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Habilitationsschrift

The role of Ras superfamily GTPases and their regulators in cellular transformation and in the development of the cerebral neocortex

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1 General introduction

1.1 The Ras superfamily of GTPases

The Ras superfamily consists of around 167 small GTPase proteins that share the ability to bind and hydrolyse GTP (1). Although similar to the alpha subunit of G proteins, Ras superfamily proteins function as monomers. The superfamily can be divided into seven main families based on sequence homology and functional similarity: the Ras, Rho, Ran, Rab, Arf, Miro and RGK families.

Ras superfamily GTPases share an overall conserved fold, called the G domain, of around 20kDa that is involved in nucleotide binding as well as, in most cases, conservation of sequences involved in GTP hydrolysis (2). In addition the majority of Ras superfamily proteins undergo specific lipid modifications including C-terminal addition of farnesyl, geranylgeranyl and palmitoyl moieties (Ras, Rho and Rab familes) or N-terminal myristoylation (Arf family) that help localise these small G proteins to cellular membranes. Some Ras superfamily members localise to membranes without an apparent lipid modification (e.g. Rit, RhoBTB, Miro, Sar1) while Ran and Rerg are neither lipid modified nor localised to membranes.

A direct consequence of these features is the ability of Ras superfamily proteins to cycle between an active GTP-bound, and an inactive GDP-bound state. GTP-binding induces a conformational change in the protein, in particular at two regions called the switch I and II domains, that highly increases affinity of the GTP-bound protein for its downstream effectors, thus enabling downstream signalling. Effectors associate mainly through a core effector domain that includes the switch I domain. Hydrolysis of GTP terminates signalling (3).

Members of particular Ras subfamilies share similarities not only in sequence but also in their function. For example, Ras family proteins have been typically associated with the regulation of cellular proliferation, survival and differentiation, Rho family members with the regulation of actin dynamics, Rab and Arf proteins with various aspects of vesicle trafficking and the smallest subfamily, Ran, with nuclear transport. These are not, however, strict divisions and functions are naturally interconnected. Rho family members, for example, also play critical roles in the regulation of cell migration, differentiation and vesicle transport not only through their roles in the regulation of cytoskeletal dynamics but also through the activation of alternative downstream signalling pathways (sections 4-5).

1.2 Regulation of the Ras superfamily

Ras proteins regulate a myriad of core signalling functions such as proliferation and differentiation, cytoskeletal dynamics and membrane and vesicular trafficking that are essential for cell survival and function. Indeed, there are suprisingly few Ras proteins when one considers the vast number of cellular functions regulated by these proteins.

It is important to note that, although the GTP-bound form is the active form for all Ras superfamily proteins, the process of GTP/GDP cycling is in itself critical for effective downstream signalling. Cycling enables effector dissociation and exchange and presents a bottleneck for activation of the multiple, mostly amplifying, downstream signalling cascades. Despite being able to bind GTP/GDP and, for the most part, possessing low intrinsic GTPase activity, Ras proteins are dependent on regulatory proteins to switch them on, off, or to maintain them in an active/inactive state. The main Ras regulators are the guanine-nucleotide exchange factors (GEFs), that promote the exchange of GDP for GTP, and the GTPase-activating proteins (GAPs) that stimulate the hydrolysis rate of GTP (Fig. 1). Guanine nucleotide dissociation inhibitors (GDIs) represent an additional class of regulators for the Rho and Rab subfamily and function by sequestering the GDP-bound form in the cytosol. These regulators, in particular the GEFs and GAPs, are essential for determining the timing, specificity and subcellular localization of Ras protein activation as well as the integration of Ras activity with other cellular activities and environmental cues. It has been estimated that around 0.5% of human genes encode functional GAPs, 60% of which are specific for Rho or Ras proteins (section 4-5) (4-6).

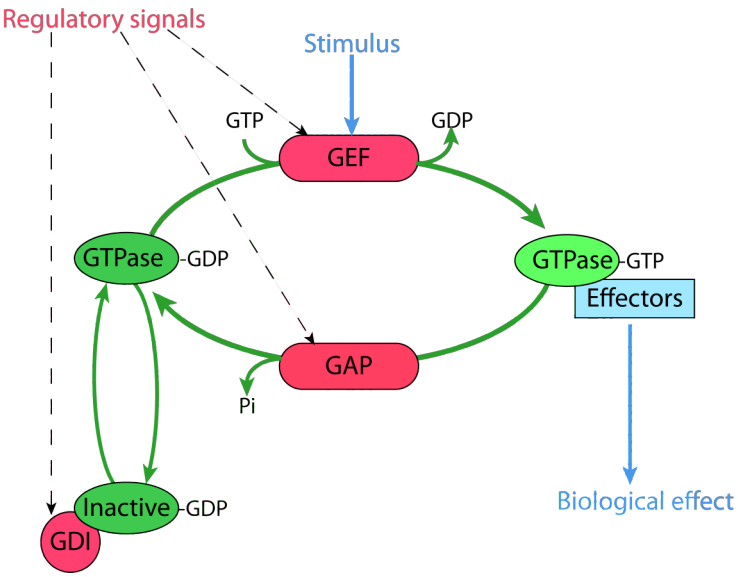

Figure 1: Regulation of the activity of Ras superfamily GTPases. Ras proteins cycle between GTP-bound active and GDP-bound inactive states. Cycling is regulated by guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Figure by Rosário (unpublished).

2 Original work 1: Signalling by TC21, a novel Ras family member

2.1 Introduction

2.1.1 The Ras family of GTPases

The Ras family of GTPases has been further subdivided into Ras, Ral, Rit, Rap, and Rheb subfamilies. The founding members of this family were the Ras sarcoma oncoproteins H-ras and K-ras. These were initially described in the cancer-causing viruses Harvey sarcoma virus and Kirsten sarcoma virus, respectively, and later found to be also be mutated in human cancer cells (7-14). Subsequently N-Ras was identified as the transforming factor in several human cancer cell lines (15-19). Mutation of these three genes is one of the most common events in tumourigenesis, and is thought to occur in around 30% of all human tumours.

Two hot spots for Ras oncogenic mutations have been identified around codons 12 and 61. Glycine 12 is required for the interaction of Ras proteins with GAPs and mutation of this site (such as G12V) render Ras insensitive to inactivation. Q61, on the other hand, is involved in stabilizing the transition state for GTP hydrolysis and thus mutation of this amino acid inhibits the intrinsic GTPase activity of the Ras protein. These residues are conserved in most Ras superfamily members and equivalent mutations result in constitutively active proteins.

Most of the other subsequently indentified Ras family proteins do not show transforming potential in the conventional NIH3T3 focus-forming assay with the exception of the R-Ras subfamily of Ras proteins (**original work 1**). In addition, a few activating mutations have by now also been identified in Rho family members (including in Rac1/2, RhoT and Cdc42) (20). De-regulation of the expression of Ras superfamily proteins (including Ras, Rho, Rab and Arf family members) in cancer cells is more common.

2.1.2 Ras signalling pathways

The ERK1/2 MAP kinase pathway is the best characterised signalling pathway downstream of Ras proteins. Activation of Ras enables binding to the cytosolic Raf family of serine/threonine kinases, thus promoting the translocation of these kinases to the plasma membrane. Additional phosphorylation events here promote full Raf kinase activation and thus initiate a kinase cascade involving first the dual specificity protein kinases MEK1/2 and subsequently the ERK1/2 mitogen-activated protein (MAP) kinases. Activated ERK1/2 can phosphorylate multiple cytosolic effectors, and is also able to translocate to the nucleus where it can induce trancriptional changes through phosphorylation of nuclear targets such as the Ets-family of transcription factors.

Other signalling pathways that have by now been shown to be activated downstream of Ras proteins include the phosphatidylinositol-3-kinase (PI3K), p38MAPkinase, atypical MAP kinase Erk5, phospholipase C gamma (PLC γ) pathways and, through the recruitment of different GEFs, a number of other Ras superfamily proteins such as Rac, Ral and Rap (4, 21).

2.2 Original work 1: Activation of the Ral and phosphatidylinositol 3' kinase signalling pathways by the ras-related protein TC21.

Rosario M, Paterson HF, Marshall CJ. 2001. **Mol Cell Biol** 21:3750-3762.

https://doi.org/10.1128/MCB.21.11.3750-3762.2001

TC21 (also called R-Ras2) is a Ras subfamily protein originally identified from an expression cDNA library derived from the human ovarian teratocarcinoma cell line A2780 (22). The mutation identified in this study, Q72L, is at the equivalent position to the hotspot codon 61 in Ras. TC21 shows most similarity to another Ras family member, R-Ras and together with M-Ras/R-Ras3 these genes constitute the R-Ras subgroup of Ras proteins. At the time of **original work 1**, another oncogenic mutation in TC21 had just been identified in an uterine leiomyosarcoma (23). Indeed, my previous research had demonstrated that TC21, like Ras, has transforming activity *in vitro* and that it can also activate the Raf/ ERK1/2 MAP kinase pathway, albeit more weakly than the classical Ras genes (24, 25). Activation of this pathway, however, did not appear to account for the transforming activity of this protein (26). We therefore, searched for other downstream effectors of TC21 that might mediate these cellular functions. **Original work 1** is the first study to identify Ral and phosphatidylinositol 3´kinase (PI3K) as dowstream effectors of TC21. In **original work 1** we demonstrated that oncogenic (V23) TC21 is a powerful activator of class I PI3K through direct association with p110, the catalytic subunit of this kinase. Activation of PI3K downstream of TC21 induces the membrane translocation and activation of the downstream effector Akt and is necessary for TC21-induced morphological changes in NIH 3T3 and PC12 rat pheochromocytoma cells. A yeast two-hybrid screen had previously identified RalGDS, a GEF for the Ras family member RalA, as a putative interactor of TC21 (27), but the significance of this interaction or the function of the Ral pathway itself was unknown. We showed for the first time that TC21 could induce the translocation to the plasma membrane and thus activation of the cytosolic RalGEF family members, RalGDS and the atypical Rlf , in epithelial cells. Furthermore, we identified residues T46 and Y51 in TC21 as essential for association with these RalGEF family members and demonstrated that interaction of TC21 with RalGEFs results in the constitutive hyperactivation of endogenous RalA in NIH 3T3 cells. We also used overexpression of the Ral-GTP binding domain (RalBD) of the Ral effector, RalBP1, as a tool to selectively inhibit Ral signalling. Using this tool or a dominant negative mutant of Ral, we demonstrated that Ral activation downstream of TC21 is essential for TC21-induced stimulation of DNA synthesis but is dispensible for the morphological

alterations in V23 TC21-transformed fibroblast cell lines as well as in human breast and uterine cancer cell lines harboring activating mutations in TC21. Furthermore, DNA synthesis in these RalBP1 RalBD-expressing cancer cell lines could be restored by expression of constitutively active Ral. This was the first demonstration that Ral signalling is involved in human tumour cell growth (section 6.1).

