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TAMBET TÕNISSOO

Identification and molecular analysis of the role of guanine nucleotide exchange factor RIC-8 in mouse development and neural function

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

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

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

III **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 239:3404–3415.

My contributions to the papers referred to in this thesis are as follows:

Ref. I Designed and performed the analyses of *Ric-8/lacZ* expression and whole mount embryo *in situ* hybridization experiments, wrote the manuscript

Ref. II Performed behavioural tests and the analyses of *Ric-8/lacZ* expression, wrote the manuscript

Ref. III Dissected and characterized the phenotype of *Ric-8*$^{-/-}$ embryos, performed immunohistochemical experiments, wrote the manuscript

ABBREVIATIONS

AC5	type V adenylate cyclase
AGS3	G-protein signalling modulator 1 (AGS3-like, *C. elegans*)
aPKC	atypical protein kinase C
A-P	anterior- posterior
AVE	anterior visceral endoderm
Baz	Bazooka
BM	basement membrane
BMP	bone morphogenetic protein family
BMP4	bone morphogenetic protein 4
Brat	translation inhibitor Brain tumour
CDC42	cell division cycle 42
CER1	Cerberus 1 homolog (*Xenopus laevis*)
DKK1	Dickkopf homologue 1 (*Xenopus laevis*)
Dlg	Discs large
CNS	central nervous system
DVE	distal visceral endoderm
E-cadherin	cadherin 1, type 1, E-cadherin (epithelial)
EGL-30	egg laying defective
EMT	epithelial to mesenchymal transition
EmVE	embryonic visceral endoderm
EOMES	eomesodermin homologue (*Xenopus laevis*)
ExE	extra-embryonic ectoderm
FGF	fibroblast growth factor
FGF8	fibroblast growth factor 8
FGFR1	fibroblast growth factor receptor 1
FOXA2	forkhead box A2
GAP	GTPase-activating proteins
GEF	guanine nucleotide exchange factor
GMC	ganglion mother cell
GOA1	G protein,O, alpha subunit
GPA-16	G protein, alpha subunit family member
GPCR	G-protein coupled receptor
GPR1/2	G-protein regulator 1/2
Gβ13F	G protein β-subunit 13F
HEX	haematopoietically expressed homeobox protein
ICM	inner cell mass
INM	interkinetic nuclear migration
IPC	intermediate progenitor cells
Insc	Inscuteable
Khc73	kinesin 73
Laminin-1	laminin, alpha 1
LEFTY1	left right determination factor 1

LGN	G-protein signalling modulator 2 (AGS3-like, *C. elegans*)
LHX1(also LIM1)	LIM homeobox protein 1
LIN-5	abnormal cell LINeage
LIS-1	lissencephaly 1
LTD	long-term depression
LTP	long-term potentiation
Mud	Mushroom body defekt
N-cadherin	cadherin 2, type 1, N-cadherin (neuronal)
NB	neuroblasts
NDEL1	nuclear distribution protein nudE-like 1
NET1	neuroepithelial-transforming protein 1
NUMBL	numb homolog (*Drosophila*)-like
OTX2	orthodenticle homologue 2, (*Drosophila*)
PAR protein	abnormal embryonic PARtitioning of cytoplasm
P-D	proximal–distal
PE	primitive endoderm
Pins	Partner of Inscuteable
PNS	peripheral nervous system
RGC	radial glia cells
RGS	regulator of G-protein signalling
RGS14	regulator of G-protein signalling 14
RhoA	ras homolog gene family member A
RIC-8	resistant to inhibitors of cholinesterase
SHH	sonic hedgehog
SMAD	a family of proteins similar to the gene products of the *Drosophila* gene 'mothers against decapentaplegic' (*Mad*) and the *C. elegans* gene *Sma*
SMAD1	MAD homolog 1 (*Drosophila*)
SMAD2	MAD homolog 2 (*Drosophila*)
SNAIL1	snail homolog 1 (*Drosophila*)
SOP	*Drosophila* sensory organ precursor cells
SVZ	subventricular zone
T	brachyury
TGF	transforming growth factor
VE	visceral endoderm
VZ	ventricular zone
WNT	wingless-type MMTV integration site family
WNT2b	wingless-type MMTV integration site family, member 2B
WNT3	wingless-type MMTV integration site family, member 3
ZO1	tight junction protein 1 (zona occludens 1)
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

I. INTRODUCTION

Heterotrimeric G-proteins transmit extracellular signals relayed by the seven transmembrane domain containing receptors to effector proteins, thus modulating a wide variety of complex cellular processes such as differentiation, division, motility, and exocytosis, which in turn regulate systemic functions such as embryonic development, learning and memory, and homeostasis. Extensive investigations into the function of G-proteins and their regulators in different model organisms are motivated by the necessity to test the relevance of the signalling mechanisms described in *in vitro* assays to those performed under normal conditions *in vivo* as well as in disease states. Such an integrated view would provide the basis for a better understanding of the physiological and pathophysiological role of G-protein-mediated signalling and allow the full exploitation of this multifaceted signalling system as a target for pharmacological interventions.

In addition to the canonical G-protein-coupled receptor (GPCR) mediated signalling, recently several non-canonical G-protein signal transduction pathways have been described, that are involved in regulating the asymmetric cell division and synaptic signalling. As a key component in the receptor-independent G-protein activation mechanism, a conserved protein – RIC-8 (Synembryn) has emerged. RIC-8 functions as a guanine nucleotide exchange factor (GEF) for Gα subunits of the G-proteins.

Based on our results and studies published by others about the expression and function of RIC-8, the first part of the present thesis gives a general overview about mammalian gastrulation, asymmetric cell division and neurogenesis and a synopsis of the function of RIC-8 in development and nervous system in different model organisms. Although, the crucial role of RIC-8 in the model organism's *C. elegans* and *D. melanogaster* is well established, its function in the mammalian embryogenesis and nervous system is still poorly characterized. The main purpose of the research presented in the dissertation was to elucidate the role of RIC-8 in the mouse development and nervous system by using *Ric-8* mutant animals. First, we mapped the expression of RIC-8 during the early development of mouse and demonstrated that the disruption of *Ric-8* gene results in multiple developmental malformations during the mouse gastrulation, leading to the early embryonic lethality at E6.5-E8.5. Second, we showed that during the early development of mouse nervous system (E9.5-E12.5) the temporal and spatial expression of *Ric-8* is restricted mainly to the neural cells and in adult mice *Ric-8* is expressed in brain regions involved in the regulation of behaviour. Third, we found that insufficiency of RIC-8 in heterozygous *Ric-8*$^{+/-}$ mice leads to the increased anxiety and causes problems with the spatial memory and relearning, suggesting that the nucleotide exchange factor RIC-8 plays an important role in the regulation of memory and emotional behaviour.

2. REVIEW OF LITERATURE

2.1. General overview of mouse gastrulation

2.1.1. Establishing the proximal–distal axis

Mouse (*Mus musculus*) blastocysts implant into the uterus at about E4.5 days after fertilization. The late blastocyst stage (E4.5) mouse embryo contains three distinct lineage-restricted subpopulations (Fig. 1A). First, the trophoectoderm which mediates implantation and then expands to form the progenitors of placenta – the extra-embryonic ectoderm (ExE) and the ectoplacental cone. The primitive endoderm (PE) gives rise to the parietal endoderm, which migrates from the surface of the ICM (inner cell mass) and the visceral endoderm (VE). VE remains in contact with the embryo and expands along the surface of the ExE and epiblast, giving further rise to the endoderm of the visceral yolk sac. The early epiblast retains pluripotency and gives rise to both the somatic tissues and the germ cell lineage of the embryo.

Shortly after implantation by E5.5 a cavity forms in the centre of the epiblast and the embryo elongates to form the radially symmetric egg cylinder comprising a bilaminar cup shaped epithelial structure with a defined proximal – distal (P–D) axis (Fig. 1B). The visceral endoderm, which surrounds the epiblast and ExE, is an important source of signals for correct embryonic patterning. The proximal part of visceral endoderm (extra-embryonic visceral endoderm, ExVE) consist of a cuboidal epithelium exhibiting a regular hexagonal packing of cells and the distal part of visceral endoderm (embryonic visceral endoderm, EmVE) contains a squamous epithelium characterized by a less organized arrangement of cells (Mesnard et al., 2006; Perea-Gomez et al., 2007). The precursors of the anterior visceral endoderm (AVE), which is crucial for the correct anterior-posterior patterning, arise at the distal tip of the egg cylinder, termed as a distal visceral endoderm (DVE). DVE cells distinguish as morphologically different from their VE neighbours cells (Rivera-Perez et al., 2003). The DVE can be regarded as a specialized derivate of EmVE (Mesnard et al., 2006; Perea-Gomez et al., 2007). These cells can also be identified by the presence of molecular markers that include *haematopoietically expressed homeobox* (*Hex*), *Cerberus-like 1* (*Cer1*), *left right determination factor 1* (*Lefty1*), and *Dickkopf homologue 1* (*Dkk1*).

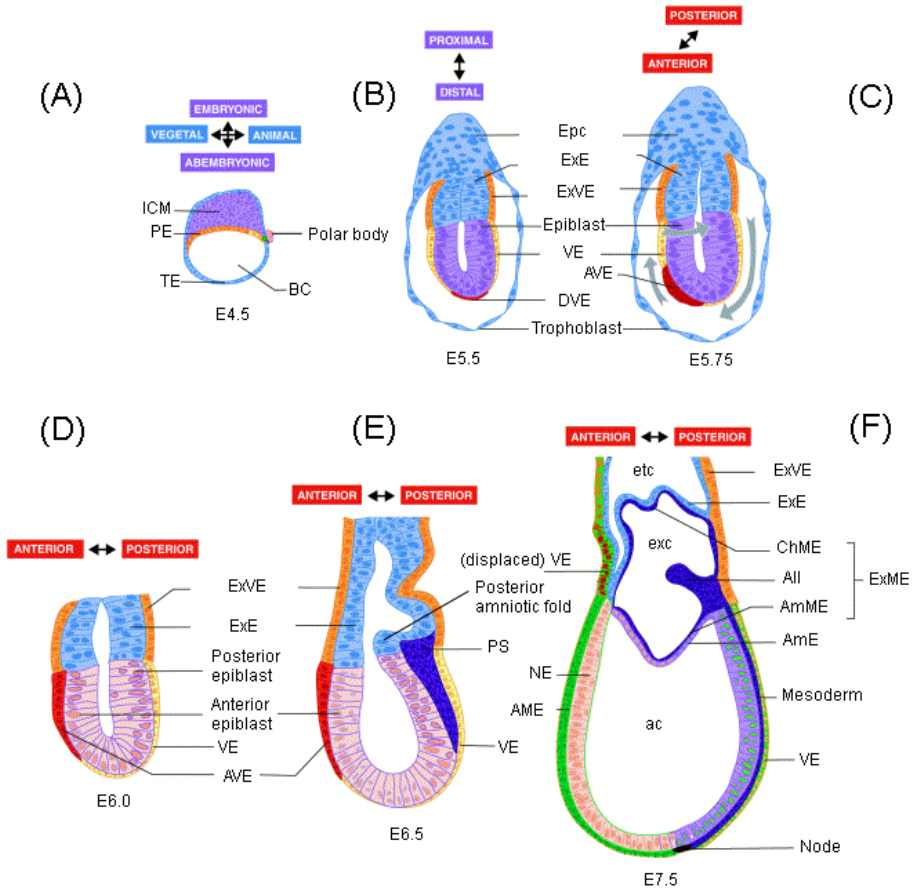

Figure 1. Overview of early axis and germ layer formation in the mouse embryo from implantation at E4.5 to the mid-streak stage at E7.5. (A) The late blastocyst stage at E4.5 mouse embryo contains the trophoectoderm (TE), the primitive endoderm (PE) and and ICM (inner cell mass). (B) The P-D axis in the egg cylinder at E5.5 is defined by a proximally located ectoplacental cone (Epc), and the distal pole at the bottom of developing embryo. (C-D) Before gastrulation the P-D axis rotates and is converted into the A-P axis. The specified visceral endoderm cells (DVE) at the distal tip of the embryo migrate proximally to the prospective anterior side toward the embryonic-extraembryonic junction to form the anterior visceral endoderm (AVE). (E) Mouse gastrulation starts between E6.0 and E6.5 by the formation of a primitive streak (PS) in the posterior region of developing embryo. (F) During gastrulation the primitive streak elongates from the rim of the cup to its distal tip and the progenitor cells form the epiblast ingress through the primitive streak giving rise to the epithelial definitive endoderm and mesenchymal mesoderm. Abbreviations: BC, blastocyst cavity; ExE, extra-embryonic ectoderm; ExVE, extra-embryonic visceral endoderm; VE, visceral endoderm; NE, neuroectoderm; AME, anterior mesendoderm; ExME, extra-embryonic mesoderm; ChME, chorionic mesoderm; AmME, amniotic mesoderm; AmE, amniotic ectoderm; All, allantois; etc, ectoplacental cavity; exc, exocoelomic cavity; ac, amniotic cavity (modified from Lu et al., 2001).

Several genetic studies indicate that NODAL/SMAD and WNT/β-catenin signalling are required to generate and maintain the proximal-distal pattering (reviewed recently by Tam and Loebel, 2007; Arnold and Robertson, 2009). NODAL is a secreted member of the transforming growth factor-β (TGF beta) superfamily of ligands (Zhou et al., 1993). Its expression is first detected at the egg cylinder stage within epiblast and visceral endoderm. Subsequently, *Nodal* expression is lost in the VE and it becomes progressively restricted to the proximal-posterior region of the embryo and localizes to the primitive streak during gastrulation (Conlon et al., 1994; Varlet et al., 1997). NODAL signalling from the epiblast is required for DVE formation at the embryonic day 5.5. With regard to the formation of the A-P axis, *Nodal*$^{-/-}$ embryos display a failure of DVE formation at E5.5 and embryos lack expression of typical DVE markers like *Hex*, *LIM homeobox protein 1* (*Lhx1*) and *orthodenticle homologue 2* (*Otx2*) (Brennan et al., 2001; Lu and Robertson, 2004). Since the visceral endoderm (VE) of *Nodal*$^{-/-}$ embryos is abnormally specified before DVE formation, the primary role of NODAL in DVE formation is to define the embryonic and extra-embryonic compartments of VE before DVE formation (Mesnard et al., 2006). NODAL signal is transduced by intracellular molecules including MAD homolog 2 (SMAD2) (Brennan et al., 2001). Recently it was shown that DVE is formed at the distal region of the embryo where SMAD2-mediated signal is present and MAD homolog 1 (SMAD1) – mediated signal is absent (Yamamoto et al., 2009). NODAL–SMAD2 signalling induces the expression of transcription factors *forkhead box A2* (*Foxa2*) and *Lhx1*. These in turn together with SMAD2 regulate the production of extracellular antagonists of WNT and NODAL signalling, including DKK1, CER1 and LEFTY1 (Arnold and Robertson, 2009).

