OLs *Rac1* mRNA reaches its total and pA2-derived expression levels (Figure 15A), and in cortical neurons where there is a peak in the expression of the longer isoform (Figure 22A). These results suggest that Nova1 could act as a *Rac1* APA regulator, by activating the distal PAS selection. Nova1 has been shown to regulate APA by binding to the flanking regions of proximal pAs, blocking the binding of the core cleavage/polyadenylation factors, thereby promoting the usage of the distal pAs that are available for the cleavage machinery assemblage[111]. Thus, it is likely that, at least in neurons, Nova1 is regulating *Rac1* APA, promoting the usage of the longer mRNA isoform at day 14 of axonal growth.

Regarding *Ptbp*, we observe a switch from *Ptbp1* to *Ptbp2* (also known as neuronal Ptb) expression during neuronal differentiation (Figure 27D and 27F), as previous described[5]. Interestingly, we also observe the same *Ptbp1/Ptbp2* switch during OL differentiation (Figure 27C and 27E). *Ptbp1* mRNA levels decrease gradually during the OLs differentiation, while *Ptbp2* levels increase up to day 5, although these changes are not statistically significant (Figure 27C and 27E). The same tendency occurs in cortical neurons during axonal growth: expression of *Ptbp1* undergoes a drastic decrease from day 0 to day 7 (Figure 27D) whilst the increase in *Ptbp2* expression is very slight (Figure 27F). The function of this switch is well established in alternative splicing where Ptbp1 downregulates Ptbp2 co- and post-transcriptionally[5, 113].

Individually, Ptb proteins can affect the mRNA stability through the 3'UTRs[114, 115], and also polyadenylation[98, 112]. In the *C2 complement* gene, Ptbp1 can activate the polyadenylation through bind to it pAs USE[98], however this effect seems to be in a dose-dependent manner whereas either *Ptbp1* knockdown or overexpression results in a decrease of C2 complement expression[112]. Our data indicates that, *Ptbp1* expression levels in OLs remains approximately stable during differentiation, whereas in neurons there is a drastic decrease of its expression.

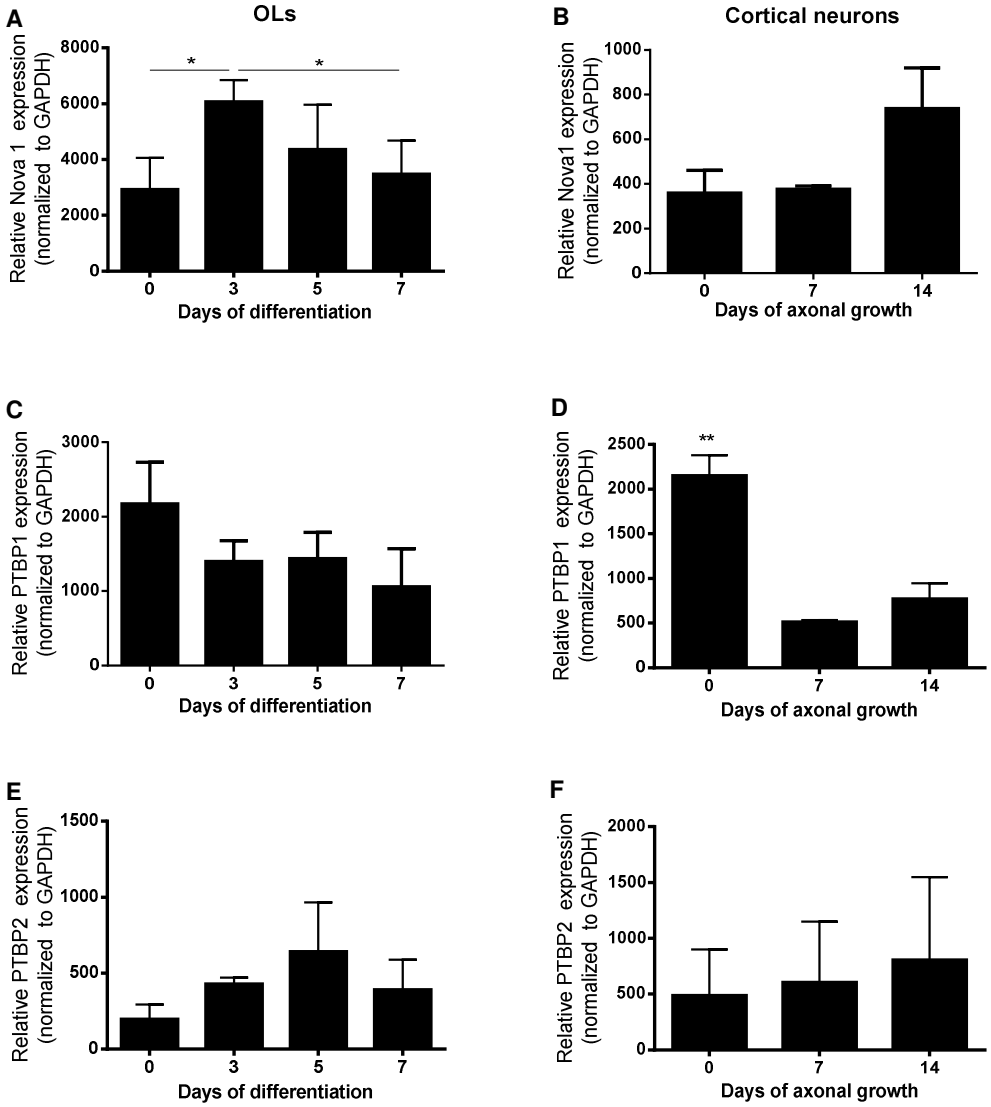



**Figure27. Quantification of mRNA expression levels of RBPs during OLs differentiation and axonal growth of neurons.** The mRNA expression of *Nova1* **(A,B)**, *Ptbp1* **(C,D)** and *Ptbp2* **(E,F)** was quantified by RT-qPCR using *in vitro* differentiated OLs **(A,C,E)** or neurons **(B,D,F)**. Error bars represent the SD of three independent experiments.*p<0,05, **p<0,01.

If Ptbp1 has the same role in regulation of PA in *Rac1* as in *C2 complement*, the differences in its expression could explain the differences in pAs selection between OLs and cortical neurons. Since the binding site for Ptbp1 is upstream of the proximal *Rac1* pAs, it can be activating during OL differentiation and in the early stages of neuronal axon growth. However the function of Ptbp1/Ptbp2 switch in 3'end processing are not known, is possible to hypothesize that the decrease in *Ptbp1* expression may be leading to the usage of the distal *Rac1* pAs accompanied by the stabilization of the longest mRNA isoform through the increase of *Ptbp2* expression in cortical neurons.

Another potential effect of the Ptbp proteins in *Rac1* regulation is at cytoplasm level. Recent studies found that Ptbp1 regulates Rac1 by promoting miRNA targeting, through the formation of a secondary mRNA structure (when Ptbp1 is bound), which makes the target site available to the miRNA 124 (miR-124)[116]. We observed that the Ptbp binding site is close to the miR-124 target site in the long *Rac1* 3'UTR. Therefore, the *Rac1* regulation in neurons could be done by this mechanism, since the increase in long *Rac1* mRNA corresponds to the time point where *Ptbp1* expression decreases. Thus, is no longer available for promoting the miRNA targeting of *Rac1* 3'UTR.