3 Original work 2: The SHP2 tyrosine phosphatase

3.1 Introduction

3.1.1 Activation of Shp2 and downstream signalling

The PTPN11 gene encodes a non-receptor tyrosine phosphatase called Shp2 (or Syp, SH-PTP2, SH-PTP3, PTP1D or PTP2C) that contains two N-terminal SH2 domains and a C-terminal phosphatase domain. Originally identified in parallel in mammals and in Drosophila (and named, Corkscrew, Csw), Shp2 was shown to become activated downstream of a subset of receptor tyrosine kinases and to promote the downstream activation of the Ras/ERK MAP kinase pathway (28-32). Analysis of the crystal structure of Shp2 and biochemical studies have revealed that activation of Shp2 is dependent on the release of an intramolecular autoinhibitory phosphotyrosine-independent interaction between the N-terminal SH2 domain and the phosphatase domain that maintains the protein in a “closed” inactive state. Release of this autoinhibitory interaction occurs upon interaction of the Shp2 SH2 domains with phosphorylated tyrosine residues on activated receptors or associated adaptor proteins. Interaction with the receptor complex thus enables substrate access to the catalytic site of Shp2 and also localizes this cytosolic protein to the receptor complex and thus to the plasma membrane (33, 34).

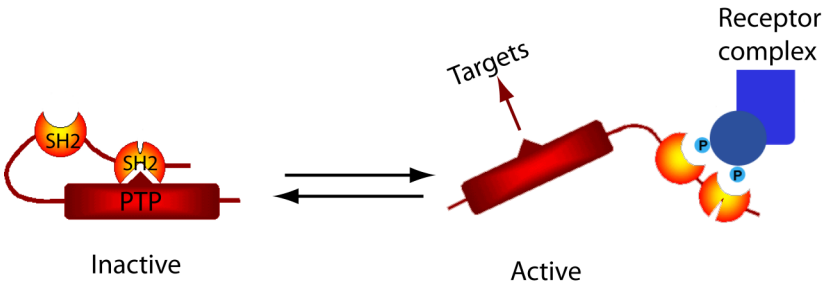

Figure 2: Activation of Shp2

An intramolecular phosphotyrosine-independent interaction between the N-terminal SH2 domain and residues in the phosphatase domain (PTP) keeps Shp2 in an inactive state. Higher affinity phosphotyrosine-based interaction of both SH2 domains with tyrosine-phosphorylated effector proteins release this inhibitory interaction. Figure by Rosário (unpublished), based on (35).

The major pathway activated downstream of Shp2 is the Ras/ ERK1/2 MAP kinase pathway. Several mechanisms have been suggested to mediate Ras activation downstream of Shp2. These include through the recruitment of the adaptor protein

Grb2 (36-41), the dephosphorylation of Ras on inhibitory sites (42), the dephosphosphorylation of binding sites for negative regulators of Ras such as RasGAP on receptors and associated adaptor proteins (43-45), the dephosphorylation of the Ras/ERK negative regulator Sprouty (46) and through the activation of Src tyrosine kinase either via direct dephosphorylation and thus activation of Src proteins or through dephosphorylation of the Src inhibitor Csk (47). Other pathways that have been described to be activated downstream of Shp2 include the Rho family of GTPases (48-50), ROCKII (51), PI3K (52), NF-kB (53) and the Jak/Stat signalling pathway (54). Nuclear and mitochondrial functions for Shp2 have also been proposed (55-58).

3.1.2 The role of Shp2 in human disease

Germline mutations in the human PTPN11 gene have been shown to cause Noonan (NS) and LEOPARD (Lentigenes, ECG abnormalities, Ocular hypertelorism, Pulmonic valvular stenosis, Abnormalities of genitalia, Retardation of growth, and Deafness) syndromes (58-61). These are distinct multisymptomatic syndromes that are both associated with intellectual disability, different craniofacial and cardiac defects and short stature but also show distinct symptoms such as webbed neck, skeletal defects and bleeding in NS but not in LEOPARD syndrome. In addition somatic mutations in PTPN11 are associated with tumourigenesis. Indeed, PTPN11 has been found to be mutated in several haematologic malignancies, most notably juvenile myelomonocytic leukemia (JMML) (62). Furthermore PTPN11 mutations have also been described in a handful of solid tumours including neuroblastomas, lung cancer, breast cancer, pilocytic astrocytoma and medulloblastomas (63-69).

It is estimated that almost 50% of NS patients, 80% of LEOPARD syndrome and 35% of JMML patients carry mutations in PTPN11 (58; 70-73). Mutations in NS and JMML are generally missense and mostly lie either in the N-terminal SH2 domain or in the phosphatase domain. These mutations are thought to interfere with the autoinhibitory interaction of the N-terminal SH2 and phosphatase domains and hence to result in gain of function (GOF) mutants (74, 75). LEOPARD-associated mutations were first thought to be loss-of-function (LOF) mutations but subsequent work has suggested that they may also possess GOF activity (76).

The major signalling event underlying these condition appears to be the hyperactivation of the Ras/ERK pathway. Mutations in other players of the Ras/ERK pathway such as Ras, the serine kinase Raf, the Ras GEF, SOS1 and in and the Ras GAP, NF1 have also been described in NS and JMML patients that lack PTPN11 mutations (58; 77-80). Indeed, NS and LEOPARD are considered to be

RASopathies. Other Shp2-regulated pathways that have been associated with these conditions include the JAK/Stat pathway (81) and PI3K/Akt pathways (82-84).

Additionally, animal models have uncovered critical functions for Shp2 in the development of several mammalian organs including the heart, CNS, bone and the hematopoietic and immune system (section 4.1.1) (58).

3.2 Original work 2: Specific inhibitors of the protein tyrosine phosphatase Shp2 identified by high-throughput docking.

Hellmuth K, Grosskopf S, Lum CT, Wurtele M, Roder N, von Kries JP, Rosario M, Rademann J, Birchmeier W. 2008. **Proc Natl Acad Sci U S A** 105:7275-7280.

https://doi.org/10.1073/pnas.0710468105

The association of human disease with the aberrant activation of Shp2 has caused great interest in the development of a specific inhibitor to Shp2. However, the similarity of the catalytic cleft among tyrosine phosphatases presents a challenge for the identification of a specific inhibitor. In **original work 2** we used a high-thoughput *in silico* screen to identify a small-molecular-weight compound that is specific for Shp2. We used available information on the 3D structure of SH2-autoinhibited Shp2 and of the phosphatase domain of other tyrosine phosphatases together with knowledge of the activation mechanism of Shp2 (Fig. 2), to model the open-configuration state of the Shp2 phosphatase domain. Using this structure, we carried out in silico docking of a library of around 2.7 million small molecular weight compounds into this catalytic site. This enabled identification of twenty compounds that inhibited Shp2 but not the closely related Shp1 phosphatase in an *in vitro* enzymatic assay. Eight of these also blocked signalling by Hepatocyte Growth Factor (HGF) in MDCK cells, which is dependent on Shp2 activity (85, 86). The most efficient compound, PHPS1 was further studied and several derivatives of this compound were generated and tested for specificity and biological activity. The predicted binding mechanism of PHPS1 in Shp2 was also confirmed *in vitro* using mutations in Shp2 and by the substitution of amino acids in the related but non-interacting tyrosine phosphatase PTP1B to those predicted to be relevant for PHPS1 binding to Shp2. We then used a panel of different biological assays to demonstrate that PHPS1 is capable of specifically inhibiting the biological activity and downstream signalling of not only wildtype but also a dominant active JMML-associated mutant of Shp2 without affecting other parallel signalling pathways or parallel routes to activation of the ERK/MAP kinase pathway. Most importantly, we showed that the compound is non-cytotoxic, cell-permeable and can inhibit the growth of a panel of human tumour cell lines, including very efficiently a lung carcinoma cell line that carries an activating mutation in Shp2.

4 Original work 3: NOMA-GAP/*ARHGAP33*: a novel neuronal regulator of Shp2 and of the RhoGTPase Cdc42

4.1 Introduction

4.1.1 The function of Shp2 during cerebral development

Noonan syndrome (NS) is associated with mild cognitive impairment and learning disability (58, 87). Mice expressing a NS GOF Shp2 mutant in the forebrain show defects in exploratory behaviour and memory specificity associated with strongly perturbed activity-induced signalling in hippocampal neurons (82).

