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UNIVERSITY OF TARTU
Press

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Dissertation is accepted for of commencement of degree of Doctor of Philosophy in Cell Biology on June 20th, 2016 by the Council of the Institute of Molecular and Cell Biology, University of Tartu

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Commencement: Room No 105, 23B Riia Street, Tartu, Estonia at 10.15 on August 22th, 2016

Publication of this dissertation is granted by the Institute of Molecular and Cell Biology, University of Tartu, Estonia

ISSN 1024-6479
ISBN 978-9949-77-179-0 (print)
ISBN 978-9949-77-180-6 (pdf)

University of Tartu Press
www.tyk.ee

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I. Tõnissoo T, Lulla S, Meier R, Saare M, **Ruisu K**, Pooga M, Karis A. 2010. Nucleotide exchange factor RIC-8 is indispensable in mammalian early development. Dev Dyn. 239(12):3404–15.
doi: 10.1002/dvdy.22480

II. Kask K, **Ruisu K**, Tikker L, Karis K, Saare M, Meier R, Karis A, Tõnissoo T, Pooga M. 2015. Deletion of RIC8A in neural precursor cells leads to altered neurogenesis and neonatal lethality of mouse. Dev Neurobiol. 75(9):984–1002.

doi: 10.1002/dneu.22264

III. **Ruisu K**, Kask K, Meier R, Saare M, Raid R, Veraksitš A, Karis A, Tõnissoo T, Pooga M. 2013. Ablation of RIC8A function in mouse neurons leads to a severe neuromuscular phenotype and postnatal death. PLoS One. 8(8):e74031.

doi: 10.1371/journal.pone.0074031

IV. **Ruisu K**, Velling T, Meier R, Tõnissoo T, Pooga M. The role of RIC8A in the organisation of actin cytoskeleton and cell-matrix adhesion. Manuscript.

My contributions to the listed articles are as follows:

Ref. I – Contributed to the collection of experimental data and to compilation of the manuscript

Ref. II – Participated in the assay design, performed and analysed a part of the experiments, participated in the manuscript drafting and finalisation

Ref. III – Participated in the design of the study, performed most the experiments, analysed the data and wrote the manuscript draft

Ref. IV – Participated in the design of the study, performed all of the experiments, analysed the data and wrote the manuscript draft

LIST OF ABBREVIATIONS

AC5	Adenylyl Cyclase 5
BM	Basement membrane
cAMP	Cyclic Adenosine Monophosphate
CDC42	Cell Division Cycle protein 42
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ExE	Extraembryonic ectoderm
FBS	Foetal bovine serum
GAP	GTPase Activating Proteins
GDI	Guanine nucleotide Dissociation Inhibitor
GEF	Guanine nucleotide Exchange Factor
GPCR	G Protein-Coupled Receptor
GPR56	G Protein-Coupled Receptor 56
GT(D)P	Guanosine tri-(di-)phosphate
ICM	Inner Cell Mass
MEF	Mouse embryonic fibroblasts
mES	Mouse embryonic stem cells
NCAM	Neural Cell Adhesion Molecule
NCS-1	Neuronal Calcium Sensor-1
PDGF	Platelet Derived Growth Factor
PKC	Protein Kinase C
PLC β	Phospholipase Cβ
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RGS	Regulators of G-protein Signalling
RTK	Receptor Tyrosine Kinase
RIC8A	Resistance To Inhibitors Of Cholinesterase 8 Homolog A
ROCK	Rho-associated Protein Kinase
VZ	Ventricular Zone

INTRODUCTION

Communication between cells is the key to the proper functioning of multi-cellular organisms. Sensing the environment for information about changes in the surrounding conditions and signals allows the cell to respond and adjust its properties in concert with the rest of the organism. G-protein coupled receptors (GPCRs), and G proteins (guanine nucleotide-binding proteins) that the former are coupled to, are critical for transducing the extracellular information to the inside of the cell, being involved in a multitude of developmental, physiological and behavioural processes. Of the three major groups of receptors – GPCRs, ion channels, and receptor tyrosine kinases – GPCRs form the largest family. They represent the most substantial class of drug targets today, acting as the primary targets of approximately half of the drugs on the market. GPCRs communicate signals acquired through the binding of hormones, neurotransmitters, ions and even light particles to name a few. The subsequent activation of G-proteins triggers a complex and a highly regulated intracellular signalling cascade, disturbance of which may result in many types of human diseases, such as cardio-vascular, neurological and metabolic, as well as cancer. RIC8, one of the indispensable components of this signalling pathway, interacts directly with G protein α subunits, regulating their activity and abundance in cells. Both RIC8 and G proteins are present in humans, but also in simple organisms like the amoeba *Dictyostelium discoideum* and the red bread mould *Neurospora grassa* indicating that they emerged quite early in the evolution of the eukaryotes. Thus, RIC8 is a component of a highly conserved cell signalling system. The research into the *in vivo* function of RIC8 in nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster* and African clawed frog *Xenopus laevis* has revealed that it plays an essential role in cell division, synaptic signal transduction, and cell adhesion and migration. However, the function of RIC8A (one of two RIC8 proteins in vertebrates) in mice is still poorly characterised. The previous studies have revealed that RIC8A is expressed in the developing nervous system during the early organogenesis phase (E9.5-E12.5) and that it is also expressed in the brain of the adult mice. Moreover, the mice heterozygous for the *Ric8a* allele display behavioural abnormalities. The research presented in this dissertation is a continuation of these studies.

The main goal of this thesis was to analyse the phenotypes of three different *Ric8a* knockout mice: a total knockout (*Ric8a*$^{-/-}$) and the specific depletions of *Ric8a* from the neural precursor cells and from differentiated neurons. Two major conclusions were drawn from this analysis. First, the ablation of RIC8A in the nervous system results in a neuromuscular phenotype and second, the lack of RIC8A results in cell migration defects during gastrulation and neuro-genesis, probably due to defective adhesion of cells to the extracellular matrix. To elaborate on the latter finding, the adhesive properties of RIC8A-deficient mouse primary cells were evaluated.

REVIEW OF LITERATURE

1. G proteins

Heterotrimeric G-proteins are fundamentally conserved from bacteria to mammals and play diverse roles in many aspects of cell regulation. They are composed of non-identical alpha, beta and gamma subunits. The basic mechanism of G-protein signalling depends on the capability of the Gα subunit to bind and hydrolyse guanosine triphosphate (GTP) to guanosine diphosphate (GDP). In its relatively inactive, GDP-bound state Gα subunit forms a complex with Gβ and Gγ subunits, and functionally dissociates from the Gβγ complex upon binding GTP. The formed Gα:GTP complex and disassociated Gβγ dimer transduce signal by modulating downstream effectors. The intrinsic GTP-hydrolysing activity of the Gα subunit leads to the reformation of the heterotrimer in its inactive GDP- and Gβγ-bound state (Alberts *et al.*, 2002). In addition to heterotrimeric G-proteins, there are monomeric GTPses called small G-proteins (aka small GTPases or Ras superfamily GTPases) that share a common architecture with a core 'GTPase domain' that is similar in structure and function to Gα subunits (Mishra and Lambright, 2016). The GTPase activity of Gα and small G-proteins is regulated by various accessory proteins: regulators of G-protein signalling (RGS, also dubbed GTPase activating proteins or GAP's), guanine nucleotide exchange factors (GEF) and guanine nucleotide dissociation inhibitors (GDI), (Siderovski and Willard, 2005). The major non-structural difference between the heterotrimeric and monomeric G-proteins is that heterotrimeric G proteins are mostly bound to a G-protein coupled receptors (GPCR) which, upon stimulation by extracellular ligands (e.g. chemokines, hormones, neurotransmitters, etc.), act as GEFs inducing the release of GDP and enabling the binding of GTP to Gα. Small G-proteins usually function several steps downstream of a membrane receptor and are activated by various intracellular GEF's (Fig. 1).

On the basis of sequence similarity, Gα subunits of heterotrimeric G-proteins have been divided into four main subfamilies: $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ (Neves *et al.*, 2002). $G\alpha_s$ pathway was the first one described in this group. Its general function is to activate the cAMP-dependent pathway by activating adenylyl cyclase (Milligan and Kostenis, 2006). $G\alpha_i$ generally functions as an inhibitory regulator of the cAMP-dependent pathway (Milligan and Kostenis, 2006). Therefore, $G\alpha_s$ and $G\alpha_i$ have inverse regulatory functions when it comes to cAMP production. $G\alpha_o$, a nervous system-specific member of the $G\alpha_i$ subfamily, has been shown to inhibit the voltage-dependent calcium channels having no effect on adenylyl cyclase activity (Jiang *et al.*, 1998). $G\alpha_{q/11}$ proteins are ubiquitously expressed and they mostly activate phospholipase Cβ, which generates two downstream messengers essential in the development of organisms, namely the water-soluble inositol phosphates that regulate intracellular Ca^{2+} mobilisation, and diacylglycerol, which activates PKC (Litosch, 2016). The major downstream effector of the $G\alpha_{12/13}$ family is the small GTPase RhoA (Buhl *et al.*, 1995), which is activated by Rho guanine nucleotide exchange factors (RhoGEFs)

that are direct targets of $G\alpha_{12/13}$ (Kozasa *et al.,* 1998). It is important to note that these subunit families (especially $G\alpha_i$ and $G\alpha_q$) have multiple subtypes with much more identified targets than the ones named here.

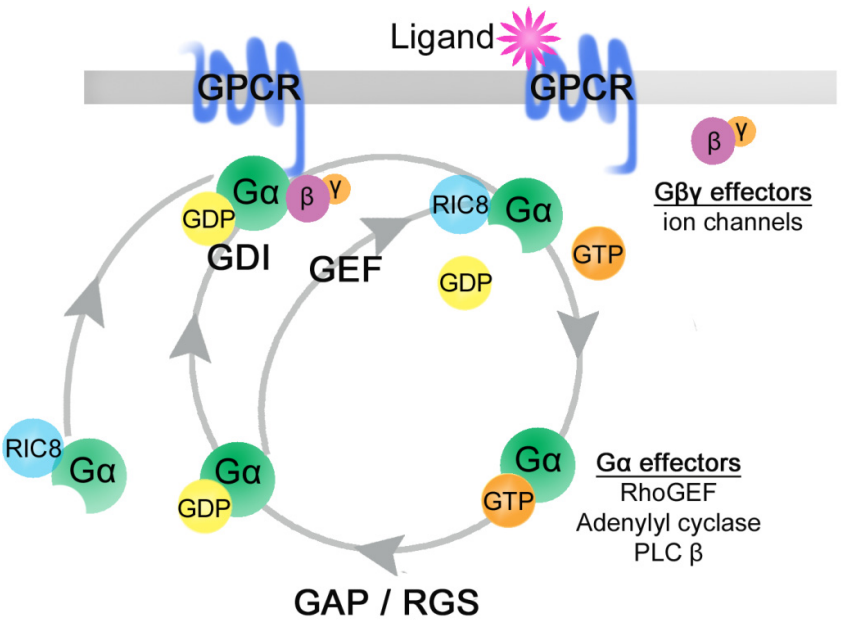

Figure 1. A standard model for the regulation of G-protein signalling. Gα:GDP in complex with Gβγ are coupled to the 7 transmembrane GPCR (G-protein-coupled receptor). GDI (guanine nucleotide dissociation inhibitor) also acts on the Gα subunits to inhibit the spontaneous release of GDP. When an extracellular ligand binds to the GPCR, the receptor triggers a conformational change in Gα enabling its dissociation from the receptor and the release of βγ subunit. Subsequently, GDP is released and GTP can associate with Gα. Both the GTP-bound Gα and released Gβγ transduce the signal from receptor inside the cell by modulating the activity of various enzymes, ion channels, and other effectors. Regulators of the G-protein signalling (RGS) act as GTPase-accelerating proteins (GAPs) that stimulate the signal termination for Gα dramatically enhancing their intrinsic rate of GTP hydrolysis to GDP. GDP-bound Gα enables the re-assembly of the receptor-bound complex. However, Gα:GDP can also be activated in a receptor-independent manner. This is mediated by GEFs (guanine nucleotide exchange factors). RIC8 has been shown to act as a receptor-independent GEF for Gα subunits. It has also been shown to act as a chaperone for Gα subunits necessary for their localisation to the plasma membrane.

2. RIC8A protein

2.1. Biochemical properties of RIC8

Resistance to **I**nhibitors of **C**holinesterase **8** (RIC8) is a regulator for a subset of G-protein α subunits. The highly conserved protein was first found in nematode *Caenorhabditis elegans* (*C.elegans*) from a mutagenesis screen that sought for animals resistant to cholinesterase inhibitors among mutants with phenotypes similar to egl-30 ($G\alpha_q$ in *C.elegans*) deficient worms (Miller *et al.*, 2000). When cholinesterase enzyme is inhibited, the level and duration of action of the neurotransmitter increases leading to a toxic effect (Risher *et al.*, 1987) that is absent in RIC8 mutants. Further research revealed that RIC8 functions upstream of, or in conjunction with, EGL-30 ($G\alpha_q$) (Miller *et al.*, 2000). *In vitro* experiments later identified mammalian RIC8 to function as a guanine nucleotide exchange factor that regulates the activity G protein α subunits (Tall *et al.*, 2003). Recent evidence has implied that RIC8 may also act as a chaperone for Gα subunits (Gabay *et al.*, 2011; Chan *et al.*, 2013) (Fig. 1). In vertebrates, two RIC8 isoforms have been identified: RIC8A and RIC8B. These homologues have the same biochemical function but they differ in the Gα subunits they associate with. RIC8A regulates $G\alpha_{12/13}$, $G\alpha_{q/11}$, and $G\alpha_{i/o}$ families and RIC8B has mostly been associated with the $G\alpha_s$ family but it also binds $G\alpha_{12/13}$ and $G\alpha_{q/11}$ weakly (Tall *et al.*, 2003; Chan *et al.*, 2011; Gabay *et al.*, 2011).

Attempts to resolve the structure of RIC8 have yielded little results so far. A group working with *Xenopus laevis* compared the xRic-8 (RIC8 in the *X.laevis*) amino acid sequence with protein databases and found that xRic-8 belongs to a unique protein family with no homology to other proteins (Figueroa *et al.*, 2009). The structural model of xRic8a, assembled by using a battery of bioinformatics approaches, is composed of 10 armadillo folding motifs organized in a right-twisted α-alpha super helix (Figueroa *et al.*, 2009). Armadillo proteins have been shown to act as "scaffold proteins" interacting with a diverse set of partners and participating in many signalling pathways (Hatzfeld, 1999). Although Gα subunits play a central role in RIC8A function, some studies have indeed found additional binding partners for RIC8A. For example, it was found to interact with neural cell adhesion molecule (NCAM) and specific NCAM isoform 180 appeared to be necessary for the recruitment of RIC8A to the cell surface (Amoureux *et al.*, 2012). In addition, type V adenylate cyclase (AC5) has also been shown to directly interact with RIC8A. By binding to the N-terminus of AC5, RIC8A suppressed the activity of AC5 via a $G\alpha_i$-dependent pathway (Wang *et al.*, 2007).