Formation of DVE is regulated by ExE and epiblast derived signals. ExE is a source of signals for patterning the VE. Removal of the extra-embryonic ectoderm leads to the expansion of DVE at the pregastrulation stage, suggesting that signals derived from ExE inhibit DVE formation (Rodriguez et al., 2005; Mesnard et al., 2006).

Genetic studies with embryos that lack β-catenin (Huelsken et al., 2000) or Adenomatous polyposis coli (APC), which modulates canonical WNT signalling (Chazaud and Rossant, 2006) indicate that the involvement of canonical WNT/β-catenin signalling from the epiblast is initially required to establish proximodistal identity, restricting the formation of the DVE to the distal tip of developing embryo.

2.1.2. Establishment of the anterior–posterior axis

A very important step in the formation of primary body axes is the generation of anterior-posterior polarity (Fig. 1C-D). The specified visceral endoderm cells at the distal tip of the embryo migrate proximally to the prospective anterior side toward the embryonic-extra-embryonic junction to form the anterior visceral

endoderm (Thomas and Beddington, 1996; Thomas et al., 1998). The cell migration from the distal tip of the egg cylinder to this endpoint is completed fairly rapidly in 4–5 h. The migration of DVE cells is an active process, during which the cells continuously change their shape and project filopodial processes in the direction of motion (Srinivas et al., 2004). On the other hand, it is proposed that NODAL activity promotes cell proliferation on the prospective posterior side, whereas the inhibition of NODAL by its antagonists LEFTY1 and CER1 suppresses cell proliferation on the future anterior side, providing a mechanism for the visceral endoderm migration (Yamamoto et al., 2004).

Several studies indicate that DVE fails to migrate in embryos that lack the function of certain components of NODAL and WNT signalling pathways (Arnold and Robertson, 2009). The migrating visceral cells might be guided by the WNT signalling that acts as a repulsive cue at the posterior side and by the WNT inhibitor DKK1 acting as an attractive signal on the anterior side (Kimura-Yoshida et al., 2005). This translocation of the DVE cells within EmVE to the anterior side rotates the proximal-distal axis converting it to an anterior-posterior axis and breaks radial symmetry by repositioning the source of NODAL and WNT antagonist. Once the AVE has migrated to the prospective anterior region, the expression of genes such as *Wnt3, Brachyury* (*T*)*, fibroblast growth factor 8* (*Fgf8*)*, Cripto*, and *Nodal* are found in the posterior epiblast opposite to *Hex*- and *Cer1*-expressing visceral endoderm on the anterior side (Thomas and Beddington, 1996; Ding et al., 1998; Brennan et al., 2001; Perea-Gomez et al., 2001). The antagonists secreted by the AVE block signalling and impart neurectodermal character, whereas signals on the prospective posterior side of the embryo instruct cells to acquire mesodermal and endodermal fates. Genes like *Cer1*, *Lefty1* and *Dkk1*, which are expressed in the AVE, encode secreted factors that antagonize or modulate TGF-β (NODAL, BMP) and WNT signalling (Glinka et al., 1998; Shawlot et al., 1998; Brennan et al., 2001; Perea-Gomez et al., 2002; Kimura-Yoshida et al., 2005). Although, the loss of DKK1, CER1, or LEFTY1 function does not seem to affect gastrulation or the primitive streak formation (Perea-Gomez et al., 2001), a simultaneous loss of CER1 and LEFTY1 leads to severe pattering defects, including occasional duplications of the primitive streak (Perea-Gomez et al., 2001; Perea-Gomez et al., 2002).

Interestingly, the molecular markers that are characteristic for the anterior and posterior poles of the embryo suggest that before the onset of gastrulation, the newly established A-P axis changes its orientation of bilateral symmetry, as the embryo remodels its shape. Precisely, in the pregastrulation stage at E5.5-5.75, the A-P axis aligns with the short transverse axis of the embryo. After E5.75, the A-P axis gradually shifts due to the tissue remodelling and eventually aligns in parallel to the long axis of the embryo (Mesnard et al., 2004; Perea-Gomez et al., 2004). Concomitant with the remodelling, both the A-P axis and the long axis of the embryo become perpendicular to the longitudinal axis of the uterine horn at E6.5 (Mesnard et al., 2004). Recently, it was shown that the

reshaping of embryo requires *Fgf8b* and *Wnt3* activity in the epiblast (Barrow et al., 2007; Guo and Li, 2007).

2.1.3. The primitive streak formation

Mouse gastrulation commences between E6.0 and E6.5 following the formation of a primitive streak within the epiblast at the extra-embryonic-embryonic boundary in the posterior region of the embryo (Fig. 1E). During gastrulation the primitive streak elongates from the rim of the cup to its distal tip and the progenitor cells from the epiblast laminating through the primitive streak give rise to the epithelial definitive endoderm and mesenchymal mesoderm. Several models suggest that NODAL/SMAD and WNT/β-catenin signals are involved in the induction of the primitive streak (Liu et al., 1999; Huelsken et al., 2000; Perea-Gomez et al., 2002; Arnold and Robertson, 2009). As the embryo progresses to gastrulation, *Nodal*, *Wnt3* and its downstream target *T* (Arnold et al., 2000), are downregulated in the prospective anterior part and upregulated in the posterior where the primitive streak forms. The T-box gene *Eomesodermin* (*Eomes*), expressed in the extra-embryonic ectoderm as well as in the posterior epiblast prior to the beginning of gastrulation, is essential for mesoderm formation (Russ et al., 2000). In *Nodal* mutant embryos, the expression of *bone morphogenetic protein 4* (*Bmp4*) and *Eomes* in the extra-embryonic ectoderm is absent and as a result of this *T*, *Fgf8*, *Wnt3* are not expressed (Brennan et al., 2001). It has been shown that embryos lacking *Nodal* or *Cripto* activity do not form the primitive streak (Conlon et al., 1994; Ding et al., 1998; Brennan et al., 2001). In the *Wnt-3*$^{-/-}$ mutant embryos, the epiblast remains viable, but does not establish a primitive streak, mesoderm or node (Liu et al., 1999). Simultaneous loss of *Cer1* and *Lefty1* leads to the formation of extra primitive streaks (Perea-Gomez et al., 2002).

2.1.4. Epithelial to mesenchymal transition (EMT)

The formation of the primitive streak and the subsequent morphogenesis of the mesodermal and endodermal tissue layers involve complex cellular dynamics. A critical component of the gastrulation process is the epithelial to mesenchymal transition (EMT). Columnar epithelial cells of the epiblast exhibit defined apical-basal polarity and lie in close contact with the basement membrane (BM). In the primitive streak the ingressing cells acquire a bottle-shaped morphology, lose their characteristic apical-basal cell polarity, detach from the basement membrane and undergo rapid and drastic cytoskeletal rearrangements that enable them to migrate (Nakaya and Sheng, 2008; Arnold and Robertson, 2009). Several signalling pathways, including FGF, TGF-β and WNT are required for the functional primitive streak and EMT (Nakaya and Sheng, 2008). For example, *Fgf 8* and *Fgf receptor 1* (*Fgfr1*) deficient embryos are able to form the primitive streak and undergo or initiate EMT, but cells either fail to

migrate away or are unable to maintain the mesenchymal state (Sun et al., 1999; Ciruna and Rossant, 2001). Very important step for undergoing EMT is down-regulation of E-cadherin expression for the disruption of the adherens junctions, allowing mesodermal cells to migrate away from the streak. When this process is interfered as in mutants for the Zn-finger transcription factor SNAIL1, the mesodermal cell exhibit abnormal morphology with apical-basal polarity and epithelial type adherence junctions (Carver et al., 2001). *E-cadherin* expression is also regulated by the T-box transcription factor EOMES. Loss of the *Eomes* function in the epiblast results in the partial downregulation of *E-cadherin* and EMT arrest, but the upstream regulator of *E-cadherin*, FGF8 and its down-stream target *Snail* are expressed at normal levels (Arnold et al., 2008). Recent studies with chick embryos suggest that the breakdown of the epithelial base-ment membrane is the first cellular event leading to EMT during gastrulation. It takes place prior to the breakdown of tight junctions and apical-basal polarity, whereas cadherins shift gradually from epithelial (E-cadherin) to mesenchymal (N-cadherin) type after ingression (Nakaya et al., 2008). The breakdown of basement membrane is induced by the loss of basally localized small GTPase Ras homolog gene family member A (RhoA) and its activator neuroepithelial-transforming protein 1 (NET1), a RhoA GEF. In the epiblast cells (lateral to the primitive streak) the BM is maintained by basally localized RhoA activity, mediated by NET1. In the medial primitive streak region loss of basal RhoA/NET1 activity leads to disruption of cell-BM interaction and BM break-down. Therefore, the failure of RhoA downregulation leads to BM retention in medial primitive streak (Nakaya et al., 2008). In addition, it was shown that BM breakdown is also mediated by RhoA regulated basal microtubule stability, in particular the destabilization of basal microtubules during ingression, causing disruption of integrin-mediated epithelial cell-BM interaction (Nakaya et al., 2008).

2.1.5. Germ layer morphogenesis

As gastrulation continues, the primitive streak extends distally along the poste-rior side to the distal tip of the developing embryo (Fig. 1E-F). Ingressing mes-enchymal cells that are formed in the primitive streak spread distally between epiblast and visceral endoderm or proximally where they displace the extra-embryonic ectoderm, which retracts proximally and anteriorly. At the mid-streak stage the amniochorionic fold is formed at the posterior side of the embryo as the extra-embryonic mesoderm accumulates between the extra-em-bryonic ectoderm and visceral endoderm, pushing the extra-embryonic ecto-derm and embryonic ectoderm into the proamniotic cavity (Fig. 1E). Subse-quently, the amniotic folds stretch across the proamniotic cavity to form the amnion that divides the proamniotic cavity into the amniotic cavity and exo-coelom, (later develops into visceral yolk sac cavity), which is formed within the extra-embryonic mesoderm (Fig. 1F). The mesoderm lining the exocoelom

differentiates into the blood islands of the yolk sac. The extra-embryonic mesoderm from the amniochorionic fold is the first and posterior-most mesoderm that leaves the streak and migrates into the extra-embryonic region and later gives rise to amnion, chorion and yolk sac mesoderm (Kinder et al., 1999; Dobreva et al., 2010). The pattering of the posterior mesoderm subpopulation is controlled by BMP-4 signalling from ExE (Winnier et al., 1995). Studies with mutant mice revealed that BMP-4 and its downstream efector SMAD1 are required for the formation of the allantois and primordial germ cells (Winnier et al., 1995; Lawson et al., 1999; Tremblay et al., 2001). Somewhat later the ingressing cells from the middle and anterior streak regions give rise to a population of the cardiac mesoderm, lateral plate and paraxial mesoderm cells. The last epiblast cell population that migrates through the anterior-most tip of the primitive streak give rise to the axial mesendoderm, comprising the prechordal plate, the notochord, the node and also definitive endoderm (Fig. 1F; Tam and Beddington, 1987; Kinder et al., 1999). Epiblast cells that fail to migrate through the primitive streak give rise to the neuroectoderm and eventually the central nervous system and also the surface ectoderm. Considerable evidence suggests that neuroectoderm represents the default state of epiblast differentiation (Arnold and Robertson, 2009). Loss of NODAL leads to precocious differentiation of the epiblast into neural progenitors (Camus et al., 2006). The pattern of gene expression in the germ layers becomes more complex at the onset of gastrulation, where the specification of progenitors for neuroectoderm, mesoderm and definitive endoderm takes place. The visceral endoderm is replaced by the definitive endoderm, which expresses *Cer1*, *SRY-box containing gene 17* (*Sox17*), *Hex*, *Disabled homolog 2* (*Dab2*) and *Dkk1*. The forming mesoderm is marked by the expression of genes like *Lhx1*, *Mix1 homeobox like 1* (*Mixl1*), *Mesoderm posterior 1* (*Mesp1*), *Smad1*, *Wnt2b*, *Wnt3*, *left-right determination factor 2* (*Lefty2*) and the primitive streak by expression of *T*, *Wnt3*, *Wnt3a*, *Fgf8*, *Mixl1* and others. In the anterior region of the primitive streak, in the node, cells express *goosecoid homeobox* (*Gsc*), *Foxa2*, *noggin* (*Nog*), *chordin* (*Chrd*) and *Nodal* (Pfister et al., 2007). In addition, the expression of *Sonic hedgehog* (*Shh*) is initiated in the node, the notochord, and the definitive endoderm at E7.5 (Zhang et al., 2001). The interference with the SHH signaling pathway leads to defective left-right axis formation (Zhang et al., 2001). Asymmetrically expressed *Nodal* is also a key regulator for the induction of the left-right asymmetry (Norris and Robertson, 1999).

2.2. Asymmetric cell division

Asymmetric cell division is crucial for generating the diversity during development and for self-renewal function of stem cells. Although, the asymmetric cell division has fascinated scientists for a long time, a thorough understanding of the mechanisms involved is only just starting to emerge. The majority of data about the molecular mechanisms that direct asymmetric cell

division in animals is derived from experiments in the fruit fly *Drosophila melanogaster* or the nematode *Caenorhabditis elegans*.