Shp2 activation has been shown to be a necessary step downstream of several receptors involved in neuronal differentiation and neocortical development. These include the fibrobast growth factor receptors (FGFRs) and the Trk family of neurotrophin receptors (58). Mutation of the Shp2 binding sites in FRS2 alpha, an adaptor protein that can mediate recruitment and activation of Shp2 downstream of both of these receptors (88, 89), disturbs neuronal progenitor division and thus development of the neocortex in mice (90). The proliferation of neuronal progenitors is also disturbed upon conditional knockout of Shp2 in neural progenitors cells using the Nestin-Cre recombinase line, and leads to defective neuronal cell fate specification and impaired corticogenesis (section 5) (91). Experiments using expression of GOF mutants of Shp2 in the neocortex similarily indicated a positive role for Shp2 in inducing the neurogenic cell fate in the neocortex (92). Shp2 also has pro-neurogenic functions during retinal development (93). In addition, Shp2 has critical roles in the mature brain in synaptic plasticity and memory formation as well as in the regulation of energy balance and metabolism (94, 95). The signalling mechanisms involved in the function of Shp2 in neuronal cells remain poorly understood (**original work 3**).

4.1.2 Neurotrophin signalling

Neurotrophins are important regulators of neuronal differentiation, morphology and synaptic maturation during brain development as well as of synaptic transmission and plasticity in the mature brain. Indeed, defective neurotrophin signalling has been associated with a number of human neurological diseases including neuropsychiatric diseases such as Autism Spectrum Disorders (ASD), obsessive-compulsive disorder, major depression and Schizophrenia, as well as neurodegenerative conditions such as Alzheimers and Parkinsons (96, 97). Downstream activation of the MAPK cascade, through multiple redundant mechanisms, is a critical and necessary step for

neurotrophin function. Shp2 has been shown to be required for the sustained activation of the ERK1/2 MAP kinase pathway downstream of neurotrophins (98).

Another important signalling pathways activated downstream of neurotrophins are the Rho family of actin regulators (**original work 3**) (21). Indeed, Rho GTPases were shown to be critical regulators of the various morphological alterations induced by neurotrophins during neuronal differentiation or in response to activity, including axon outgrowth and dendritic maturation and dendritic tree and spine maturation (99-102).

4.1.3 The Rho family of GTPases

The Ras homologous (Rho) family consists of over 20 members that have been divided into 8 subgroups based on amino acid similarity (Fig. 3). The best studied members are RhoA, Rac1 and Cdc42. Like other Ras proteins, classical RhoGTPases act as small molecular switches by cycling between active GTP-bound and inactive GDP-bound states. Specificity in the timing, subcellular location and kinetics of signalling is thought to be achieved through the differential action and properties of Rho regulators, in particular GEFs and GAPs, respectively encoded by the *ARHGEF* and *ARHGAP* families of genes, which far outnumber the Rho proteins themselves.

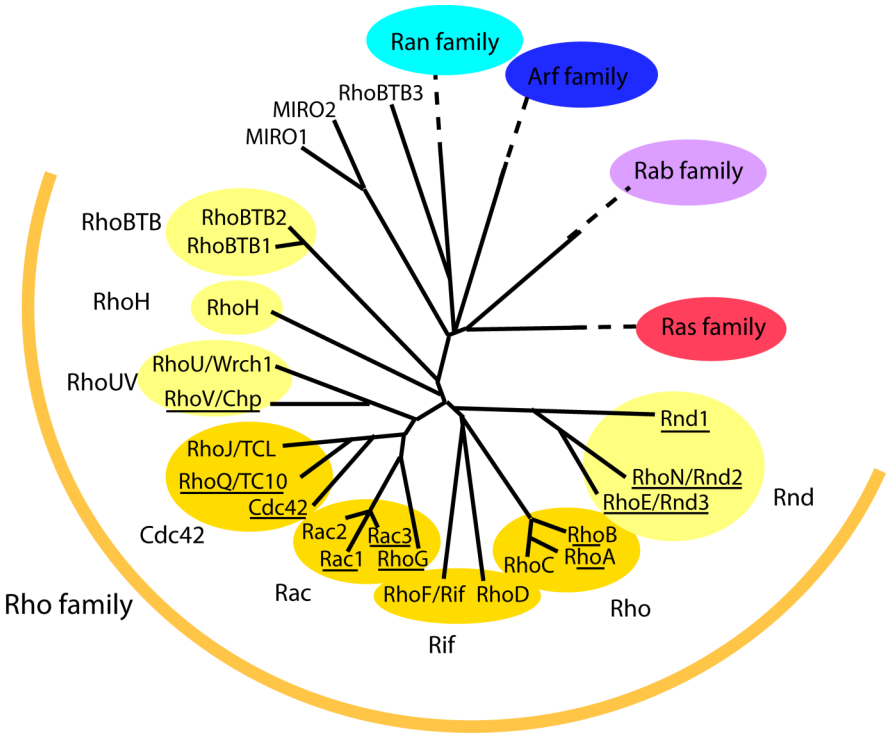

Figure 3: Phlyogenetic tree of the mammalian RhoGTPases

The names of the Rho subfamilies are shown next to the grouped members. RhoGTPases with known expression in the developing cerebral cortex are underlined (103). Figure by Rosário (unpublished), based on (104).

RhoGTPases are best known for their ability to direct actin dynamics and thereby regulate the cytoskeletal structure. Activation of RhoA is associated with the assembly of contractile actin:myosin filaments while Rac1 and Cdc42 both promote peripheral actin polymerization and respectively induce the formation of lamellipodia and filopodia. Critical for these functions is the activation of two actin polymerization factors: the Arp2/3 complex downstream of Rac and Cdc42 and the Formins downstream of Rho (105).

In addition, RhoGTPases regulate several signalling cascades that result in changes in gene expression, enzymatic activity and the regulation of microtubule dynamics. RhoGTPases are thereby essential regulators of various essential cellular functions such as cell proliferation, differentiation, survival, morphology, polarity and movement (105, 106). Unsurprisingly, germ line deletion of Rac1 or Cdc42 leads to very early embryonic lethality associated with severe morphological defects and cell death (107). A large number of tissue-specific deletions in different Rho proteins have since been generated that underscore the critical importance of these GTPases for the development and function of most tissues analysed (section 5.1.3) (108).

4.2 Original work 3: NOMA-GAP/*ARHGAP33*: The neurite outgrowth multiadaptor RhoGAP, NOMA-GAP, regulates neurite extension through SHP2 and Cdc42.

Rosario M, Franke R, Bednarski C, Birchmeier W. 2007. **J Cell Biol** 178:503-516.

https://doi.org/10.1083/jcb.200609146

Original work 3 describes the first identification and characterisation of a novel neuron-specific RhoGAP and multiadaptor protein, NOMA-GAP encoded by the *ARHGAP33* gene (Fig. 4; **original works 4 and 5**). We identified and cloned NOMA-GAP through two different routes: by screening expression profile data of E12-E14 mouse spinal cords and DRGs for sequences predicted to encode GAPs or GEFs for Rho family GTPases and by cross-referencing this data to a modified yeast two-hybrid screen in a human brain cDNA library for novel protein interactors of Shp2. **Original work 3** also describes the first full-length sequence of PX-RICS encoded by the *ARHGAP32* gene (other names used for partial sequences, include, RICS, GC-GAP or GRIT) (109-111). Both NOMA-GAP/*ARHGAP33* and PX-RICS/*ARHGAP32* possess an N-terminal Phox (PX)-like domain, a src-homology domain 3 (SH3), a RhoGAP domain specific for Cdc42 and a large divergent tyrosine and serine-rich C-terminus. Given the homology between these proteins in the N-terminal domain-containing half, we proposed that PX-RICS/*ARHGAP32* and NOMA-GAP/*ARHGAP33* form a subfamily of Rho GAPs. This is now accepted (112).

NOMA-GAP expression during development is restricted to neuronal tissue. Using siRNA to downregulate NOMA-GAP expression in the neuronal precursor PC12 phaechromocytoma cell line and through overexpression of NOMA-GAP in the developing chick spinal cord, we showed that NOMA-GAP is essential for NGF-induced neuronal differentiation and, when overexpressed, is sufficient to induce neuronal differentiation in different systems. Furthermore, we showed that NOMA-GAP directly associates with the NGF receptor, TrkA, and is a constitutive component of this receptor complex. Neurotrophin activation leads to the phosphorylation of NOMA-GAP/*ARHGAP33* on multiple tyrosine residues, two of which act as binding and thus activation sites for Shp2 (Fig. 2 and 4). Phosphorylation of NOMA-GAP/*ARHGAP33* also enables the recruitment of other downstream signalling molecules such as the small adaptor proteins and MAP kinase activators, Shc and Grb2. Using point mutations in the binding sites for these

different downstream effectors, we demonstrated that Shp2-binding is essential for NOMA-GAP-induced neuronal differentiation. Moreover, we showed that activation of Shp2 downstream of NOMA-GAP and TrkA leads to the sustained activation of the atypical MAP kinase, ERK5. In addition, we demonstrated that NOMA-GAP/*ARHGAP33* possesses GAP activity against the Rho family member, Cdc42, and is involved in restricting Cdc42 activation and downstream signalling following NGF stimulation. Finally, we showed that the GAP activity of NOMA-GAP is also necessary for NGF-stimulated neurite outgrowth (Fig 4; **original work 3**).