Nevertheless, a vast majority of published data on the biochemical function of RIC8A places it into the G protein signalling pathway (Miller and Rand, 2000; Tall *et al.*, 2003; Tall and Gilman, 2005; Woodard *et al.*, 2010; Gabay *et al.*, 2011; Boularan *et al.*, 2015). Tall and his group have been the front-runners in this field. They first described RIC8A protein as a GEF for a subset of Gα subunits that potentiates and prolongs the signals received from membrane

GPCRs (Tall *et al.*, 2003; Tall and Gilman, 2005). Later they discovered that RIC8A proteins also act as molecular chaperones that control the initial association of nascent Gα subunits with cellular membranes (Gabay *et al.*, 2011; Chan *et al.*, 2013). Indeed, the membrane localisation of different Gα subunits has been shown to be regulated by RIC8A in multiple studies (Hampoelz *et al.*, 2005; Nishimura *et al.*, 2006; Saare *et al.*, 2015). In a recent review paper, it has been proposed that the function of RIC8A protein in cells is to promote Gα protein abundance and that RIC8A GEF activity *per se* is not necessarily the purpose of producing activated Gα-GTP in order to engage downstream effectors (Tall, 2013).

2.2. Function of RIC8A in cells and organisms

RIC8A has also been called Synembryn for its identified physiological activities in *C. elegans*: synaptic transmission and embryogenesis (Miller *et al.*, 2000). The embryonic functions that RIC8 has been found to participate in have often been associated with cell division and the function of $G\alpha_i$ in cells. RIC8 has been assigned a role as a regulatory component of an evolutionarily conserved heterotrimeric Gα-mediated mechanism that controls spindle orientation and asymmetric cell division in *C. elegans* embryos (Miller and Rand, 2000) and *D. melanogaster* neuroblasts (David *et al.*, 2005; Hampoelz *et al.*, 2005; Wang *et al.*, 2005). These results were substantiated by findings in HeLa cells, where reduced *Ric8a* expression prolonged mitosis, caused occasional mitotic arrest, and decreased mitotic spindle movements in a $G\alpha_i$-dependent manner (Woodard *et al.*, 2010). The RIC8A localization in oocytes was similar to that of HeLa cells and its inhibition interfered with the recruitment of $G\alpha_i$ to the plasma membrane (Saare *et al.*, 2015).

In mouse embryos (E9.5–12.5), the expression of *Ric8a* was detected mostly in the developing nervous system (Tõnissoo *et al.*, 2003). RIC8A was also expressed in the adult brain, where it was found in the hippocampus, neocortex, and cerebellum (Tõnissoo *et al.*, 2003). In accordance with this neurospecific expression, *Ric8a* haploinsufficiency was found to affect the behavioural physiology and spatial memory of mice (Tõnissoo *et al.*, 2006). In the nervous system of *C. elegans,* RIC8 has been shown to affect the $G\alpha_q$–$G\alpha_o$ pathway by mediating the release of neurotransmitters in the neuromuscular synapse (Miller *et al.*, 2000). Nematodes with suppressed RIC8 function were shown to display decreased locomotion and body flexion (Miller *et al.*, 2000). In the nervous system of *D. melanogaster*, Ric8 binds to the Ca^{2+} sensor NCS-1 to regulate the synapse number and neurotransmitter release (Romero-Pozuelo and Dason, 2014).

At *Xenopus tropicalis* early developmental stages, *xRic-8a* is expressed in the animal hemisphere of an embryo whereas later its expression is restricted to neural tissues (neural tube and developing brain) (Maldonado-Agurto *et al.*, 2011). Recently, there has been an accumulation of evidence linking RIC8A

with focal adhesions and actin cytoskeleton remodelling. These processes are reviewed in detail in chapter 3.2.

Information about the *in vivo* roles of RIC8A is fragmentary. It is clear, however, that RIC8A functions in concert with Gα subunits performing crucial cellular tasks, since its absence is lethal in *D. melanogaster* (Hampoelz *et al.*, 2005; Wang *et al.*, 2005), *C. elegans* (Miller and Rand, 2000) and mice (Tõnissoo *et al.*, 2006).

3. Connection between cells and the extracellular matrix

The extracellular matrix (ECM) is synthesized and secreted from the earliest stages of development on, and it is critically important for cell growth, survival, differentiation, morphogenesis and the maintenance of tissues. Macromolecules that constitute the ECM are mainly produced by the cells in the matrix (fibroblasts in most connective tissues) and are linked to each other through diverse protein-, cell- and carbohydrate-binding domains. These macromolecules are divided into two classes: glycosaminoglycans (GAGs), and fibrous proteins, including collagen, fibronectin, laminin, elastin, etc. (Alberts *et al.*, 2002). GAGs are polysaccharide chains that carry a high negative charge, are strongly hydrophilic and attract a lot of water into the matrix, which helps the connective tissues to maintain elasticity. In addition to structural functions, GAGs participate in the regulation of different cellular processes like cell migration, cell division, etc., and interact with a variety of secreted proteins that regulate their activity. Collagens are the most abundant proteins of the ECM. Collagen molecules are generally long and form cross-linked fibrils with the extent and type of cross-linking varying from tissue to tissue (Gelse *et al.*, 2003). Laminins, the heterotrimeric proteins composed of α, β, and γ chains, are found in multiple genetic variants that form different combinations (Domogatskaya *et al.*, 2012). Laminins are the major components of the basal lamina, a specialized form of the ECM on the basal side of polarized epithelial cell sheets separating them from the underlying connective tissue. The basal lamina together with the underlying layer of reticular lamina forms the basement membrane (BM). Basal lamina consists mostly of collagen IV and laminins, and it is secreted by the epithelial cells whereas the reticular lamina consists of collagen fibres and is produced by the underlying connective tissue cells (Sanes, 2003). Fibronectin is present in both layers and is thought to anchor them to each other and to the cells (Singh *et al.*, 2010). The BM is characterised in more detail in chapter 4.

Most cells need to attach to the extracellular matrix to grow, proliferate and survive, which is known as anchorage dependence (Frisch and Francis, 1994), and this interaction is mediated mainly by integrins and regulated by the intracellular signals they generate. Integrins are transmembrane proteins that mediate the adhesion and bidirectional signalling between the cell and the ECM. They

are heterodimeric proteins consisting of α and β subunits with relatively large extracellular domains, a single transmembrane domain (TMD), and a short cytoplasmic tail (Hynes, 2002). There are 18 α subunits and 8 β subunits that can combine to form 24 functionally different integrins expressed in tissue-specific fashion. In addition to this tissue specificity, each integrin exhibits a distinct binding affinity to a particular ligand or a set of ligands (Humphries *et al.*, 2006). Therefore, a cell with its set of integrins can adhere to, or migrate toward, a specific region where the respective ligands are present. Integrins mediate signals bidirectionally: "outside-in" signalling is initiated by the ligand binding allowing the cell to sense the extracellular environment and react correspondingly, and "inside-out" signalling is activated by the intracellular signalling molecules to regulate the ligand binding characteristics of integrins (Qin *et al.*, 2004).

Akin to the integrin receptors is the dystroglycan, another receptor complex that consists of an extracellular α- and transmembrane β-subunits linking ECM components to the cytoskeletal network (Henry and Campbell, 1999). Originally isolated from skeletal muscles and associated with muscular dystrophy (Ervasti *et al.*, 1990), dystroglycan is now recognised as a laminin receptor in all tissues (Durbeej *et al.*, 1998). α-dystroglycan interacts with the BM and generates intracellular signals, which are transmitted by a transmembrane β-dystroglycan. The signalling pathways activated by dystroglycan partially overlap with those regulated by integrins (Belkin and Smalheiser, 1996; Spence *et al.*, 2004; Thompson *et al.*, 2010).

3.1. The role of actin cytoskeleton in cell-ECM adhesion

The physical spreading of cells on the matrix has a strong influence on intracellular events. Cells that are forced to spread over a large surface area survive better and proliferate faster than those that are not spread (Chen *et al.*, 1997). Cells adhere to, spread and migrate on substrates by exerting mechanical forces to the inner face of the membrane, which are generated by polymerising actin, the main component of the cytoskeleton, and its coupling to a motor protein, myosin. Polymerisation of actin drives the membrane protrusion and extension through the formation of filopodia and lamellipodia. This is considered as the first step in cell migration. Filament polymerization from the (+) end is enhanced by the family of proteins known as formins (Dia) and the Ena/VASP homology proteins, and the disassembly of actin at its (–) end is mediated by ADF/cofilin (Pollard and Borisy, 2003). Branching of the actin network is enabled by the Arp2/3 complex, which stabilises the connections between the filaments (Goley and Welch, 2006). In order to generate adhesion strength (traction force), actin cytoskeleton needs to be connected to the extracellular matrix. Following the cell-ECM interaction, large molecular complexes called focal adhesions are assembled. These contain clustered integrins in the membrane and different cytoplasmic proteins including e.g. vinculin, talin, paxillin, etc., that bind, either directly or indirectly, to the cytoplasmic tails of integrins (Calderwood *et al.*,

2000). Actin filaments organised in a special manner called stress fibres are anchored to focal adhesions. Stress fibres form contractile structures in cells together with myosin II. Interestingly, on soft substrates cells do not form focal adhesion complexes or stress fibres (Gupta *et al.*, 2015).

3.2. G proteins and cell adhesion to the ECM

Rho, Rac, and Cdc42 form a subfamily of Rho GTPases, small G-proteins that regulate actin and cell adhesion. Notably, these proteins control many cellular processes involved in cell adhesion and the regulation of the cytoskeleton dynamics. Cdc42 is activated in protrusions, filopodia, and at the Golgi, Rac1 activity is required for the formation of protrusions and ruffles, but also controls disassembly of invadopodia, whereas RhoA activity is associated with membrane protrusion, tail retraction, ruffling and cell polarity (Martin *et al.*, 2016). RhoA has been shown to regulate the actin cytoskeleton with the activation of downstream targets formin (mDia) and ROCK (Leung *et al.*, 1995; Watanabe *et al.*, 1997). Formins produce straight, unbranched actin fibres by accelerating the incorporation of actin monomers as well as protecting the ends from the capping proteins (Goode and Eck, 2007). ROCK has been shown to mediate the activation of actomyosin-mediated contraction through inhibition of myosin light chain phosphatases (Riento and Ridley, 2003). Another target of ROCK is ADF/cofilin that has been shown to be one of the key regulators of actin severing, nucleation, and capping within the protrusive machinery (Song *et al.*, 2006).

Out of the heterotrimeric G proteins, $G\alpha_{12/13}$ has mostly been associated with the regulation of cell motility and changes in morphology of cells via the direct activation of RhoGEF's, which in turn activate the RhoA GTPase.

A number of different GPCRs have been reported to couple with the $G\alpha_{12/13}$ family (Riobo and Manning, 2005), but not in an exclusive manner. Receptors that are coupled to $G\alpha_{12}$ and/or $G\alpha_{13}$ invariably couple to one or more other G proteins. For example, many $G\alpha_q$/$G\alpha_{11}$-coupled receptors are reported to induce stress fibre assembly in the absence of $G\alpha_q$ and $G\alpha_{11}$ and that this involves either a $G\alpha_{12}$ or a $G\alpha_{13}$ coupled receptor-mediated pathway (Gohla *et al.*, 1999). Recently, it was found that in addition to GPCRs, $G\alpha_{13}$ also interacts with integrins, in particular, $G\alpha_{13}$ binds integrin β3 subunit in platelets (Gong *et al.*, 2010) as well as the ubiquitous β1 integrin subunit (Shen *et al.*, 2015). These interactions were suggested to be necessary for integrin-mediated "outside-in" signalling, transient inactivation of RhoA, and Src activation required for the initial cell spreading and migration (Gong *et al.*, 2010; Shen *et al.*, 2015). Therefore, it seems that $G\alpha_{13}$ plays a dual role in the regulation of RhoA both by stimulating it through GPCR-activated pathways and inhibiting it via integrin-mediated outside-in signalling (Shen *et al.*, 2015). In addition to the outside-in integrin signalling, G-proteins have also been associated with the inside-out signalling in platelets. Adhesion of platelets to the site of vascular injury is dependent on the recruitment of additional platelets into a growing thrombus

and this requires mediators such as ADP, thromboxane A2, and thrombin, which act through GPCRs (Offermanns, 2006). $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12}/G\alpha_{13}$ have been associated with these processes. $G\alpha_{13}$ has been shown to be involved in the RhoA-mediated induction of change in the platelet shape (Moers *et al.*, 2003), and co-stimulation of $G\alpha_i$- and $G\alpha_{12}/G\alpha_{13}$ induces the activation of integrin αIIbβ3 (Nieswandt *et al.*, 2002). Therefore both Rho GTPases and Gα proteins play crucial roles in cell adhesion and migration processes.

3.3. RIC8A and cell adhesion to ECM

Recently, analogous results have been published independently by several research groups that have been using different model systems, where RIC8A has been implicated in the assembly of focal adhesions and organisation of the actin cytoskeleton. The *in vivo* transplantation experiments with *X. laevis* demonstrated that xRic-8A deficit causes impaired migration of the cranial neural crest cells, a strong reduction in cell spreading and focal complex formation, and reduced adhesion to fibronectin (Fuentealba *et al.*, 2013). Similarly, a study focusing on mouse cerebellum reported that in the absence of RIC8A in neural progenitors the adhesion of these cells to laminin was reduced, and therefore the specialised astrocytes called Bergmann glia were unable to attach to the basement membrane (Ma *et al.*, 2012). In addition, RIC8 has been linked to growth factor-induced cell migration in mouse embryonic fibroblasts (MEF) (Wang *et al.*, 2007). Downregulation of *Ric8a* by RNA interference inhibited platelet-derived growth factor (PDGF)-initiated cell migration and slowed down PDGF-induced dorsal ruffle turnover (Wang *et al.*, 2007). Dorsal ruffles or waves (aka actin ribbons) are structures consisting of polymerised cortical actin that assemble on the dorsal plasma membrane in response to growth factors (Buccione *et al.*, 2004). RIC8A has also been linked with actin remodelling in another study that concerns *D. melanogaster* gastrulation, where mutation of *ric-8* resulted in perturbation of cortical actin and formation of blebs on the ventral cellular surface of the blastoderm cells (Kanesaki *et al.*, 2013). Moreover, it was found that the amount of total and polymerised actin, and the filopodia-like structures were reduced in mouse *Ric8*$^{-/-}$ embryonic stem (ES) cells, as was the activation of RhoA GTPase, an important regulator of the actin cytoskeleton organisation (Gabay *et al.*, 2011). All these cytoskeletal defects in RIC8-deficient conditions were associated with the function of G proteins in the respective studies.