In addition, the switch from Ptbp1 to Ptbp2 in neuronal differentiation was also described as being mediated by miR-124, since it can suppress *Ptbp1* expression.[117] This down regulation of Ptbp1 allows the increase of Ptbp2 expression, as Ptbp2 is sufficient to induce the neuronal phenotype in fibroblasts[116], it is likely that the switch Ptbp1/Ptbp2 have a role in the neuronal axon growth. In addition, in OLs the basal level of *Ptbp1* that is maintained throughout differentiation could modulate the degradation of the longest 3'UTR by miRNAs. Thus the switch of Ptbp proteins may be leading to the differences between OLs and neurons in *Rac1* isoforms expression.

Further studies are required to confirm this hypothesis, however this cross regulation through Ptbp1/Ptbp2 and miRNAs can be fundamental players in the regulation of the *Rac1* expression.

Only two RBPs were predicted to bind to the shortest *Rac1* 3'UTR, one of which is Elavl2 (ELAV like neuron-specific RNA binding protein 2) (Table 4). There is a high conservation of the consensus binding sites among Elavl (also known as Hu) proteins, which are the AU-rich elements (AREs)[69, 118]. Elavl1 was recently described as a mediator of neuronal 3'UTRs extension in *Drosophila*[119]. Interestingly, in one genome-wide study, using brain tissue and searching for Elav4 targets, one binding site in *Rac1* 3'UTR was found (Toma Tebaldi, personal communication). We therefore quantified the expressions of *Elavl1* and *Elavl4* in cortical neurons (Figure 28). However, our results have shown that *Elavl1* and *Elavl4* expression levels do not change during axonal growth.

Elavl proteins are known for their multiple functions on mRNA: local transcription rate enhancement, translation repression or enhancement, alternative splicing, nuclear export of mRNA, mRNA stabilization (Elavl1 also affect mRNA destabilization) and APA. While Elavl1 is ubiquitously expressed, Elavl4 is neuron-specific and have critical roles in neuronal development and function. Elavl4 have indeed an essential role in the terminal differentiation of the neurons, specifically in dendritic and axonal outgrowth.[120, 121] Thus, the absence of variation in *Elavl1* and *Elavl4* mRNA levels does not imply a

lack of function in the *Rac1* 3'UTR regulation. They may affect *Rac1* mRNA by modulating their cellular localization, for example. Further studies are required to address this issue.

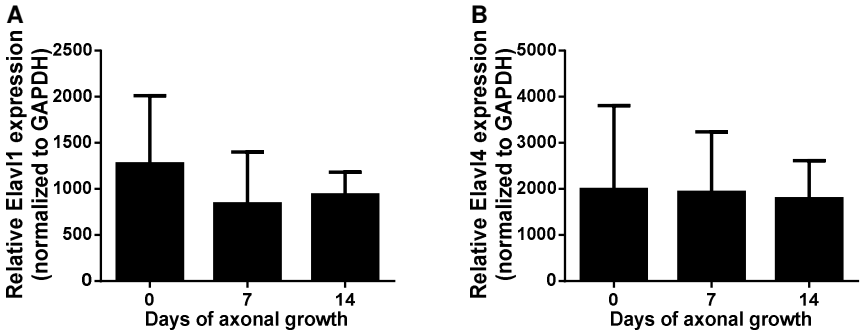



**Figure 28. Quantification of mRNA expression levels of RBPs during OLs axonal growth of cortical neurons.** The mRNA expression of **A)** *Elavl1*, **B)** *Elavl4* was quantified by RT-qPCR using cultured cortical neurons. Error bars represent the SD of three independent experiments.

Concerning the putative RBPs present only in the longest *Rac1* 3'UTR, we decided to further study Ybx1 (Y box binding protein1). Ybx1 binds to the mRNA and participate in pre-mRNA transcription and splicing, mRNA packaging and regulation of its stability and translation[122]. Our results have revealed an interesting pattern in OLs differentiation. *Ybx1* mRNA present a peak of expression at day 3 of differentiation, followed by a drastic decrease at day 5 (Figure 29A). This mRNA expression pattern is accompanied by the protein levels (Figure 29B). To confirm that Ybx1 is able to bind to *Rac1* mRNA, we performed a RNA-immunoprecipitation (RIP) assay. Our results have shown that Ybx1 clearly binds to *Rac1* mRNA (Figure 29C). Although this technique does not allow us to map the binding site of Ybx1 to *Rac1* mRNA, it is very likely through 3'UTR, as our in silico data indicates.

The involvement of Ybx1 in mRNA translation regulation has been described as result of variations in Ybx1/mRNA ratio: low ratios promote mRNA translation whereas high ratios make the mRNA inaccessible to translation and also to degradation[122]. Thus, the Rac1 could be regulated in a similar manner, *i.e.* the increase at day 3 of Ybx1 corresponds to an increase in *Rac1* expression (increasing the ratio), which could indicate that part of the *Rac1* mRNA at this stage is inaccessible to translation, and we can hypothesized that is being transported into the processes, since Ybx1 is a major component of the mRNPs, the structures that transport the mRNAs in the cell[122]. At day 5 the Ybx1 protein levels decrease and *Rac1* mRNA expression continue to increase (decreasing the ratio). It is possible that at this stage the mRNA is being locally translated.

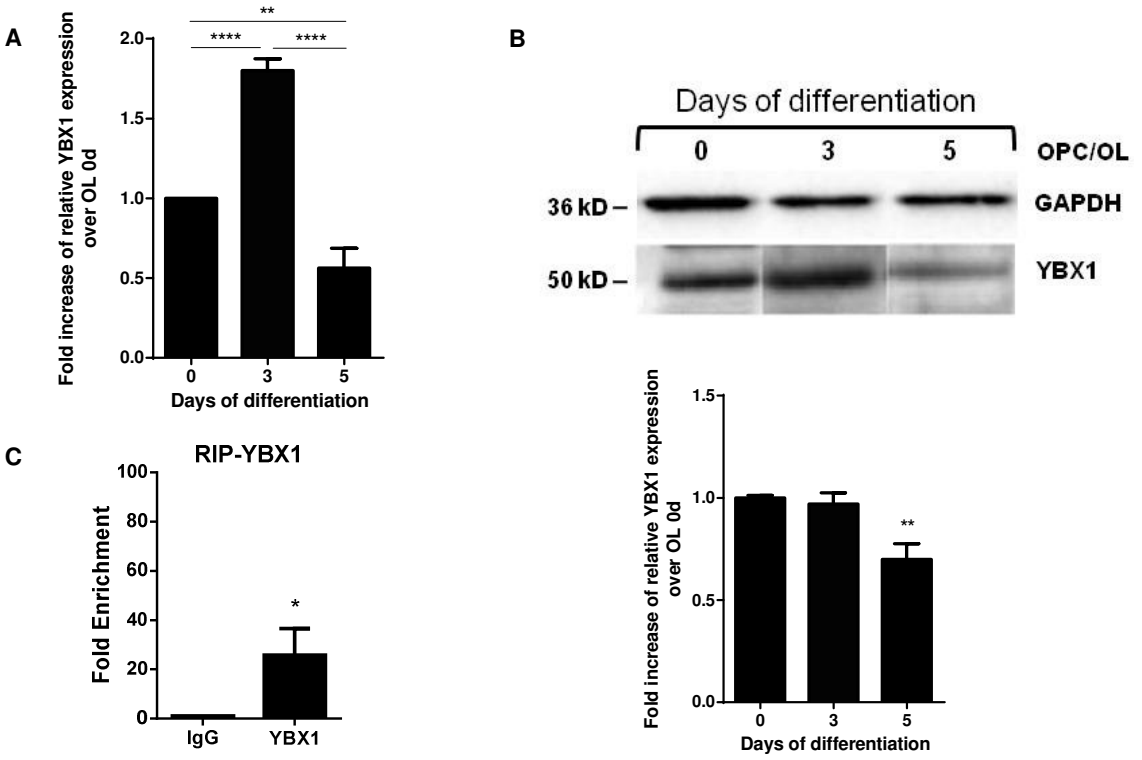



**Figure 29. YBX1 expression and *Rac1* mRNA binding during OL differentiation. A)** The *YBX1* mRNA expression was quantified by RT-qPCR in OPC/OL at day 0, day3 and day 5of differentiation**. B)** The proteins levels were measured by Western blot and the respective bands quantification**. C)** YBX1-Rac1 mRNA interaction was assessed by RIP assays**.** *p<0.05; **p<0.01; ****p<0.0001.