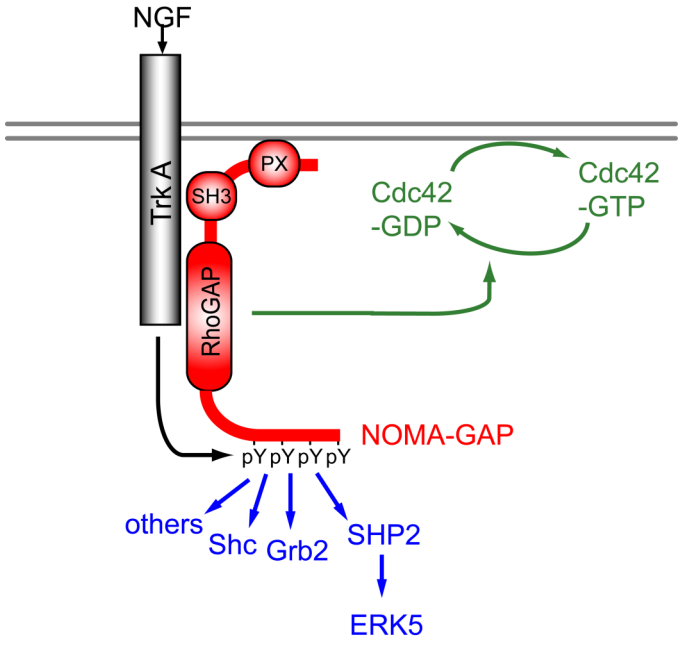

Figure 4: Signalling by NOMA-GAP downstream of the neurotrophin receptor, TrkA.

NOMA-GAP is constitutively associated with the neurotrophin receptor TrkA and becomes heavily phosphorylated following receptor activation. This enables recruitment of multiple downstream signalling effectors including Shp2, Grb2 and Shc. Both the Shp2 binding site and RhoGAP activity are essential for NOMA-GAP and NGF-induced of neurite outgrowth. Figure by Rosário (unpublished).

5 Original works 4 and 5: the role of NOMA-GAP/*ARHGAP33* and the RhoGTPase Cdc42 in the development of the cerebral neocortex.

5.1 Introduction:

5.1.1 Development of the cerebral neocortex

The human neocortex is a complex six-layered structure responsible for directing higher order brain functions including cognition, advanced motor functions, language, social interactions and sensory perception. These functions depend on the correct development of two main populations of neurons, the inhibitory GABAergic interneurons that are involved in local circuitry and the more abundant excitatory projection or pyramidal neurons that extend axons to more distant intracortical, subcortical and subcerebral targets (113, 114). Interneurons are born laterally in the medial and caudal ganglionic eminences and must migrate tangentially to reach the cortex. The pyramidal neurons that occupy the different cortical layers possess distinct molecular characteristics, functions and connectivity. They are however, all generated by tightly-timed, sequential divisions of progenitor cells found in the ventricular and subventricular zones (VZ/SVZ) that overlie the lateral ventricles of the dorsal telencephalon (115). This process occurs in an inside-out manner so that earlier born neurons occupy the deepest cortical layers while later born neurons must undergo radial migration past these earlier born neurons to settle in more superficial layers. Newly-born neurons have been described to go through four steps during this migratory process: first the acquistion of a bipolar morphology and movement to the SVZ, secondly an approximately 24h pause in migration and acquisition of a dynamic multipolar morphology, thirdly the extension of a leading process and cellular movement towards the ventricle and finally reaquisition of bipolar morphology, reversal of polarity and resumption of migration towards the cortical plate (116). Migration into the cortical plate occurs along the parallel radial fibers that serve as the attachment of radial glia, a progenitor cell type, to the pial surface (Fig. 5) (114). Axon extension and determination likely occurs upon cellular contact with the ventricle, before start of the migratory stage, and continues during migration (116). Formation of the dendritic tree, the highly branched structure that acts as the site of information input to the neuron, is suppressed during these early stages and during migration and only starts after the neuron has reached its correct position in the cortical plate. The mechanism of suppression of dendritic development during neuronal differentiation in the neocortex was first addressed in **original work 4** (section 5.2).

Upon detachment from the radial fibres, the migratory leading process of the neuron extends, contacts the pia and branches to form the apical dendrite. At the same time basal dendrites extend and branch from the base of the neuronal soma. The branched dendritic tree subsequently undergoes further maturation and dendritic spines, the sites of synaptic contact, form along the dendrites. This enables activity-dependent remodelling of the dendritic tree leading to pruning of some dendrites and the strengthening of others (117). Thus, unlike axon determination and outgrowth, dendritic tree branching and maturation occurs mainly in the postnatal period and is only completed upon adulthood. During adulthood the dendritic tree is mostly stable. Dendritic spines, on the other hand, can be dynamically remodelled throughout the life of the neuron (118).

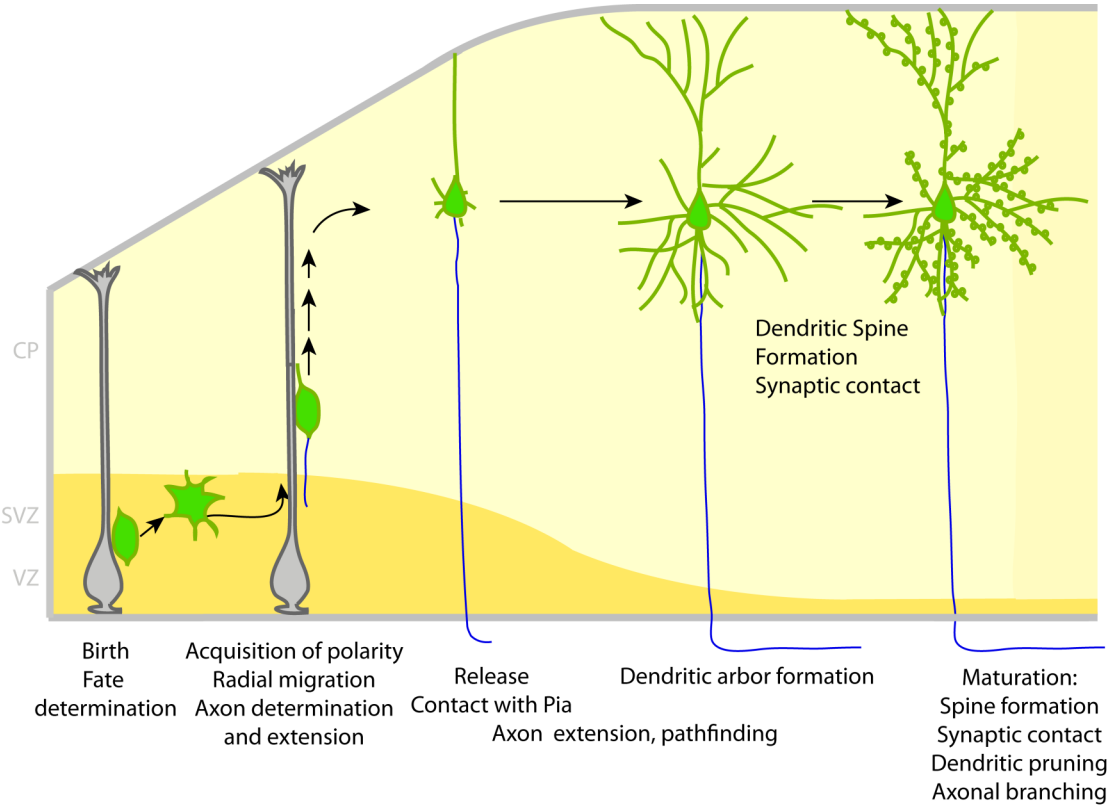

Figure 5: Neuronal differentiation during mammalian neocortical development

In the mammalian neocortex, neurons (green) arise from the asymmetric division of progenitor cells (grey) in the ventricular and subventricular zones (VZ/SVZ). The neuron must undergo dramatic morphological changes until it has reached its final laminar position in the neocortex, differentiated fully and established synaptic contact. Dendritic tree and axon morphology are relatively stable in the mature neuron. Dendritic spines, however, are continuously remodelled in response to activity and environmental cues in the mature neuron requiring dynamic regulation of the cytoskeleton throughout the life of the neuron. Figure by Rosário (unpublished).

5.1.2 Studying the role of RhoGTPases

Many of the cellular functions of RhoGTPases have been investigated using dominant negative and constitutively active mutants of these GTPases (**original work 3**). Constitutively active mutants contain mutations in sites originally identified as hotspot oncogenic mutations in Ras proteins (section 2) and possess a slower rate of GTP hydrolysis together with a faster intrinsic GTP/GDP exchange. Dominant negative mutants such as S17N, were generated based on equivalent mutations in Ras proteins and function by sequestering upstream GEFs, thus preventing activation of endogenous Rho proteins (119-122). Given that many GEFs can act on multiple Rho proteins, these mutants can affect the activation of multiple Rho proteins. Bacterial C3-like ADP-ribosyltransferases that preferentially modify and inhibit Rho A, B and C have also been used to examine the function of RhoGTPases (123).

More recently, genetical engineering of mice and the generation of tissue-specific deletions of genes encoding Rho proteins and their regulators, has provided an alternative and powerful tool for the analysis of the role of these proteins and their regulators in normal physiology and during embryonic development (Table 1; **original work 4**). An additional technique that has enabled *in vivo* analysis of the function of RhoGTPases and their regulators is *in utero* electroporation. Here DNA constructs, for example overexpression or downregulation constructs, are injected into the lateral ventricles of mouse embryos *in utero* and are taken up by the neuronal progenitor cells lining the ventricle walls following a short electrical pulse applied across the developing neocortex. The embryos are allowed to develop normally *in utero* so that the constructs are passed onto all the neurons born from these modified progenitors after the time of electroporation (**original works 4 and 5**) (124).