4. The Basement membrane-cell contact in development

Basement membranes (BM) are thin sheets of specialised extracellular matrix that surround epithelia, endothelia, muscle cells, fat cells, Schwann cells and peripheral nerves, as well as the entire central nervous system. They affect the survival and differentiation of adherent cells by playing important roles in maintaining tissue integrity and compartmentalisation, filtration and diverse developmental processes. BM mainly contain type IV collagen, nidogen, perlecan, agrin, collagen XVIII, sulphated proteoglycans, and members of the laminin family (Erickson and Couchman, 2000). However, the composition of the BM is highly divergent depending on its precise location within the body and the type of tissue which it supports.

4.1. BM in early embryonic development

A dramatic reorganisation of cells takes place in the early mammalian embryo immediately after implantation where the non-polar stem cells of the inner cell mass (ICM) will become specialized and give rise to the three germ layers (ectoderm, mesoderm, endoderm) that later form all the tissues in an organism. By the time of implantation, the mouse embryo has developed into a blastocyst (E4.5) that contains three distinct cell populations: ICM has differentiated into epiblast (primitive ectoderm) and primitive endoderm (hypoblast), which are surrounded by the trophectoderm (Belousov, 2011). During the peri-implantation period of mammalian blastocyst development, the first BM to appear in the inner cell mass is deposited beneath the primitive endoderm and the trophoblast cells so that the epiblast is surrounded by the BM (Bedzhov *et al.*, 2014). Laminin-1 (α1β1γ1) is the earliest laminin expressed during mouse embryonic development (Cooper and MacQueen, 1983). The endodermal BM induces the epiblast development. Primitive endoderm cells remaining in contact with BM differentiate into visceral endoderm and epiblast cells in contact with the BM become polarised and accumulate F-actin to the apical side of the cells (Sakai *et al.*, 2003) to form the columnar epiblast epithelium. Cells at the centre of the epiblast that are not connected to the BM undergo apoptosis and thereby give rise to the proamniotic cavity in a process called cavitation (Coucouvanis and Martin, 1995) (Fig. 2). Mutation of laminin γ1 subunit causes embryonic death prior to gastrulation (Smyth *et al.*, 1999) most likely because BM regulates the development of epiblast epithelial cells directly and the aforementioned programmed cell death indirectly (Murray and Edgar, 2000). After cavitation (E5.5) the polar trophectoderm and the epiblast develop into an elongated structure that is made up of the ectoplacental cone (which connects the embryo to the uterus), the extraembryonic ectoderm (ExE), the epiblast, and a layer of visceral endoderm.

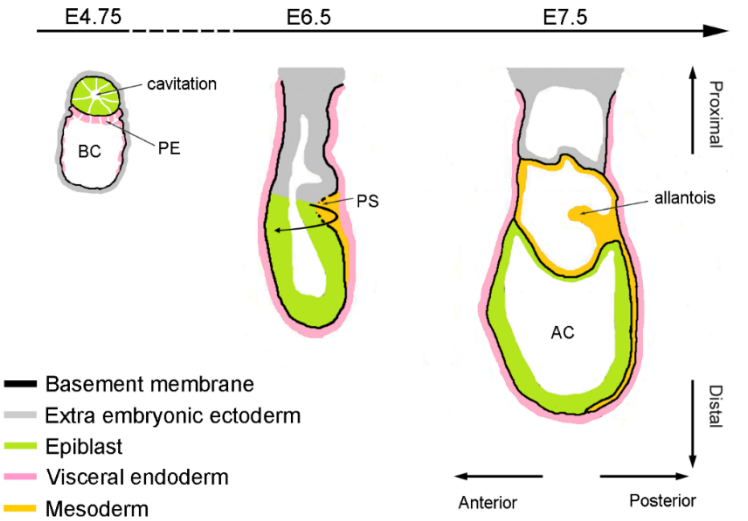

Figure 2. Basement membrane during the different stages of the early embryonic development. At E4.75, during the peri-implantation stage, the first BM is established. Epiblast cells become polarised and form the columnar epiblast epithelium. Cells that are not connected to the BM undergo apoptosis in a process called cavitation. Gastrulation starts with the formation of primitive streak (E6.0–6.5). The ingressing cells lose their polarity, the BM brakes down and cells pass through the primitive streak. After which they become mesenchymal stem cells (Lim and Thiery, 2012), in a process called epithelial-mesenchymal transition (EMT). In a late gastrulation embryo (E7.5), the ingressing mesenchymal cells spread between the epiblast and visceral endoderm, or proximally where they displace the extra-embryonic ectoderm. Visceral endoderm secretes a new BM between itself and the mesenchymal cells that emerge from the primitive streak. Abbreviations: PE – primitive endoderm, BC – blastocyst cavity, PS – primitive streak, AC – amniotic cavity

Gastrulation then commences with the formation of the primitive streak (E6.0–6.5) at the junction between the extraembryonic tissue and the epiblast on the posterior side of the embryo. This becomes the site of ingression through which epiblast cells ingress to form the mesoderm and the endoderm (Fig. 2). These two tissues and the ectoderm (the descendants of epiblast cells that do not pass through the primitive streak) constitute the three primary germ layers (Tam and Loebel, 2007). When primitive streak forms, the ingressing cells lose their polarity, detach from the BM and undergo cytoskeletal rearrangements that enable migration, and after passing the primitive streak, they become mesenchymal stem cells (Lim and Thiery, 2012). This process is called epithelial-mesenchymal transition (EMT). The first step of EMT is the breakdown of BM in the location of the primitive streak, which is controlled by the loss of basally localised RhoA activity (Nakaya *et al.*, 2008). Tight junctions and apical-basal polarity are both maintained throughout epiblast cells and are lost immediately after ingression (Nakaya *et al.*, 2008). Ingressing mesenchymal cells spread distally between the epiblast and the visceral endoderm or proximally where

they displace the extra-embryonic ectoderm. Visceral endoderm secretes a new BM between itself and the mesenchymal cells emerging from the primitive streak (Fig. 2). Cell fate determination is coupled to morphogenetic movements during mammalian embryogenesis. Morphogenesis is orchestrated by a small number of modular mechanical properties: cell-cell adhesion, cell-matrix adhesion, protrusion, and contractility, all of which require appropriate regulation and dynamics of the cytoskeleton (Montell, 2008).

4.2. BM in neurogenesis

During development of the nervous system, a limited number of neural precursor cells give rise to a high number of diverse neural cell types. Neocortical neurons arise from a small set of progenitor cells that locate in the ventricular zone (Miyata *et al.*, 2001; Noctor *et al.*, 2002). The cortical plate of the mouse cerebral cortex develops between E12 and E18 with postmitotic neurons migrating away from VZ in an inside-out manner with the earliest-generated neurons populating the deepest neocortical layers and later-generated neurons occupying the superficial layers (Hatten, 1999; Nadarajah *et al.*, 2001). Defects in these events can cause severe neural defects and are associated with various diseases like lissencephaly, microcephaly, polymicrogyria, different heterotopias, epilepsy and others (Olson and Walsh, 2002; Manzini and Walsh, 2011). The organizing framework for cortex histogenesis is provided by the spindle-shaped radial glia cells that serve as the substrate for the migrating neuroblasts. Establishment of the pial-glial barrier is one of the earliest histogenetic events in neurogenesis. This is accomplished by coordinated interaction among the processes of radial glia, various ECM components, and mesenchymal cells at the pial surface, with the formation of a BM that tightly abuts the glia limitans. Radial glia interacts with the pial BM through the endfeet of the characteristic radial processes extending from the ventricular zone to the pial surface. Therefore, the pial BM acts as both an anchor point for the endfeet of radial processes, and as a physical barrier to migrating neurons. Alterations in pial BM composition and the function of ECM-associated proteins, including the laminin γ1 chain (Halfter *et al.*, 2002), GPR56 (Li *et al.*, 2008), perlecan (Costell *et al.*, 1999), and collagen type III (Luo *et al.*, 2011), result in cortical lamination defects accompanied by the fragility of the pial BM and detachment of RG from the BM. Moreover, mutations in genes encoding BM components (e.g. laminin α5 or γ1, perlecan), as well as their cellular receptors (dystroglycan, β1 or α6 integrin), disrupt normal deposition of the cortical BM and result in a disorganized cortex (Costell *et al.*, 1999; De Arcangelis *et al.*, 1999; Graus-Porta *et al.*, 2001; Halfter *et al.*, 2002). These studies demonstrate that an intact BM and ECM binding proteins are an absolute requirement for a proper cortical development. Interestingly, mutations in $G\alpha_{12}/G\alpha_{13}$ and RhoA GTPase in the developing nervous system result in similar morphological defects as the mutations in BM components and their binding receptors (Moers *et al.*, 2008; Cappello *et al.*, 2012) indicating that their activity is essential for the glial-BM contact.

AIMS OF THE STUDY

The overall aim of this thesis was to study the role of RIC8A in the mouse development using knockout mouse models and primary cell cultures. The thesis summarises the results of four linked studies with three main objectives:

- The first goal of the research presented in this thesis was to assess the in vivo function of RIC8 in mammals by analysing the consequences of the absence of RIC8A in a mouse. Since *Ric8a*$^{-/-}$ embryos died at the gastrulation stage, we set an aim to describe the morphological defects occurring during the aberrant gastrulation process.
- The second goal of this thesis was inspired by the earlier work of our group, and others, showing that RIC8A might be important in the development of the nervous system and in the synaptic signal transduction. To study the role of RIC8A in these processes and to circumvent the embryonic lethality of the *Ric8a*$^{-/-}$ mice, we analysed the effect of the targeted depletion of Ric8a in neural progenitors and in differentiated neurons.
- The third goal was impelled by the observed defects in cell migration and basement membrane in gastrulation and neurogenesis of RIC8A deficient animals. To give a functional mechanistic context to the obtained in vivo results, we analysed the embryonic stem cells and fibroblasts isolated from RIC8A-deficient mice for their adhesive and migratory properties and the molecular mechanisms responsible for the regulation of these functions.

RESULTS AND DISCUSSION

1. *Ric8a* knockout mouse lines

1.1. Ablation of RIC8A causes defective morphogenetic movements during gastrulation in mice (Ref. I)

In order to gain insights into RIC8A function, *Ric8a* knockout mouse model was generated. Although *Ric8a* haploinsufficiency in mice does not cause any apparent morphological defects or changes in viability or reproductive capabilities (Tõnissoo *et al.*, 2006), the homozygous deletion of *Ric8a* is lethal at a very early stage of the embryonic development. RIC8A deficient ($Ric8a^{-/-}$) embryos are able to implant and initiate gastrulation but are unable to complete gastrulation and die between E6.5 and E8.5. This is in accordance with earlier results in other model organisms, where the *ric-8* reduction of function mutants in *C. elegans* (Miller and Rand, 2000) and *D. melanogaster* (Hampoelz *et al.*, 2005; Wang *et al.*, 2005) exhibit embryonic lethality. Moreover, Ric-8 deficiency in *D. melanogaster* led to multiple gastrulation defects (Hampoelz *et al.*, 2005; Wang *et al.*, 2005). Similarly, varying gastrulation defects could be observed in $Ric8a^{-/-}$ mutant mouse at the ages E6.5–E7.5. In order to better understand the nature of these defects, we analysed the expression of known marker genes for gastrulation. As an overall result, all of the tested markers were present in the $Ric8a^{-/-}$ embryos, suggesting that RIC8A does not influence the transcription of genes that are essential for gastrulation (Ref. I, Fig. 6). In addition, since marker genes for different germ layers were also expressed, RIC8A is probably not involved in the differentiation of the epiblast along embryonic and extra-embryonic lineages. However, there were significant alterations in the expression levels and temporal localisation of these lineage markers. For example, staining for *Bmp4*, a marker for ExE (trophoblast-derived extraembryonic ectoderm), that also marks epiblast-derived extra-embryonic mesoderm (Fujiwara *et al.*, 2001), revealed that in $Ric8a^{-/-}$ embryos the expression was restricted to the area of ExE, and did not extend to the area of the extraembryonic mesoderm as in normal embryos (Ref. I, Fig. 6P). In addition, a mesoderm marker Lim1 (Tsang *et al.*, 2000) had also very restricted expression which was confined to one side of the embryo (Ref. I, Fig. 6Q, Q'). These results indicate that although epiblast cells had differentiated into mesoderm and ectoderm, they did not pass the correct morphogenetic movements during the gastrulation process. Closer inspection of the histology of the $Ric8^{-/-}$ embryos revealed that the extra-embryonic mesoderm-driven early structures that contribute to the amnion, allantois, and yolk sac, were either malformed or had not formed at all (Ref. I, Fig. 2). In $Ric8a^{-/-}$ embryos that had survived longest (E8.5), anterior structures like head folds and rudimentary trunk regions were severely underdeveloped, although had partially formed. Their littermates at that age already displayed a head region with a neural ectoderm and a neural tube along with somites (Ref. I, Fig. 3). These morphological defects are in accordance with the expression of

lineage markers suggesting that the process of cell migration is impaired in the absence of RIC8A. Since the BM is essential for the early embryonic development (Miner *et al.*, 2004; Nakaya *et al.*, 2008), we also studied the expression of laminin in *Ric8a*$^{-/-}$ embryos. Immunohistochemical analysis of laminin-1 expression revealed that in *Ric8a*$^{-/-}$ embryos the Reichert's membrane was intact, but the labelling of the surface of visceral endoderm and of the basal surface of embryonic ectoderm was discontinuous and the BM appeared to be disorganised (Ref. I, Fig. 5). The results of laminin-1 staining have come into focus in the light of new evidence connecting RIC8A with the cell-ECM adhesion and therefore are elaborated upon in chapter 2 of this section.

Since RIC8A is functionally linked to a subset of G protein α subunits, the pathologies in the respective knockout mice that are similar to those of *Ric8a*$^{-/-}$ embryos could help to identify the Gα subunit(s) that regulate(s) this particular morphogenic event. Pinpointing a sole Gα is improbable because RIC8A has been shown to interact with $G\alpha_{i/o}$, $G\alpha_{q}$, and $G\alpha_{12/13}$ subunits, which all have multiple subtypes that in some cases can compensate for one-another (Worzfeld *et al.*, 2008). Also, more than one Gα is probably involved in these highly complex processes. Most of the characterised Gα knockout mice are viable or at least develop past gastrulation. The mutant mouse that is the most similar to *Ric8a*$^{-/-}$ is the $G\alpha_{12}$ and $G\alpha_{13}$ double knockout that dies between E8 and E8.5 (Gu *et al.*, 2002). The study where those mutants are described does not elaborate on the morphological details but does state that the allantois in these mice was short and thick and was not fused to the chorion (Gu *et al.*, 2002). $G\alpha_{12/13}$ strongly activates the small GTPase RhoA (Buhl *et al.*, 1995), and it has been shown that the loss of basal RhoA activity during the epithelial-mesenchymal transition in chick embryos leads to disruption of cell-BM interaction and subsequently to the breakdown of BM (Nakaya *et al.*, 2008). Thus, $G\alpha_{12/13}$ and RhoA might be the links through which RIC8A regulates mouse gastrulation.