Altogether, our results indicate that the expression of the RBPs studied is cell type dependent. As we found that these proteins putatively bind to *Rac1* 3'UTR and confirmed experimentally this binding for Ybx1, it is plausible that their expression variations observed may impact on the regulation of *Rac1* mRNA isoforms.

# 2. Cdc42 isoforms functions in CNS and PNS myelination

## 2.1. Potential different roles of Cdc42 isoforms in OL and SC differentiation.

The myelination is a common process of both CNS and PNS, which requires the full differentiation and maturation of the OLs or SC respectively. This process is Cdc42-dependent in both cell types[38, 40], however *Cdc42* present two alternative spliced isoforms (Figure 30) with different features[31, 32, 123], whose role in myelination remains unrevealed.

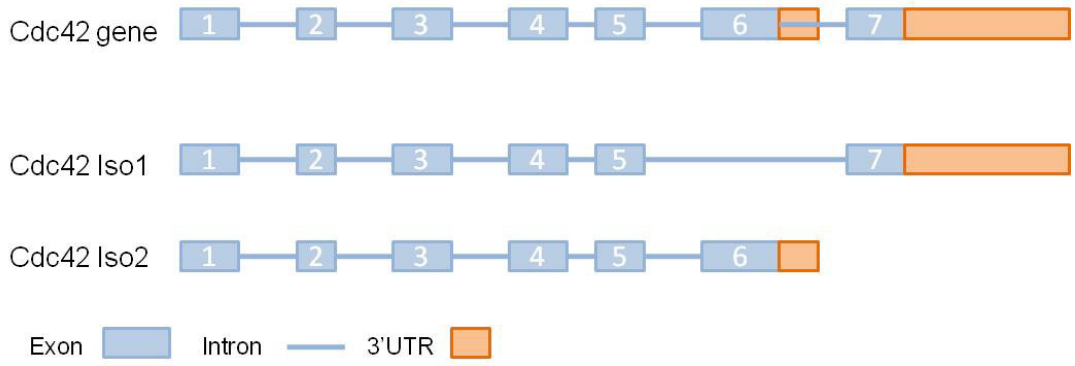



**Figure 30. Schematic representation of the *Cdc42* gene splicing variants.** *Cdc42* gene has 7 exons, and exons 6 and 7 undergo exon skipping to generate Cdc42 Iso1 or Iso2, respectively, containing different 3'UTRs.

These two *Cdc42* mRNA isoforms are translated in two different proteins with the same size (191 aa.), which only vary in the a.a. sequence from position 182 to 191, the region that corresponds to the membrane localization domain[29]. To start to investigate the biological relevance of these two Cdc42 Isoforms in differentiation and myelination, we quantified their mRNA levels in OLs, in ON and in SN. Since *Cdc42 Iso2* mRNA expression levels in non-neuronal cells is lower than those of *Cdc42 Iso1*, and to highlight the variation in the relative expression of the two isoforms during the cell differentiation, the ratio between the mRNA expression of the 2 isoforms was determined (Figure 31). In OLs we observed that during differentiation *Cdc42 Iso1* expression increases relatively to *Cdc42 Iso2* (Figure 31A). However, in the *in vivo* system (ON) this difference is not observed (Figure 31B), probably due to the influence of the axonal portion present in ON, since *Cdc42 Iso2* is highly expressed in neurons and its expression increases with axonal growth (Figure 22A). Surprisingly, the result from SN shows a significant increase in the relative expression of the *Cdc42 Iso1* (Figure 31C) during myelination (from P2 to P30).

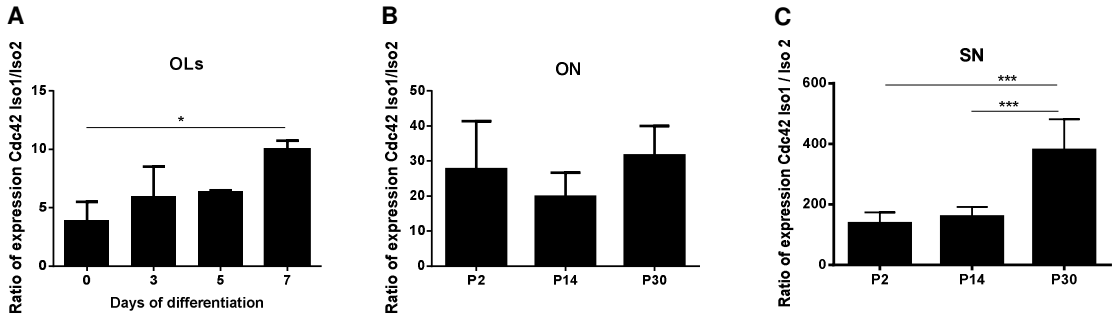



**Figure 31. mRNA expression ratios of the *Cdc42* isoforms during OL, ON and SC differentiation.** The ratio *Cdc42 Iso1/Cdc42 Iso2* expression was determined in **A)** OLs, **B)** ON and **C)** SN at different time points of differentiation. Error bars represent the SD of at least three independent experiments. *p<0.05; ***p<0.001

These observations strongly support the hypothesis that the two spliced isoforms of *Cdc42* have different roles in differentiation and myelination of both OLs and SN, with a more pronounced role in the last one. From these results we hypothesize that Cdc42 Iso2 is more required in the early stages, while the Cdc42 Iso1 is more preponderant in the later stages of differentiation. It was recently demonstrated that in SC, Cdc42 is recruited and activated at the plasma membrane by Frabin/Fgd4 (a Cdc42 GEF) and, this function is critical to proper myelin maintenance in the adulthood[124], however in the pup stage Frabin/Fgd4 is not able to activate Cdc42. On the other hand, Cdc42 knockout mice show a decrease in SC proliferation (unpublished data from João B. Relva's Lab). However, in both situations was considered the total Cdc42. Altogether, these results are in accordance with the suggestion that Cdc42 Isoforms have different roles, at least in SC. Thus Cdc42 Iso1 could be affecting differentiation and myelination, and Cdc42 Iso2 might be involved in the proliferation of myelinating cells.

To unravel the potential function of the two Cdc42 proteins, the CG4 cell line (a cell line of OLs) was transfected with constitutively active Cdc42 Iso1 and Cdc42 Iso2 plasmids with a GFP tag (Figure 33). CG4 cells were used in order to obtain more transfection efficiency, since the primary OLs are difficult to transfect. Of note, the levels of Cdc42 mRNAs expression was also measured in this cell line to confirm that they express similar levels as primary OLs (data not shown). Albeit the lower percentage of the transfected cells, it is possible to analyse the results due to the selection of the positive cells that express GFP. The preliminary immunofluorescence images show that the Cdc42 Iso2 seems to cause an arrest in the cell differentiation with a simultaneous decrease in process complexity (Figure 32C-E). This phenotype is very clear when

comparing GFP-positive cells with non-transfected cells at day 3 and 5. At day 3, non-transfected cells already present numerous processes while GFP-positive are still bipolar (Figure 32D). Also, at day 5, the delay in differentiation is visible, with the transfected cell having some processes extensions. However, these processes seem to be shortest and have less branching complexity than the non-transfected cells (Figure 32E). The results from Cdc42 Iso1 transfection have shown that at day 0 the cells remain phenotypically normal (Figure 32A), whereas at day 5 GFP-positive cells appear to present longest processes when compared with negative cells (Figure 32B). Regarding the localization, the results have suggested that the exogenous proteins localize differentially, at least at day 5. Thus, at the later stages of the differentiation, the GFP signal of Cdc42 Iso1 transfected cells is localized in the soma (Figure 32B), whereas in the Cdc42 Iso2 transfection the signal is present in the soma and processes, and the protein seem to be in the cytoplasmatic membrane (Figure 32E).