2.2.1. Asymmetric cell division in *C. elegans* embryogenesis

The early one cell-stage embryo of *C. elegans* is an attractive model to investigate the mechanisms of unequal cell division. In the first cell division, in response to anterior-posterior polarity cues, the mitotic spindle is displaced towards the posterior part by the end of anaphase, resulting in unequal cleavage into larger anterior, a founder cell (AB) and a smaller posterior stem cell (P1). During the second division, the anterior founder cell (AB) divides equatorially (longitudinally; at 90° angle to the anterior-posterior axis), while the P1 cell divides meridionally (transversely) to produce another founder cell (EMS) and a posterior stem cell (P2) (Schneider and Bowerman, 2003). The position of the cleavage plane is highly regulated in *C. elegans* and critical in establishing the body axes as well as in generating cell lineages throughout development. The asymmetric cell division involves several coordinated steps, like establishment of cell polarity, asymmetric localization of determinants and determination of the cell cleavage plane. The establishment of cell polarity in animal cells depends on the evolutionarily conserved PAR complexes. The anterior PAR complex PAR-3 and PAR-6 in association with atypical protein kinase C (PKC-3) is localized to the anterior cortex of the zygote. The posterior cortex is defined by the PAR1 kinase and the PAR2 ring-finger protein (Cuenca et al., 2003). PAR proteins of the anterior and posterior complex antagonize each other's localization, which results in the establishment of stable cortical domains. The anterior PAR domain extends toward the posterior when *par-2* is inactive and vice versa – mutations in the anterior *par* genes allow PAR-1 and PAR-2 to localize uniformly around the cortex (Cheng et al., 1995; Cuenca et al., 2003). During asymmetric division cortical polarity modulates interactions between microtubules and the cortex, so that different pulling forces act on the two centrosomes of the mitotic spindle, and leads to a displacement of the spindle toward the posterior end. Asymmetric spindle displacement results from the unbalanced cortical force generators that act on astral microtubules and pull the spindle poles (Grill et al., 2001; Grill et al., 2003). Several studies demonstrate that heterotrimeric G-proteins and their binding partners play a key role in this process. All three subunits (GOA-1 or GPA-16, Gβ and Gγ) exhibit homogeneous cortical localization during the first division and are required for the proper centrosome migration, spindle orientation and spindle positioning (Miller and Rand, 2000; Gotta and Ahringer, 2001). Gα and GPR-1/2 are required for regulating the spindle positioning downstream of the PAR proteins. PAR polarity cues result in the enrichment of G-protein regulators GPR-1/2 (Pins in *Drosophila*; LGN/AGS3 in mammals) at the anterior cortex during prophase when the rotation of nucleus-centrosome complex occurs, and at the posterior cortex during anaphase spindle positioning (Gotta and Ahringer, 2001;

Colombo et al., 2003; Gotta et al., 2003). GPR-1 and GPR-2 contain the TPR-GoLoco domain that binds to the GDP-bound Gα and activates GPR1/2 by disrupting intramolecular TPR-GoLoco interactions. Activated Gα-GDP:GPR1/2 uses its TPR domain to bind LIN-5 (the *C. elegans* homologue of the nuclear mitotic apparatus protein 1 (NuMA) in mammals and Mud in *Drosophila*). LIN-5 and GPR1/2 can associate with the microtubule minus-end-directed motor dynein and dynein activator LIS-1 (lissencephaly 1) (Couwenbergs et al., 2007; Nguyen-Ngoc et al., 2007). LIN-5, dynein and LIS-1 form a complex in the cytoplasm and are recruited to the plasma membrane by binding to Gα-GDP:GPR1/2, forming an attachment site for the plus ends of astral microtubules, thereby exerting pulling force on the mitotic spindle. As the concentration of GPR-1 and GPR-2 is higher at the posterior cortex, the mitotic spindle is pulled towards this end (Gonczy, 2008; Siller and Doe, 2009; Knoblich, 2010). Inactivation of *goa1*/*gpa-16*, *gpr1/2*, *lin-5* genes leads to the same mitotic spindle positioning defects as the loss of dynein-dynactin function, caused by reduced cortical pulling forces on the astral microtubules (Miller and Rand, 2000; Gotta and Ahringer, 2001; Colombo et al., 2003; Gotta et al., 2003; Srinivasan et al., 2003; Couwenbergs et al., 2007; Galli and van den Heuvel, 2008).

2.2.2. Asymmetric cell division in *Drosophila* neuroblasts

Drosophila sensory organ (SOP) cells (the progenitors of the peripheral nervous system) and neuroblasts (the progenitors of the central nervous system) are the commonly used model systems in studies of asymmetrical cell division. SOP cells give rise to the four cells in external sensory organs. SOP cells delaminate from a polarized epithelium and divide asymmetrically along the anterior-posterior axis to generate an anterior pIIb and a posterior pIIa cell. Both cells divide once more asymmetrically forming two inner and two outer cells of sensory organ (Gho et al., 1999). Another popular model to assess the asymmetric cell division in *Drosophila* is development of neuroblasts. Embryonic neuroblasts of *Drosophila* delaminate from the surface of an apical-basal polarized neuroectoderm and inherit their apical-basal polarity. Neuroblasts divide asymmetrically perpendicular to the plane of the neuroectoderm, giving rise to a larger apical neuroblast, which retains the neuroblast identity and a smaller basal ganglion mother cell (GMC). The GMC divides terminally to generate two postmitotic neurons or glia cells, whereas the apical daughter cells continue to divide asymmetrically (Knoblich, 2008). The asymmetric division of neuroblast consist of several consecutive steps (Fig. 2): 1) establishment of apico-basal cortical polarity during late interphase/early prophase; 2) the mitotic spindle orientation along the cell polarity axis, which is established by prometaphase, and finally 3) asymmetrical spindle positioning towards the basal cortex during anaphase, leading to unequal sized daughter cells.

The following part of overview focuses on the mechanisms of asymmetric cell division in embryonic neuroblast of *Drosophila*.

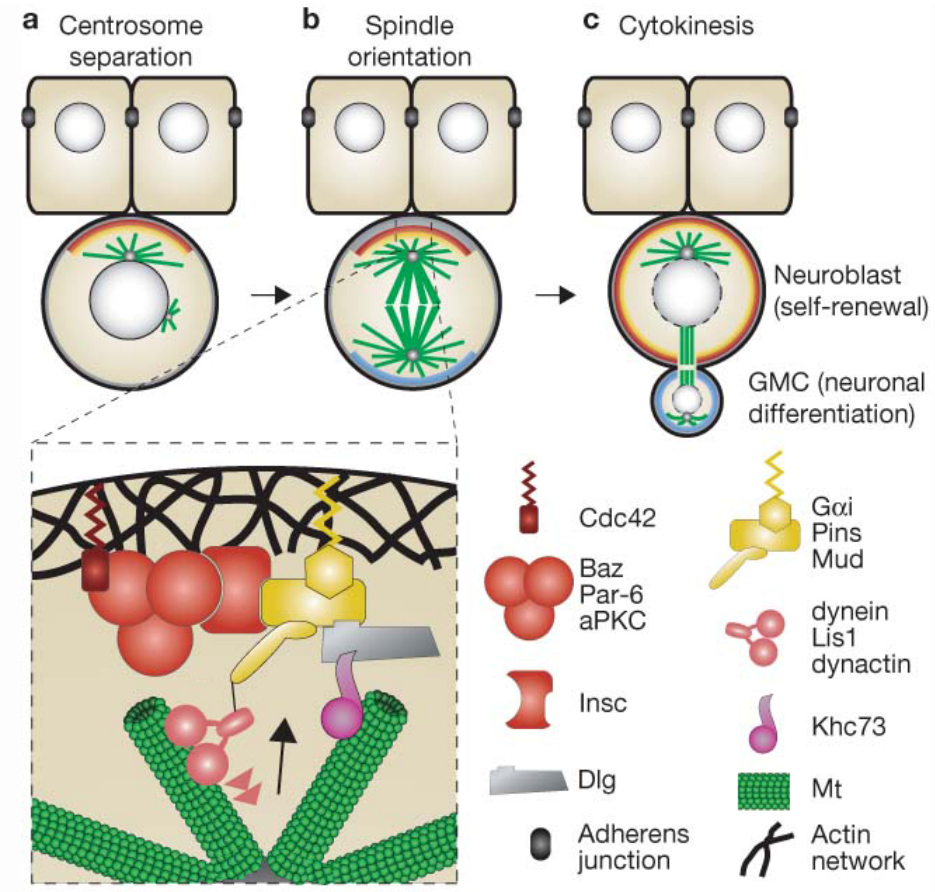

Figure 2. Orientation and positioning of mitotic spindle, establishment of polarity and division of *Drosophila melanogaster* neuroblast. (a) Late interphase/prophase. Par-proteins (Baz, Par-6 and aPKC) and Cdc42 (associated through Par-6) are enriched at the apical cortex. One centrosome is anchored to the apical cortex by Gα–Pins–Mud and by Pins–Dlg–Khc73. The second centrosome nucleates few microtubules (Mt) and migrates basally. (b) Prometaphase/metaphase. Neuroblasts have established apical and basal domains. During mitosis, the apical complex directs the orientation of the mitotic spindle and the asymmetric localization of the adaptor proteins Partner of Numb (PON) and Miranda and, consequently, of the cell fate determinants Numb, Brain tumour (BRAT) and Prospero to the basal cell cortex (light blue). Motor protein kinesin 73 (Khc73) binds Dlg and may facilitate cortical microtubule anchoring, whereas spindle positioning forces on microtubules are probably applied by dynein complex activity. The Insc protein directly binds Baz and Pins, thereby coupling Par polarity with Mud- and Dlg–Khc73-dependent spindle positioning pathways. (**c**) Anaphase. The mitotic spindle becomes asymmetric leading to unequal sized daughter cells. After mitosis, Numb, BRAT and Prospero act together to abolish self-renewal and induce cell cycle exit and differentiation. Light red arrowheads indicate direction of dynein motion; bold black arrows indicate the direction of the net spindle positioning force (adapted from Siller and Doe, 2009).

The different fate of two neuroblast daughter cells is caused by the asymmetric localization of the segregating determinants. Several basally localized proteins like endocytic protein Numb (acts as a tissue-specific repressor of the Notch pathway) (Rhyu et al., 1994; Spana et al., 1995), translation inhibitor Brain tumour (Brat), which associates with Miranda (Lee et al., 2006) and another Miranda cargo protein, transcription factor Prospero (Shen et al., 1997) are subsequently localized at prometaphase (Fig. 2B). After the appropriate localization of basal determinants the mitotic spindle is oriented in an apical-basal direction and the respective determinants are inherited by the basal daughter cells (Fig. 2C). The asymmetric localization of basal determinants requires two apically-enriched cortical complexes, which establish the apical-basal polarity.

Apical cortical polarity is first detectable at late interphase/early prophase stage for the Par-complex of proteins Bazooka (Par-3), Par-6, aPKC (atypical protein kinase C) and the associated Inscuteable (Insc), Pins and $G\alpha_i$ proteins (Knoblich, 2008) (Fig. 2). The initial apical localization of Par-3, Par-6 and aPKC is inherited from epithelial cells where Par proteins establish and maintain apical-basal polarity. The apical Par-3, Par-6 and aPKC mediate the asymmetric localization of the cell fate determinants to the basal cortex (Rolls et al., 2003), whereas the G-protein related complexes are required in orienting the mitotic spindle (Siller and Doe, 2009). The C-terminal region of Pins (Partner of Inscuteable) contains GoLoco domains which binds $G\alpha_i$ (Schaefer et al., 2000) and acts as a guanine nucleotide dissociation inhibitor for $G\alpha_i$. Binding via the first GoLoco domain recruits Pins to the plasma membrane to facilitate its apical concentration. The binding of $G\alpha_i$ to the other GoLoco domains induces the conformational changes in Pins and its N-terminus binds via TPR domain with Mud protein (*Dorsophila* homolog of the microtubule and dynein binding nuclear mitotic apparatus protein 1 – NuMa in mammals and LIN-5 in *C. elegans*) (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Nipper et al., 2007). In the absence of Mud, the cortical polarity is normal, but mitotic spindles fail to align with the polarity axis. This leads to a symmetric segregation of the cell fate determinants and symmetric division of neuroblasts resulting in a tumor-like overproliferation in the nervous system (Bowman et al., 2006; Siller et al., 2006). $G\alpha_i$-Pins-Mud protein complex is linked to the apical Par complex by the adaptor protein Insc, which binds Bazooka (Par-3) and Pins (Schaefer et al., 2000; Yu et al., 2000). Insc localizes to the apical cell cortex in the neuroblast. In the absence of Insc mitotic spindles in neuroblasts are misoriented (Kraut et al., 1996), reminiscent defects of *pins* mutants (Yu et al., 2000). Bazooka (Baz) is required for Insc apical localization, in the absence of Baz Insc is uniformly distributed in the cytoplasm, the mitotic spindles are randomly oriented and basal determinants like Numb, Miranda, Prospero fail to localize asymmetrically in metaphase (Schober et al., 1999). Loss of $G\alpha_i$ causes Pins to localize to the cytosol, and mutant neuroblasts exhibit phenotypes which are very similar to *pins* mutants (Yu et al., 2000; Yu et al., 2003a), suggesting that receptor-independent G-protein activation by Pins at the apical cell

membrane is responsible for spindle orientation, regulation of daughter cell size and maintenance of polarity in neuroblasts. In metaphase Pins binds to the protein Mud (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Nipper et al., 2007), which associates with components of the dynein-dynactin-Lis1 complex and provides a cortical attachment site for astral microtubules to ensure the apical-basal orientation of the mitotic spindle (Siller and Doe, 2008) (Fig. 2B). Loss of Lis1/dynactin leads to spindle/cortical polarity alignment defects at metaphase in *Drosophila* larval neuroblasts (Siller and Doe, 2008). However, interaction between Mud and the dynein-dynactin complex in *Drosophila* neuroblasts is still not documented (Siller and Doe, 2009).

Loss of single members of the apical complex, such as *baz*, *insc*, and *pins*, results in a defective basal protein localization and spindle misorientation in mitotic neuroblasts up to metaphase, although these defects can be partially corrected late in mitosis, a phenomenon called „telophase rescue“ (Knoblich, 2008). In metaphase, G-protein-Pins and Mud establish a cortical attachment site for astral microtubules to orient the mitotic spindle. In telophase rescue is mediated by an interaction of the mitotic spindle with the overlying cell cortex and is regulated by the second spindle orientation pathway that involves Pins, membrane associated tumour suppressor Discs large (Dlg) and kinesin 73 (Khc73) (Siegrist and Doe, 2005; Siller and Doe, 2009; Knoblich, 2010). Khc73, which is transported on astral microtubules, localizes to plus ends of astral microtubules and binds to an adaptor protein Dlg at the cell cortex (Siegrist and Doe, 2005). Dlg binds to Pins and these interactions lead to a clustering of the polarity complexes over one the spindle pole and a polarization of the cell cortex in the direction of the mitotic spindle (Knoblich, 2010). Normally, the telophase pathway is not essential. In *mud* mutants the misoriented mitotic spindle can use this mechanism to reorient cortical polarity during late mitosis and thereby rescue the determinant segregation in many neuroblasts (Siegrist and Doe, 2005).

2.2.3. Mammalian neurogenesis

Asymmetric cell division of neural progenitor cells in the developing central nervous system is the best understood asymmetric cell division process in mammals.