1.2. Ablation of RIC8A in the neural precursor cells of mice causes cortical migration defects and is fatal in the embryonic and perinatal stages (Ref. II)

In order to circumvent the embryonic lethality and to study the developmental processes following gastrulation, the cre-lox gene knockout system was utilised to create conditional mouse lines. Since in the developing mouse embryo (E9.5–E14.5) RIC8 is mainly expressed in the nervous system, and in the adult brain, RIC8 expression is detected in a number of locations (Tõnissoo *et al.*, 2003) we focused our attention on the nervous system. To study the role of RIC8A in mouse neurogenesis, we used *Nestin* promoter-driven Cre-recombinase (Tronche *et al.*, 1999) to achieve the deletion of *Ric8a* in neural precursor cells. We were able to first detect *Nestin-Cre* expression in the neural tube from E9.5 onwards (data not shown), which is at the very beginning of neural development, and suggests that the deletion of *Ric8a* probably took place at that stage. Already at

E10.5 some of the *Nes;Ric8a*^{CKO} embryos (about 40%) displayed neural tube closure and craniofacial defects and morphologically defective brain vesicles (Ref. II Fig. 1B–D). Visual inspection suggested that the rest of the mutants (60%) developed in a rather normal manner until birth, after which they died (or were killed by their mother) within 12 h. They displayed a strong neuromuscular phenotype (Ref. II, Fig. 1J, L), were relatively immobile, but responded to tactile stimuli. Upon dissecting the brain, we noticed the enlarged cortical hemispheres in the *Nes;Ric8a*^{CKO} pups (Ref. II, Fig. 1N). This was due to the swelling of the ventricles as it was evident that the cortices were actually thinner in mutant animals (Ref. II, Fig. 2F, F'). We also observed column-like cell clusters that had invaded into the marginal zone in *Nes;Ric8a*^{CKO} mice (Ref. II, Fig. 2D, F'). Such ectopias were first observed at E14.5, the age that is considered to be the peak phase of neurogenesis (Kwan *et al.*, 2012). Visualisation of the markers for the cortical layers revealed that the ectopias in *Nes;Ric8a*^{CKO} mutants mostly contained cells from the upper layers (II–IV), although the cells from deeper layers (V and VI) were also present, especially in bigger heterotopias (Ref. II, Fig. 4). Since the neuronal overmigration could be the consequence of the discontinuity of pial BM, the coronal sections of embryonic head regions were stained with anti-laminin-1 antibody. At E12.5 no apparent defects in the BM of *Nes;Ric8a*^{CKO} embryos could be detected (Ref. II, Fig. 5A–D). However, from E14.5 (time of the appearance of first ectopias) onwards the BM was discontinuous and scattered between the pial cells (Ref. II, Fig. 5E–P). The pial BM is discussed in more detail in Chapter 2 along with the defects seen in BM of *Ric8a*$^{-/-}$ embryos during gastrulation.

Analogous experiments have been conducted by conditionally ablating $G\alpha_{12}$ and $G\alpha_{13}$ in neural precursor cells using the same *Nestin cre* construct (Tronche *et al.*, 1999). Depletion of $G\alpha_{12/13}$ results in similar neuronal overmigration as does the depletion of RIC8A. In accordance with this, the $G\alpha_{13}$ expression in *Nes;Ric8a*^{CKO} brain structures was downregulated (Ref. II, Supplementary fig. 2). Interestingly, Moers *et. al.* observed the first ectopias at E15.5 but did not find any at E14.5 (the time of the appearance of ectopias in *Nes;Ric8a*^{CKO} mice) (Moers *et al.*, 2008). In the same study, Cre under NEX promoter (Goebbels *et al.*, 2006), which restricts recombination in the principal neurons of the forebrain (cortical plate neurons), was used in addition to Nestin Cre. These mice displayed similar ectopias, thus strongly suggesting that this defect is brought about by the lack of $G\alpha_{12/13}$ in neurons and not in glial cells (Moers *et al.*, 2008). Authors of this study hypothesised that the deficiency of $G\alpha_{12}/G\alpha_{13}$ makes the neurons incapable of receiving stop signals from $G\alpha_{12}/G\alpha_{13}$ coupled receptors.

One of the candidates for mediating such stop signal is GPR56 since the lack of this orphan G protein receptor in neural precursor cells caused similar overmigrations (Li *et al.*, 2008). Later it was suggested that collagen III, a ligand for GPR56, is the major component of the BM and that the interaction of collagen III and GPR56 inhibits neuronal migration by activating the RhoA pathway in a $G\alpha_{12/13}$-dependent manner (Luo *et al.*, 2011). Since $G\alpha_{12/13}$ stimulates RhoA-dependent actomyosin-based contractility (Buhl *et al.*, 1995) it is highly likely

that the loss of this regulatory pathway interferes with the normal regulation of cell migration.

Indeed, when RhoA was ablated in the developing cerebral cortex similar overmigrations of neurons were found. In addition, heterotopias developed on the apical side creating a subcortical band heterotopia (Cappello *et al.*, 2012). The formation of the apical heterotopias was attributed to the lack of RhoA in glial cells rather than in neurons, and basal ectopias were considered to be the result of a defective neural migration since the targeted mutation of RhoA in neurons caused somewhat faster radial migration of these neurons in WT cortex (Cappello *et al.*, 2012; Cappello, 2013). Therefore, one could hypothesise that the cortical ectopias of *Nes;Ric8a*^{CKO} mice are caused by the defective $G\alpha_{12/13}$ – RhoA signalling pathway.

1.3. Ablation of RIC8A in differentiated neurons of mice results in a neuromuscular defect and early postnatal lethality (Ref. III)

Ric8a haplodeficient (*Ric8a*$^{+/-}$) mice subjected to behavioural tests displayed impaired spatial memory and increased anxiety but coordination and locomotor activity tests showed no significant differences (Tõnissoo *et al.*, 2006). Since RIC8A expression in specific regions of the adult brain (like the hippocampus, cerebellum, neocortex, etc.) (Tõnissoo *et al.*, 2003) would indicate even more severe neurological impairment in its absence we set out to gain more insight into the impact of RIC8A on the behaviour and motility of the transgenic mice. With the aim of circumventing the embryonic lethality (Ref. I) and severe mutations brought about by RIC8A deficiency in neural precursor cells (Ref. II) a conditional mouse strain with RIC8A depleted only in postmitotic differentiated neurons was generated. To this end, a *Synapsin I* promoter-driven Cre transgenic mouse strain (*SynCre*) was introduced into the floxed *Ric8a* (*Ric8aF/F*) background. In analogy with *Nes;Ric8a*^{CKO} mutants, the *Syn;Ric8a*^{CKO} mice died shortly after birth (surviving until the postnatal day 6 (P6)), and the overall appearance of these animals was also very similar to *Nes;Ric8a*^{CKO} mutants. The *Syn;Ric8a*^{CKO} displayed a strong neuromuscular phenotype: they lied on their sides, exhibited spontaneous convulsions and spasms, and were generally hypoactive, thus, our initial goal to perform behavioural experiments could not be met. In an attempt to evaluate the neuro-motor performance of *Syn;Ric8a*^{CKO} pups we performed simple handling assays. Tail suspension test revealed that the littermates spread their limbs adequately whereas *Syn;Ric8a*^{CKO} mice remained almost completely immobile (Ref. II, Fig. 2B, C). When pups were placed on their backs, the littermates turned around but mutant mice were unable to right themselves. However "pinching test" revealed that the responses to tactile and pain stimuli were not lost in mutant animals. To find a reason for the reduced mobility of *Syn;Ric8a*^{CKO} pups, we first carried out a thorough histological examination but found no morphological changes in any of the included

central nervous system regions (Ref. III, Fig. 3). We did, however, find skeletal muscle atrophy (Ref. III, Fig. 2G, H, I) and heart muscle hypoplasia (Ref III, Fig. 4B, H), which were probably caused by insufficient signalling from the nervous system where RIC8A was absent. Furthermore, we found that sinoatrial node, a pacemaker tissue in the right atrium of the heart that is responsible for the generation of normal sinus rhythm, was smaller and misplaced in *Syn;Ric8a*^{CKO} mice (Ref. III, Fig. 4D, F). Moreover, electrocardiography (ECG) measurements indicated a slower heart rate in mutants (Ref. III, data not shown). The aforementioned defects in the heart and the skeletal muscle of *Syn;Ric8a*^{CKO} pups might perhaps be the result of impaired functioning of signalling neurons – either deficient transmission in the peripheral nervous system, insufficient release of neurotransmitters in the neuromuscular junction, the absence of inhibitory signals, or a combined effect of some of these factors.

Genetic ablation of G-protein α subunits that associate with RIC8A also yields abnormalities that resemble the neuromuscular phenotype of *Nes;Ric8a*^{CKO} and *Syn;Ric8a*^{CKO} mice. For instance, in addition to neural overmigration, the ablation of $G\alpha_{12}$ and $G\alpha_{13}$ in neural precursor cells also resulted in postnatal death between P10 and P40, reduced body size, and ataxia (Moers *et al.*, 2008). Interestingly, ablation of RhoA in neural precursors caused no effect on the viability or behaviour of mice (Cappello *et al.*, 2012). Therefore, the interference with the $G\alpha_{12/13}$-RhoA signalling pathway is probably not the underlying reason for the neuromotor defects seen in *Nes;Ric8a*^{CKO} and *Syn;Ric8aCKO* mice, and $G\alpha_{12/13}$ may have functions in the nervous system that are not coupled to RhoA signalling.

To date, other RIC8A-regulated Gα subunits ($G\alpha_{q/11}$, $G\alpha_{i,o}$) have not been specifically knocked out from the nervous system, but since their mutations are not lethal at the embryonic stage, the total knockouts do provide some clues. $G\alpha_o$ is highly expressed in neurons being one of the most abundant proteins in neurons in general (Sternweis and Robishaw, 1984) where it mediates effects of a group of rhodopsin-like receptors that include the opioid, α2-adrenergic, M2 muscarinic and somatostatin receptors (Cerione *et al.*, 1986; Kleuss *et al.*, 1991). $G\alpha_o^{-/-}$ mice had poor survival with 50% surviving offspring for less than 2 months (Jiang *et al.*, 1998). In addition, although the $G\alpha_o^{-/-}$ mice had impaired motor control they were hyperactive and were continuously running in circles (Jiang 1998). Although the $G\alpha_q$/PLC-β proteins are ubiquitously expressed, the $G\alpha_q$/PLC-β mediated signalling pathway has mostly been studied within a context of cardiac function and development (LaMorte *et al.*, 1994; Wettschureck *et al.*, 2001). $G\alpha_q/G\alpha_{11}$ double knockout animals died at E11.5 whereas mutants with single active allele survived until birth and then died within a couple of hours (Offermanns *et al.*, 1998). As expected, they displayed numerous cardiac malformations, however, they also were runty and anoxic, were poorly responsive to tactile stimuli but did not have any obvious brain defects (Offermanns *et al.*, 1998). Mice lacking only $G\alpha_q$ were viable but suffered from ataxia and motor discoordination (Offermanns *et al.*, 1997). All of the Gα subunits described above seem to regulate neural functions and therefore may contribute to the neuromuscular phenotype and early lethality seen in *Nes;Ric8a*^{CKO} and *Syn;Ric8a*^{CKO} pups.

2. Basement membrane defects in *Ric8A* mutant mice (Ref. I, Ref. II)

Over the past few years, there has been a surge of new evidence linking RIC8A to cell adhesion and migration processes (see Review of literature, Chapter 3.2). Both gastrulation and neurogenesis are major events during embryonic development that involve active cell migration and depend on the proper association of cells with the ECM. Therefore, I decided to re-analyse the BM defects of both the *Ric8a*$^{-/-}$ gastrula and *Nes;Ric8a*^{CKO} cortex looking for similar tendencies in order to provide clues for the role of RIC8A in maintaining BM integrity.

In *Ric8a*$^{-/-}$ embryo we visualised laminin-1 at E7.5, the stage where there are three membranes enclosing the forming embryo. The innermost one is between the embryo and primitive streak derivatives, mesoderm, and definitive endoderm, the latter two being separated from the visceral endoderm also by a BM. The outermost membrane is the Reichert's membrane. Laminin-1 was abundantly expressed in Reichert's membrane (RM) both in the wild-type and *Ric8a*$^{-/-}$ mutant embryos at E7.5 (Ref. I, arrows in Fig. 5A–D). The recently performed 3D image analyses confirmed that the Reichert's membrane is intact in *Ric8a*$^{-/-}$ embryos (data not shown). However, the laminin-1 localisation encircling the *Ric8a*$^{-/-}$ embryos was discontinuous and disorganised and in some places even absent (Ref. I, Fig. 5B–D). In the region of the primitive streak, the BM was also fragmentary in normal embryos (Ref. I, Fig. 5A) due to detachment of the primitive streak cells from the BM during the EMT. In *Ric8a*$^{-/-}$ embryos the primitive streak was defined by the expression of its markers (Ref. I, Fig. 6I, J), therefore the fragmentary BM did not interfere with its positioning. The first step of EMT in gastrulation is the breakdown of the BM in the location of the primitive streak, which is controlled by the loss of basally localised RhoA activity (Nakaya *et al.*, 2008). Interestingly, in *Ric8a*$^{-/-}$ mES cells reduced RhoA activity has been detected (Gabay *et al.*, 2011). If the RhoA activation was impaired in *Ric8a*$^{-/-}$ embryos, that could be the reason for the breakdown of the BM in regions outside the primitive streak. It has been shown that when epithelial cells lose their connection with the BM, apoptosis is induced (Frisch and Francis, 1994) and a substantial amount of cells undergo apoptosis in *Ric8a*$^{-/-}$ embryos (Ref. I, Fig. 5F). Whether the apoptotic cells align with the breaches in the BM is not clear yet, since we did not examine the co-localisation of laminin-1 and apoptosis marker Caspase-3.

Defective BM may also be the underlying cause for the neuronal overmigration detected in forebrains of mice with RIC8A lacking in neural progenitors. In analogy with the BM defects in *Ric8a*$^{-/-}$ embryos, we found the discontinuous laminin-1 structures in *Nes;Ric8a*^{CKO} cortices starting from E14.5, when laminin-1 staining in RIC8A-deficient embryos was fragmentary, had large gaps and displayed aberrant positioning (Ref. II; Fig. 4H, white arrows). At P0, furthest that the mutants were able to develop, laminin-1-positive fragments were only detectable in BM above subarachnoid space but the pial

BM was mostly undetectable (Ref. II; Fig. 4J and 4N). The laminin-1 positive fragments were also found scattered between the cells, especially in the regions where the extent of ectopias was considerable (Fig. 4L and 4P). In conclusion, our results suggest that the lack of RIC8 in neural precursors influences the deposition of the BM component laminin-1 and the integrity of the BM.