These results fit with our previous hypothesis of the different functions for the Cdc42 protein isoforms. Moreover, they advance a role for Cdc42 Iso1 in differentiation, since transfected cells present longer processes, and a role for Cdc42 Iso2 in proliferation, since the positive cells retain for a longer time the proliferative phenotype, even in the presence of differentiation factors. The results from the membrane localization of the Cdc42 Iso2 are also in concordance with previous studies. This demonstrates in one hand, that the specific dual lipidification of this protein increases its affinity for the membranes[31] and, in the other hand that the a.a. changes (relative to the Cdc42 Iso1) in the membrane localization domain prevents the interaction with the Rho GDIα, that is an inhibitor of the Rho GTPases activation [33]. Although these are preliminary results, they highlight the relevance of both protein isoforms during OLs differentiation, and the existence of different roles in this process. Further studies need to be done to confirm and dissect the role of the Cdc42 isoforms in CNS and PNS during differentiation and myelination.

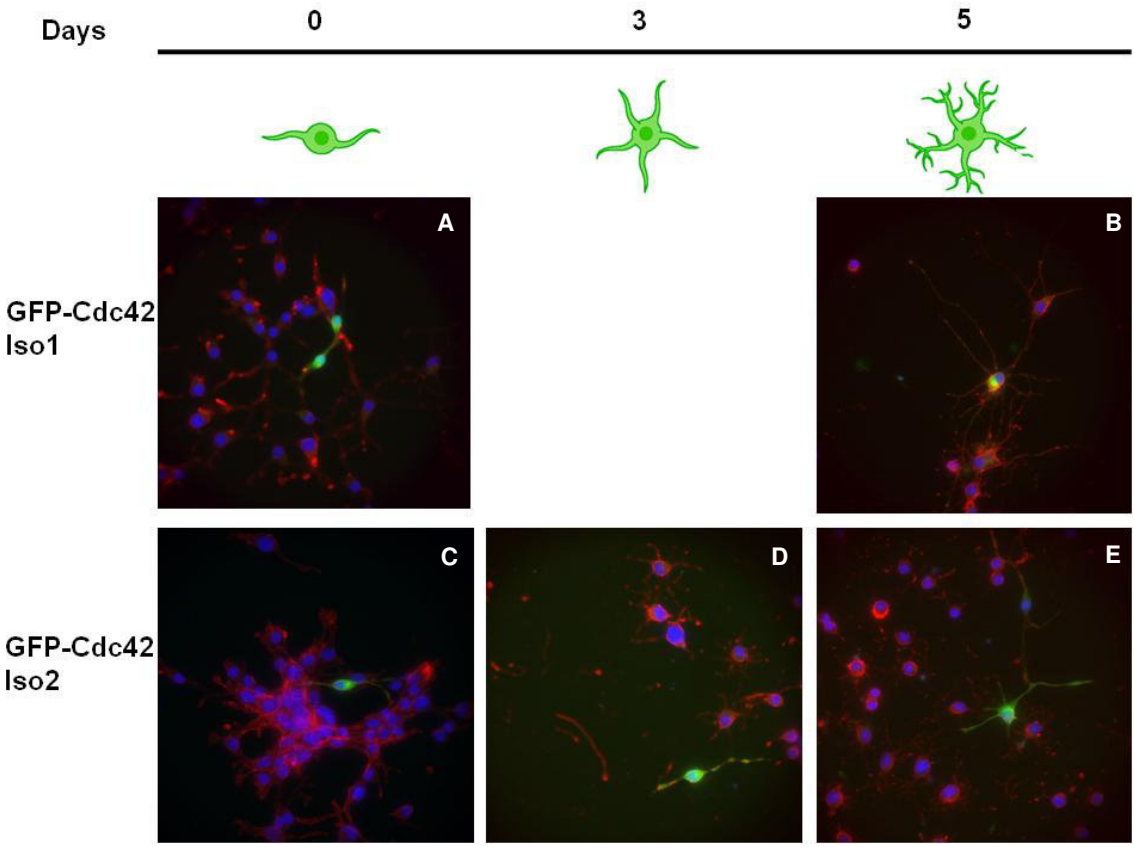



**Figure 32. Constitutive expression of Cdc42 Isoforms in CG4 cells. (A, B)** Cdc42 Iso1 and **(C, D, E)** Cdc42 iso2 plasmids with GFP tag were constitutively expressed in CG4 cells. CG4 cells were collected at **(A, C)** day 0, **(D)** day 3 and **(B, E)** day 5. The green staining marks the Cdc42 proteins, the red tubulin and in blue the DAPI. Amplification 40x.

## 2.2. Role of miRNAs in regulation of the *Cdc42* isoforms expression.

The *Cdc42* mRNAs not only have differences in the alternative exons that are included (by alternative splicing), but also contain different 3'UTRs (Figure 30). Consequently, *Cdc42 Iso1* and *Cdc42 Iso2* could be differential regulated through its 3'UTRs, by RBP and miRNAs targeting. Since the shortest 3'UTRs of the two *Cdc42* mRNA isoforms are the most expressed, we used this 3'UTRs to perform an *in silico* analysis to search for putative miRNAs target sites.


**Figure 33. Representation of the *Cdc42 Iso1* and *Cdc42 Iso2* shortest 3'UTR highlighting the putative target sites for miRNAs.** The target sites are colored in the same color of the respective miRNA, in red are represented the pAs.

Three miRNAs databases were assessed and only those miRNAs that were present in all of them were considered and represented in Figure 33. Curiously the two *Cdc42* isoforms have completely different putative binding site for miRNAs. Furthermore, to restrict the number of miRNAs, we selected those that have some described relation with Cdc42 or OLs. The miR-137 and miR-195-5p were demonstrated as regulators of *Cdc42* in some carcinoma models[81, 125, 126], and the cluster miR-17-92 was involved in the control of cell number of OLs[81]. In order to understand if *Cdc42 Iso1* and *Cdc42 Iso2* expression could be modulated by these miRNAs during the OL differentiation, their expression was quantified by RT-qPCR using specific probes. Our results have shown that the expression of miR-195 increases at 3 days post differentiation (Figure 34), the time point from each the expression of *Cdc42 Iso2* starts to decrease (Figure 15). Interestingly, the expression of this miRNA decreases from day 3 to day 7, accompanying the decrease in *Cdc42 Iso2* expression. These results may indicate that the increase in miR-195 expression in the first 3 days of differentiation downregulates *Cdc42 Iso2* expression.

Regarding the expression of the miR-137 and miR-92a, the results show a similar expression pattern, with an increase at day 3 followed by a decrease up to day 7 (Figure 34). Analysing the expression pattern of *Cdc42 Iso1* (Figure 15), we observed that until day 3 the relative expression is not altered, and from day 3 onwards there is an increase in *Cdc42 Iso1* expression. This suggests that miR-92a and miR-137 may regulate *Cdc42 Iso1* basal levels in the first days of differentiation, and only upon a decrease in the expression levels of these miRNA, the expression of *Cdc42 Iso1* starts to increase. However, further studies are needed to dissect the potential role of these miRNAs in the regulation of *Cdc42* isoforms.