Before the onset of mouse neurogenesis the neural plate and the neural tube are composed of a single-cell layer of pseudostratified neuroepithelium. The nuclei of neuroepithelial cells move up and down the apical-basal axis in a cell cycle-dependent fashion (interkinetic nuclear migration, INM). The mitotic spindle of neuroepithelial cells is oriented parallel to the epithelial surface resulting in symmetric divisions that expand the progenitor pool. These highly polarized neuroepithelial cells show typical epithelial features and contain tight and adherens junctions (Gotz and Huttner, 2005). When the neural tube is closed (at E9), tight junction markers disappear (but not adherens junctions) and

the neuroepithelium no longer acts as diffusion barrier (Aaku-Saraste et al., 1996). After the onset of neurogenesis at E9-E10 (the peak of neurogenesis is at E14-E15) the neuroepithelial cells start to express characteristic features of glial cells and transform into the radial glial cells (RGC) (Fig. 3). RGC express several astroglial markers, like astrocyte-spetcific glutamate transporter (GLAST) and brain lipid-binding protein (BLBP), but also intermediate filament proteins like Nestin and Vimentin, which are typical for neural precursor cells (Campbell and Gotz, 2002). RGC are radially oriented and exhibit apical-basal polarity, and thin processes that maintain contact with both, the ventricular lumen and the pial surface of the developing brain, and undergo INM like neuroepithelial cells (their nuclei migrate only within the ventricular zone). RGCs can be regarded as neural stem cells. At the onset of neurogenesis they undergo self-renewing stem cell-like asymmetric divisions producing also either a neuron or a further type of neural progenitor, called basal or intermediate progenitor cells (nIPC) (Fig. 3; Noctor et al., 2004; Noctor et al., 2007).

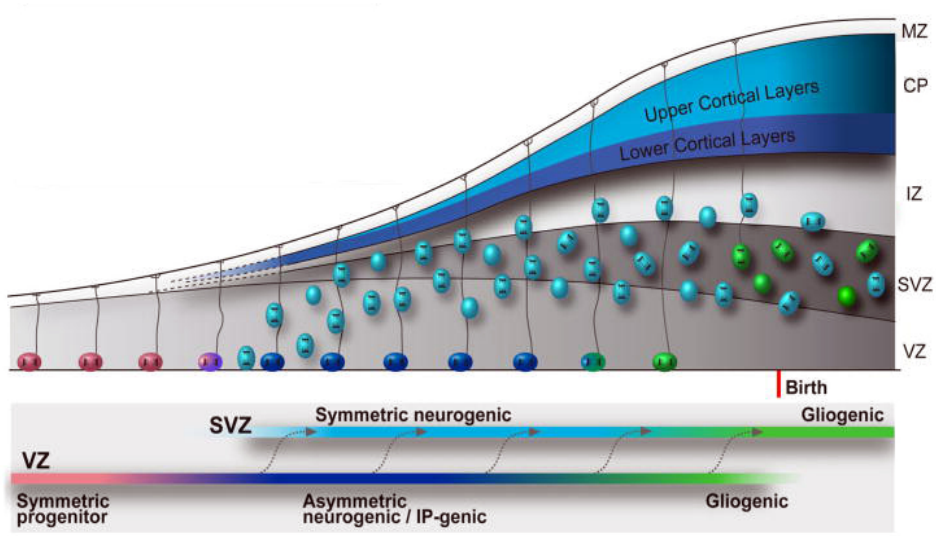

Figure 3. The location, mitotic spindle orientation and division mode of neural stem cells and intermediate progenitor cells during cortical development. Before the neurogenesis most divisions of RGC (radial glial cells) are planar symmetric self-renewing (red), expanding the population of neural stem cells. At the onset of neurogenesis RGC undergo "planar" asymmetric self-renewing (dark blue) division and produce neurons or intermediate progenitor (IP) cells (light blue). Daughter neurons which are produced directly by RGC may form the lower cortical layers. IP cells divide horizontally and produce symmetric pairs of neurons that form the upper cortical layers. After producing neurons RGC translocate away from the ventricle and produce glial progeny (green). Abbreviations: VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, marginal zone (modified from Noctor et al., 2008).

IPCs lose their glia identity and migrate to the basal side of the ventricular zone where they populate the embryonic subventricular zone (SVZ). Unlike neuroepithelial and RGC, IPCs do not undergo INM. IPCs division is symmetric and produces two neurons but they also may divide symmetrically to produce two additional IPCs (Noctor et al., 2004; Noctor et al., 2007). Oligodendrocytes and astrocytes are also derived form RGC through IPC (Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009).

2.2.4. Asymmetric cell division in mammalian neurogenesis

The majority of VZ cells exhibit planar spindle orientation, it means that the mitotic spindle is aligned in the plane of the neuroepithelium. It has been suggested that the planar vertical divisions are symmetric proliferative divisions of neuroepithelial and radial glia cells. Whereas the horizontal divisions, with the spindle aligned along the apical-basal axis, are asymmetric neurogenic divisions, because the apical and basal components will be inherited unequally by the daughter cells (Gotz and Huttner, 2005; Sanada and Tsai, 2005). Recently, it was shown that RGC divide primarily with a vertical orientation throughout development producing symmetric self-renewing daughter cells during early stages of development and asymmetric daughter cells at the onset of cortical neurogenesis (Konno et al., 2008; Noctor et al., 2008). RGC undergo asymmetric cell divisions producing neurons, which may form the lower cortical layers, or intermediate progenitor cells (IPC). Most IPC divide horizontally and produce symmetric pairs of neurons, or additional IPC that form the upper cortical layers (Fig. 3; Noctor et al., 2004; Noctor et al., 2007; Noctor et al., 2008).

The multipotent neuroepithelial progenitor cells are highly polarized. They possess a very small apical cortical domain containing CDC42-PAR3-aPKC-PAR-6 and the transmembrane protein Prominin (CD133), and also extensive basolateral domain containing LGN (named after ten Leucine-Glycine-Asparagine tripeptides in its N-terminal region) (Gotz and Huttner, 2005; Morin et al., 2007; Konno et al., 2008). The apical end-feet of RGC are anchored to each other through adherens junctions (E/N-cadherin, α and β-catenin), which separate two domains as mentioned above. These junctions maintain apical attachment and VZ integrity. The mammalian homologs of the *Drosophila* Numb, namely Numb and Numbl (numb-like) colocalize with the cadherin-catenin adhesion complex and are required to maintain cadherin-mediated adhesion and polarity of RGC during neurogenesis (Rasin et al., 2007). Inactivation of *Numb/Numbl* in RGCs decreases basolateral insertion of cadherins, disrupts adherens junctions and polarity, and leads to progenitor dispersion and disorganized cortical lamination (Rasin et al., 2007).

A model proposed by Kosodo and colleagues (Kosodo et al., 2004) suggests that the cleavage furrow bisects equally the thin apical plasma membrane producing two symmetrically fated daughter cells during planar cell divisions.

However, even a minor change in the cleavage plane angle in planar division causes unequal inheritance of apical plasma membrane, resulting in asymmetrically fated daughter cells. Additionally, adherens junctions and PAR-3 (also called ACIP in vertebrates) a protein that is localized to the apical cortex of mammalian neuroepithelial cells and is concentrated in adherens junctions (Manabe et al., 2002), seem to be inherited equally or unequally in the proliferative and neurogenic divisions respectively (Kosodo et al., 2004; Gotz and Huttner, 2005). However, recent results provide new evidence that the adherens junction comprises three microdomains (ZO1 and Afadin positioned centrally, PAR3/aPKC enriched apicalmost domain and N-cadherin/β-catenin form basal domain). During asymmetric cell division these domains are split in a way that both daughter cells retain the adhesive proteins, but only one inherits the polarity proteins (PAR3/aPKC) along with the apical membrane (Marthiens and ffrench-Constant, 2009). Recent findings in radial glial progenitor cells demonstrate that dynamically distributed PAR-3 is enriched at the lateral membrane domain in the ventricular end-feet during interphase and then dispersed during mitosis, which can lead to an asymmetric inheritance of PAR-3 by the two daughter cells and result in distinct daughter cell fate specification. PAR-3 acts through NUMB (a negative regulator of NOTCH signalling) and NUMB-LIKE in regulating NOTCH signalling activity. The daughter cells that inherit a higher amount of PAR3 develop high NOTCH signalling activity and remain radial glial cells. On the contrary, the daughter cell that inherits less PAR-3 with low NOTCH activity develops either neuronal or an IPC fate (Bultje et al., 2009). Loss of *Numb* and *Numblike* in the developing mouse forebrain leads to the hyperproliferation of neural progenitors and impaired neuronal differentiation causing defects in cortical morphogenesis (Li et al., 2003; Petersen et al., 2004).

The basic components (for example G-proteins, LGN/AGS3 and NuMa), which regulate the asymmetric cell division in *Drosophila* and *C. elegans* are also conserved in vertebrates. However, the function of these proteins in the process of asymmetric cell division in vertebrates is still far from being completely understood.

Planar spindle orientation in mammalian neurogenesis requires the basolateral LGN protein (vertebrate homolog of Pins). Inactivation of *Lgn* randomizes the spindle orientation during the early proliferative phase of planar neuroepithelial divisions in mouse (Konno et al., 2008) and chicken (Morin et al., 2007). Subsequently, the resultant loss of the apical membrane from daughter cells frequently converted them to become abnormally localized basal progenitor cells. However, these defects do not affect the progenitor cell proliferation and neuron production (Morin et al., 2007; Konno et al., 2008). In contrast, the LGN homologue AGS3 regulates spindle movements to promote oblique and apico-basal spindle orientation. In the mouse embryonic cortex the interference with Gβγ function or depletion of AGS3 results in a switch from the apical-basal to planar spindle orientation, and cause hyperdifferentiation of the pro-

genitors into neurons (Sanada and Tsai, 2005). However, apical-basal spindle orientation seems to be relatively rare during cortical neurogenesis (Konno et al., 2008; Noctor et al., 2008). Several studies suggest that during mitosis LGN binds NuMa, which associates with dynein-dynactin complex and GDP-$G\alpha_i$ and the trimolecular complex localizes to the cell cortex, where the dynamic release of NuMa from LGN may regulate aster microtubule pulling (Du et al., 2002; Du and Macara, 2004). Although, it has not been shown yet, LGN may recruit NuMa-dynein-dynactin to the basolateral domain of RGC (Siller and Doe, 2009).

Reduction of the dynein regulators LIS1 and NDEL1 (binding partner of both LIS1 and dynein) level in mutant mice disrupts the planar spindle orientation in neuroepithelial progenitors, suggesting that LIS1/NDEL1/dynein mediated cortical microtubule capture is essential to control the spindle orientation in the self-renewal division during the early phase of corticogenesis (Pawlisz et al., 2008; Yingling et al., 2008).

Whether Gα subunit is involved in the regulation of selfrenewal *versus* differentiation during mammalian neurogenesis is largely unclear. Genetic studies with *C.elegans* (Miller and Rand, 2000; Schaefer et al., 2000) and *Drosophila* (Schaefer et al., 2000) indicated that the $G\alpha_i$ subfamily plays a key role in the asymmetric cell division. The members of $G\alpha_i$ subunits ($G\alpha_{i1}$, $G\alpha_{i3}$ and especially $G\alpha_{i2}$) are preferentially expressed in the cortical VZ by neural progenitor cells during neurogenesis (Murai et al., 2010), indicating their possible role in asymmetric/symmetric cell divisions. It was shown recently that activation of $G\alpha_{i2}$ signalling in the cortical neural progenitor cells promotes cell cycle exit and neuronal differentiation (Murai et al., 2010).

In conclusion, data from several genetic studies suggest that the mechanisms controlling the mitotic spindle orientation and cell fate determination in symmetric/asymmetric division are evolutionally conserved. However, the majority of questions about the detailed mechanisms remain still open.

2.3. Guanine nucleotide exchange factor RIC-8

ric-8 (*r*esistant to *i*nhibitors of *c*holinesterase) was first identified in a genetic screen designed to find genes that facilitate synaptic transmission in *C. elegans* nervous system (Miller et al., 2000). *ric-8* encodes an evolutionally conserved 63 kDa cytoplasmatic protein RIC-8 (also called SYNEMBRYN) (Miller et al., 2000; Miller and Rand, 2000). In mammals, two paralogues of RIC-8 exist, RIC-8A (frequently called just RIC-8) and RIC-8B have been described (Tall et al., 2003). The RIC-8 structure contains 10 armadillo folding motifs, which are organized in right-twisted α-super helix (Figueroa et al., 2009). RIC-8A functions as a guanine nucleotide exchange factor (GEF) for a subset of Gα proteins (Tall et al., 2003). It has been suggested to have two different main physiological functions: regulation of signalling through G-proteins in the nervous system

(Miller et al., 2000; Reynolds et al., 2005; Schade et al., 2005) and the control of asymmetric cell division in embryogenesis (Miller and Rand, 2000; Afshar et al., 2004; Couwenbergs et al., 2004; Afshar et al., 2005; David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005). The above-mentioned functions also gave name to its synonym SYNEMBRYN.

2.3.1. Biochemical function of RIC-8

Heterotrimeric G-proteins, composed of α, β and γ subunits, transduce signals from the membrane-bound receptors to intracellular effectors through a cycle of guanine nucleotide exchange and hydrolysis (Gilman, 1987). Based on the sequence similarity, mammalian Gα subunits have been divided into four families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12}$ (Simon et al., 1991). Each of the four classes of Gα proteins regulates distinct effector proteins and downstream second messengers. Briefly, $G\alpha_s$ proteins activate adenylyl cyclase to stimulate the production of cyclic adenosine monophosphate (cAMP). $G\alpha_q$ proteins activate phospholipase Cβ (PLCβ) to produce diacylglycerol and inositol-1,4,5-trisphosphate (IP3), which facilitates the controlled release of Ca^{2+} from intracellular stores. $G\alpha_i$ proteins inhibit adenylyl cyclase, regulate ion channels, and activate some isoforms of PLCβ via release of Gβγ subunits. $G\alpha_{12}$ proteins activate a small family of RHO GEFs termed RHO GEF(rg)RGS proteins, which control cell motility (Neves et al., 2002; Siderovski and Willard, 2005)**.**

Canonical G-protein signalling pathways are activated by the binding of agonist to a heptahelical receptor (also known as G-protein coupled receptors; GPCR), which acts as a guanine-nucleotide exchange factor (GEF) stimulating the exchange of GDP for GTP on the α subunit. This facilitates dissociation of Gα:GTP from the βγ dimer and dissociated G-protein subunits can interact with a variety of effectors. Hydrolysis of GTP by Gα restores Gα:GDP, which then reassociates with Gβγ and receptor to terminate signalling (Fig. 4; Coleman et al., 1994; Tall and Gilman, 2004). GTPase-activating proteins (GAPs), like RGS (regulator of G-protein signalling proteins) facilitate the inactivation of G-proteins, act as negative regulators (Watson et al., 1996). In noncanonical G-protein signalling the GPCR is replaced by an intracellular guanine nucleotide exchange factor (GEF), e. g. RIC-8, which may re-activate Gα subunit (Tall et al., 2003; Reynolds et al., 2005), and thereby act as positive regulator of G-protein pathways (Fig. 4). Phylogenetic analysis indicates that the genes encoding the GPCRs, Gαβγ subunits and RGS proteins are found within higher eukaryotic organisms (animals, plants, fungi) and also in *Dictyostelium* and *Entamoeba*. By contrast, divergent orthologs of RIC-8 are found only in animals and some fungi, suggesting that RIC-8 mediated pathway appeared evolutionally after GPCR signalling (Wilkie and Kinch, 2005).