Considering that the pial BM was assembled at the early stage of cortical development, and that it finally broke in the absence of RIC8A, it is possible that the BM cannot sustain the tension generated by the overmigrating neurons. Interestingly, when $G\alpha_{12}$ and $G\alpha_{13}$ were specifically ablated in neural precursor cells no BM defect was found at the age when neuronal overmigrations were first detected (E15.5). However when the ectopias were more prominent (E16.5) the fragmentation of the laminin structures was also observed in $G\alpha_{12/13}$ mutant mice. From these results, the authors concluded that appearance of ectopias precedes the defects in the BM (Moers *et al.*, 2008). Another study focusing on a $G\alpha_{12/13}$ coupled receptor GPR56 revealed that the loss of mouse *Gpr56* gene leads to neuronal ectopias in the cerebral cortex, and that the leading causal events are most likely the breaches in the pial BM, which in turn are associated with abnormal anchorage of radial glial endfeet (Li *et al.*, 2008). Our results also indicate that the breaches in the BM enable the overmigration and not vice versa. Since the BM is discontinuous in regions where no overmigrations can be detected in *Nes;Ric8a*^{CKO} forebrains (Ref. II, Fig. 5H and N), it is highly likely that the formation of the BM breaks precedes the ectopias and therefore is the cause and not the end result of the overmigration of neurons.

Although the gastrulation and neurogenesis are two completely different processes in development, we did observe some common characteristic BM defects in RIC8A deficient mutant mice. (1) In the absence of RIC8A laminin-1 is synthesised and secreted since the BM forms in both *Ric8a*$^{-/-}$ mice and *Nes;Ric8a*^{CKO} brains. In *Nes;Ric8a*^{CKO} mouse cortices it also seems to be correctly incorporated into the BM at first, since at E12.5 no brakes in the BM could be detected. In *Ric8a*$^{-/-}$ embryos, however, it is difficult to distinguish whether the BM is assembled correctly, because the laminin-1 defects are of varying severity, and we did not stain embryos for laminin-1 at early BM forming age (E5.5). However, the *Ric8a*$^{-/-}$ embryos were able to go through cavitation, a highly BM-dependent process (Murray and Edgar, 2000), undisturbed. In addition, in some regions of the *Ric8a*$^{-/-}$ embryo, the BM was intact and the Reichert's membrane was unaffected in all cases, which indicates that there is definitely a possibility for the correct BM assembly under RIC8A deficient conditions. (2) At E7.5 there are obvious breaks in the BM (Fig. 3, white arrows) and the laminin-1 localisation is severely disorganised in *Ric8a*$^{-/-}$ embryos. Also, in *Nes;Ric8a*^{CKO} E14.5 embryos and P0 pups the BM is similarly discontinuous and disorganised (Fig. 3F). At P0, the pial BM that is observable in the littermates (Fig. 3G) had completely disappeared in *Nes;Ric8a*^{CKO} mice and only laminin-1-positive fragments remained scattered between the cells (Fig. 3H). (3) Interestingly, in both transgenic models of RIC8A deficiency, laminin-1 localised in areas in between and around the cells (yellow arrowheads) instead

of forming a continuous layer. Although this was most prominent in the pial BM of the *Nes;Ric8a*^{CKO} E14.5 cortices and in the visceral endoderm BM of the *Ric8*$^{-/-}$ embryos, it was noticeable in other regions as well. Cells surrounded by laminin-1 in an analogous manner were also present in the primitive streak region of the WT E7.5 embryo (Fig. 3C). Cells in the primitive streak region lose polarity, which induces the breakdown of the BM (Nakaya *et al.*, 2008). Thus, aberrant laminin localisation accompanying the RIC8A deficiency also implies a defect in the epithelial tissue polarity associated with the malfunctioning of the RhoA pathway (Cappello *et al.*, 2012; Daley *et al.*, 2012).

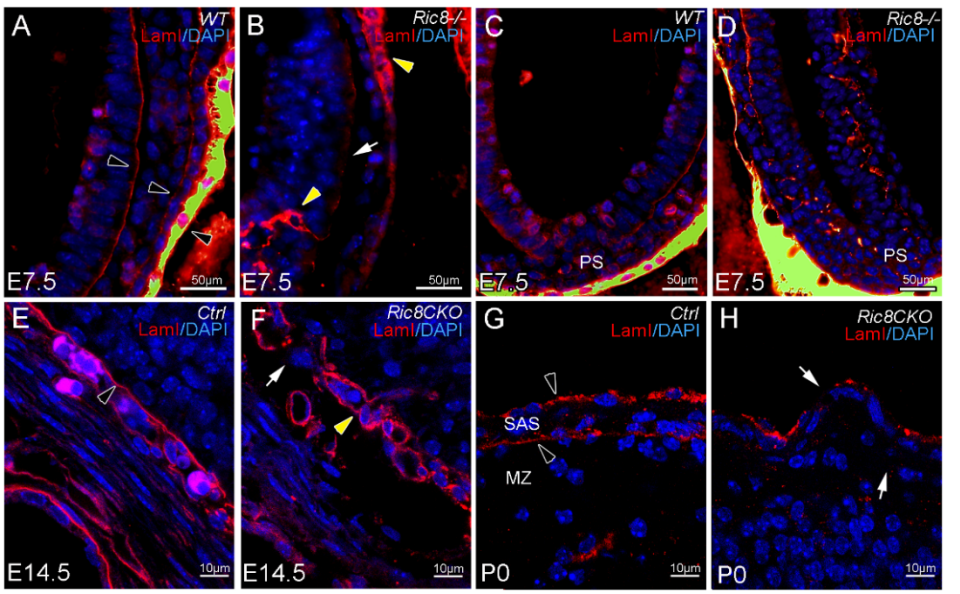

Figure 3. Laminin-1 localisation in E7.5 gastrula and in E14.5 and P0 cortices. (A) Laminin-1 staining showing an intact BM in wt embryo and (B) various BM defects in *Ric8a*$^{-/-}$ embryos. (C) Discontinuous laminin-1 staining at the site of the primitive streak and (D) highly aberrant laminin staining outside of the primitive streak region. (E) The correctly aligned laminin-1 in the meninges of E14.5 littermate controls and (F) discontinuous laminin deposited between the cells in the meninges of *Nes;Ric8a*^{CKO} mice from the same age. (G) Aligned laminin-1 of the P0 littermate cortex visualising the pial BM (lower) and the BM above the subarachnoidal space. (H) The highly discontinuous BM above the subarachnoidal space and the barely visible pial BM of the P0 *Nes;Ric8a*^{CKO} forebrains. Black arrowheads – intact laminin 1 staining, yellow arrowheads – the misaligned laminin 1 staining, white arrow – the breaks in the laminin 1 expression. Red – Laminin I, yellow – overexposed Laminin I blue – DAPI. Abbreviations: MZ – marginal zone, PS – primitive streak, SAS – subarachnoidal space.

3. The role of RIC8A in the regulation of actin cytoskeleton and cell-matrix adhesion in mES and MEF cells (Ref. IV)

In order to expand on the results obtained *in vivo*, primary mouse embryonic stem cells (mES) and mouse embryonic fibroblasts (MEF) were utilised to study the role of RIC8A on a cellular level. Previously we tried to derive neural stem cells from *Ric8a*$^{-/-}$ mES cells. Indeed, the *Ric8a*$^{-/-}$ mES cells were able to differentiate as verified by the expression of neural stem cell markers (data not shown). However, during the feeder-free culturing of mES cells that is necessary for the differentiation process, we noticed that while the control cells formed a monolayer on the gelatine-coated plastic most of the *Ric8a*$^{-/-}$ cells grew in round multilayer colonies (Ref. VI, Fig. 1G). This sparked our interest since *Ric8*$^{-/-}$ colonies showed no apparent morphological defects when cultured on feeders indicating that the absence of RIC8A somehow might influence the cell-matrix adhesion. We visualised the actin cytoskeleton of the cells on gelatine with phalloidin and saw that while cells at the edges of the wt colonies spread out and formed polymerised actin bundles called stress fibres, no such structures could be found in *Ric8a*$^{-/-}$ cells (Ref. VI, Fig. 2B, F). In order to further expand our studies on the role of RIC8A in cell-matrix adhesion-dependent cellular events we established RIC8A-deficient primary MEF cells and used them in cellular and biochemical experiments. Similarly to the *Ric8a*$^{-/-}$ mES cells, no polymerised actin structures formed in MEFs except at the cortical regions of the adherent cells (Ref. IV, Fig. 2D). Outside stimulus in the form of PDGF did not induce actin cytoskeleton remodelling in *Ric8a*$^{-/-}$ mES cells (Ref. IV, Fig. 2J) or in RIC8A deficient MEFs (Ref. IV, data not shown). It is, therefore, evident that RIC8A is required for the correct organisation of the actin cytoskeleton in both mES and MEFs. The reduction of polymerised actin level in *Ric8a*$^{-/-}$ mES cells has been quantified in an earlier study showing a 70% drop in F-actin levels (Gabay *et al.*, 2011). To gain further insight into the role of RIC8A in the adhesion-related events we analysed the formation and presence of focal adhesion complexes and the localisation of integrin β1 upon cell adhesion to ECM components. Our results showed that in the absence of RIC8A focal adhesion complexes did not form and the cell surface integrin β1 was distributed randomly in the plasma membrane (Reg. IV, Fig. 3 B, D), whereas in the RIC8A-expressing cells under same conditions it had accumulated into aggregates (Ref. IV, Fig. 3A, C). In accordance with our results, the reduction of focal adhesion complexes in RIC8A-deficient conditions was also detected in *X. laevis* neural crest cells (Fuentealba *et al.*, 2013).

Aforementioned results suggest that the lack of RIC8A severely impairs the general adhesion-induced cellular processes. However, we did not find significant differences in the adhesion rates of RIC8A-deficient cells on any tested substrates (Ref. IV, Fig. 4A, B). Thus, the substrate recognition of RIC8A-

deficient cells under our experimental conditions was not defective. However, previously it has been shown that the downregulation of RIC8A in *X. laevis* resulted in reduced adhesion of neural crest cells to fibronectin (Fuentealba *et al.*, 2013), and upon its depletion from mouse neural progenitor cells reduced adhesion to laminin was observed (Ma *et al.*, 2012). Therefore, it seems that in addition to changes in the adhesive properties, the deficiency of RIC8A can also impair the association of cells with the substrate under certain experimental conditions. In order to analyse whether the observed defects in the organisation of actin cytoskeleton and the formation of focal adhesions affect cell migration, we used a modified Boyden chamber assay in which the lower side of the polycarbonate membrane was coated with an integrin ligand and serum-starved cells were seeded to the upper compartment. To assess haptotaxis, the lower compartment of the chamber contained serum-free media so that cell migration was driven exclusively by the substrate. Under these conditions the migration of *Ric8a*$^{-/-}$ mES cells was reduced on laminin 521 but not on collagen IV or fibronectin (Ref. IV, Fig. 4C). Likewise, the migration of RIC8A-deficient MEFs was unaffected on fibronectin, however, compared to the RIC8A-expressing cells it was reduced on collagen I (Ref. IV, Fig. 4D). In addition, we tested the chemotactic response of these cells to stimulation with foetal bovine serum (FBS). Surprisingly, we found that the migration of RIC8A-deficient cells was enhanced on type IV and type I collagen, and on laminin 521 compared to control cells but, similarly to the haptotaxis experiments, not on fibronectin. These results indicate that RIC8A has a role in the regulation of cell migration, which is dependent on the ECM substrate. Laminins, collagens, and fibronectin all bind different sets of integrins, and this might be the underlying reason for the observed differences. Integrin heterodimers that bind to laminin and collagen belong to the β1 integrin subfamily, fibronectin, on the other hand, mostly binds receptors belonging to RGD-binding integrins that differ from those interacting with laminins and collagens (Humphries *et al.*, 2006). Interestingly, the downregulation of RIC8A in MEFs has been shown to reduce the receptor tyrosine kinase (RTK) mediated cell migration on fibronectin in response to PDGF (Wang *et al.*, 2011). We repeated the experiment in our experimental set-up and saw a similar tendency but this did not reach a statistical significance. However, in addition to fibronectin, we assayed the PDGF-induced migration on collagen I and found that it was also reduced in RIC8A deficient conditions (Ref. IV, data not shown). These results suggest that RIC8A might have an additional, RTK-dependent and substrate-independent role in the regulation of cell migration.

Considering the lack of detailed information about the possible connection between RIC8A and integrins, we chose to study the putative role of RIC8A in the regulation of β1 integrin function. We found that activation of integrin β1 upon the binding of MEFs to type I collagen was decreased in the absence of RIC8A, and the activating phosphorylation of AKT downstream of integrins was also decreased, which correlates with the reduction of integrin activity (Ref. IV, Fig. 5). This indicates that RIC8A is required for the regulation of integrin

adhesive and signalling functions in MEFs. $G\alpha_{13}$, one of the target proteins of RIC8A, has been shown to interact with integrins. Namely, it has been shown to bind to the cytoplasmic domains of the platelet integrin β3 subunit (Gong *et al.*, 2010), and the β1 integrin subunit (Shen *et al.*, 2015). These type of interactions were suggested to lead to the transient inactivation of RhoA and the concomitant activation of Src required for the initial cell spreading and migration (Gong *et al.*, 2010; Shen *et al.*, 2015). Therefore, it seems that $G\alpha_{13}$ plays a dual role in the regulation of RhoA by both stimulating it through GPCR-activated pathways and inhibiting it via integrin outside-in signalling (Shen *et al.*, 2015). The RhoA activity assay using our experimental setup produced similar initial results: RIC8A deficiency leads to the downregulation of $G\alpha_{13}$ (Ref. IV, Fig. 1F), the reduction of RhoA activity, and it precludes the transient inhibition of RhoA activity upon integrin β1 stimulation (Ref. IV, data not shown).