5.1.3 RhoGTPases and their GAPs in neuronal development and disease

Neuronal differentiation involves dramatic morphological changes (Fig. 5) which are dependent on precisely controlled and dynamic alterations to the cellular cytoskeleton. *In vitro* studies have shown that RhoGTPases play central roles in most neuronal functions including during early neuronal differentiation steps such as neuritogenesis and the acquisition of polarity, as well as during axon formation and guidance, during the development and maturation of the dendritic tree and during synaptic transmission (section 4) (125). Furthermore, *in vivo* studies using Cre-mediated deletion of floxed Cdc42, Rac1 or RhoA alleles in the mouse brain have

emphasized the critical importance of these proteins for neuronal development and function (Table 1).

Rac1	
Mouse Cre Line	Phenotype
Foxg1-Cre	Embyonic lethal (late), microcephaly, defects in all commissures and projections, cortical laminar disorganisation including cobblestone lisencephaly associated with defects in radial glial attachment, survival and differentiation (126-128).
Emx1-Cre	Absence of the corpus callosum and anterior commissure (129).
Nestin-Cre	Early postnatal lethal, hydrocephalus and cerebellar defects (130).
Cdc42	
Mouse Cre Line	Phenotype
Foxg1-Cre	Progenitor cells in the neuroepithelium show defects in cell polarity and adhesion that may underly the observed holoprosencephaly and thicker neuroepithelium (131).
Emx1-Cre	Neuronal progenitor cells show defects in cell polarity and adherens junctions. Increased cortical thickness associated with premature neurogenesis and increased number of intermediate progenitors (132).
Nestin-Cre	Lethal at birth. Loss of axonal tracks and defective growth cones (133).
RhoA	
Mouse Cre Line	Phenotype
Foxg1-Cre	Enlarged forebrain, exencephaly-like protrusions. Proliferation and cell polarity defects (134).
Emx1-Cre	Megalencephaly associated with increased proliferation, subcortical band heterotopia and cobblestone lisencephally associated with defective radial glial fibres (135).

Table 1: Summary of the phenotypes observed upon neuronal-specific deletion of the RhoGTPases RhoA, Rac1 or Cdc42.

Several cre lines have been used to study the role of RhoGTPases during cerebral development. The Foxg1-Cre line is active in early forebrain cells (136), the Emx1-cre in the dorsal telencephalon (137) and the Nestin-cre in CNS progenitor cells including radial glia. Table by Rosário (unpublished), based on (108).

While mutation of these critical RhoGTPases has not been reported in human neurological disease, mutations of genes encoding GEFs and GAPs for RhoGTPases have been increasingly identified in association with human neurological conditions in particular with intellectual disability and neuropsychiatric conditions (138). These include the Rho GAPs oligophrenin-1/*ARHGAP41*(139) and MEGAP/srGAP3/*ARHGAP14* (140) as well as the Rho GEFs *ARHGEF6* (141) and *FGD1* (142) all of which have been linked to intellectual disability in humans. Many more RhoGTPase regulators have been identified in numerous screens for human neurodevelopmental disease-associated single nucleotide polymorphisms (SNPs) and copy-number variations (CNVs), but remain further uncharacterised.

GEFs and GAPs are generally large proteins containing multiple additional protein and lipid domains that enable the integration of RhoGTPase regulation with other signalling cascades. Of the 70 mammalian Rho GAPs identified so far, we have estimated that 50 are expressed in the brain, reflecting the importance of tight regulation of RhoGTPases in neuronal cells. Their association with neurological disease has heightenened interest in these signalling molecules and the function of some Rho GAPs has been analysed *in vitro* or *in vivo* using mouse models (**original work 4 and 5**) (103). The emerging picture is that Rho GAPs are critical regulators of *in vivo* neuronal differentiation which direct distinct cellular events or cell types during the formation of the brain.

5.2 Original work 4: Neocortical dendritic complexity is controlled during development by NOMA-GAP-dependent inhibition of Cdc42 and activation of cofilin.

Rosario M, Schuster S, Juttner R, Parthasarathy S, Tarabykin V, Birchmeier W. 2012. **Genes & Dev** 26:1743-1757.

https://doi.org/10.1101/gad.191593.112

In **original work 4** we addressed the *in vivo* function of NOMA-GAP in the mouse during development. We inserted the LacZ gene into the *ARHGAP33* locus, thus disrupting expression of NOMA-GAP. Beta-galactosidase reporter activity in these mice is therefore under the control of the endogenous NOMA-GAP promoter. Similar to what we observed using *in situ* hybridisation, beta-galactosidase activity indicates that NOMA-GAP is principally expressed in neuronal tissue. In the neocortex, NOMA-GAP expression is very strong in all postmitotic layers from around E14.5 but absent in the proliferative VZ and SVZ.

NOMA-GAP-deficient mice are born at the expected Mendelian ratio and are indistinguishable from littermate wildtype mice in terms of their size and outward appearance. MRI scans showed however, that NOMA-GAP-deficient mice have a reduced brain volume associated predominantly with a reduced cortical thickness. Overall brain anatomy appears unaltered. Cortical thinning becomes apparent in the first postnatal week but there are no changes in total cell number or in the rates of cell death or proliferation in the neocortex. The fate specification, migration and final laminar position of the different cortical neurons is also unaltered. **Original work 4** demonstrates however, that dendritic arborization, particularly that of upper layer neurons, is strongly disturbed in NOMA-GAP-deficient mice and neurons develop only very simple, poorly ramified dendritic trees. The number of primary dendrites, that is those emerging directly from the soma, is not altered. This indicates that the defect is specific to the acquisition of branching complexity. In upper layer neocortical neurons, dendritic branching occurs primarily during the first postnatal week in mice, after migration of these neurons to their correct laminar position is complete (section 5.1.1). This process is associated with a decrease in cellular density and a corresponding increase in cortical volume. Loss of NOMA-GAP interferes with dendritic arborization and thus postnatal cortical volume expansion does not occur normally. **Original work 4** further shows that NOMA-GAP is a major negative regulator of Cdc42 in the developing cortex. Loss of NOMA-GAP is associated with

hyperactivation of the Cdc42 and the downstream PAK1/2 signalling pathway in the developing neocortex. Indeed, we could restore dendritic branching in NOMA-GAP-deficient primary neurons in culture by expression of a dominant negative mutant of Cdc42 or by re-expression of full-length, but not RhoGAP-defective, NOMA-GAP.

In **original work 4** we also used the technique of *in utero* electroporation first described by Saito *et al* (143) to selectively modify upper layer neurons *in vivo* in the developing mouse neocortex. Using this technique we showed that hyperactivation of Cdc42 in postmitotic neurons, is sufficient to disturb dendritic branching during neocortical development.

To address the molecular mechanism by which Cdc42 activity inhibits dendritic arborization, we used an immunoprecipitation (IP)-mass spectrophotometric approach to identify changes in phosphorylated proteins in the cortex of NOMA-GAP-deficient embryos. Through this approach we found that loss of NOMA-GAP is associated with increased phosphorylation of the actin polymerization regulator cofilin. Phosphorylation of cofilin on serine 3 has been previously shown to be catalysed by the kinases LIMK1/2 which can themselves be activated by the Cdc42 effectors PAK kinases (144-146). PAK1/2 and LIMK1/2 activities are both elevated in the developing cortex of NOMA-GAP-deficient mice.

In order to demonstrate that hyperactivation of Cdc42 is responsible for the hyperactivation of PAK/LIMK and the inactivation of cofilin, we used genetic crosses between NOMA-GAP-deficient mice, mice carrying a floxed Cdc42 allele (147) and mice expressing cre recombinase from the Nex1 cortical postmitotic promoter (148) to lower the levels of Cdc42 protein in NOMA-GAP-deficient neurons *in vivo* (N-cdc-cre line). Heterozygous deletion of Cdc42 in these mice, restores PAK1/2 and LIMK1/2 activity levels as well as cofilin serine 3 phosphorylation levels to those observed in wildtype mice. This indicates that hyperactivation of Cdc42 upon loss of NOMA-GAP is responsible for the downstream elevated activation of PAK1/2 and LIMK1/2 and hyperphosphorylation of cofilin (Fig. 6). Phosphorylation of cofilin on serine 3 inhibits the ability of this protein to interact with G or F-actin, thus inactivating it (149).

Heterozygous deletion of Cdc42 in NOMA-GAP deficient mice through this approach also partially restored the complexity of the dendritic tree of neocortical neurons *in vivo*.

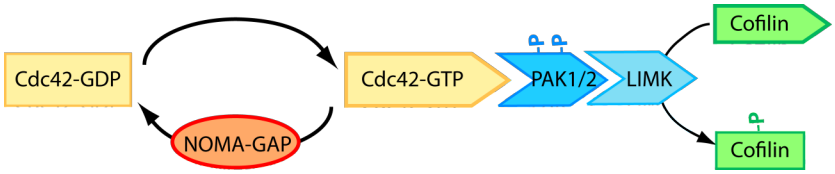

Figure 6: Cdc42-regulated signalling downstream of NOMA-GAP in the developing neocortex. Activation of Cdc42 (conversion to the GTP bound form) initiates a kinase cascade involving the activation of the kinases PAK1/2 and LIMK that results in the phosphorylation on serine 3 and consequent inactivation of cofilin. NOMA-GAP counteracts this pathway by direct inactivation of Cdc42. Figure by Rosário (unpublished).