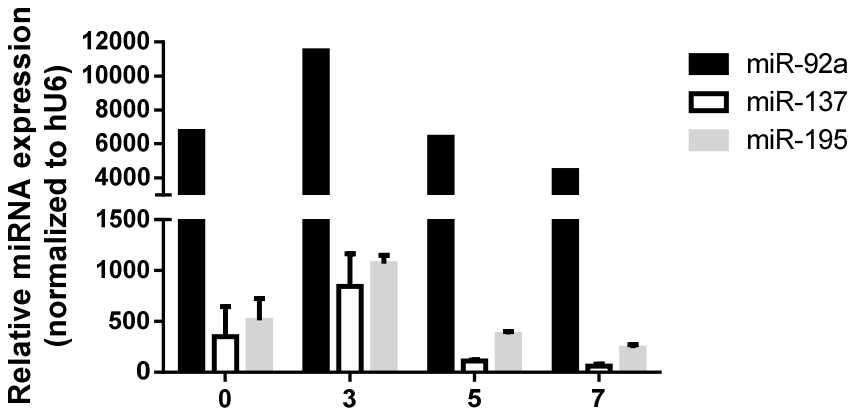



**Figure 34. miRNAs expression during OLs differentiation.** The mRNA expression of miR-92a, miR-137, and miR-195 was quantified by RT-qPCR using OLs. Error bars represent the SD of two independent experiments for miR-137 and miR-195. The expression of miR-92a was analyzed once.

The *Cdc42* isoforms can also being regulated either by other miRNAs or competition between miRNAs and RBPs or, by differential expression of RBPs.

Thus, an important issue to be addressed in the future is the existence of different RBPs binding sites in one or another *Cdc42* isoforms. Potential candidates are the PTBP proteins, since they control *Cdc42* splicing events[117]. Ptbp1 is responsible for the inclusion of the exon 7 in *Cdc42 Iso1*, and Ptbp2 for the inclusion of the exon 6 in the *Cdc42 Iso2*[117]. Given this, it is likely that *Cdc42* isoforms are being regulated by the interaction of various mechanisms in a time-dependent manner. Thereby, the *Cdc42* isoforms appears to have a critical function in OL differentiation. However, the role of each isoform and the molecular mechanisms behind its differential expression during differentiation still remains elusive.

# CONCLUSION

APA is a co-transcriptional mechanism that allows gene regulation through the differential expression of different 3'UTRs in a very specific manner. Therefore, APA is used to regulate diverse cellular processes and conditions.

From the results of this work it is possible to conclude that the 'classically activated' *Rac1*, *Cdc42* and *RhoA* have different mRNA isoforms produced by APA in the 3'UTR, which can be subject of regulation by RBPs and miRNAs. We found that the relative expression of the different mRNA isoforms during OL differentiation do not change, and the proximal signal is always the most used signal, in contrast with the data obtained from genome-wide studies[64]. Therefore, it is tempting to speculate that this pattern of APA is due to the specificity of the classical Rho GTPAses, since their function is fine-tune regulated at the protein level through activation/inactivation by GEFs, GAPs and GDIs. Consequently they choose preferentially the proximal site to produce enough amounts of protein that can be regulated itself. This hypothesis was further supported by the results from the atypical *RhoBTB2*, which have showed a total usage of the distal pA throughout OL differentiation. Since RhoBTB2 proteins are constitutively active, they may require an upstream regulatory mechanism at the mRNA level, and for that reason the distal pAs is selected for production of the longest 3'UTRs, more prone to regulation. Remarkably, the APA pattern of the Rho GTPases studied in this thesis seems to be transversal to all glia cells, revealing a cell specific behavior of this process, a mechanism not yet described.

Interestingly, when APA of *Rac1* Rho GTPase was analyzed in cortical neurons, we observed that *Rac1* 3'UTR undergoes a complete lengthening through the usage of the distal pAs during axonal growth. This is in contrast to other previously described genes in other cell types and conditions that express the shortest mRNAs to produce higher amounts of protein. In neurons, lengthening of the *Rac1* mRNA leads to higher protein levels, according to what is described in high throughput studies for APA in the brain. This thesis also provides indications for the mechanism of regulation of the 3'UTRs extension in neurons by the differential expression of RBPs, namely the brain specific Nova1. Albeit the APA process was characterized, the biological function of the expression of long *Rac1* 3'UTR stills requires a deep investigation. This work demonstrated for the first time, that APA is a gene specific process that allows the achievement of a cell specific regulation of genes that are ubiquitously expressed.

Altogether, we provide for the first time evidences that APA is a gene and cell specific mechanism, and that we need to have caution in the extrapolation of the genome wide studies when we are dealing with a mixture of cells.

Additionally, this work provides for the first time evidences for distinct functions of Cdc42 alternative isoforms in OL differentiation and myelination. Although the molecular mechanism underlying the differential expression of *Cdc42* isoforms and their functional relevance remains unknown, these findings open the door towards a more complete understanding of the myelination and remyelination process in CNS and PNS.

# FUTURE PERSPECTIVES

### Alternative Polyadenylation in Rho GTPases

In contrast with the genome-wide data about APA[63, 64], this work puts forward a mechanism that is more gene and cell specific. Nevertheless, several issues need to be address in the future:

- What is the biological importance of the longest Rac1 mRNA in cortical neurons?
- Is Rac1 3'UTR affecting its cellular localization?
- What are the molecular regulators of the Rac1 3' end extension?

### Cdc42 isoforms functions in CNS and PNS myelination

Cdc42 plays an important role in CNS and PNS myelination[38, 40]. We have found that the two isoform are expressed in both CNS and PNS glial cells, still the relevance of this findings remains elusive. Therefore, several important questions keep unanswered:

- What is the function of both Cdc42 Isoforms in the CNS and PNS?
- How are the Cdc42 isoforms gene regulated in the CNS and PNS?
- Different Cdc42 isoforms play different roles in the remyelination process of the PNS?

To unravel these points will open new avenues for the development of new strategies for the treatment of neurological diseases.