4. Impaired cell-matrix adhesion as one of the underlying causes for phenotypic defects seen in Ric8a-deficient mice (Ref. I, Ref. II, Ref. IV)

Focal adhesion complexes did not form in RIC8A-deficient cells. Therefore analysing the expression of the different focal adhesion components in *Ric8a*$^{-/-}$ knockout mice could provide insights into the observed developmental defects. Talin is one of the cytoplasmic proteins that links cell adhesion molecules to the actin cytoskeleton (Jockusch *et al.*, 1995; Priddle *et al.*, 1998), and is responsible for integrin activation (Tadokoro *et al.*, 2003). In analogy with *Ric8a*$^{-/-}$ mES cells, the *talin*$^{-/-}$ mES cells also lacked stress fibres, focal adhesions, and the colonies displayed a round morphology on gelatine (Monkley *et al.*, 2000). Developmental defects of *talin*$^{-/-}$ embryos were very similar to *Ric8*$^{-/-}$ embryos and the lack of talin resulted in severe disorganisation of embryos at gastrulation and death around E8.5–9.5. Similarly, the *talin*$^{-/-}$ embryos were smaller in size, and though the mesoderm and extraembryonic tissues had formed, they were completely disorganised referring to defective cell migration during gastrulation (Monkley *et al.*, 2000). Interestingly, in the absence of vinculin, a binding partner of talin, mES cells were still able to form stress fibres and talin-containing focal adhesion complexes on fibronectin but formed similar round colonies on gelatine as the *talin*$^{-/-}$ (Priddle *et al.*, 1998) and *Ric8a*$^{-/-}$ mES cells. The embryos lacking another major protein of actin cytoskeleton formation, vinculin, had a somewhat milder phenotype. The *vinculin*$^{-/-}$ embryos started lagging behind in size at E8.5 and survived until E10.5 with the most prominent defects in organogenesis being the defective neural tube closure and an underdeveloped heart (Xu and Baribault, 1998). Interestingly, *vinculin*$^{-/-}$ MEFs, similarly to the RIC8A-deficient MEFs, migrated at about twice the rate of WT cells towards fibronectin in response to stimulation with FBS (Xu *et al.*, 1998). Paxillin deficient embryos also died around E9.5 although the malforma-

tions were again not as severe as in *Ric8a*$^{-/-}$ and *talin*$^{-/-}$ embryos: *paxillin*$^{-/-}$ embryos were smaller from E8.5 onward having no obvious heart structure and displaying an abnormal headfold (Hagel *et al.*, 2002). The absence of focal adhesion kinase (FAK) also leads to a rather similar phenotype: *FAK*$^{-/-}$ embryos die at around E8.5–E9.0 and the malformations that are obvious at E8.5 manifest as deficits in the development of mesenchymal derivatives including the absence of somites, a rudimentary non-beating heart or no heart at all, and the lack of functional blood vessels (Ilic *et al.*, 2003). Mice with a null mutation in Tensin, another component of focal adhesion complexes, were viable (Lo *et al.*, 1997). Nevertheless, these null mutations of focal adhesion proteins further confirm that the defects seen in *Ric8a*$^{-/-}$ embryos are related to the impaired cell-matrix adhesion. Importantly, the strongest similarity to *Ric8a*$^{-/-}$ embryos was that of the embryos lacking talin, a protein directly interacting with and mediating the activation of integrins (Tadokoro *et al.*, 2003).

RIC8A deficiency led to defects in cell migration on integrin substrates, flawed clustering of activated β1 integrins on the plasma membrane and the reduced activation of cell-surface β1 integrins. Therefore, it is highly likely that the ablation of RIC8A *in vivo* would result in some form of a deficit in integrin regulation. Integrin family contains an array of α and β subunits that are expressed in a tissue-specific manner. β1 integrin is ubiquitously expressed and its knockout mice die already before gastrulation (Fässler and Meyer, 1995; Stephens *et al.*, 1995). This is probably due to a complete absence of BM in β1-null embryos (Aumailley *et al.*, 2000) since the laminin γ1-deficient embryos also lack BM and die in the same peri-implantation stage (Smyth *et al.*, 1999). The presence of β1 integrin thus appears to be crucial for the formation of the BM. Although the absence of β1 integrin results in a more severe embryonic phenotype than that of RIC8A, it is possible that the BM defect in *Ric8*$^{-/-}$ embryos may be a result of interfered regulation of integrin recruitment and activation. Laminin-binding α3 and α6 integrins have been shown to have a function in the brain tissue and their ablation results in rather similar defects in the brain as those found in the brains of *Nes;Ric8a*^{CKO} mice. *α3*$^{-/-}$ mice display a defect in neuronal migration and a disorganized layering of the cerebral cortex suggesting that this integrin is involved in the radial migration of neuronal cells (Anton *et al.*, 1999). *α6*$^{-/-}$ mice also have abnormalities in the laminar organization of the developing cerebral cortex, in addition, they display ectopic clusters of overmigrated neurons and an abnormal laminin deposition (Georges-Labouesse *et al.*, 1998). Therefore, the misregulation of α6 integrin may be one of the reasons for the cortical ectopias seen in *Nes;Ric8a*^{CKO} mice.

Our results on the downregulation of $G\alpha_{13}$ levels and reduced activation of RhoA observed in RIC8A-deficient cells confirmed those obtained earlier (Gabay *et al.*, 2011). The *in vivo* impact of the downregulation of both $G\alpha_{13}$ and RhoA have been already discussed in chapters 1.2 and 1.3. RIC8A may be involved in the regulation of many cellular processes, and its absence in mouse results in developmental defects seen during gastrulation and neurogenesis: insufficient recognition of migratory clues mediated by $G\alpha_{12/13}$ coupled receptors; impaired

RhoA-mediated actomyosin contractility; failure in the formation of RhoA-dependent epithelial tissue polarity and the consequent defective deposition of the BM; and faulty interaction of cells with extracellular substrates. More studies are needed to unravel the exact processes RIC8A is involved in on a molecular level. However, based on the published research and on our own studies it can be concluded that the regulation of cell-BM contact appears to include a similar general mechanism during gastrulation and neurogenesis, both of which comprise major events in cell migration during development. In addition, integrins, RhoA, $G\alpha_{12/13}$ and RIC8A all seem to be the key components involved in a pathway mediating the cell-ECM contact and the proper organisation of actin cytoskeleton in these processes.

CONCLUSIONS

The main objective of the research presented in this dissertation was to elucidate the role of RIC8A in the mouse development by utilising *Ric8a* knockout mice and to substantiate the *in vivo* findings with assays in cell culture using RIC8A deficient primary mouse cells.

The main results of this thesis can be summarised as follows:

1. Homozygous deletion of *Ric8a* results in an impaired gastrulation process and embryonic lethality at E6.5–E8.5. The *Ric8a*$^{-/-}$ epiblast differentiated into cells expressing mesoderm and ectoderm markers, but the localisation of relevant cells was disorganised because of flawed morphogenetic movements during gastrulation.
2. The deletion of *Ric8a* in neural precursor cells leads to a cortical over-migration defect. Starting from E14.5 the column-like cell clusters that had invaded into the marginal zone could be observed in the developing cortices in RIC8A deficient mice.
3. The expression of RIC8A is necessary for the integrity of the basement membrane (BM). The aforementioned migration defects were accompanied by a defective BM. The BM marker laminin-1 was synthesised and secreted, but its expression was discontinuous and disorganised.
4. The deletion of *Ric8a* form differentiated neurons leads to early postnatal death (P6) of mutant mice and causes a severe neuromuscular defect characterised by reduced movement, skeletal muscle atrophy, heart defects, and deceleration of the heart rate. Lack of RIC8A in neural precursor cells results in neonatal death (P0) and a similar phenotype.
5. RIC8A deficient cells were unable to form focal adhesion complexes and to organise actin into stress fibres. In addition, the activation of β1 integrin, its downstream signalling, and integrin β1 dependent cell migration were impaired in RIC8A deficient cells.

Investigation of the function of RIC8A on the level of cells and an organism was partly motivated by the necessity to test the relevance of its biochemical properties described in *in vitro* assays. Our results support the proposed function for RIC8A as a chaperone for Gα subunits since $G\alpha_{13}$ levels were downregulated under RIC8A-deficient conditions, both *in vivo* and in the primary cells. Moreover, β1 integrins, RhoA, $G\alpha_{13}$, and RIC8A are most likely involved in a signalling pathway or pathways that mediate the contact between the cell and the extracellular matrix and the proper organisation of the actin cytoskeleton. Therefore, the migration and BM defects seen in RIC8A deficient mouse models might be caused by defective RhoA and/or integrin β1 activation. The neuromuscular defects seen in the nervous system-specific *Ric8a*$^{-/-}$ mutants are probably induced by the downregulation of various Gα proteins and subsequent signalling defects in the peripheral nervous system.

SUMMARY IN ESTONIAN

RIC8A roll hiire arengus ja funktsioon rakk-maatriks adhesioonis ning aktiini tsütoskeleti organiseerimises

Hulkrakse organismi normaalse elutegevuse tagamiseks on oluline, et rakud saaksid omavahel vahetada infot kas otsese või kaudse kommunikatsiooni vahendusel. Transmembraansed G-valk-seoselised retseptorid (GPCR-d) ja nendega interakteeruvad G valgud (guaniin-nukleotiidi siduvad valgud) on üks levinumaid väliskeskkonnast saadava info kanaleid läbi mille reguleeritakse mitmeid arengulisi, füsioloogilisi ja käitumuslikke protsesse. G valgud on heterotrimeersed valgud, mis koosnevad kolmest erinevast subühikust (α, β, γ) ning moodustavad GPCR-iga plasmamembraani siseküljel kompleksi. GPCR-d võtavad vastu infot mitmetelt signaalmolekulidelt nagu näiteks hormoonid, neurotransmitterid ja kasvufaktorid aktiveerides seeläbi G valgud, mis omakorda käivitavad rakusisese signaalikaskaadi. Sellisel viisil kutsutakse rakkudes esile vastus, milleks võib olla näiteks migratsioon, jagunemine, või aktsioonipotentsiaali tekkimine. RIC8 on nende signaalikaskaadide asendamatu komponent. Täpsemalt on RIC8-l kirjeldatud kahte peamist funktsiooni: heterotrimeerse G valgu α subühiku aktiivsuse reguleerimine (nukleotiidivahetusfaktori roll) ning Gα korrektse koguse ja membraanse asetuse tagamine rakus (*chaperon*'i roll). Selgroogsetes on kaks RIC8 homoloogi, RIC8A ja RIC8B, mis on funktsioonilt sarnased, kuid erinevad mõningal määral selles, milliste Gα subühikutega nad seonduvad. (Tall *et al.,* 2003, Chan *et al.,* 2011, Gabay *et al.,* 2011).

Käesoleva töö esimene eesmärk oli uurida RIC8A-puudulike hiirte arengut selgitamaks selle valgu rolli hiire organismis. Leidsime, et RIC8A-puudulikud hiired surevad gastrulatsiooni faasis (E6.5–8.5) ning seega seadsime sihi analüüsida *Ric8a*$^{-/-}$ embrüotel esinevaid morfoloogilisi häireid. Selle käigus avastasime, et RIC8A puudulikud embrüod on võimelised gastrulatsiooni alustama, sest neil moodustub ürgjutt, millest epiblasti rakud ka läbi migreeruvad. Samuti olid epiblasti rakud võimelised diferentseeruma, kuna nad ekspresseerisid mesodermi ja ektodermi markereid. Siiski ei suutnud *Ric8a*$^{-/-}$ looted gastrulatsiooni läbida, sest mesoderm ei organiseerunud korrektselt ning olulised mesodermaalsed struktuurid olid kas defektsed või ei moodustunud üldse. Seega on RIC8A-puudulike hiirte arengu peatumine gatrulatsioonis tõenäoliselt tingitud häiretest morfogeneetilistes liikumistes.

Doktoritöö teine eesmärk oli ajendatud varasematest tulemustest, mis näitasid, et hiire lootes (E9.5–E12.5) on RIC8A peamiselt ekspresseeritud arenevas närvisüsteemis (Tõnissoo *et al.*, 2003). Ka täiskasvanud hiire ajus on RIC8A ekspresseeritud kindlates piirkondades nagu hipokampus ja eesaju koor (Tõnissoo *et al.*, 2003). Lisaks selgus *Ric8a* heterosügootsete hiirtega tehtud käitumiskatsetest, et geeni puudumine ühelt alleelilt põhjustab suurenenud ärevust ning ruumilise mälu halvenemist (Tõnissoo *et al.*, 2006). Nendele tulemustele tuginedes võib oletada, et RIC8A mängib närvisüsteemi arengus ja toimimises olulist rolli, mille uurimiseks kasutasime edasises töös kahte erinevat RIC8A puudulikku

hiireliini. RIC8A rolli kirjeldamiseks närvisüsteemi arengus lõigati geen välja neuraalsetes eellasrakkudes. Need hiired sündisid, kuid surid päeva jooksul pärast sündi. Teise mutantse hiireliini tekitasime selleks, et täpsemalt uurida *Ric8a*$^{+/-}$ hiirtel ilmnenud häireid käitumises. Selleks lõime hiireliini kus *Ric8a* oli inaktiveeritud diferentseerunud neuronites. Ka need mutantsed hiired surid mõni päev pärast sündi ning seega ei saanud käitumiskatseid teostada. Mõlema närvisüsteemi-spetsiifilise mutandi puhul tekkis RIC8A puudusel tugev neuromuskulaarne fenotüüp, mida iseloomustas liikumisvõime puudumine, tõmblused ning treemor. Hiirtes, kus RIC8A oli puudu diferentseerunud neuronites, kujunes välja skeleti- ja südamelihaste atroofia kuid aju morfoloogias kõrvalekaldeid ei leitud. Neuraalsete eellasrakkude-spetsiifilises *Ric8a* mutandis oli ajukoor õhem ning kohati olid närvirakud migreerunud oma loomulikust anatoomilisest lookusest välja marginaaltsooni moodustades nn kortikaalsed ektoopiad.

Seega oli RIC8A puudusel rakkude migratsioon häiritud nii gastrulatsiooni kui neurogeneesi käigus. Basaalmembraani (BM) ühe peamise komponendi laminiin-1 ekspressioon näitas, et BM oli nii *Ric8a*$^{-/-}$ hiires kui RIC8A-puudulikus ajukoores katkendlik ning paiknes kaootiliselt rakkude ümber, mitte ühe kihina epiteelrakkude basaalsel poolel. Ülaltoodust võib järeldada, et RIC8A puudusel tekkivad migratsioonidefektid võivad olla põhjustatud häiretest raku ja ekstratsellulaarse maatriksi (ECM) vahelistes interaktsioonides. Sellest lähtuvalt püstitus käesoleva töö kolmas eesmark – uurida RIC8A rolli rakk-ECM-i adhesioonis kasutades hiire RIC8A-puudulikke primaarseid rakke. RIC8A puudusel ei koondunud rakupinna β1 integriinid klastritesse ning samuti ei moodustunud rakk-maatriks adhesioonil tekkivaid olulisi struktuure nagu fokaalse adhesiooni kompleksid ning aktiini stressi fiibrid. Lisaks oli RIC8A puudusel häiritud rakupinna β1 integriinide aktiveerumine ning sellest sõltuva signaaliraja aktiveerumine rakus.