Finally, we used *in utero* electroporation of an active mutant of cofilin (S3A) into the developing neocortex of NOMA-GAP-deficient mice to address the role of cofilin downstream of NOMA-GAP. *In vivo* expression of S3A cofilin restored the complexity of the neuronal dendritic tree in NOMA-GAP-deficient animals, indicating that positive regulation of cofilin downstream of NOMA-GAP is essential for the development of this important neuronal structure.

Original work 4 was the first study to establish the role of Cdc42 and downstream signalling in the suppression of dendritic branching during the early migratory stage of neuronal differentiation and to provide a mechanism for how this suppression is relieved upon completion of migration (section 6.3.2).

5.3 Original work 5: NOMA-GAP/*ARHGAP33* regulates synapse development and autistic-like behavior in the mouse.

Schuster S, Rivalan M, Strauss U, Stoenica L, Trimbuch T, Rademacher N, Parthasarathy S, Lajko D, Rosenmund C, Shoichet SA, Winter Y, Tarabykin V, Rosario M. 2015. **Mol Psychiatry** 20:1120-1131.

https://doi.org/10.1038/mp.2015.42

Original work 5 addresses the role of NOMA-GAP and Cdc42 signalling during the formation of dendritic spines and the establishment of connectivity in the mouse neocortex as well as the consequences of loss of NOMA-GAP/*ARHGAP33* for mouse behaviour.

NOMA-GAP is found in small vesicles along the dendritic shaft and in large accumulations at the heads of spines where it colocalizes with postsynaptic proteins suggesting that it may have a function in the development and/or function of the dendritic spine. Indeed, **orginal work 5** shows that loss of NOMA-GAP in the mouse is associated with neocortical neurons that possess dendritic spines with longer necks, an indication of immaturity. These neurons also form fewer synapses in culture. In line with these observations, we observed that the electrophysiological properties of these neurons in acute slices are strongly altered. Interestingly, we could restore dendritic spine morphology *in vivo* by re-expression of full length NOMA-GAP by *in utero* electroporation in the developing mouse neocortex, indicating that this is a cell intrinsic defect. Surprisingly however, expression of a mutant of NOMA-GAP that lacks the RhoGAP domain *in vivo* also restored spine morphology suggesting that the RhoGAP activity of NOMA-GAP is dispensible for the regulation of spine development. We confirmed this by analysing the N-cdc-cre mouse line where one copy of the Cdc42 is postmitotically deleted in neocortical neurons deficient in NOMA-GAP. Lowering the levels of Cdc42 expression through this approach however, did not improve spine morphology or synaptic transmission, although the same manipulation is sufficient to restore dendritic branching in these mice (**original work 4**).

These results suggest that NOMA-GAP exerts its effects on spine morphology through a pathway other than Cdc42. **Original work 5** shows that NOMA-GAP also directly interacts with several members of the MAGUK family of postsynaptic scaffold proteins, including PSD-95. Furthermore, we demonstrated that correct subcellular localization and phosphorylation of PSD-95 is dependent on the expression of

NOMA-GAP. Loss of NOMA-GAP is associated with the hyperphosphorylation of PSD-95 on the regulatory serine 295, and relocation of PSD-95 away from the spine head and into the dendritic shaft. Interestingly, association with PSD-95 appears to negatively regulate the Cdc42 GAP activity of NOMA-GAP.

Surface expression of the AMPA receptor subunit GluR1 is also dependent on expression of NOMA-GAP as shown both by immunofluorescent and surface biotinylation studies. The AMPA receptor is an ionotropic transmembrane heterotetrameric glutamate receptor that is responsible for most of the fast excitatory synaptic transmission in the CNS. Its localization and retention at the dendritic spine is indirectly dependent on PSD95 (sections 6.3.3-6.3.4) (150-152).

Original work 5 also addressed the consequences of loss of NOMA-GAP for animal behaviour. Following SHIRPA protocols we first carried out a general characterisation of animal health and well-being and then addressed general animal behaviour as well as sensory function in a battery of different tests. NOMA-GAP-deficient mice are indistinguishable from wildtype littermates in terms of their general health, sensory perception and gross overall behaviour. However, **original work 5** demonstrates that male NOMA-GAP-deficient mice have strong defects in social interaction and recognition. Specifically, male NOMA-GAP-deficient mice spend less time interacting with a new mouse, fail to show a preference for spending time with another mouse as opposed to an object and fail to distinguish between a familiar and an unfamiliar mouse. These alterations in social behaviour were not restored by genetically lowering the levels of Cdc42 (N-cdc-cre line).

Interestingly, we did not see a significant change in the social behaviour of female NOMA-GAP-deficient mice. However, NOMA-GAP-deficient, particularly the female, mice, showed signs of increased anxiety in a number of the tests undertaken. This included increased number of freezing and grooming bouts in a novel environment, the building of more complex nests and faster burrowing of foreign objects.

Increased anxiety and altered social behaviour are characteristics of neuropsychiatric disorders such as Autism spectrum disorders and Schizophrenia (section 6.3.5).

6 Discussion

6.1 Signalling by the Ras-related protein TC21

At the time of **original work 1,** TC21 was a relatively new Ras subfamily protein, that exibited transforming potential but that had not been further characterised. Since, TC21 has been found to be mutated at the hotspot codons 23/24 and 70/72 (equivalent to positions 12 and 61 respectively in Ras) in only a small number of tumours (153). De-regulation of TC21 expression is much more common and has been reported in several human carcinomas including of the oral cavity, esophagus, stomach, skin and breast, and in lymphomas and a wide range of CNS tumours including glioblastomas, astrocytomas and oligodendrogliomas (153-157). Indeed overexpression of TC21 in pre-malignant hyperplasias of the brain has lead to the suggestion that this is a key step in triggering transformation in this organ (156).

Activation of PI3K by TC21, as first identified in **original work 1**, has since been shown to be a critical step for tumourigenesis (158-160). Indeed, in the mammary gland, whose normal development is dependent on TC21 activity, TC21 function and the downstream activation of PI3K is necessary for both primary tumorigenesis and metastasis (161, 162).

Original work 1 also demonstrated for the first time that TC21 is a powerful activator of at the time relatively unknown, Ral pathway and thereby demonstrated a new mechanism for crosstalk between different Ras family members. Furthermore, **original work 1** provided the first demonstration that this pathway was necessary for the proliferation of human tumours. Indeed, we showed that activation of the Ral pathway although necessary is, by itself, insufficient for tumourigenesis. Subsequent work has confirmed these initial observations (163) and established the Ral pathway as a critical but not sufficient player in tumourigenesis, in particular downstream of Ras signalling. Recently, multiple efforts to selectively target this pathway in human cancers have been initiated (164, 165).

6.2 Rational design of Shp2 inhibitors

Hyperactivation of the Ras/ERK MAP kinase pathway has been estimated to be involved in the malignant transformation of more than 90% of human cancers. Considerable effort has therefore been directed at designing specific inhibitors to different components of this cascade (166). In **original work 2** we identified and characterised a new inhibitor (PHPS1) of Shp2, a tyrosine phosphatase that is necessary for the prolonged activation of this pathway. PHPS1 was the first inhibitor that could distinguish between Shp2 and the closely related phosphatase, Shp1. This was an important criterion. Shp1 is predominantly expressed in the hematopoietic system and its deletion or inhibition in these cells can lead to the development of myelomonocytic leukemia-like disease and systemic autoimmunity (167).

PHPS1 is now commercially available from several large pharmaceutical companies and has since been used in numerous studies addressing the role of Shp2 in different biological contexts including tumourigenesis (42, 168, 169), LEOPARD syndrome-associated hypertrophic cardiomyopathy (170) and pulmonary fibrosis (171) among others. Several other Shp2 inhibitors have by now been published, but PHPS1 remains among the most specific and potent inhibitors for Shp2 available (172).

6.3 The RhoGAP and multiadaptor protein NOMA-GAP/*ARHGAP33* in the development of the neocortex and in disease

6.3.1 Rho GAPs

Interest in *ARHGAPs* has increased considerably in recent years due to the identification of mutated forms of these genes in association with human neurodevelopmental disorders. Most Rho GAPs are still uncharacterised and the functional consequences of identified disease-associated SNPs or mutations are unknown. Nevertheless, it is acknowledged that a precisely timed activation of the different Rho family members is essential for the generation, migration, differentiation and cellular functions of neurons (106, 125). Integration of this signalling with environmental cues and cellular status is critical to achieve the appropriate cellular responses. The large variety and number of protein and lipid interaction and regulatory domains present on Rho GAPs enables this precise and integrative activation of RhoGTPases and remodelling of the cytoskeleton (Fig. 7) (112).