It has been demonstrated by biochemical studies that RIC-8A (rat homolog) has GEF activity for $G\alpha_i$, $G\alpha_q$, $G\alpha_o$ and $G\alpha_{12}$ but not for $G\alpha_s$ (Tall et al., 2003). RIC-8A interacts with GDP:Gα proteins, stimulates the release of GDP, and

forms a stable nucleotide-free transition state complex upon binding of GTP to Gα, which disrupts the complex, releasing RIC-8A and the activated Gα proteins (Tall et al., 2003). In contrast to GPCR that act on the heterotrimeric form of G-proteins, RIC-8A interacts and stimulates the GDP/GTP exchange on the monomeric Gα subunit in absence of Gβγ subunits (Tall et al., 2003).

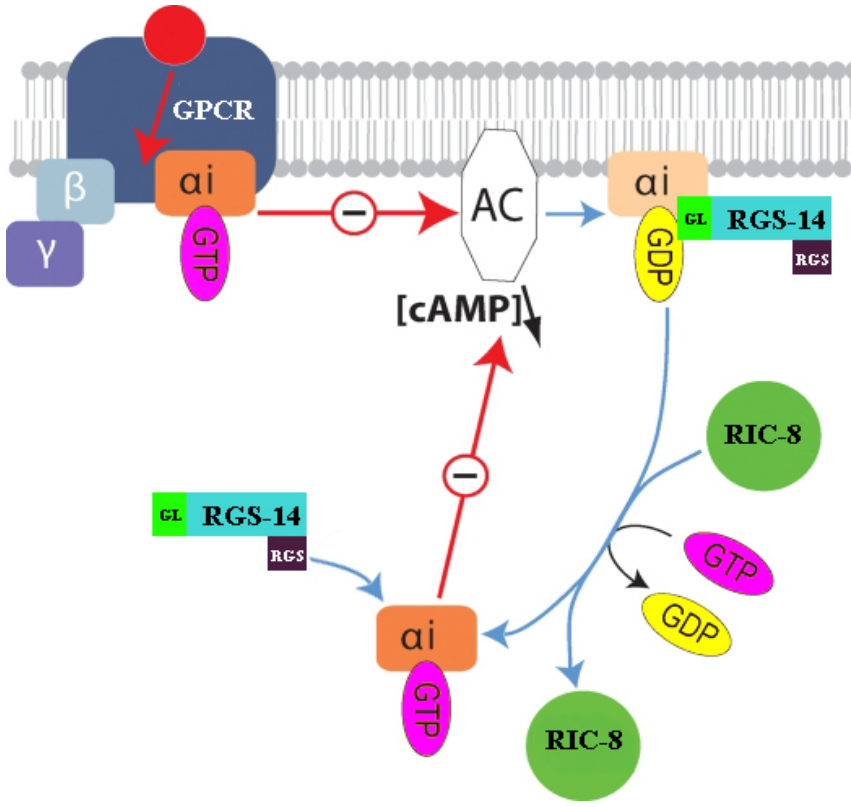

Figure 4. Schematic representation of $G\alpha_i$ protein mediated signalling pathway regulated by RIC-8 and RGS-14. Canonical G-protein signalling pathways are activated by the binding of agonist to G-protein coupled receptors (GPCR), which acts as a guanine nucleotide exchange factor (GEF) that exchanges GDP for GTP on the Gα subunit and facilitates heterotrimeric complex dissociation. Activated G-protein subunits can interact with a variety of effectors, for example $G\alpha_i$ inhibits the activity of adenylyl cyclase (AC) and thereby the production of cAMP from ATP. Subsequently the hydrolysis of GTP by $G\alpha_i$ restores $G\alpha_i$-GDP, which can then reassociate with Gγβ and receptor to terminate signalling. A GTPase-activating protein RGS-14 facilitates the inactivation of G-proteins by its RGS domain promoting reassociation of heterotrimer. RGS-14 also contains the GoLoco domain (GL) that binds $G\alpha_i$-GDP to inhibit the dissociation and exchange of guanine nucleotide. RIC-8 recognizes the RGS14:$G\alpha_i$-GDP complex to stimulate nucleotide exchange and GTP binding. This reactivates $G\alpha_i$ subunit and promotes the dissociation of RGS14 (because the GL domain does not bind $G\alpha_i$-GTP) amplifing thereby the duration of a signal from $G\alpha_i$ (adapted from Fenech et al., 2009; Vellano et al., 2010).

2.3.2. RIC-8 in the asymmetric cell division and embryogenesis

In addition to mediating the signal from GPCR into cell interior, the G-proteins regulate the mitotic spindle and aster microtubule pulling forces during asymmetric cell division, which is essential for the establishment and maintenance of differentiated tissues and cell types in animals. Numerous genetic studies have uncovered the key components of evolutionally conserved mechanisms regulating these processes in model organisms (reviewed in chapter 2.2.). Among the positive regulators of Gα GEF RIC-8 is especially attractive, since it is irreplaceable for asymmetric cell divisions to occur. It assists in the alignment of the mitotic spindle, nuclear migration and other centrosome-mediated events during early embryogenesis in *C. elegans* (Miller and Rand, 2000; Afshar et al., 2004; Couwenbergs et al., 2004; Afshar et al., 2005; Wilkie and Kinch, 2005). RIC-8 is also required for generating pulling forces on spindle poles (Afshar et al., 2004; Couwenbergs et al., 2004). *ric-8* reduction of function mutants cause embryonic lethality in *C. elegans* (Miller et al., 2000; Miller and Rand, 2000). Inactivation of *ric-8* in *C. elegans* results in an essentially identical phenotype to that of inactivation of both *goa* and *gpa16* (Miller and Rand, 2000; Afshar et al., 2004; Couwenbergs et al., 2004). The embryonic lethality of *ric-8* mutants increased to almost 100% by the reduction of maternal *goa-1* gene dosage in *goa-1/+; ric-8/ric-8* parents. These embryos primarily contained a disorganized mass of tissues, wherein some differentiated cell types like cells of pharynx, gut, hypodermis and body wall muscle were also present (Miller and Rand, 2000). Reduction of *ric-8* expression in *C.elegans* embryos by RNAi caused disturbance of the posterior centrosome migration resulting in a symmetrical division of the zygote and the ultimate death by the first larval stage (Miller and Rand, 2000; Afshar et al., 2004; Hess et al., 2004). Three research groups have discovered in parallel that *Drosophila* Ric-8 is similarly essential factor for the proper mitotic spindle orientation and differences in daughter cell size. They demonstrated the involvement of Ric-8 in the localization of the asymmetric cell-fate determinants in the formation of neuroblasts and sensory organ precursor cells of *Drosophila* (David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005; Yu et al., 2006). Concerning the size difference of neuroblast daughter cells in *ric-8* mutant embryos two highly contradictory reports are published. In one case, Hampoelz and colleagues concluded that the *ric-8* mutant phenotype is similar to that observed in *Gβ13F* mutants and significantly differs from $G\alpha_i$ mutants, since most of *ric-8* mutant NB divided into almost equal-sized daughter cells (Hampoelz et al., 2005). On the contrary, Wang and colleagues argue that the neuroblast daughter-cell from *ric-8* and *Gαi* mutants have similar phenotypes, whereas the phenotype of *Gβ13F* mutants is more severe (Wang et al., 2005).

The cellular localization of RIC-8 in the early *C. elegans* embryo is similar to the localization of GOA-1, for instance in the cell cortex and on the asters of

mitotic spindle (Afshar et al., 2004; Couwenbergs et al., 2004), although the majority of RIC-8 is cytoplasmic in early embryos. Additionally, RIC-8 is localized on the central spindle, at the nuclear envelope, around the chromatin, and at the junctions between cells (Afshar et al., 2004; Couwenbergs et al., 2004; Hess et al., 2004). Interestingly, depletion of *Gα*, *gpr-1/2* and *lin-5,* which act in the same pathway as RIC-8 does not significantly affect the localization of RIC-8 (Couwenbergs et al., 2004), analogously to *Gαi* and *Gβ13F* mutants in *Drosophila*. Ric-8 was localized to the cytoplasm of NB and pI (sensory precursor cells) cells of *Drosophila* throughout the cell cycle and seems to be concentrated at the mitotic spindle (Hampoelz et al., 2005; Wang et al., 2005). Recently, it was shown that, depending on the cell cycle phase RIC-8A localizes at the cell cortex, spindle poles, centromeres, central spindle or midbody in mammalian cells (Woodard et al., 2010).

Several groups have shown that RIC-8 can interact with both GOA-1 and GPA-16 in *C. elegans* and it acts as a guanine nucleotide exchange factor for GOA-1 (Afshar et al., 2004; Hess et al., 2004) but not for GPA-16 (Afshar et al., 2005). RIC-8 of *C. elegans* and *Drosophila* can bind to GPA-16 ($G\alpha_i$ in *Drosophila*) both in the GDP and the GTP bound state, whereas the association with GOA-1 *in vitro* can take place in GDP bound state only (Afshar et al., 2004; Afshar et al., 2005; Hampoelz et al., 2005).

RIC-8 is required for the GPA-16 cortical localization (Afshar et al., 2005), whereas the deficiency of RIC-8 did not influence localization of GOA-1 in the cell cortex (Afshar et al., 2004). In analogy, *Drosophila* Ric-8 is necessary for plasma-membrane localization of $G\alpha_i$, $G\alpha_o$ and Gβ13F (David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005). Moreover, the level of $G\alpha_i$ protein and Gβ13F was shown to be significantly reduced in *Drosophila ric-8* mutants, but $G\alpha_o$ was present in normal amounts (Hampoelz et al., 2005). However, in another study the $G\alpha_i$ level remained mostly unaffected (Wang et al., 2005). Since RIC-8A binds the Gα subunit in the absence of Gβγ (Tall et al., 2003), it is speculated that RIC-8 may operate as a chaperone for promoting the assembly of heterotrimer at the plasma membrane (Hampoelz et al., 2005). Others suggests that RIC-8 is only required for the membrane targeting of $G\alpha_i$ but not for its stability, however, both are relevant RIC-8 functions in the case of Gβ13F (Wang et al., 2005).

Several models have been proposed for describing the receptor-independent G-protein cycle that is involved in asymmetric cell division and generation of spindle force. In early embryogenesis of *C. elegans* GPR1/2 (GDP dissociation inhibitor) probably competes with Gβγ for binding to Gα to form a Gα-GDP:GPR1/2 complex, which is as substrate for RIC-8 (Couwenbergs et al., 2004; Hess et al., 2004). However, it was shown that RIC-8 functions before GPR1/2 in the Gα activation cycle. In *C. elegans* embryo GPA-16 and GOA-1 subunits associate in a RIC-8-dependent manner with GPR1/2:LIN-5 to generate pulling force (Afshar et al., 2004; Afshar et al., 2005). In *Drosophila* the $G\alpha_i$-Pins complex binds to Insc or Mud in order to control the asymmetric cell

division (see also chapter 2.2.2.), and it was shown that Ric-8 positively regulates the $G\alpha_i$ activity and binds to the $G\alpha_i$-GDP:Pins complex (David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005). Biochemical studies with mammalian RIC-8A suggest that it dissociates the complex of $G\alpha_i$-GDP:LGN:NuMa, releasing GTP-$G\alpha_i$ and causing liberation of NuMa from LGN, which regulates the microtubule pulling forces on centrosomes during cell division (Tall and Gilman, 2005). RIC-8A can also catalyze the rapid release of GDP from $G\alpha_i$:AGS3 (activator of G-protein signalling 3; a paralog of LGN) complex (Thomas et al., 2008). Recent studies to investigate the function of mammalian RIC-8A in the HeLa and MDCK cells show, that RIC-8A is a key regulator of the dynamic localization, spatial interactions and functions of a $G\alpha_i$-GDP:LGN:NuMa complex in cell division. Reduction of RIC-8A expression impairs nucleotide exchange on $G\alpha_i$ and thereby inhibits liberation of $G\alpha_i$-GTP and NuMa from tertiary complex, which causes prolonged mitosis, occasional mitotic arrest and decreased mitotic spindle movements (Woodard et al., 2010).

In addition to defects in asymmetric division, *ric-8* mutants of *Drosophila* exhibit embryonic lethality and have various defects during gastrulation (for instance cuticle phenotype that is characteristic for defects in mid-gut invagination), and their phenotypes resembled that of *Gβ13F* and *Gγ1* (but not $G\alpha_i$) mutant embryos (Schaefer et al., 2001; Hampoelz et al., 2005; Wang et al., 2005). However, the function of Ric-8 in gastrulation of *Drosophila* is still unclear.

2.3.3. RIC-8 function in the nervous system

In *C. elegans* RIC-8 is localized similarly to other components of the $G\alpha_o$-$G\alpha_q$ signalling network in the nervous system of both juveniles and adults suggesting that RIC-8 could interact with them. RIC-8 is expressed in the majority of neurons of *C.elegans*, although, the intensity of its signal varies between individual neurons. RIC-8 is concentrated in neuronal soma and is also detected in neuronal processes (Miller et al., 2000). *ric-8* mutants resemble the *egl-30* mutants and exhibit profoundly altered neuronal phenotype – such as resistance to cholinesterase inhibitor aldicarb and decreased egg laying. Furthermore, the locomotion rate of *ric-8* mutants and the body flexion is reduced, suggesting that RIC-8 functions upstream of or parallel with EGL-30 and is unlikely to function as a negative regulator of GOA-1. Interestingly, in the early embryonic development *goa-1* and *ric-8* mutants had similar phenotypes (Miller and Rand, 2000; Afshar et al., 2004), whereas in the nervous system the same mutants had opposite phenotypes (Miller et al., 2000). Miller and colleagues proposed that since EGL-30 acts downstream of GOA-1 in the nervous system, decrease in RIC-8 level results in *egl-30* reduction of function mutant phenotype rather than a *goa-1* reduction of function phenotype; and on the other hand, EGL-30 presumably does not play a role in the embryogenesis (Miller et al., 2000). Nevertheless, in *C. elegans* nervous system RIC-8 and EGL-30/GOA-1 partici-

pate in the signalling networks, which regulate neurotransmitter release by controlling the production and consumption of diacylglycerol (Miller et al., 2000). The epistasis analysis of a *ric-8* null mutant *C. elegans* revealed that RIC-8 is required for the activity of both the $G\alpha_q$ and the $G\alpha_s$ pathway of the synaptic signalling networks (Reynolds et al., 2005; Schade et al., 2005). In contrast to invertebrates, which have only one RIC-8, mammals possess two homologues: RIC-8A (or RIC-8) and RIC-8B, respectively. RIC-8A functions as a GEF for $G\alpha_q$, $G\alpha_i$, $G\alpha_o$ and $G\alpha_{13}$ subunits *in vitro*, but not for $G\alpha_s$. RIC-8B on the other hand was shown to interact with $G\alpha_s$ and $G\alpha_q$, but not with $G\alpha_i$ and $G\alpha_{12}$ (Klattenhoff et al., 2003; Tall et al., 2003). Mammalian RIC-8B is specifically expressed in olfactory sensory neurons, and potentiates olfactory specific $G\alpha_{olf}$ mediated signalling (Von Dannecker et al., 2005; Von Dannecker et al., 2006; Kerr et al., 2008). Recently, it was shown that RIC-8B specifically and positively regulates the $G\alpha_s$ signalling by stabilizing the $G\alpha_s$ protein (Nagai et al., 2010). These results support the idea that the vertebrate RIC-8A and RIC-8B are specialized for different Gα proteins, whereas the one isoform RIC-8 in *C.elegans* for example retains a broad Gα specificity.