RIC8A funktsiooni uurimine rakkudes ja hiire organismis on osaliselt motiveeritud vajadusest testida selle valgu *in vitro* tingimustes kirjeldatud biokeemiliste omaduste paikapidavust. Leidsime, et RIC8A puudusel on $G\alpha_{13}$ tase alla reguleeritud nii hiire koes *in vivo* kui primaarsetes rakkudes, mis kinnitab RIC8A rolli Gα subühiku korrektse hulga tagamises (*chaperon*'ina). Käesoleva doktoritöö tulemused koos eelnevalt avaldatud materjaliga viitavad sellele, et β1 integriinid, RhoA, $G\alpha_{13}$ ja RIC8A on omavahel seotud signaalirajas (või radades), mis reguleerivad raku adhesiooni ECM-ile ning sellest sõltuvat aktiini tsütoskeleti organiseerumist. Seega *Ric8a* mutantidel esinenud kõrvalekalded rakumigratsioonis ja BM terviklikkuses võivad olla tingitud RhoA ja β1 integriinide häiritud aktivatsioonist. Neuromuskulaarsed defektid, mis esinesid närvisüsteemi-spetsiifilisel *Ric8a* mutantidel, on tõenäoliselt põhjustatud ühe või mitme Gα subühiku taseme alla reguleerimisest ja sellest tingitud puudulikust signaaliülekandest perifeerses närvisüsteemis.

REFERENCES

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell.

Amoureux, M. C., Nicolas, S., and Rougon, G. (2012). NCAM180 regulates Ric8A membrane localization and potentiates β-adrenergic response. PLoS One *7*.

Anton, E. S., Kreidberg, J. A., and Rakic, P. (1999). Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. Neuron *22*, 277–289.

De Arcangelis, A., Mark, M., Kreidberg, J., Sorokin, L., and Georges-Labouesse, E. (1999). Synergistic activities of alpha3 and alpha6 integrins are required during apical ectodermal ridge formation and organogenesis in the mouse. Development *126*, 3957–3968.

Aumailley, M., Pesch, M., Tunggal, L., Gaill, F., and Fässler, R. (2000). Altered synthesis of laminin 1 and absence of basement membrane component deposition in (beta)1 integrin-deficient embryoid bodies. J. Cell Sci. *113 Pt 2*, 259–268.

Bedzhov, I., Graham, S. J. L., Leung, C. Y., and Zernicka-Goetz, M. (2014). Developmental plasticity, cell fate specification and morphogenesis in the early mouse embryo. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *369*.

Belkin, A. M., and Smalheiser, N. R. (1996). Localization of cranin (dystroglycan) at sites of cell-matrix and cell-cell contact: recruitment to focal adhesions is dependent upon extracellular ligands. Cell Adhes. Commun. *4*, 281–296.

Belousov, L. V. (2011). Scott F. Gilbert – Developmental Biology, 2010, Sinauer Associates, Inc., Sunderland, MA Ninth Edition. Russ. J. Dev. Biol. *42*, 349–349.

Boularan, C., Hwang, I.-Y., Kamenyeva, O., Park, C., Harrison, K., Huang, Z., and Kehrl, J. H. (2015). B Lymphocyte-Specific Loss of Ric-8A Results in a Gα Protein Deficit and Severe Humoral Immunodeficiency. J. Immunol. *195*, 2090–2102.

Buccione, R., Orth, J. D., and McNiven, M. A. (2004). Foot and mouth: podosomes, invadopodia and circular dorsal ruffles. Nat. Rev. Mol. Cell Biol. *5*, 647–657.

Buhl, A. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995). G alpha12 and G alpha13 Stimulate Rho-dependent Stress Fiber Formation and Focal Adhesion Assembly. J. Biol. Chem. *270*, 24631–24634.

Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2000). Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling. J. Biol. Chem. *275*, 22607–22610.

Cappello, S. Böhringer C. R., Bergami M., Conzelmann K. K., Ghanem A., Tomassy G. S., Arlotta P., Mainardi M., Allegra M., Caleo M., van Hengel J., Brakebusch C., Götz M. (2012). A Radial Glia-Specific Role of RhoA in Double Cortex Formation. Neuron *73*, 911–924.

Cappello, S. (2013). Small Rho-GTPases and cortical malformations: fine-tuning the cytoskeleton stability. Small GTPases *4*, 51–56.

Cerione, R. A., Regan, J. W., Nakata, H., Codina, J., Benovic, J. L., Gierschik, P., Somers, R. L., Spiegel, A. M., Birnbaumer, L., and Lefkowitz, R. J. (1986). Functional reconstitution of the alpha 2-adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. J. Biol. Chem. *261*, 3901–3909.

Chan, P., Gabay, M., Wright, F. a, and Tall, G. G. (2011). Ric-8B is a GTP-dependent G protein alphas guanine nucleotide exchange factor. J. Biol. Chem. *286*, 19932–19942.

Chan, P., Thomas, C. J., Sprang, S. R., and Tall, G. G. (2013). Molecular chaperoning function of Ric-8 is to fold nascent heterotrimeric G protein α subunits. Proc. Natl. Acad. Sci. U. S. A. *110*, 3794–3799.

Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D. E. (1997). Geometric control of cell life and death. Science *276*, 1425–1428.

Cooper, A. R., and MacQueen, H. A. (1983). Subunits of laminin are differentially synthesized in mouse eggs and early embryos. Dev. Biol. *96*, 467–471.

Costell, M., Gustafsson, E., Aszódi, A., Mörgelin, M., Bloch, W., Hunziker, E., Addicks, K., Timpl, R., and Fässler, R. (1999). Perlecan maintains the integrity of cartilage and some basement membranes. J. Cell Biol. *147*, 1109–1122.

Coucouvanis, E., and Martin, G. R. (1995). Signals for death and survival: A two-step mechanism for cavitation in the vertebrate embryo. Cell *83*, 279–287.

Daley, W. P., Gervais, E. M., Centanni, S. W., Gulfo, K. M., Nelson, D. A., and Larsen, M. (2012). ROCK1-directed basement membrane positioning coordinates epithelial tissue polarity. Development *139*, 411–422.

David, N. B., Martin, C. a, Segalen, M., Rosenfeld, F., Schweisguth, F., and Bellaïche, Y. (2005). Drosophila Ric-8 regulates Gαi cortical localization to promote Gαi-dependent planar orientation of the mitotic spindle during asymmetric cell division. Nat. Cell Biol. *7*, 1083–1090.

Domogatskaya, A., Rodin, S., and Tryggvason, K. (2012). Functional diversity of laminins. Annu. Rev. Cell Dev. Biol. *28*, 523–553.

Durbeej, M., Henry, M. D., and Campbell, K. P. (1998). Dystroglycan in development and disease. Curr. Opin. Cell Biol. *10*, 594–601.

Erickson, A. C., and Couchman, J. R. (2000). Still more complexity in mammalian basement membranes. J. Histochem. Cytochem. *48*, 1291–1306.

Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature *345*, 315–319.

Figueroa, M., Hinrichs, M. V., Bunster, M., Babbitt, P., Martinez-Oyanedel, J., and Olate, J. (2009). Biophysical studies support a predicted superhelical structure with armadillo repeats for Ric-8. Protein Sci. *18*, 1139–1145.

Frisch, S. M., and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. *124*, 619–626.

Fuentealba, J Toro-Tapia G., Arriagada C., Riquelme L., Beyer A., Henriquez J. P., Caprile T., Mayor R., Marcellini S., Hinrichs M. V., Olate J., Torrejón M.. (2013). Ric-8A, a guanine nucleotide exchange factor for heterotrimeric G proteins, is critical for cranial neural crest cell migration. Dev. Biol. *378*, 74–82.

Fujiwara, T., Dunn, N. R., and Hogan, B. L. (2001). Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. Proc. Natl. Acad. Sci. U. S. A. *98*, 13739–13744.

Fässler, R., and Meyer, M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev. *9*, 1896–1908.

Gabay, M., Pinter, M. E., Wright, F. A., Chan, P., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., and Tall, G. G. (2011). Ric-8 Proteins Are Molecular Chaperones That Direct Nascent G Protein Subunit Membrane Association. Sci. Signal. *4*, ra79–ra79.

Gelse, K., Pöschl, E., and Aigner, T. (2003). Collagens – structure, function, and biosynthesis. Adv. Drug Deliv. Rev. *55*, 1531–1546.

Georges-Labouesse, E., Mark, M., Messaddeq, N., and Gansmüller, A. (1998). Essential role of alpha 6 integrins in cortical and retinal lamination. Curr. Biol. *8*, 983–986.

Goebbels, S., Bormuth, I., Bode, U., Hermanson, O., Schwab, M. H., and Nave, K.-A. (2006). Genetic targeting of principal neurons in neocortex and hippocampus of NEX-Cre mice. Genesis *44*, 611–621.

Gohla, a, Offermanns, S., Wilkie, T. M., and Schultz, G. (1999). Differential involvement of Galpha12 and Galpha13 in receptor-mediated stress fiber formation. J. Biol. Chem. *274*, 17901–17907.

Goley, E. D., and Welch, M. D. (2006). The ARP2/3 complex: an actin nucleator comes of age. Nat. Rev. Mol. Cell Biol. *7*, 713–726.

Gong, H., Shen, B., Flevaris, P., Chow, C., Lam, S. C.-T., Voyno-Yasenetskaya, T. A., Kozasa, T., and Du, X. (2010). G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling. Science *327*, 340–343.

Goode, B. L., and Eck, M. J. (2007). Mechanism and function of formins in the control of actin assembly. Annu. Rev. Biochem. *76*, 593–627.

Graus-Porta, D., Blaess, S., Senften, M., Littlewood-Evans, A., Damsky, C., Huang, Z., Orban, P., Klein, R., Schittny, J. C., and Müller, U. (2001). Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron *31*, 367–379.

Gu, J. L., Muller, S., Mancino, V., Offermanns, S., and Simon, M. I. (2002). Interaction of G 12 with G 13 and G q signaling pathways. Proc. Natl. Acad. Sci. *99*, 9352–9357.

Gupta, M., Sarangi, B. R., Deschamps, J., Nematbakhsh, Y., Callan-Jones, A., Margadant, F., Mège, R.-M., Lim, C. T., Voituriez, R., and Ladoux, B. (2015). Adaptive rheology and ordering of cell cytoskeleton govern matrix rigidity sensing. Nat. Commun. *6*, 7525.

Hagel, M., George, E. L., Kim, A., Tamimi, R., Opitz, S. L., Turner, C. E., Imamoto, A., and Thomas, S. M. (2002). The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol. Cell. Biol. *22*, 901–915.

Halfter, W., Dong, S., Yip, Y.-P., Willem, M., and Mayer, U. (2002). A critical function of the pial basement membrane in cortical histogenesis. J. Neurosci. *22*, 6029–6040.

Hampoelz, B., Hoeller, O., Bowman, S. K., Dunican, D., and Knoblich, J. a (2005). Drosophila Ric-8 is essential for plasma-membrane localization of heterotrimeric G proteins. Nat. Cell Biol. *7*, 1099–1105.

Hatzfeld, M. (1999). The armadillo family of structural proteins. Int. Rev. Cytol. *186*, 179–224.

Hatten, M. E. (1999). Central nervous system neuronal migration. Annu. Rev. Neurosci. *22*, 511–539.

Henry, M. D., and Campbell, K. P. (1999). Dystroglycan inside and out. Curr. Opin. Cell Biol. *11*, 602–607.

Humphries, J. D., Byron, A., and Humphries, M. J. (2006). Integrin ligands at a glance. J. Cell Sci. *119*, 3901–3903.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell *110*, 673–687.

Ilic, D., Kovacic, B., McDonagh, S., Jin, F., Baumbusch, C., Gardner, D. G., and Damsky, C. H. (2003). Focal adhesion kinase is required for blood vessel morphogenesis. Circ. Res. *92*, 300–307.

Jiang, M., Gold, M. S., Boulay, G., Spicher, K., Peyton, M., Brabet, P., Srinivasan, Y., Rudolph, U., Ellison, G., and Birnbaumer, L. (1998). Multiple neurological

abnormalities in mice deficient in the G protein Go. Proc. Natl. Acad. Sci. U. S. A. *95*, 3269–3274.

Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rüdiger, M., Schlüter, K., Stanke, G., and Winkler, J. (1995). The molecular architecture of focal adhesions. Annu. Rev. Cell Dev. Biol. *11*, 379–416.

Kanesaki, T., Hirose, S., Grosshans, J., and Fuse, N. (2013). Heterotrimeric G protein signaling governs the cortical stability during apical constriction in Drosophila gastrulation. Mech. Dev. *130*, 132–142.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G., and Wittig, B. (1991). Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature *353*, 43–48.

Kozasa, T., Jiang X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G., Sternweis P.C. (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. Science *280(5372)*, 2109-11.

Kwan, K. Y., Sestan, N., and Anton, E. S. (2012). Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex. Development *139*, 1535–1546.

LaMorte, V. J., Thorburn, J., Absher, D., Spiegel, A., Brown, J. H., Chien, K. R., Feramisco, J. R., and Knowlton, K. U. (1994). Gq- and ras-dependent pathways mediate hypertrophy of neonatal rat ventricular myocytes following alpha 1-adrenergic stimulation. J. Biol. Chem. *269*, 13490–13496.

Leung, T., Manser, E., Tan, L., and Lim, L. (1995). A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J. Biol. Chem. *270*, 29051–29054.

Li, S., Jin, Z., Koirala, S., Bu, L., Xu, L., Hynes, R. O., Walsh, C. A., Corfas, G., and Piao, X. (2008). GPR56 Regulates Pial Basement Membrane Integrity and Cortical Lamination. J. Neurosci. *28*, 5817–5826.

Lim, J., and Thiery, J. P. (2012). Epithelial-mesenchymal transitions: insights from development. Development *139*, 3471–3486.

Litosch, I. (2016). Decoding Gαq signaling. Life Sci. *152*, 99–106.

Lo, S. H., Yu, Q. C., Degenstein, L., Chen, L. B., and Fuchs, E. (1997). Progressive kidney degeneration in mice lacking tensin. J. Cell Biol. *136*, 1349–1361.

Luo, R., Jeong, S.-J., Jin, Z., Strokes, N., Li, S., and Piao, X. (2011). G protein-coupled receptor 56 and collagen III, a receptor-ligand pair, regulates cortical development and lamination. Proc. Natl. Acad. Sci. U. S. A. *108*, 12925–12930.

Ma, S., Kwon, H. J., and Huang, Z. (2012). Ric-8a, a Guanine Nucleotide Exchange Factor for Heterotrimeric G Proteins, Regulates Bergmann Glia-Basement Membrane Adhesion during Cerebellar Foliation. J. Neurosci. *32*, 14979–14993.

Maldonado-Agurto, R., Toro, G., Fuentealba, J., Arriagada, C., Campos, T., Albistur, M., Henriquez, J. P., Olate, J., Hinrichs, M. V., and Torrej??n, M. (2011). Cloning and spatiotemporal expression of RIC-8 in Xenopus embryogenesis. Gene Expr. Patterns *11*, 401–408.

Manzini, M. C., and Walsh, C. A. (2011). What disorders of cortical development tell us about the cortex: one plus one does not always make two. Curr. Opin. Genet. Dev. *21*, 333–339.