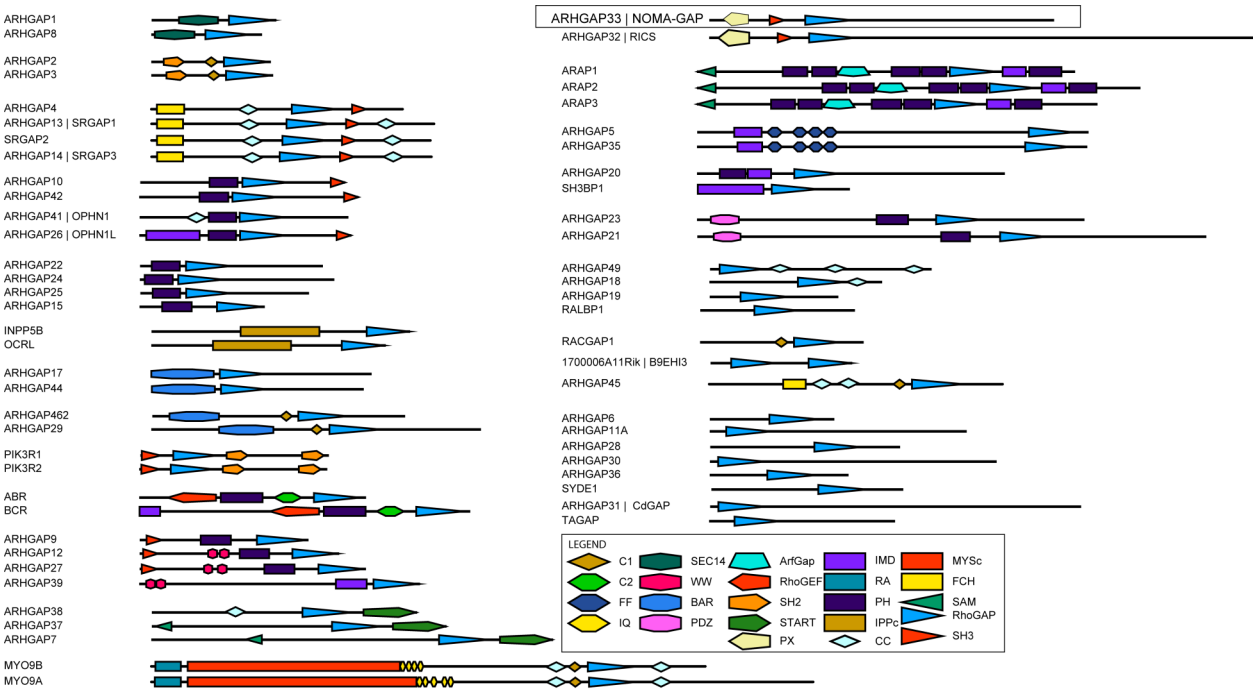

Figure 7: Domain structure of the Rho GAPs predicted to be expressed in neuronal tissue. The structure of NOMA-GAP is highlighted by a box. Domain prediction using SMART v9. Figure assembled by A. Newmann and M. Rosário (unpublished).

Original works 3 to 5 describe the identification of a novel Rho GAP, NOMA-GAP (encoded by the *ARHGAP33* gene), the characterisation of its signalling and of its

cellular functions in the developing neocortex and finally the consequences of loss of this protein to animal behaviour. *ARHGAP33* encodes, through alternative splicing, several proteins of around 137-140kDa that contain a PX (Phox-like) domain, a SH3 (Src-Homology 3) domain that may be involved in association with receptors, a Cdc42-specific RhoGAP domain and a large C-terminal tail rich in phosphorylated tyrosine and serine residues (**original work 3**).

Many Rho GAPs contain lipid binding motifs that enable localisation to the plasma membrane and thus proximity to their targets (Fig. 7). Canonical PX domains have been shown to bind phosphoinositides (PIs) (173). Although the PX-like domain of NOMA-GAP lacks key residues involved in lipid binding, *in vitro* association with several phosphoinositides has been reported (174). Membrane association may also be stabilised through interaction with membrane proteins (**orginal work 3, 5**) (174, 175).

Like other Rho GAPs, NOMA-GAP possesses several protein-interaction domains in addition to the required RhoGAP domain. In NOMA-GAP these enable interaction with both multiple downstream effectors such as Shp2, Cdc42 and Grb2 but also with upstream activators such as the Trk receptors (**original work 3**). In **original work 3** we showed that NOMA-GAP is constitutively associated with TrkA and that it is essential for the activation of signalling downstream of this receptor. Interaction with TrkA occurs in a region conserved among other neurotrophin receptors. Indeed, we have observed interaction of NOMA-GAP also with TrkB. Interaction of NOMA-GAP with TrkB was recently confirmed by another group that further suggested that NOMA-GAP is essential for the trafficking and correct subcellular localization of this receptor (175, 176). In fact, **original work 4**, demonstrated that NOMA-GAP is localised not only to the plasma membrane but also to intracellular vesicles. Furthermore, as demonstrated in **original work 5**, NOMA-GAP is required for the correct surface localisation not only of the neurotrophin receptors but also of the AMPA receptors, suggesting a broader role in directing the intracellular transport of various dendritic proteins.

The specificity and catalytic potency of the RhoGAP domain varies among Rho GAP proteins. Rho GAPs often show *in vitro* catalytic activity towards multiple Rho family GTPases although *in vivo* activity is often more restrictive (177). We have shown that both in PC12 cells and in the developing mouse neocortex, the major catalytic function of NOMA-GAP is the regulation of Cdc42 (**original work 3 and 4**). In accordance, activation of Rac and Rho proteins do not appear to be altered in the neocortex upon loss of NOMA-GAP. Furthermore, the reduced complexity of the

dendritic tree in these mice can be partially restored by genetic manipulation that decrease Cdc42 protein levels (**original work 4**).

The GAP activity of NOMA-GAP does not, however, seem to be required for all NOMA-GAP–dependent cellular activities. Adipocytes express a variant isoform of NOMA-GAP (named TCGAP) that negatively regulates insulin-stimulated GLUT4 translocation and glucose uptake through a RhoGAP-independent mechanism (174). Similarly, we have shown that regulation of dendritic spine morphology by NOMA-GAP is independent of the RhoGAP domain (**orginal work 5**).

6.3.2 NOMA-GAP and the regulation of dendritic branching

Orginal work 4 uncovered the first molecular mechanism that explains how dendritic branching is timed *in vivo* during formation of the neocortex. When primary neurons are placed in culture, dendritic branching and axon extension occur roughly at the same time. During neocortical development however, axogenesis is initiated much earlier, before the migration of neurons into the cortical plate takes place. Dendritic branching, on the other hand, starts only once migration is finished and contact to the pial surface is established (178). The mechanisms that prevent dendritic branching during the early stages of neuronal differentiation had not been previously addressed. **Original work 4** indicates that regulation of the RhoGTPase Cdc42 is essential for the control of dendritic branching. Activation of Cdc42 has been previously shown to play an essential role in maintaining the polarity and thus the fate of apical progenitor cells in the VZ of the neocortex (132). In addition, Cdc42 appears to be required very early during neuronal differentiation for the aquisition of polarity and axonogenesis (133, 179, 180). **Original work 4** shows, however, that continued activation of Cdc42 after these early stages acts as a brake for dendritic arborization by leading to the inhibition of the actin-binding molecule, cofilin. Prevention of dendritic arborization in these early stages is essential to enable the migration of these neurons out of the VZ/SVZ and into their correct laminar position in the cortical plate. Upon reaching the cortical plate, however, neurons induce the expression of NOMA-GAP which then directly inhibits Cdc42 activity, thus enabling the activation of cofilin and initiation of dendritic arborization (Fig. 8). **Original work 4** was the first work to address the postmitotic functions of Cdc42, independently of its function in progenitor cells (103). Our observations have stressed the importance of addressing each neuronal developmental stage singly and, together with **original work 5**, have highlighted that molecules such as Cdc42 can play distinct roles at different timepoints in the differentiation of the neuron.

It is still unclear how Cdc42 becomes activated in the neocortex and why upper layer neurons are particularly sensitive to loss of the negative Cdc42 regulator, NOMA-GAP. Upper layer neurons, as the last born neurons, have a longer migratory distance to cover than the first-born deeper layer neurons and may for this reason be more sensitive to factors that influence their migration or timing of contact with the pia. In addition, the developing neocortex expresses a number of other RhoGAPs, including PX-RICS/*ARHGAP32* which shares close N-terminal homology to NOMA-GAP (**original work 3**). These may act as redundant regulators of Cdc42 in deeper layer neurons. Although loss of PX-RICS in the mouse does not appear to affect neocortical development (181), defects in the extension of primary dendrites have been reported following the knockdown of this protein using shRNA in cultured neurons (182). In addition, Cdc42 may become more strongly activated in upper layer neurons following stimulation of these neurons from pial/upper layer-derived diffusible factors such as Reelin and Semaphorin 3A, both of which have been shown to regulate actin dynamics through activation of Cdc42 and regulation of cofilin (183-188). Finally, upper and deeper layers neurons have unique molecular characteristics which may modulate their responses to external stimuli. The neurotrophins BDNF and NT-3 have, for example, been shown to exert layer-specific effects on the dendritic morphology of neocortical neurons (189).

Live-imaging studies in hippocampal slices have indicated that neurons first form primary dendrites, followed by branches that arise from the sides of the dendritic shaft and not from the growing tip (190). These branches start off as short unstable actin-rich projections called filopodia that eventually stabilize and extend. Dendritic branching therefore requires a dynamic reorganisation of the actin structure in the primary dendrite. Cofilin is an actin-severing molecule whose function in creating breaks in F-actin may not only act to generate a site for branching to occur but also generate a local pool of free actin available for recycling and novel actin polymerization. Activation of cofilin in neocortical neurons upon suppression of the Cdc42/PAK/LIMK pathway by NOMA-GAP may thus promote branching by increasing actin turnover and dynamics (191). In support of our observations, LIMK has been shown to stabilize exisiting actin filaments through inhibition of cofilin and thereby to inhibit actin dynamics and the growth of new actin filaments (145, 146).

6.3.3 Spine formation, connectivity and animal behaviour

Dendritic branching is followed by the formation of specialized cell contact structures, the dendritic spines, that enable neurons to receive signals from other neurons. Spines are therefore specialized signalling compartments that must be equipped to

maintain contact with the axon, to receive and further transmit the signal into the cell and to regulate their molecular components in response to the strength, frequency and duration of signal transmission as occurs during long-term potentiation (LTP) and depression (LTD).