Based on their biochemical results with mammalian RIC-8A, Tall and colleagues proposed that RIC-8A may function as a signal amplifier (Tall et al., 2003). Briefly, before rebinding Gβγ to Gα-GDP to terminate signalling, RIC-8A could bind a monomeric Gα and reactivate it, thereby amplifying the duration of a signal that comes from an individual G-protein (Fig. 4; Tall et al., 2003). This idea is supported by the experiments which showed that RIC-8A positively regulates $G\alpha_q$-coupled receptor-mediated signalling in the membrane and functions as a signal amplifier in intact cells. RIC-8A is located in the cytosol, but significant amount of it translocates to the plasma membrane and co-localizes with $G\alpha_q$ in response to $G\alpha_q$-coupled receptor stimulation. Reduction of RIC-8 level by siRNA decreases $G\alpha_q$-coupled receptor-mediated ERK (extracellular-signal-regulated kinase) activation and intracellular calcium mobilization (Nishimura et al., 2006). In addition, it was shown recently that RIC-8A is expressed in a large proportion of mouse taste bud cells, including mostly IPR3 positive (a marker of type II cells) sweet, umami and bitter taste receptor cells. RIC-8A interacts directly with Gα-gustducin and $G\alpha_{i2}$, and thereby RIC-8A is able to promote the signal transduction of hTas2R16 (the human receptor for bitter tastant salicin) through $G\alpha_{i2}$. Thus, RIC-8A might be a general modulator of GPCR signalling for bitter taste (Fenech et al., 2009). RIC-8A interacts also with AC5 (type V adenylate cyclase) by its N-terminus. AC5 and RIC-8A co-localize in many regions of mouse brain, including the striatum and in the primary striatal neurons. Chern's research group demonstrated that GEF RIC-8A suppresses in highly selective and isoform-specific manner the activity of AC5 in a $G\alpha_i$-dependent pathway (Wang et al., 2007).

Several studies suggest that mammalian RIC-8A catalyzes the release of GDP from $G\alpha_i$:AGS3/or LGN (GoLoco domain containing proteins) complexes (Tall and Gilman, 2005; Thomas et al., 2008). Furthermore, RIC-8A function-

ally interacts with the $G\alpha_{i1}$-GDP:RGS14 signalling complex to regulate its activation state as was shown recently (Fig. 4; Vellano et al., 2010). Like other RGS (regulators of G-protein signalling) proteins, RGS-14 is a GTPase activating protein (GAP) that regulates the intrinsic GTPase activity of the Gα subunit and promotes reassociation of heterotrimer that terminates $G\alpha_{i/o}$ signalling. However, in contrary to other RGS proteins, RGS14 also contains the GoLoco domain that binds $G\alpha_{i1/3}$ to inhibit the binding and exchange of guanine nucleotide (Kimple et al., 2002). RIC-8A and RGS-14 colocalize at the plasma membrane with $G\alpha_{i1}$, where RIC-8A acts as GEF stimulating the dissociation of RGS14:GDP-$G\alpha_i$ complex in cells and *in vitro*. RIC-8A and RGS-14 seem to compete for the Gα-GDP binding, and the functional response depends on the molar ratio of RGS-14 relative to RIC-8A (Vellano et al., 2010). Similarly, excess amounts of other GoLoco proteins like LGN and AGS3 were shown to suppress the effect of RIC-8A on $G\alpha_{i1}$ (Tall and Gilman, 2005; Thomas et al., 2008; Vellano et al., 2010). In addition, RGS-14 and RIC-8A coexist and colocalize in the same neurons of the CA2 and CA1 regions of the mouse hippocampus (Lee et al., 2010; Vellano et al., 2010).

3. RESULTS AND DISCUSSION

3.1. Aims of the study

The G-protein guanine nucleotide exchange factor RIC-8 is a conserved protein, which plays an important role in regulating asymmetric cell divisions and nervous system development in different organisms. The present dissertation is focused on the investigation of the biological function of RIC-8 in mammals. We used *Ric-8* mutant mice as a model for these studies. The main objectives of the study are:

1. To characterize the temporal and spatial expression profile of *Ric-8* in mouse development and adult nervous system.
2. To elucidate the function of RIC-8 in the mouse behavioural physiology by using haploinsufficient *Ric-8*$^{+/-}$ mice.
3. To examine the function of RIC-8 during mammalian development by using *Ric-8* knockout animals. A more specific goal was to map the lethality profile of *Ric-8*$^{-/-}$ embryos and to characterize the phenotype of these embryos.

3.2. The expression profile of *Ric-8* in the mouse embryogenesis and adult nervous system (paper I, II, III)

The nucleotide exchange factor *ric-8* was first identified during a genetic screen to find genes that facilitate synaptic transmission in *C. elegans* nervous system (Miller et al., 2000). Studies with *ric-8* mutant nematodes suggest, that in *C. elegans* RIC-8 participates in the $G\alpha_o$-$G\alpha_q$ signalling networks, which regulate neurotransmitter release by controlling the production and consumption of diacylglycerol (Miller et al., 2000). Although, the importance of RIC-8 in the *C. elegans* nervous system is appreciated, the function of RIC-8 in the mammals was unknown. Therefore, we decided to generate *Ric-8* knockout mice by homologues recombination, replacing first 5 exons of *Ric-8* gene with β-geo cassette (described more detailed manner in paper II, chapter Material and Methods).

3.2.1. Neural expression of *Ric-8* in the mouse development (paper I)

To assess the temporal and spatial expression of *Ric-8* in the early embryogenesis we used heterozygous *Ric-8*$^{lacZ/+}$ (lacZ knock-in) and wild type (C57Bl6/J) embryos. First we analyzed embryos at different developmental days (starting at E8.5-E12.5). This is the period from the onset of neurogenesis up to the active neurogenesis in different parts of developing nervous system of

mouse (Noctor et al., 2004; Gotz and Huttner, 2005). The earliest expression of *Ric-8/lacZ* was detected at E9.5 in the trigeminal (V) and facio-acustic (VII–VIII) cranial ganglia (I, Fig. 1A), and was in a good concordance with the results from *in situ* hybridization on wild type embryo from the same developmental stage (data not shown). At E10.5 X-gal staining was prominent in the cranial ganglia (V, VII–VIII, IX, X), in the sympathetic trunk, dorsal root ganglia, neural tube and also weakly detectable in the developing brain (I, Fig. 1B). One day later, at E11.5, *Ric-8/lacZ* was additionally expressed in multiple areas of the developing brain, in the vomeronasal organ and eye (I, Fig. 1C). The expression pattern of *Ric-8*$^{+/lacZ}$ embryos resemble closely the results from the whole mount *in situ* hybridization on wild type embryos of this age (I, Fig. 1D and data not shown). In order to study the expression of *Ric-8* in a higher resolution, we analysed the cross-sections of embryos at E12.0. We found that *Ric-8/lacZ* expression was restricted to several distinct regions in the developing brain (I, Fig. 2A and B) and also in the neural tube, such as motoneurons localization site in the ventral part of the neural tube (I, Fig. 2E). Pronounced X-gal staining was also detected in the lens of developing eyes (I, Fig.2D), in the vomeronasal organ (I, Fig. 2C) and in the peripheral nervous system including cranial,– sympathetic and dorsal root ganglia (I, Fig. 2B, D-F). These data indicate that *Ric-8* expression is highly neurospecific in the early mouse development (at E9.5-E12.5) and it is mainly located to the active area of neurogenesis. These findings are supported by our recent results from immunofluorescence analysis of wild type embryos with RIC-8 at E12.5 (Fig. 5). RIC-8 is expressed widely in the developing central nervous system (Fig. 5A-H), in the peripheral nervous system (Fig. 5E), in the developing eye (Fig. 5I and J) and in the vomeronasal organ and olfactory epithelium (Fig. 5K and L). In addition, RIC-8 is also expressed in the ventricular zone of the developing central nervous system (Fig. 5A-H, white arrowheads), which indicates that RIC-8 might have a function in the mouse neurogenesis. Several studies demonstrate that RIC-8 plays a key role in the receptor-independent G-protein cycle involved in the asymmetric cell division in the early embryogenesis of *C. elegans* and in the formation of neuroblasts (the progenitors of the CNS) and sensory organ precursor cells (the progenitors of the PNS) in *Drosophila* neurogenesis (Miller and Rand, 2000; Afshar et al., 2004; Couwenbergs et al., 2004; Afshar et al., 2005; David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005). Asymmetric cell division of the neural progenitor cells in the developing central nervous system is the best understood asymmetric cell division process in mammals. The basic components (for example G-proteins, LGN/AGS3 and NuMa), which regulate the asymmetric cell division in *C. elegans* and *Drosophila* are also conserved in vertebrates.

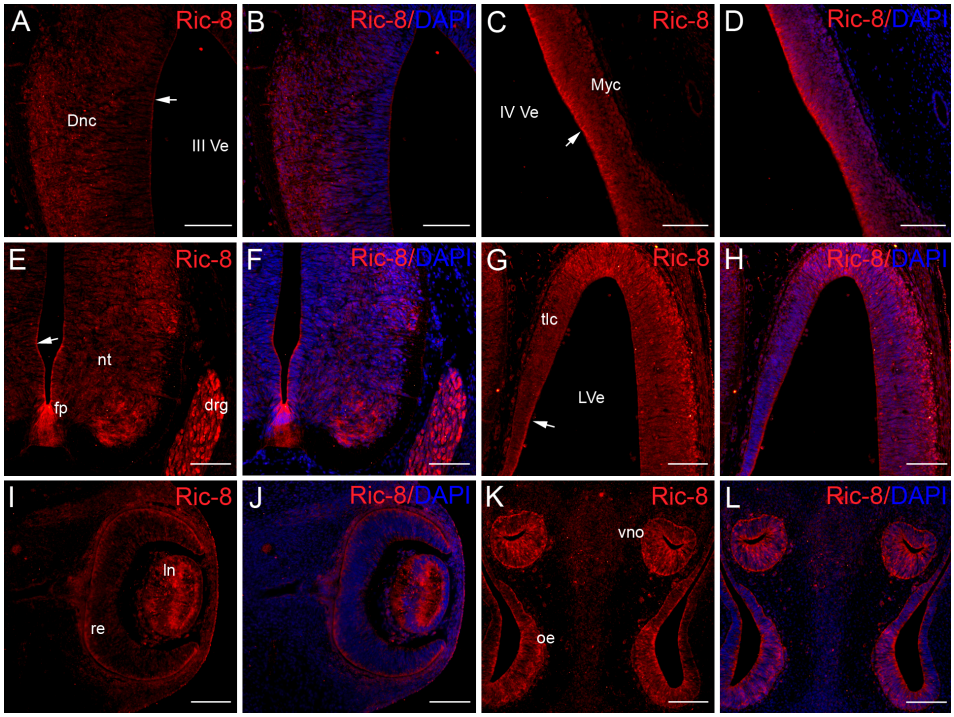

Figure 5. Neurospecific expression of RIC-8 in the mouse embryogenesis. Paraffin embedded cross sections of wild type embryos at E12.5 are shown. RIC-8 was stained with rabbit polyclonal antibodies to RIC-8 (Proteintech Group, Inc., Cat No: 11138-1) and Alexa Fluor®594 (red) labelled secondary antibody, and cell nuclei were visualized with DAPI (blue). White arrowheads indicate the ventricular layer of developing nervous system. Abbreviations: dnc, diencephalon; myc, myelencephalon; fp, floor plate; nt, neural tube; drg, dorsal root ganglia; tlc, telencephalon; re, retina; ln, lens; vno, vomeronasal organ; oe, olfactory epithelium; III Ve, third ventricle; IV Ve, fourth ventricle; LVe, lateral ventricle. Scale bars: 100 μm.

Recent studies in the HeLa (Henrietta Lacks) and MDCK (Madin-Darby Canine Kidney) cells reveal, that RIC-8A is a key regulator of the dynamic localization, spatial interactions and functions of a $G\alpha_i$:LGN:NuMa complex in cell division (Woodard et al., 2010). The members of $G\alpha_i$ subunits ($G\alpha_{i1}$, $G\alpha_{i3}$ and especially $G\alpha_{i2}$) are preferentially expressed in the cortical VZ by neural progenitor cells during neurogenesis (Murai et al., 2010). Mouse *Lgn* expression was highly enriched in the ventricular zone of the developing central nervous system, whereas *Ags3* is broadly expressed (Konno et al., 2008). *Lgn* was also detected in the ganglia of PNS, like dorsal root ganglia, cranial sensory neurons and the sympathetic chain at E12.5 (Yu et al., 2003b). How mouse RIC-8 participates in asymmetric cell division during mammalian neurogenesis is still unclear. However, the expression pattern of RIC-8, $G\alpha_i$ and LGN allow us to speculate that

RIC-8 is one of the regulators of the symmetric/asymmetric cell division in mammalian neurogenesis.