Martin, K., Reimann, A., Fritz, R. D., Ryu, H., Jeon, N. L., and Pertz, O. (2016). Spatio-temporal co-ordination of RhoA, Rac1 and Cdc42 activation during prototypical edge protrusion and retraction dynamics. Sci. Rep. *6*, 21901.

Miller, K. G., Emerson, M. D., McManus, J. R., and Rand, J. B. (2000). RIC-8 (Synembryn): a novel conserved protein that is required for G(q)alpha signaling in the C. elegans nervous system. Neuron *27*, 289–299.

Miller, K. G., and Rand, J. B. (2000). A role for RIC-8 (synembryn) and GOA-1 (G(o)??) in regulating a subset of centrosome movements during early embryogenesis in Caenorhabditis elegans. Genetics *156*, 1649–1660.

Milligan, G., and Kostenis, E. (2006). Heterotrimeric G-proteins: a short history. Br. J. Pharmacol. *147 Suppl* , S46–S55.

Miner, J. H., Li, C., Mudd, J. L., Go, G., and Sutherland, A. E. (2004). Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. Development *131*, 2247–2256.

Mishra, A. K., and Lambright, D. G. (2016). Small GTPases and their GAPs. Biopolymers *105*, 431–448.

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron *31*, 727–741.

Moers, A., Nieswandt B., Massberg S., Wettschureck N., Grüner S., Konrad I., Schulte V., Aktas B., Gratacap M. P., Simon M. I., Gawaz M., Offermanns S. (2003). G13 is an essential mediator of platelet activation in hemostasis and thrombosis. Nat. Med. *9*, 1418–1422.

Moers, A., Nürnberg, A., Goebbels, S., Wettschureck, N., and Offermanns, S. (2008). Galpha12/Galpha13 deficiency causes localized overmigration of neurons in the developing cerebral and cerebellar cortices. Mol. Cell. Biol. *28*, 1480–1488.

Monkley, S. J., Zhou, X. H., Kinston, S. J., Giblett, S. M., Hemmings, L., Priddle, H., Brown, J. E., Pritchard, C. A., Critchley, D. R., and Fässler, R. (2000). Disruption of the talin gene arrests mouse development at the gastrulation stage. Dev. Dyn. *219*, 560–574.

Montell, D. J. (2008). Morphogenetic cell movements: diversity from modular mechanical properties. Science *322*, 1502–1505.

Murray, P., and Edgar, D. (2000). Regulation of programmed cell death by basement membranes in embryonic development. J. Cell Biol. *150*, 1215–1221.

Nadarajah, B., Brunstrom, J. E., Grutzendler, J., Wong, R. O., and Pearlman, A. L. (2001). Two modes of radial migration in early development of the cerebral cortex. Nat. Neurosci. *4*, 143–150.

Nakaya, Y., Sukowati, E. W., Wu, Y., and Sheng, G. (2008). RhoA and microtubule dynamics control cell-basement membrane interaction in EMT during gastrulation. Nat. Cell Biol. *10*, 765–775.

Neves, S. R., Ram, P. T., and Iyengar, R. (2002). G protein pathways. Science *296*, 1636–1639.

Nieswandt, B., Schulte, V., Zywietz, A., Gratacap, M.-P., and Offermanns, S. (2002). Costimulation of Gi- and G12/G13-mediated signaling pathways induces integrin alpha IIbbeta 3 activation in platelets. J. Biol. Chem. *277*, 39493–39498.

Nishimura, A., Okamoto, M., Sugawara, Y., Mizuno, N., Yamauchi, J., and Itoh, H. (2006). Ric-8A potentiates Gq-mediated signal transduction by acting downstream of G protein-coupled receptor in intact cells. Genes to Cells *11*, 487–498.

Noctor, S. C., Flint, A. C., Weissman, T. A., Wong, W. S., Clinton, B. K., and Kriegstein, A. R. (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. J. Neurosci. *22*, 3161–3173.

Offermanns, S. (2006). Activation of platelet function through G protein-coupled receptors. Circ. Res. *99*, 1293–1304.

Offermanns, S., Hashimoto, K., Watanabe, M., Sun, W., Kurihara, H., Thompson, R. F., Inoue, Y., Kano, M., and Simon, M. I. (1997). Impaired motor coordination and persistent multiple climbing fiber innervation of cerebellar Purkinje cells in mice lacking Gαq. Proc. Natl. Acad. Sci. U. S. A. *94*, 14089–14094.

Offermanns, S., Zhao, L. P., Gohla, A., Sarosi, I., Simon, M. I., and Wilkie, T. M. (1998). Embryonic cardiomyocyte hypoplasia and craniofacial defects in Gα(q)/Gα11-mutant mice. EMBO J. *17*, 4304–4312.

Olson, E. C., and Walsh, C. A. (2002). Smooth, rough and upside-down neocortical development. Curr. Opin. Genet. Dev. *12*, 320–327.

Pollard, T. D., and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453–465.

Priddle, H., Hemmings, L., Monkley, S., Woods, A., Patel, B., Sutton, D., Dunn, G. A., Zicha, D., and Critchley, D. R. (1998). Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells. J. Cell Biol. *142*, 1121–1133.

Qin, J., Vinogradova, O., and Plow, E. F. (2004). Integrin bidirectional signaling: a molecular view. PLoS Biol. *2*, e169.

Riento, K., and Ridley, A. J. (2003). Rocks: multifunctional kinases in cell behaviour. Nat. Rev. Mol. Cell Biol. *4*, 446–456.

Riobo N. A., Manning D. R.. (2005) Receptors coupled to heterotrimeric G proteins of the G12 family. Trends Pharmacol. Sci. *26*, 146–154

Risher, J. F., Mink, F. L., and Stara, J. F. (1987). The toxicologic effects of the carbamate insecticide aldicarb in mammals: a review. Environ. Health Perspect. *72*, 267–281.

Romero-Pozuelo, J., and Dason, J. (2014). The guanine-exchange factor Ric8a binds the calcium sensor NCS-1 to regulate synapse number and probability of release. J. Cell … *5*, 4246–4259.

Saare, M., Lulla, S., Tõnissoo, T., Meier, R., Kask, K., Ruisu, K., Karis, A., Salumets, A., and Pooga, M. (2015). Expression Pattern and Localization Dynamics of Guanine Nucleotide Exchange Factor RIC8 during Mouse Oogenesis. PLoS One *10*, e0129131.

Sakai, T., Li, S., Docheva, D., Grashoff, C., Sakai, K., Kostka, G., Braun, A., Pfeifer, A., Yurchenco, P. D., and Fässler, R. (2003). Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. Genes Dev. *17*, 926–940.

Sanes, J. R. (2003). The basement membrane/basal lamina of skeletal muscle. J. Biol. Chem. *278*, 12601–12604.

Shen, B., Estevez, B., Xu, Z., Kreutz, B., Karginov, A., Bai, Y., Qian, F., Norifumi, U., Mosher, D., and Du, X. (2015). The interaction of Gα13 with integrin β1 mediates cell migration by dynamic regulation of RhoA. Mol. Biol. Cell *26*, 3658–3670.

Siderovski, D. P., and Willard, F. S. (2005). The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int. J. Biol. Sci. *1*, 51–66.

Singh, P., Carraher, C., and Schwarzbauer, J. E. (2010). Assembly of fibronectin extracellular matrix. Annu. Rev. Cell Dev. Biol. *26*, 397–419.

Smyth, N., Vatansever, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M., and Edgar, D. (1999). Absence of basement membranes after targeting the LAMC1 gene results in

embryonic lethality due to failure of endoderm differentiation. J. Cell Biol. *144*, 151–160.

Song, X., Chen, X., Yamaguchi, H., Mouneimne, G., Condeelis, J. S., and Eddy, R. J. (2006). Initiation of cofilin activity in response to EGF is uncoupled from cofilin phosphorylation and dephosphorylation in carcinoma cells. J. Cell Sci. *119*, 2871–2881.

Spence, H. J., Dhillon, A. S., James, M., and Winder, S. J. (2004). Dystroglycan, a scaffold for the ERK-MAP kinase cascade. EMBO Rep. *5*, 484–489.

Stephens, L. E., Sutherland, A. E., Klimanskaya, I. V, Andrieux, A., Meneses, J., Pedersen, R. A., and Damsky, C. H. (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Dev. *9*, 1883–1895.

Sternweis, P. C., and Robishaw, J. D. (1984). Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J. Biol. Chem. *259*, 13806–13813.

Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003). Talin binding to integrin beta tails: a final common step in integrin activation. Science *302*, 103–106.

Tall, G. G. (2013). Ric-8 regulation of heterotrimeric G proteins. J. Recept. Signal Transduct. Res. *33*, 139–143.

Tall, G. G., and Gilman, A. G. (2005). Resistance to inhibitors of cholinesterase 8A catalyzes release of Galphai-GTP and nuclear mitotic apparatus protein (NuMA) from NuMA/LGN/Galphai-GDP complexes. Proc. Natl. Acad. Sci. U. S. A. *102*, 16584–16589.

Tall, G. G., Krumins, A. M., and Gilman, A. G. (2003). Mammalian Ric-8A (synembryn) is a heterotrimeric G?? protein guanine nucleotide exchange factor. J. Biol. Chem. *278*, 8356–8362.

Tam, P. P. L., and Loebel, D. A. F. (2007). Gene function in mouse embryogenesis: get set for gastrulation. Nat. Rev. Genet. *8*, 368–381.

Thompson, O., Moore, C. J., Hussain, S.-A., Kleino, I., Peckham, M., Hohenester, E., Ayscough, K. R., Saksela, K., and Winder, S. J. (2010). Modulation of cell spreading and cell-substrate adhesion dynamics by dystroglycan. J. Cell Sci. *123*, 118–127.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., Bock, R., Klein, R., and Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. *23*, 99–103.

Tsang, T. E., Shawlot, W., Kinder, S. J., Kobayashi, A., Kwan, K. M., Schughart, K., Kania, A., Jessell, T. M., Behringer, R. R., and Tam, P. P. (2000). Lim1 activity is required for intermediate mesoderm differentiation in the mouse embryo. Dev. Biol. *223*, 77–90.

Tõnissoo, T., Kõks, S., Meier, R., Raud, S., Plaas, M., Vasar, E., and Karis, A. (2006). Heterozygous mice with Ric-8 mutation exhibit impaired spatial memory and decreased anxiety. Behav. Brain Res. *167*, 42–48.

Tõnissoo, T., Meier, R., Talts, K., Plaas, M., and Karis, A. (2003). Expression of ric-8 (synembryn) gene in the nervous system of developing and adult mouse. Gene Expr. Patterns *3*, 591–594.

Wang, H., Ng, K. H., Qian, H., Siderovski, D. P., Chia, W., and Yu, F. (2005). Ric-8 controls Drosophila neural progenitor asymmetric division by regulating heterotrimeric G proteins. Nat. Cell Biol. *7*, 1091–1098.

Wang, L., Guo, D., Xing, B., Zhang, J. J., Shu, H. B., Guo, L., and Huang, X. Y. (2011). Resistance to inhibitors of cholinesterase-8A (Ric-8A) is critical for growth factor

receptor-induced actin cytoskeletal reorganization. J. Biol. Chem. *286*, 31055–31061.

Wang, S.-C., Lai, H.-L., Chiu, Y.-T., Ou, R., Huang, C.-L., and Chern, Y. (2007). Regulation of type V adenylate cyclase by Ric8a, a guanine nucleotide exchange factor. Biochem. J. *406*, 383–388.

Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997). p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. EMBO J. *16*, 3044–3056.

Wettschureck, N., Rütten, H., Zywietz, a, Gehring, D., Wilkie, T. M., Chen, J., Chien, K. R., and Offermanns, S. (2001). Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes. Nat. Med. *7*, 1236–1240.

Woodard, G. E., Huang, N.-N., Cho, H., Miki, T., Tall, G. G., and Kehrl, J. H. (2010). Ric-8A and Gi alpha recruit LGN, NuMA, and dynein to the cell cortex to help orient the mitotic spindle. Mol. Cell. Biol. *30*, 3519–3530.

Worzfeld, T., Wettschureck, N., and Offermanns, S. (2008). G12/G13-mediated signalling in mammalian physiology and disease. Trends Pharmacol. Sci. *29*, 582–589.

Xu, W., and Baribault, H. (1998). Vinculin knockout results in heart and brain defects during embryonic development. Development *125*, 327–337.

Xu, W., Coll, J. L., and Adamson, E. D. (1998). Rescue of the mutant phenotype by re-expression of full-length vinculin in null F9 cells; effects on cell locomotion by domain deleted vinculin. J. Cell Sci. *111 (Pt 1*, 1535–1544.

AKNOWLEGEMENTS

First and foremost I would like to thank my superb supervisors prof. Margus Pooga and Tambet Tõnisso. I would like to thank Margus for keeping the RIC8 workgroup afloat and for continuously having good advice to give. I would like to thank Tambet for all his help throughout my academic pursuit and for always believing in me (even when I did not deserve it). I would also like to thank my non-official supervisor Riho Meier, who has always been there for me to discuss random new ideas, methodical specifics, politics, family, etc. For help with the last project and for editing this dissertation I owe the greatest thanks to Teet Velling.

Members of The Department of Developmental Biology (and its extended family), thank you for creating such a fun working environment. Special mention to my labmates Sirje and Keiu, it is always nice not to be going insane alone.

Since by now I have been studying in the University of Tartu for 11 years, I could not have done it without the moral and financial support from my family to whom I am forever grateful. I also want to thank my friends for helping me to maintain a social life outside of the lab.

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Ruisu K., Kask K., Meier R., Saare M., Raid R., Veraksitš A., Karis A., Tõnissoo T., Pooga M. (2013) Ablation of RIC8A function in mouse neurons

leads to a severe neuromuscular phenotype and postnatal death. PLoS One. 2013 Aug 16;8(8):e74031.

Kask K., Ruisu K., Tikker .L, Karis K., Saare M., Meier R., Karis A., Tõnissoo T., Pooga M. (2015). Deletion of RIC8A in neural precursor cells leads to altered neurogenesis and neonatal lethality of mouse. Dev Neurobiol. 2015 Sep;75(9):984–1002.

Saare, M., Lulla, S., Tõnissoo, T., Meier, R., Kask, K., Ruisu, K., Karis, A., Salumets, A., and Pooga, M. (2015). Expression Pattern and Localization Dynamics of Guanine Nucleotide Exchange Factor RIC8 during Mouse Oogenesis. PLoS One 10, e0129131.

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Saare, M., Lulla, S., Tõnissoo, T., Meier, R., Kask, K., Ruisu, K., Karis, A., Salumets, A., and Pooga, M. (2015). Expression Pattern and Localization Dynamics of Guanine Nucleotide Exchange Factor RIC8 during Mouse Oogenesis. PLoS One 10, e0129131.

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