# BIBLIOGRAPHY

1. PURVES D. Neuroscience. In: PURVES D, AUGUSTINE GJ, FITZPATRICK D, HALL WC, LAMANTIA A-S, MCNAMARA JO, et al., eds. Neuroscience: Sinauer Associates, Inc., 2004:1-26.
2. Dotti CG, Sullivan CA, Banker GA. THE ESTABLISHMENT OF POLARITY BY HIPPOCAMPAL-NEURONS IN CULTURE. Journal of Neuroscience 1988; 8:1454-68.
3. Polleux F, Snider W. Initiating and Growing an Axon. Cold Spring Harbor Perspectives in Biology 2010; 2.
4. Meza-Sosa KF, Pedraza-Alva G, Perez-Martinez L. microRNAs: key triggers of neuronal cell fate. Frontiers in Cellular Neuroscience 2014; 8.
5. Coutinho-Mansfield GC, Xue Y, Zhang Y, Fu X-D. PTB/nPTB switch: a post-transcriptional mechanism for programming neuronal differentiation. Genes & Development 2007; 21:1573-7.
6. Hobert O, Carrera I, Stefanakis N. The molecular and gene regulatory signature of a neuron. Trends in Neurosciences 2010; 33:435-45.
7. Norris AD, Gao S, Norris ML, Ray D, Ramani AK, Fraser AG, et al. A Pair of RNA-Binding Proteins Controls Networks of Splicing Events Contributing to Specialization of Neural Cell Types. Molecular Cell 2014; 54:946-59.
8. Nave K-A, Werner HB. Myelination of the nervous system: mechanisms and functions. Annual review of cell and developmental biology 2014; 30:503-33.
9. Pfeiffer SE, Warrington AE, Bansal R. The oligodendrocyte and its many cellular processes. Trends in cell biology 1993; 3:191-7.
10. Jackman N, Ishii A, Bansal R. Oligodendrocyte Development and Myelin Biogenesis: Parsing Out the Roles of Glycosphingolipids. Physiology 2009; 24:290-7.
11. Liu J, Casaccia P. Epigenetic regulation of oligodendrocyte identity. Trends in Neurosciences 2010; 33:193-201.
12. Jacob C, Lebrun-Julien F, Suter U. How Histone Deacetylases Control Myelination. Molecular Neurobiology 2011; 44:303-12.
13. Swiss VA, Nguyen T, Dugas J, Ibrahim A, Barres B, Androulakis IP, et al. Identification of a Gene Regulatory Network Necessary for the Initiation of Oligodendrocyte Differentiation. Plos One 2011; 6.
14. Emery B. Transcriptional and post-transcriptional control of CNS myelination. Current Opinion in Neurobiology 2010; 20:601-7.
15. Simons M, Trajkovic K. Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. Journal of Cell Science 2006; 119:4381-9.
16. Madaule P, Axel R. A NOVEL RAS-RELATED GENE FAMILY. Cell 1985; 41:31-40.
17. Bar-Sagi D, Hall A. Ras and Rho GTPases: A family reunion. Cell 2000; 103:227-38.
18. Jaffe AB, Hall A. Rho GTPases: Biochemistry and biology. Annual Review of Cell and Developmental Biology 2005; 21:247-69.
19. Boureux A, Vignal E, Faure S, Fort P. Evolution of the Rho family of Ras-like GTPases in eukaryotes. Molecular Biology and Evolution 2007; 24:203-16.
20. Bustelo XR, Sauzeau V, Berenjeno IM. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions *in vivo*. Bioessays 2007; 29:356-70.
21. Aspenstrom P, Ruusala A, Pacholsky D. Taking Rho GTPases to the next level: The cellular functions of atypical Rho GTPases. Experimental Cell Research 2007; 313:3673-9.
22. Cherfils J, Zeghouf M. REGULATION OF SMALL GTPases BY GEFs, GAPs, AND GDIs. Physiological Reviews 2013; 93:269-309.
23. Govek EE, Newey SE, Van Aelst L. The role of the Rho GTPases in neuronal development. Genes & Development 2005; 19:1-49.
24. Feltri ML, Suter U, Relvas JB. The Function of RhoGTPases in Axon Ensheathment and Myelination. Glia 2008; 56:1508-17.
25. Ng JL, Luo LQ. Rho GTPases regulate axon growth through convergent and divergent signaling pathways. Neuron 2004; 44:779-93.

26. Auer M, Hausott B, Klimaschewski L. Rho GTPases as regulators of morphological neuroplasticity. Annals of Anatomy-Anatomischer Anzeiger 2011; 193:259-66.
27. Bosco EE, Mulloy JC, Zheng Y. Rac1 GTPase: A "Rac" of All Trades. Cellular and Molecular Life Sciences 2009; 66:370-4.
28. Heasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from *in vivo* studies. Nature Reviews Molecular Cell Biology 2008; 9:690-701.
29. Johnson DI. Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. Microbiology and Molecular Biology Reviews 1999; 63:54-+.
30. Chen F, Ma L, Parrini MC, Mao X, Lopez M, Wu C, et al. Cdc42 is required for PIP2-induced actin polymerization and early development but not for cell viability. Current Biology 2000; 10:758-65.
31. Kang R, Wan J, Arstikaitis P, Takahashi H, Huang K, Bailey AO, et al. Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. Nature 2008; 456:904-9.
32. Wirth A, Chen-Wacker C, Wu Y-W, Gorinski N, Filippov MA, Pandey G, et al. Dual lipidation of the brain-specific Cdc42 isoform regulates its functional properties. Biochemical Journal 2013; 456:311-22.
33. Nishimura A, Linder ME. Identification of a Novel Prenyl and Palmitoyl Modification at the CaaX Motif of Cdc42 That Regulates RhoGDI Binding. Molecular and Cellular Biology 2013; 33:1417-29.
34. Zhou X, Zheng Y. Cell Type-specific Signaling Function of RhoA GTPase: Lessons from Mouse Gene Targeting. Journal of Biological Chemistry 2013; 288:36179-88.
35. Gualdoni S, Albertinazzi C, Corbetta S, Valtorta F, de Curtis I. Normal levels of Rac1 are important for dendritic but not axonal development in hippocampal neurons. Biology of the Cell 2007; 99:455-64.
36. Hall A, Lalli G. Rho and Ras GTPases in Axon Growth, Guidance, and Branching. Cold Spring Harbor Perspectives in Biology 2010; 2.
37. Luo LQ, Hensch TK, Ackerman L, Barbel S, Jan LY, Jan YN. Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature 1996; 379:837-40.
38. Benninger Y, Thurnherr T, Pereira JA, Krause S, Wu X, Chrostek-Grashoff A, et al. Essential and distinct roles for cdc42 and rac1 in the regulation of Schwann cell biology during peripheral nervous system development. Journal of Cell Biology 2007; 177:1051-61.
39. Guo L, Moon C, Niehaus K, Zheng Y, Ratner N. Rac1 Controls Schwann Cell Myelination through cAMP and NF2/merlin. Journal of Neuroscience 2012; 32:17251-61.
40. Thurnherr T, Benninger Y, Wu X, Chrostek A, Krause SM, Nave K-A, et al. Cdc42 and Rac1 signaling are both required for and act synergistically in the correct formation of myelin sheaths in the CNS. Journal of Neuroscience 2006; 26:10110-9.
41. Liang XQ, Draghi NA, Resh MD. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. Journal of Neuroscience 2004; 24:7140-9.
42. Kim Y, Sung JY, Ceglia I, Lee K-W, Ahn J-H, Halford JM, et al. Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology. Nature 2006; 442:814-7.
43. Ramos S, Khademi F, Somesh BP, Rivero F. Genomic organization and expression profile of the small GTPases of the RhoBTB family in human and mouse. Gene 2002; 298:147-57.
44. Lutz CS, Moreira A. Alternative mRNA polyadenylation in eukaryotes: an effective regulator of gene expression. Wiley Interdisciplinary Reviews-Rna 2011; 2:22-31.
45. Di Giammartino DC, Nishida K, Manley JL. Mechanisms and Consequences of Alternative Polyadenylation. Molecular Cell 2011; 43:853-66.
46. Tian B, Manley JL. Alternative cleavage and polyadenylation: the long and short of it. Trends in Biochemical Sciences 2013; 38:312-20.
47. Tollervey D, Caceres JF. RNA processing marches on. Cell 2000; 103:703-9.
48. Proudfoot NJ, Furger A, Dye MJ. Integrating mRNA processing with transcription. Cell 2002; 108:501-12.