Spine formation is initiated along dendrite branches through the formation of unstable actin-rich filopodia, in a manner similar to the formation of new branches (192). Stabilisation of the spine structure occurs upon contact with an incoming axon. Further maturation into a mushroom-shaped structure involves the transport of postsynaptic proteins into the immature spine and the assembly of the postsynaptic density and is accompanied by spine head growth (193).

Most studies indicate that Cdc42 activity is necessary for the formation of dendritic spines (194-196) and that, like other RhoGTPases, it becomes strongly activated at the spine head following stimulation (197). Interestingly, unlike other RhoGTPases, activation of Cdc42 following stimulation is restricted to the spine head and does not diffuse out of the spine head along the neck and into the dendrite (197), suggesting the existence of a restricting mechanism. **Original work 5** shows that NOMA-GAP, a potent inhibitor of Cdc42, is enriched at the postsynaptic site, where it could potentially prevent activation of Cdc42. However, **original work 5** also shows that association of NOMA-GAP with PSD-95, a major postsynaptic scaffold protein, inhibits the GAP activity of NOMA-GAP, thereby presumably preventing inhibition of Cdc42 at this subcellular location (Fig. 8).

6.3.4 Regulation of PSD-95 and AMPAR by NOMA-GAP

Spine maturation and responses to the electrical transmission require the recruitment of the glutamatergic AMPARs to the postsynaptic site. This step is dependent on the indirect association of AMPARs with PSD-95 through the stargazin family of co-receptor molecules (150, 152, 198). Loss of PSD-95 is associated with a decrease in AMPAR-containing synapses. This has been suggested to result in the synaptic defects seen in PSD-95-deficient animals (151, 199). Interestingly, we observe the same defects in synaptic transmission namely, a strongly reduced mEPSC frequency with no alterations in amplitude in upper layer neurons in acute neocortical slices of NOMA-GAP-deficient animals (**original work 5**). **Original work 5** also demonstrates that NOMA-GAP directly associates with and regulates PSD-95 (Fig. 8). Aberrant phosphorylation and subcellular localization of PSD-95 away from dendritic spine heads, as observed upon loss of NOMA-GAP, is expected to result in the mislocalisation of AMPARs. Indeed, **original work 5** shows that there is a 50% loss

in the level of AMPARs at the cell surface of neurons lacking NOMA-GAP. This likely explains the aberrant synaptic transmission observed in these animals.

Many animal models for neuropsychiatric disorders such as ASD, show a dysregulation of AMPAR signalling or localisation. Thus, for example surface expression of the AMPAR has been shown to be regulated by the ASD and Schizophrenia-associated Neurexin-Neuregulin signalling complex (200, 201), the Angelmann syndrom-associated E3 ubiquitin ligase Ube3A (202) and the fragile X syndrome associated FMRP proteins (203) among others. Disturbances in both ionotropic AMPAR and NMDAR signalling as well as in metabotropic glutamate signalling have been associated with ASD in humans. These include the elevation of serum levels of glutamate in ASD children (204, 205), alterations in the levels of AMPA, NMDA and metabotropic glutamate receptors (206) and mutations in several NMDAR subunits (207, 208). Several therapeutic approaches aimed at altering ionotropic and/or metabotropic glutamatergic neurotransmission have thus been developed and are currently been evaluated (208, 209). Interestingly, both NMDAR and metabotropic glutamatergic receptor signalling have been shown to regulate the trafficking and surface expression of AMPARs (210).

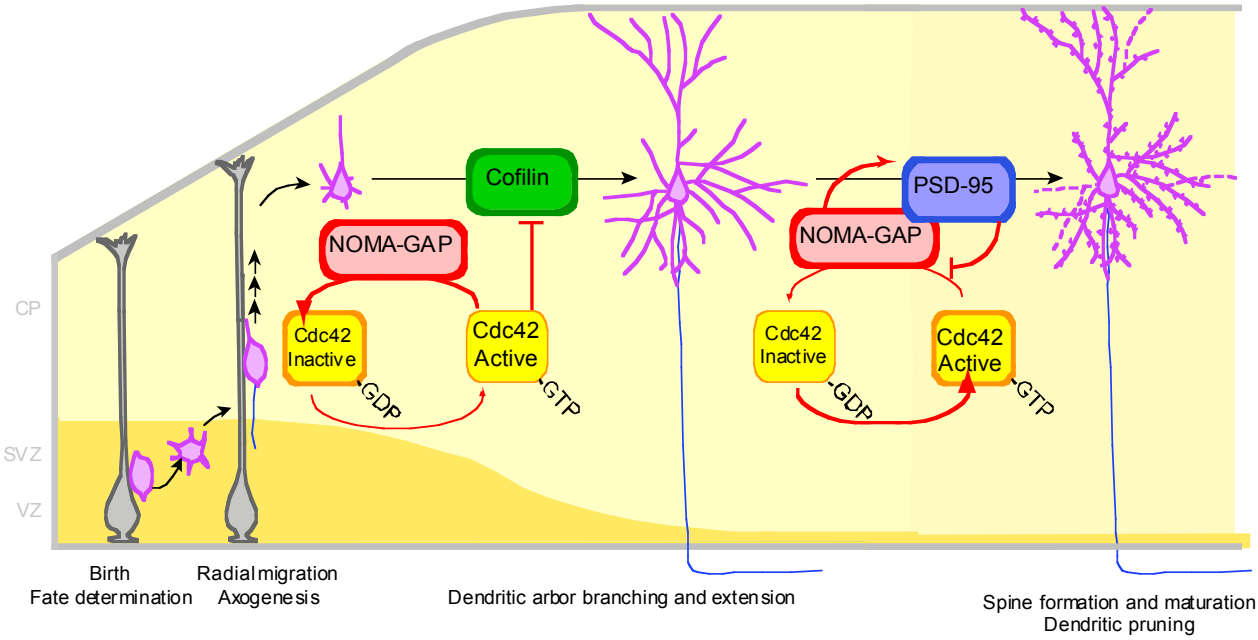

Figure 8. Regulation of Dendritic arborisation and Spine maturation during neocortical development

During cortical development, newly born neurons (pink) migrate out of the VZ/SVZ and into the cortical plate. Migration occurs along radial glia tracks (grey cells). Dendritic arborisation is suppressed by Cdc42-mediated inactivation of the actin binding protein cofilin probably through a PAK/LIMK dependent pathway. Upon completion of migration, neurons upregulate

the expression of *ARHGAP33*/NOMA-GAP, a multiadaptor protein with RhoGAP activity, which suppresses Cdc42 activity, thus enabling cofilin activation. Cofilin activity is essential to promote the branching of dendrites (**original work 4**). Cdc42 activation is enabled during maturation of the dendritic spines through the association of NOMA-GAP with PSD-95, which inhibits the RhoGAP activity of NOMA-GAP (**original work 5**). In turn, NOMA-GAP promotes the correct subcellular localization and phosphorylation state of PSD-95 and thus spine maturation (**original work 5**). VZ ventricular zone; SVZ subventricular zone; CP cortical plate. Figure by Rosário (unpublished).

6.3.5 Neuropsychiatric disorders and NOMA-GAP/*ARHGAP33*

Disorders in dendritic branching and spine formation are associated with cognitive alterations including intellectual disability and neuropsychiatric disorders such as Autism Spectrum Disorders (ASD) and Schizophrenia (211, 212). The candidate molecules identified so far are, like NOMA-GAP, predominantly postsynaptic proteins. *ARHGAP33*/NOMA-GAP-deficient mice show a sexual dimorphic behavioural phenotype typically seen in mouse models for neuropsychiatric diseases such as ASD and schizophrenia (**orginal work 5**). Male NOMA-GAP-deficient mice have strong defects in social behaviour in standard tests for identifying autism-like alterations in the mouse and the female mice show signs of increased anxiety. Both genders exhibit normal general behaviour, health and motor and sensory function. Importantly, NOMA-GAP-deficient mice show no olfactory deficits, ruling out the possibility that their lack of preference for a novel social interaction is due to an inability to detect and recognize olfactory cues that are at the basis of all social interactions in mice. Given the absence of physical, sensory and motor defects, the marked deficits in the preference for the stranger mouse, and the failure to distinguish between stranger and familiar mouse, shown by NOMA-GAP-deficient male mice can be interpreted as autism-like behaviour (213, 214). Furthermore, NOMA-GAP-deficient mice show normal exploration of novel environments, further emphasizing the social-nature of the phenotype.

Anxiety is the most common comorbidity with neuropsychiatric disorders in humans (215). Signs of increased animal anxiety, such as aberrant nest building, has also been observed in other rodent models for ASD (214). Similarly, NOMA-GAP-deficient mice, in particular the females, show signs of increased anxiety. The prevalence of anxiety in human patients with neuropsychiatric disorders is also higher in affected females. Indeed this leads to a higher initial misdiagnosis of ASD-affected female patients with conditions such as anxiety disorder, depression or borderline personality disorder (216, 217).

Although highly heritable, neuropsychiatric conditions are more frequently associated with changes in multiple genes through copy-number variations (CNVs), the accumulation of high-risk single-nucleotide polymorphisms (SNPs) or epigenetic changes than with single gene mutations. Interestingly, there is a large overlap in the genes so far identified in association with ASD, Schizophrenia, intellectual disability and epileptic encephalopathy (218, 219). Indeed, recently *ARHGAP33* was identified as a candidate for severe intellectual disability (220). The mutation identified, Val499Met, lies in the RhoGAP domain of NOMA-GAP and is predicted to destabilize or deform the GTP-binding site thus lead to the inactivation of this function (220).

7 List of included original works

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Eidesstattliche Versicherung

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