3.2.2. The expression of *Ric-8* in the adult mouse brain (paper I, II)

Since, our results clearly indicate that the expression profile of *Ric-8* at the developmental stages E9.5-E12.5 is neurospecific, we next asked whether *Ric-8* is also expressed in the adult nervous system. In order to study the expression of *Ric-8* in the adult central nervous system we used X-gal staining on brain sections of *Ric-8*$^{lacZ/+}$ animals. We observed *Ric-8/lacZ* activity in several regions of the brain, including the neocortex and cingulate cortex (I, Fig. 3A; II, Fig. 4B), caudate putamen (I, Fig. 3I), subiculum (I, Fig. 3F), the pyramidal and dentate granule cells of the hippocampus (I, Fig. 3C, F; II, Fig. 4 A, B), the cerebellum (I, Fig. 3D, G; II, Fig. 4B) and also in the ependymal layer of the lateral ventricle and the aqueduct of Sylvius (I, Fig. 3B, E; II, Fig. 4B). In addition, the expression of *Ric-8/lacZ* was detectable in the pineal gland (I, Fig. 3H). Consistent with our results, it has been recently shown that RIC-8 protein localizes in the neurons of subregions of cornu Ammonis and dental gyrus of mouse hippocampus (Vellano et al., 2010). However, our recent expression analysis of RIC-8 by using immunohistochemistry indicates that RIC-8 is more broadly expressed in different brain regions than we detected earlier by X-gal staining (K. Ruisu unpublished data). In line with this, Wang and colleagues demonstrated that RIC-8 is expressed in the majority of brain areas examined, including neocortex and striatum (Wang et al., 2007). The RIC-8 protein localization pattern very closely matches the mRNA distribution pattern of *Ric-8* in mouse brain, which is described in Allen Institute for Brain Science (http://mouse.brain-map.org), and also our *in situ* hybridisation results with *Ric-8* (K. Ruisu unpublished data).

However, besides neural expression of *Ric-8* in the CNS, it is also expressed in many other organs of adult mouse. The analysis of its expression in mouse cDNA RT PCR (CLONTECH cDNA Panels, Mouse MTC^{TM} Panel I) revealed high *Ric-8* expression in the brain, spleen, skeletal muscle and testis. Somewhat lower expression of *Ric-8* was detected in the heart, liver, kidney and lung (T. Tõnissoo, unpublished data). Analogous results were obtained using the immunoblot analysis, demonstrating that RIC-8 is expressed in the brain, retina, heart, liver, lung, kidney, spleen, placenta, pancreas and adipose of adult mouse (Nishimura et al., 2006). In addition, RIC-8A is expressed in a majority of mouse taste bud cells, including mostly IPR3 (a marker of type II cells) positive sweet, umami and bitter taste receptor cells (Fenech et al., 2009).

3.2.3. Expression of RIC-8 in the early embryogenesis of mouse (paper III)

Although, we previously had characterized the temporal and spatial expression pattern of *Ric-8* in the developing mouse nervous system at E9.5-E12.5 (I paper), the expression pattern of RIC-8 protein in the earlier stages of mouse embryogenesis had not been mapped so far. Therefore, we analyzed embryos in the uteri by using immunohistochemistry starting from E5.5 until E8.5. We found that the expression level of RIC-8 in embryonic tissues significantly increases at the beginning of gastrulation at E6.5 when the formation of primitive streak is initiated (III, Fig. 1). At the gastrulation stage (E7.0-7.5), high RIC-8 expression levels were detected all over the developing embryo, including the embryonic ectoderm, definitive mesoderm and endoderm (III, Fig. 1C and D). In addition, RIC-8 was also expressed at high levels in the extra-embryonic tissues like extra-embryonic ectoderm, extra-embryonic mesoderm with primitive blood islets, amnion and allantois; and visceral endoderm (III, Fig. 1C-F). These results agree with the results from the whole mount *in situ* hybridization on wild type embryos at E7.5 (data not shown). By day E8.5, the expression of RIC-8 was localized more to the neuroectoderm and mesenchyme of the forming head region, structures of developing heart and somites (III, Fig. 1E). However, we were not able to detect X-gal staining at E6.5-E8.5 in the developing *Ric-8*$^{+/-}$ (*Ric-8*$^{lacZ/+}$) embryos. This method is probably not sensitive enough to visualize the expression of *Ric-8* in embryo at that stage. In addition, to characterize the *Ric-8* expression level during the early stages of embryogenesis (at E4.5–E9.5) we used real-time PCR. The real-time PCR analysis revealed that *Ric-8* is expressed throughout this period but it is higher at E4.5 and E7.5 (unpublished data). Interestingly, RIC-8 seems to localize to the cell cortex of embryonic and extra-embryonic ectoderm (III, Fig. 1 D and data not shown). Recent studies with HeLa cells showed that depending on the stage of cell cycle RIC-8A can localize to the cell cortex, spindle poles, centromeres, central spindle or midbody in mammalian cells (Woodard et al., 2010). Biochemical studies suggest that RIC-8A acts on a tertiary complex of GDP-Gα_i, LGN (mammalian Pins homolog) and NuMA (nuclear mitotic apparatus protein) inducing the release of GTP-Gα_i and dissociation of NuMA from LGN (Tall and Gilman, 2005). This tertiary complex initially localizes at the cell cortex, where the dynamic release of NuMA from LGN may promote aster microtubule pulling during cell division (Du et al., 2001; Du et al., 2002; Du and Macara, 2004). Furthermore, RIC-8A is necessary for the assembly of a cortical signalling complex that orients the mitotic spindle during mammalian cell division (Woodard et al., 2010). In addition, RIC-8A functionally also interacts with the Gα_{i1}:GDP:RGS14 (regulator of G-protein signalling) signalling complex to regulate its activation state (Vellano et al., 2010). RGS-14 localizes in the nucleus during interphase and segregates to the centrosomes and astral microtubules during mitosis and plays an essential role in mitosis (Martin-

McCaffrey et al., 2004). During gastrulation (E7.5) RGS-14 is expressed in the primitive streak and neuroectoderm and by E9.5 in almost all cell types (Martin-McCaffrey et al., 2005). In conclusion, the expression pattern of RIC-8 characterized in our study lets us assume that it is involved in the cell division during mouse gastrulation.

3.3. *Ric-8* haploinsufficiency results in mouse behavioural abnormalities (paper II)

Our results about the temporal-spatial expression profile of *Ric-8* (paper I, II) clearly indicated that RIC-8 is located in several areas of the brain (for example in the neocortex, cingulate cortex, piriform cortex, caudate putamen, hippocampus, cerebellum and pineal gland), which are important for regulation of different behavioural patterns of mouse. Based on the results of expression profile of *Ric-8* we aimed to assess the potential role of RIC-8 in the regulation of mouse behaviour. Therefore, we performed series of behavioural test with heterozygous *Ric-8*$^{lacZ/+}$ (from now on named as *Ric-8*$^{+/-}$) mice aiming to characterize the phenotypes related to the motor activity, coordination and balance, exploratory activity and anxiety, learning and memory. Since the homozygous *Ric-8*$^{-/-}$ mice are not viable and die in early embryonic development during gastrulation (paper III), we used heterozygous *Ric-8*$^{+/-}$ animals for behavioural analysis. Adult heterozygous *Ric-8*$^{+/-}$ mice are viable, fertile and do not possess evident morphological aberrations. In addition, their brain- and body-weight was similar to that of wild type littermates.

3.3.1. Heterozygous *Ric-8* mice exhibit impaired spatial memory and increased anxiety

To minimize the variability in the results of behavioural studies, groups of animals for behavioural test were matched by age, gender and genetic background. We analyzed *Ric-8*$^{+/+}$ (wild type) and *Ric-8*$^{+/-}$ mice in the battery of behavioural tests (II, Material and Methods), which were performed in the following order (light-dark box, motility box, rota-rod, Morris water maze).

Light-dark compartment test (Crawley and Goodwin, 1980) is an unconditioned test of anxiety-like behaviour designed for mice. These test are based on the natural aversion by rodents to open or brightly lit spaces. We observed that the wild-type mice made significantly more transitions between two compartments than heterozygous *Ric-8*$^{+/-}$ mice (II, Fig. 2A). The amount of time spent until the first entry to the light compartment of *Ric-8*$^{+/-}$ mice was longer compared to wild type littermates (II, Fig. 2C) and *Ric-8*$^{+/-}$ mice exhibited considerable avoidance of light compartment (II, Fig. 2B). In addition, heterozygous *Ric-8*$^{+/-}$ mice (especially if males are compared) performed significantly less

rearings (indicator of explorative behaviour) in the light part than the wild-type animals (II, Fig. 2D). These findings clearly demonstrated an increased anxiety-like behaviour of *Ric-8*$^{+/-}$ mice.

The Morris water maze test (Morris et al., 1982) was used to analyze spatial learning and memory. In this task, rodents navigate in a swimming arena using the visual cues to locate a submerged escape platform. We found that escape latency of *Ric-8*$^{+/-}$ mice and their wild type littermates across the training trials decreased considerably in a similar manner (II, Fig. 3A and B, sessions 1–6). In order to estimate the selectivity of spatial navigation two transfer tests were carried out. Navigation strategy in the first transfer test (after six sessions of training, 18 trials) was similar in both *Ric-8*$^{+/-}$ and wild type mice (II, Fig. 3C and D). Effect of training session indicated that both *Ric-8*$^{+/-}$ and wild type mice learned to find the hidden platform. According to these results, the behavioural pattern of *Ric-8*$^{+/-}$ mice during the training session in rota-rod test did not differ significantly from their wild-type littermates. However, significant differences between *Ric-8*$^{+/-}$ and wild-type mice were observed in the reversal training of the reference memory in water maze task. *Ric-8*$^{+/-}$ mice were less efficient to find the platform in the novel position (II, Fig. 3A and B, sessions 7–10), which reveals that *Ric-8*$^{+/-}$ mice could defectively acquire new spatial information. The second transfer test was performed after tenth training session (30 trials). *Ric-8*$^{+/-}$ mice spent significantly more time for swimming in the opposite quadrant (which contained the platform in initial learning) than wild-type animals, indicating a poor cognitive flexibility and impaired extinction (II, Fig. 3E and F). In addition, we noticed that the thigmotactic behaviour (swimming or floating along the walls) of mice was not different in transfer tests. Taken together, our data suggest that *Ric-8*$^{+/-}$ mice have an increased anxiety-like behaviour and modestly impaired spatial memory in the reversal paradigm, suggesting the importance of RIC-8 in the regulation of memory and emotional behaviour.

Although, the physiological function of RIC-8 in the mammalian nervous system has not been sufficiently assessed yet, some hints about its role can be deducted from the studies in other model animals. In *C. elegans* RIC-8 and EGL-30/GOA-1 participate in the signalling networks, which regulate neurotransmitter release by controlling the production and consumption of diacylglycerol. Reduction of function of *ric-8* mutants resembled the *egl-30* mutants of *C. elegans*, and exhibited profoundly altered neuronal phenotypes, including decreased locomotion and body flexion (Miller et al., 2000). Biochemical studies indicate that mammalian RIC-8 is a guanine nucleotide exchange factor for $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, and $G\alpha_{12}$ subunits and may function as an amplifier of signal, which is transduced by an individual G-protein (Tall et al., 2003). This idea is supported by the experiments, which showed that RIC-8A positively regulates $G\alpha_q$-coupled receptor-mediated signalling in the membrane and functions as a signal amplifier in intact cells (Nishimura et al., 2006). RIC-8A also interacts directly with Gα-gustductin and $G\alpha_{i2}$, and thereby is able to amplify the signal

transduction from hTas2R16 (the human receptor for bitter tastant salicin) through $G\alpha_{i2}$. Thus RIC-8A might be a general modulator of bitter taste GPCR signalling (Fenech et al., 2009). The experiments with $G\alpha_q$ and $G\alpha_o$ indicate, that the absence of $G\alpha_q$ in $G\alpha_q^{-/-}$ mutant mice results in motor coordination deficit, which is associated with cerebellar dysfunction (Offermanns et al., 1997a). In addition, $G\alpha_o^{-/-}$ mutant mice also display severe motor control impairment (Jiang et al., 1997). However, in the rota-rod test (model to explore coordinated locomotor functions in mice) and motility boxes (model for investigating locomotor activity of mice) we did not detect any significant difference in the behaviour between *Ric-8*$^{+/-}$ and wild-type mice suggesting that *Ric-8*$^{+/-}$ mice are not different from their wild type littermates with regard to locomotor activity, coordination and balance. Moreover, results from Morris water maze test confirmed the intact motor behaviour because the swimming speed and climbing ability to the platform was not markedly affected in *Ric-8*$^{+/-}$ mice. Therefore, the differences observed in behaviour were probably limited to those tasks which can be linked to the hippocampus. *Ric-8* is expressed in the pyramidal and dentate granule cells of hippocampus at high level (I, Fig. 3C, F; II, Fig. 4 A, B). Analogously, $G\alpha_q$ and $G\alpha_{11}$, are co-localized with mGluR5 (metabotropic glutamate group 1 receptors) in the hippocampal pyramidal cells and primarily localize in the postsynaptic extrajunctional membrane (Tanaka et al., 2000). Long-term potentiation (LTP) and long-term depression (LTD) in the area CA1 of hippocampus are considered to be the cellular bases for certain forms of learning and memory. Experiments with mutant mice showed, that mGluR-dependent long-term depression in the hippocampal CA-1 was absent in $G\alpha_q^{-/-}$ mice whereas they exhibited normal LTP (Kleppisch et al., 2001). As LTD is essential for properly working memory (Nakao et al., 2002), one might speculate that *Ric-8*$^{+/-}$ mice have deficit in extinction due to aberrant LTD. Recently it was demonstrated that RIC-8A interacts directly with the $G\alpha_{i1}$:GDP:RGS14 signalling complex to regulate its activation state (Vellano et al., 2010). Interestingly, RIC-8A and RGS14 coexist and colocalize within the same neurons of the CA2 and CA1 subregions of the hippocampus, which indicates the functional interplay between RIC-8A and RGS14 in hippocampal signalling pathways (Lee et al., 2010; Vellano et al., 2010). Moreover, genetic deletion of RGS14 in mouse brain results in animals with a targeted enhancement of hippocampal-based learning and memory and synaptic plasticity in CA2 neurons (Lee et al., 2010; Vellano et al., 2010). Lee et al. proposed that loss of RGS14 and with it, its capacity to limit the $G\alpha_{i/o}$ signalling may alter the postsynaptic cAMP and/or calcium levels to enhance learning and memory (Lee et al., 2010). In addition, several studies suggest that mammalian RIC-8A catalyzes the release of GDP from $G\alpha_i$:AGS3/or LGN (also GoLoco domain containing proteins) complexes (Tall and Gilman, 2005; Thomas et al., 2008). LGN and AGS3 are highly enriched in various brain regions (Blumer et al., 2002) and both these are important for synaptic plasticity of brain (Bowers et al., 2004; Wiser et al., 2006). However, some recent studies with *Ags3*$^{-/-}$ mice unexpectedly showed

that the elimination of AGS3 did not alter a basal behaviour or brain morphology (Blumer et al., 2008).