49. Moore MJ, Proudfoot NJ. Pre-mRNA processing reaches back to transcription and ahead to translation. Cell 2009; 136:688-700.
50. Chang H, Lim J, Ha M, Kim VN. TAIL-seq: Genome-wide Determination of Poly(A) Tail Length and 3 ' End Modifications. Molecular Cell 2014; 53:1044-52.
51. Subtelny AO, Eichhorn SW, Chen GR, Sive H, Bartel DP. Poly(A)-tail profiling reveals an embryonic switch in translational control. Nature 2014; 508:66-+.
52. Zhang HB, Lee JY, Tian B. Biased alternative polyadenylation in human tissues. Genome Biology 2005; 6.
53. Colgan DF, Manley JL. Mechanism and regulation of mRNA polyadenylation. Genes & Development 1997; 11:2755-66.
54. Shi Y, Di Giammartino DC, Taylor D, Sarkeshik A, Rice WJ, Yates JR, III, et al. Molecular Architecture of the Human Pre-mRNA 3 ' Processing Complex. Molecular Cell 2009; 33:365-76.
55. Curinha A, Braz SO, Pereira-Castro I, Cruz A, Moreira A. Implications of polyadenylation in health and disease. Nucleus 2014; 5.
56. Derti A, Garrett-Engele P, Macisaac KD, Stevens RC, Sriram S, Chen R, et al. A quantitative atlas of polyadenylation in five mammals. Genome Res 2012; 22:1173-83.
57. Hoque M, Ji Z, Zheng D, Luo W, Li W, You B, et al. Analysis of alternative cleavage and polyadenylation by 3 ' region extraction and deep sequencing. Nature Methods 2013; 10:133-9.
58. Elkon R, Ugalde AP, Agami R. Alternative cleavage and polyadenylation: extent, regulation and function. Nature Reviews Genetics 2013; 14:496-506.
59. Sandberg R, Neilson JR, Sarma A, Sharp PA, Burge CB. Proliferating cells express mRNAs with shortened 3 ' untranslated regions and fewer microRNA target sites. Science 2008; 320:1643-7.
60. Mayr C, Bartel DP. Widespread Shortening of 3 ' UTRs by Alternative Cleavage and Polyadenylation Activates Oncogenes in Cancer Cells. Cell 2009; 138:673-84.
61. Ji Z, Tian B. Reprogramming of 3 ' Untranslated Regions of mRNAs by Alternative Polyadenylation in Generation of Pluripotent Stem Cells from Different Cell Types. Plos One 2009; 4.
62. Ji Z, Lee JY, Pan Z, Jiang B, Tian B. Progressive lengthening of 3 ' untranslated regions of mRNAs by alternative polyadenylation during mouse embryonic development. Proceedings of the National Academy of Sciences of the United States of America 2009; 106:7028-33.
63. Smibert P, Miura P, Westholm JO, Shenker S, May G, Duff MO, et al. Global Patterns of Tissue-Specific Alternative Polyadenylation in Drosophila. Cell Reports 2012; 1:277-89.
64. Miura P, Shenker S, Andreu-Agullo C, Westholm JO, Lai EC. Widespread and extensive lengthening of 3 ' UTRs in the mammalian brain. Genome Research 2013; 23:812-25.
65. Glisovic T, Bachorik JL, Yong J, Dreyfuss G. RNA-binding proteins and post-transcriptional gene regulation. Febs Letters 2008; 582:1977-86.
66. Ray D, Kazan H, Cook KB, Weirauch MT, Najafabadi HS, Li X, et al. A compendium of RNA-binding motifs for decoding gene regulation. Nature 2013; 499:172-7.
67. Hu W, Liu Y, Yan J. Microarray Meta-Analysis of RNA-Binding Protein Functions in Alternative Polyadenylation. Plos One 2014; 9.
68. Miura P, Sanfilippo P, Shenker S, Lai EC. Alternative polyadenylation in the nervous system: To what lengths will 3 ' UTR extensions take us? Bioessays 2014; 36:766-77.
69. Darnell RB. RNA Protein Interaction in Neurons. Annual Review of Neuroscience, Vol 36 2013; 36:243-70.
70. Jung H, Yoon BC, Holt CE. Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair (vol 13, pg 308, 2012). Nature Reviews Neuroscience 2012; 13.
71. Larocque D, Galarneau A, Liu HN, Scott M, Almazan G, Richard S. Protection of p27(Kip1) mRNA by quaking RNA binding proteins promotes oligodendrocyte differentiation. Nature Neuroscience 2005; 8:27-33.

72. Dobson NR, Zhou Y-X, Flint NC, Armstrong RC. Musashi1 RNA-binding protein regulates oligodendrocyte lineage cell differentiation and survival. Glia 2008; 56:318-30.
73. Laursen LS, Chan CW, Ffrench-Constant C. Translation of myelin basic protein mRNA in oligodendrocytes is regulated by integrin activation and hnRNP-K. Journal of Cell Biology 2011; 192:797-811.
74. Torvund-Jensen J, Stensgaard J, Fihl L, Reimer L, Laursen L. TRANSPORT AND TRANSLATION OF MBP MRNA IS DIFFERENTIALLY REGULATED BY DISTRINCT HNRNP PROTEINS. Glia 2013; 61:S143-S.
75. White R, Gonsior C, Bauer NM, Kraemer-Albers E-M, Luhmann HJ, Trotter J. Heterogeneous Nuclear Ribonucleoprotein (hnRNP) F Is a Novel Component of Oligodendroglial RNA Transport Granules Contributing to Regulation of Myelin Basic Protein (MBP) Synthesis. Journal of Biological Chemistry 2012; 287:1742-54.
76. Ho JJD, Marsden PA. Competition and collaboration between RNA-binding proteins and microRNAs. Wiley Interdisciplinary Reviews-Rna 2014; 5:69-86.
77. Ciafre SA, Galardi S. microRNAs and RNA-binding proteins A complex network of interactions and reciprocal regulations in cancer. Rna Biology 2013; 10:935-43.
78. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010; 11:597-610.
79. Leung AK, Sharp PA. Function and localization of microRNAs in mammalian cells. Cold Spring Harb Symp Quant Biol 2006; 71:29-38.
80. Ameres SL, Zamore PD. Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol 2013; 14:475-88.
81. Budde H, Schmitt S, Fitzner D, Opitz L, Salinas-Riester G, Simons M. Control of oligodendroglial cell number by the miR-17-92 cluster. Development 2010; 137:2127-32.
82. Zhao X, He X, Han X, Yu Y, Ye F, Chen Y, et al. MicroRNA-Mediated Control of Oligodendrocyte Differentiation. Neuron 2010; 65:612-26.
83. Lin S-T, Huang Y, Zhang L, Heng MY, Ptacek LJ, Fu Y-H. MicroRNA-23a promotes myelination in the central nervous system. Proceedings of the National Academy of Sciences of the United States of America 2013; 110:17468-73.
84. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 2012; 28:1647-9.
85. Cruz A, Teixeira D, Braz SO, Camacho R, Relvas JB, Moreira A. PBS Finder, a bioinformatic tool for clustering analysis of protein binding to mRNA UTRs. Under revision in RNA, 2014.
86. Cook KB, Kazan H, Zuberi K, Morris Q, Hughes TR. RBPDB: a database of RNA-binding specificities. Nucleic Acids Research 2011; 39:D301-D8.
87. Paz I, Akerman M, Dror I, Kosti I, Mandel-Gutfreund Y. SFmap: a web server for motif analysis and prediction of splicing factor binding sites. Nucleic Acids Research 2010; 38:W281-W5.
88. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Research 2008; 36:D149-D53.
89. Dweep H, Sticht C, Gretz N. In-Silico Algorithms for the Screening of Possible microRNA Binding Sites and Their Interactions. Current Genomics 2013; 14:127-36.
90. McCarthy KD, Devellis J. PREPARATION OF SEPARATE ASTROGLIAL AND OLIGODENDROGLIAL CELL-CULTURES FROM RAT CEREBRAL TISSUE. Journal of Cell Biology 1980; 85:890-902.
91. Syed YA, Baer AS, Lubec G, Hoeger H, Widhalm G, Kotter MR. Inhibition of oligodendrocyte precursor cell differentiation by myelin-associated proteins. Neurosurgical focus 2008; 24:E5-E.
92. Gomes JR, Lobo AC, Melo CV, Inacio AR, Takano J, Iwata N, et al. Cleavage of the Vesicular GABA Transporter under Excitotoxic Conditions Is Followed by Accumulation of the Truncated Transporter in Nonsynaptic Sites. Journal of Neuroscience 2011; 31:4622-35.
93. Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiological Genomics 2005; 21:389-95.

94. Nelissen K, Smeets K, Mulder M, Hendriks JJA, Ameloot M. Selection of reference genes for gene expression studies in rat oligodendrocytes using quantitative real time PCR. Journal of Neuroscience Methods 2010; 187:78-83.

95. Pinto PAB, Henriques T, Freitas MO, Martins T, Domingues RG, Wyrzykowska PS, et al. RNA polymerase II kinetics in polo polyadenylation signal selection. Embo Journal 2011; 30:2431-44.

96. Chen D, Pan X, Xiao P, Farwell MA, Zhang B. Evaluation and Identification of Reliable Reference Genes for Pharmacogenomics, Toxicogenomics, and Small RNA Expression Analysis. Journal of Cellular Physiology 2011; 226:2469-77.

97. Nunes NM, Li W, Tian B, Furger A. A functional human Poly(A) site requires only a potent DSE and an A-rich upstream sequence. Embo Journal 2010; 29:1523-36.

98. Moreira A, Takagaki Y, Brackenridge S, Wollerton M, Manley JL, Proudfoot NJ. The upstream sequence element of the C2 complement poly(A) signal activates mRNA 3 ' end formation by two distinct mechanisms. Genes & Development 1998; 12:2522-34.

99. Moreira A, Wollerton M, Monks J, Proudfoot NJ. UPSTREAM SEQUENCE ELEMENTS ENHANCE POLY(A) SITE EFFICIENCY OF THE C2 COMPLEMENT GENE AND ARE PHYLOGENETICALLY CONSERVED. Embo Journal 1995; 14:3809-19.

100. Hall-Pogar T, Zhang H, Tian B, Lutz CS. Alternative polyadenylation of cyclooxygenase-2. Nucleic Acids Research 2005; 33:2565-79.

101. Lianoglou S, Garg V, Yang JL, Leslie CS, Mayr C. Ubiquitously transcribed genes use alternative polyadenylation to achieve tissue-specific expression. Genes & Development 2013; 27:2380-96.

102. Raff MC, Ffrenchconstant C, Miller RH. GLIAL-CELLS IN THE RAT OPTIC-NERVE AND SOME THOUGHTS ON REMYELINATION IN THE MAMMALIAN CNS. Journal of Experimental Biology 1987; 132:35-41.

103. Murk K, Suarez EMB, Cockbill LMR, Banks P, Hanley JG. The antagonistic modulation of Arp2/3 activity by N-WASP, WAVE2 and PICK1 defines dynamic changes in astrocyte morphology. Journal of Cell Science 2013; 126:3873-83.

104. An JJ, Gharami K, Liao G-Y, Woo NH, Lau AG, Vanevski F, et al. Distinct role of long 3 ' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell 2008; 134:175-87.

105. Nakielny S, Dreyfuss G. Transport of proteins and RNAs in and out of the nucleus. Cell 1999; 99:677-90.

106. EdwaldsGilbert G, Veraldi KL, Milcarek C. Alternative poly(A) site selection in complex transcription units: Means to an end? Nucleic Acids Research 1997; 25:2547-61.

107. Blechingberg J, Lykke-Andersen S, Jensen TH, Jorgensen AL, Nielsen AL. Regulatory mechanisms for 3'-end alternative splicing and polyadenylation of the glial fibrillary acidic protein, GFAP, transcript. Nucleic Acids Research 2007; 35:7636-50.

108. Li Z, Aizenman CD, Cline HT. Regulation of rho GTPases by crosstalk and neuronal activity in vivo. Neuron 2002; 33:741-50.

109. Tolias KF, Bikoff JB, Burette A, Paradis S, Harrar D, Tavazoie S, et al. The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines. Neuron 2005; 45:525-38.

110. Wang L, Yi R. 3 ' UTRs take a long shot in the brain. Bioessays 2014; 36:39-45.

111. Licatalosi DD, Mele A, Fak JJ, Ule J, Kayikci M, Chi SW, et al. HITS-CLIP yields genome-wide insights into brain alternative RNA processing. Nature 2008; 456:464-U22.

112. Castelo-Branco P, Furger A, Wollerton M, Smith C, Moreira A, Proudfoot N. Polypyrimidine tract binding protein modulates efficiency of polyadenylation. Molecular and Cellular Biology 2004; 24:4174-83.

113. Boutz PL, Stoilov P, Li Q, Lin C-H, Chawla G, Ostrow K, et al. A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. Genes & Development 2007; 21:1636-52.

114. Irwin N, Baekelandt V, Goritchenko L, Benowitz LI. Identification of two proteins that bind to a pyrimidine-rich sequence in the 3'-untranslated region of GAP-43 mRNA. Nucleic Acids Research 1997; 25:1281-8.

115. Tillmar L, Welsh N. Glucose-induced binding of the polypyrimidine tract-binding protein (PTB) to the 3 '-untranslated region of the insulin mRNA (ins-PRS) is inhibited by rapamycin. Molecular and Cellular Biochemistry 2004; 260:85-90.

116. Xue Y, Ouyang K, Huang J, Zhou Y, Ouyang H, Li H, et al. Direct Conversion of Fibroblasts to Neurons by Reprogramming PTB-Regulated MicroRNA Circuits. Cell 2013; 152:82-96.

117. Makeyev EV, Zhang J, Carrasco MA, Maniatis T. The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative Pre-mRNA splicing. Molecular Cell 2007; 27:435-48.

118. Okano HJ, Darnell RB. A hierarchy of Hu RNA binding proteins in developing and adult neurons. Journal of Neuroscience 1997; 17:3024-37.

119. Hilgers V, Lemke SB, Levine M. ELAV mediates 3 ' UTR extension in the Drosophila nervous system. Genes & Development 2012; 26:2259-64.

120. Pascale A, Amadio M, Quattrone A. Defining a neuron: neuronal ELAV proteins. Cellular and Molecular Life Sciences 2008; 65:128-40.

121. Bronicki LM, Jasmin BJ. Emerging complexity of the HuD/ELAVl4 gene; implications for neuronal development, function, and dysfunction. Rna-a Publication of the Rna Society 2013; 19:1019-37.

122. Lyabin DN, Eliseeva IA, Ovchinnikov LP. YB-1 protein: functions and regulation. Wiley Interdisciplinary Reviews-Rna 2014; 5:95-110.

123. Marks PW, Kwiatkowski DJ. Genomic organization and chromosomal location of murine cdc42. Genomics 1996; 38:13-8.

124. Horn M, Baumann R, Pereira JA, Sidiropoulos PNM, Somandin C, Welzl H, et al. Myelin is dependent on the Charcot-Marie-Tooth Type 4H disease culprit protein FRABIN/FGD4 in Schwann cells. Brain 2012; 135:3567-83.

125. Tamim S, Vo DT, Uren PJ, Qiao M, Bindewald E, Kasprzak WK, et al. Genomic Analyses Reveal Broad Impact of miR-137 on Genes Associated with Malignant Transformation and Neuronal Differentiation in Glioblastoma Cells. Plos One 2014; 9.

126. Fu M-g, Li S, Yu T-t, Qian L-j, Cao R-s, Zhu H, et al. Differential expression of miR-195 in esophageal squamous cell carcinoma and miR-195 expression inhibits tumor cell proliferation and invasion by targeting of Cdc42. Febs Letters 2013; 587:3471-9.