In conclusion, our findings suggest the importance of RIC-8 in the regulation of memory and emotional behaviour. However, the mechanism how RIC-8 is involved in the regulation of mouse behaviour is still unclear and needs to be studied more thoroughly.

3.4. Nucleotide exchange factor RIC-8 is indispensable in mammalian early development (paper III)

Previous studies in model organisms, like *C. elegans* and *Drosophila* have associated RIC-8 protein with two main physiological functions: signalling regulation through G-proteins in nervous system (Miller et al., 2000; Reynolds et al., 2005; Schade et al., 2005) and asymmetric cell division in embryogenesis (Miller and Rand, 2000; Afshar et al., 2004; Couwenbergs et al., 2004; Afshar et al., 2005; David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005). To characterize the function of the nucleotide exchange factor RIC-8 in the mammals we used mice with a mutant *Ric-8* gene (II, Material and Methods). Heterozygous *Ric-8*$^{+/-}$ mice were indistinguishable from their wild type littermates and had no evident morphological or anatomical deviations and they were fertile as we have shown earlier (II paper). However, homozygous *Ric-8*$^{-/-}$ mice are not viable and die in the early embryonic development. In order to characterize the misdevelopment of these embryos, we analysed the morphology of *Ric-8*$^{-/-}$ embryos at different stages of gestation.

3.4.1. Loss of *Ric-8* results in early embryonic lethality in mice

Mating of heterozygous *Ric-8*$^{+/-}$ (on C57Bl/6J genetic background) mice produced both the wild type *Ric-8*$^{+/+}$ and heterozygous *Ric-8*$^{+/-}$ pups, but none of newborns had homozygous *Ric-8*$^{-/-}$ genotype, suggesting that disruption of *Ric-8* leads to embryonic lethality (III, Table 1). This result is in compliance with the genetic studies performed in other model organisms, where the *ric-8* mutants of *C. elegans* and *Drosophila* exhibited embryonic lethality (Miller and Rand, 2000; Afshar et al., 2004; Hampoelz et al., 2005; Wang et al., 2005). In order to map the lethality profile of *Ric-8*$^{-/-}$ embryos, we analyzed the embryos at different developmental stages. We discovered that the *Ric-8*$^{-/-}$ embryos reached the gastrulation stage but did not develop further and died progressively from E6.5 on (III, Table 1).

3.4.2. *Ric-8*$^{-/-}$ embryos are able to implant and initiate gastrulation

Genotyping analysis of the dissected embryos suggested that the absence of RIC-8 activity did not lead to major preimplantation defects (III, Table 1). Moreover, we observed that the majority of *Ric-8*$^{-/-}$ blastocysts hatched, induced division and migration of trophoblast cells in *in vitro* culture, demonstrating that they are competent for the initiation of implantation. Still some of the mutant blastocysts could not completely hatch from *zona pellucida* (T. Tonissoo unpublished data). After implantation, at embryonic day E5.5-E6.5, the *Ric-8*$^{-/-}$ embryos had a highly varying size and were significantly smaller compared to their wild type littermates (III, Fig. 2A and B; Fig. 3A-D). Moreover, about 20% of *Ric-8* knockout embryos were being resorbed (III, Table 1). Histological analysis of embryos at E5.5-E6.5 revealed that the development of *Ric-8*$^{-/-}$ embryos is obviously retarded (III, Fig. 3A-D). Although, the RIC-8 expression in the embryonic tissues is reliably detectable by immunohistochemistry only starting from beginning of the gastrulation on (III, Fig. 1), the earlier growth retardation of the *Ric-8*$^{-/-}$ embryos, compared to the control littermates, may be caused by defective mitotic division. This speculation is in line with the experiments in HeLa cells, where the reduced expression of RIC-8A was shown to prolong mitosis, cause occasional mitotic arrest and decreased movement of the spindle (Woodard et al., 2010).

In order to characterize the phenotype of *Ric-8*$^{-/-}$ embryos better, we examined whether the marker genes for different stages of embryonic development were expressed in correct spatial-temporal manner. Before the onset of gastrulation the specified visceral endoderm cells (DVE) at the distal tip of the embryo migrate proximally to the prospective anterior side toward the embryonic-extra-embryonic junction to form the anterior visceral endoderm (AVE) (Thomas and Beddington, 1996; Thomas et al., 1998). AVE is crucial for the correct anterior-posterior pattering and is functioning as a source of antagonists (CER-1, LEFTY-1) for posteriorizing signals (NODAL, WNT-3) and for the correct positioning of the primitive streak (Brennan et al., 2001; Srinivas, 2006). To assess whether the anterior part is properly specified we analyzed the expression of *Nodal* and *Cer-1* at E6.5 in wild type and *Ric-8*$^{-/-}$ embryos. Similarly to wild type embryos (Conlon et al., 1994; Varlet et al., 1997), in the *Ric-8*$^{-/-}$ embryos *Nodal* was expressed throughout the epiblast and the overlying visceral endoderm, with the intensity gradient towards the posterior region where the primitive streak would form (III, Fig. 6F). The expression of NODAL antagonist *Cer-1*, a marker gene for the anterior visceral endoderm (AVE) (Shawlot et al., 1998), showed that *Cer-1* expression was slightly more distal in *Ric-8*$^{-/-}$ embryos compared to the wild type (III, Fig. 6B and G). These results indicated that AVE formed in *Ric-8*$^{-/-}$ embryos and embryos were competent to initiate gastrulation, although further development of *Ric-8* mutants was markedly retarded.

3.4.3. The orientation of the *Ric-8*$^{-/-}$ embryos in the uterus is abnormal

In the pregastrulation stage at E.5.5-5.75, the (A-P) anterior-posterior axis aligns with the short transverse axis of the embryo. After E5.75, the A-P axis gradually shifts due to the tissue remodelling and eventually aligns in parallel to the long axis of the embryo (Mesnard et al., 2004; Perea-Gomez et al., 2004). Concomitant with the remodelling, both the A-P axis and the long axis of the embryo become perpendicular to the longitudinal axis of the uterine horn at E6.5 (Smith, 1985; Mesnard et al., 2004). Surprisingly, we discovered that the A-P axis (and the long axis) in the majority of gastrulating *Ric-8*$^{-/-}$ embryos was almost parallel with the longitudinal axis of the uterine horn, and not perpendicular as in normal embryos (III, Fig. 4). Therefore, the standard sagittal sectioning of uteri with embryos, rather yielded a frontal plane sections (III, Fig. 3) and the interpretation of *Ric-8*$^{-/-}$ phenotype was complicated. In addition, the expression pattern of different marker genes (for example *Cer-1, Brachyury, Hex, Lim1*; III, Fig. 6) corroborated that the A-P axis and longitudinal axis of *Ric-8*$^{-/-}$ embryos coincided. These results suggest that A-P axis and the embryonic long axis seem to be organised in normal manner in *Ric-8*$^{-/-}$ embryos, but have not aligned correctly in relation to the anatomical axes of uterus. Although, we do not know the reason of this phenomenon, one possible explanation for this is that the orientation of the *Ric-8*$^{-/-}$ embryo in the uterine cavity may be affected by the formation of disproportionately large and asymmetrically located exocoelomic cavity (III, Fig. 3F-H; Fig. 4E, E´).

3.4.4. Deficiency in *Ric-8* leads to multiple gastrulation defects

In analogy with the earlier stage, at E7.5 *Ric-8*$^{-/-}$ embryos differed in size and were markedly smaller than their normal littermates (III, Fig. 2 and Fig. 3), in addition, 24% of *Ric-8*$^{-/-}$ embryos were undergoing resorption (III, Table 1). Gastrulation seems to be a very critical stage for *Ric-8* mutants, which reflected in a variety of different malformations during gastrulation (III, Fig. 3).

The formation of the primitive streak is a morphological indication of the onset of the gastrulation, which results in the formation of the primary germ layers and the establishment of the basic body plan of the embryo. Histological analyses of *Ric-8*$^{-/-}$ embryos at E7.5 demonstrated the presence of poorly organized embryonic and extra-embryonic mesoderm, suggesting that gastrulation had been initiated and the primitive streak had formed in *Ric-8*$^{-/-}$ embryos, although in an erratic manner (III, Fig. 3F-H; Fig. 4E and E`). To confirm these results, we mapped the expression of posterior marker *Brachyury (T)* gene. *T* should be expressed by E7.5-E8.5 in cells ingressing into the primitive streak, in nascent mesoderm, the node and the notochord (III, Fig. 6D, D´ and E; Inman and Downs, 2006). However, in *Ric-8*$^{-/-}$ embryos the expression of *T* was

markedly shifted to more posterior region and failed to extend anteriorly at E7.5. Still, by E8.5 the expression of *T* in the most advanced *Ric-8*$^{-/-}$ embryos had broadened and also elongated anteriorly (III, Fig. 6). During gastrulation the primitive streak elongates from the rim of the cup to its distal tip, and the progenitor cells form the epiblast migrating through the primitive streak give rise to the epithelial definitive endoderm and mesenchymal mesoderm. In order to evaluate, whether the nascent endoderm and anterior definitive endoderm (ADE) of *Ric-8*$^{-/-}$ embryos form, we analyzed the expression of endoderm markers *Cer-1* (Shawlot et al., 1998) and *Hex* (Thomas et al., 1998). The expression of both *Cer-1* and *Hex* were detectable in the anterior part of *Ric-8*$^{-/-}$ embryos corroborating the formation of the definitive endoderm (III, Fig. 6H, H′ and data not shown).

The extra-embryonic mesoderm from the amniochorionic fold is the first and posterior-most mesoderm that leaves the primitive streak and migrates into the extra-embryonic region, which later gives rise to amnion, allantois, chorion and yolk sac mesoderm (Kinder et al., 1999; Dobreva et al., 2010). The extra-embryonic mesoderm together with ectoderm contributes to the formation of the amniotic folds. These folds stretch across the proamniotic cavity to form the amnion that divides the proaminotic cavity into the amniotic cavity and exo-coelom, which is formed within the extra-embryonic mesoderm (see chapter 2.1; Fig. 1E, F). In *Ric-8*$^{-/-}$ embryos amnion had not formed by E7.5. According to the lack of aminiotic cavity, the extra-embryonic and embryonic ectoderm of *Ric-8*$^{-/-}$ embryos lay in a close proximity and probably were separated by the basement membrane (III, Fig. 3F-H and Fig. 5B), suggesting that the initial amnion folding process is highly interfered in *Ric-8*$^{-/-}$ embryos (III, Fig. 3). At the same time the cavitation of *Ric-8*$^{-/-}$ embryos was also highly erratic. *Ric-8*$^{-/-}$ embryos had disproportionately large exocoelom-like cavity which extended from the extra-embryonic part to the distal part of the embryo, and was lined with mesodermal cells as expected in normal exocoelom (III, Fig. 3; Fig. 4). Furthermore, in the most advanced *Ric-8*$^{-/-}$ embryos the yolk sac vasculature was malformed (III, Fig. 2 and Fig. 3). Moreover, the formation of allantois was disturbed in *Ric-8*$^{-/-}$ embryos. These results suggest that the extra-embryonic mesoderm does not develop correctly in *Ric-8*$^{-/-}$ embryos. In addition, multiple defects in the basement membrane that were present in *Ric-8* mutants (III, Fig. 5) suggest that the morphogenetic migration and adhesion of cells during gastrulation were interfered. In line with this, the aberrant cell adhesion was detected in *Ric-8*$^{-/-}$ embryos, for instance in the exocoelomic cavity between the mesoderm and ectoderm, and between the extra-embryonic visceral endoderm and extra-embryonic mesoderm (III, Fig. 3). The pattering of the posterior mesoderm subpopulation is controlled by BMP-4 (bone morphogenetic protein) signalling from ExE (Winnier et al., 1995). Inactivation of *Bmp-4* in the mouse is known to cause gastrulation defects, like a very tiny allantois, and a complete lack of primordial germ cells (PGC) (Fujiwara et al., 2001). Therefore we monitored the development of extra-embryonic ectoderm (ExE) based on the

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expression of *Bmp-4*. However, the expression of *Bmp-4* in *Ric-8*$^{-/-}$ embryos at E7.5 was clearly visible in the extra-embryonic ectoderm (III, Fig. 6P). Based on these results, we propose that the malformations of the yolk sac, amnion and allantois of the *Ric-8*$^{-/-}$ embryos may be caused by the interfered migration and differentiation of mesodermal cells.

In order to characterize the embryonic mesoderm formation in *Ric-8*$^{-/-}$ embryos we used a mesoderm marker *Lim homeobox protein 1 (Lim 1)*. *Lim 1* was limited to the nascent mesoderm and visceral endoderm in the *Ric-8*$^{-/-}$ embryos at E7.5 (III, Fig. 6Q and Q`), whereas in their wild type littermates, a prominent expression of *Lim-1* took place in the anterior midline mesendoderm (Tsang et al., 2000). These results indicated that although nascent mesoderm forms in *Ric-8*$^{-/-}$ embryos, they were still in the mid-streak stage, whereas the wild type littermates had already reached the late streak stage. In the anterior region of the primitive streak, in the node, cells express *Shh* (Zhang et al., 2001) and *Nodal* (Norris and Robertson, 1999) in the wild type embryos at E7.5, but neither of these markers was detectable in *Ric-8*$^{-/-}$ littermates at that stage (III, Fig. 6R and data not shown). It is notable that in the most advanced *Ric-8*$^{-/-}$ embryos (E8.5) the expression of *Nodal* and *Shh* were still detectable in the area of node, but probably the function of the node was interfered (III, Fig. 6S-T). Altogether, these results demonstrated that *Ric-8*$^{-/-}$ embryos, despite of several malformations, were able to express all the examined marker genes. However, the expression profile of these genes was shifted in space and lagged in time, which was probably caused by the retarded development of *Ric-8*$^{-/-}$ embryos. Therefore, we can conclude that RIC-8 does not affect directly the expression of genes that are essential for gastrulation, but in its absence the remodelling and morphogenesis of embryonic tissues is interfered.

Based on histological analysis, we may presume that the migration and adhesion of cells might be interfered in *Ric-8*$^{-/-}$ embryos. This suggestion is supported by the observation that the basement membrane of *Ric-8*$^{-/-}$ embryos was often discontinuous and disorganized (III, Fig. 5B-D). Basement membrane regulates different biological activities, including development, proliferation, differentiation, growth, migration of cells, cell polarity and adhesion (Smyth et al., 1999; Erickson and Couchman, 2000; Kruegel and Miosge, 2010). Reichert's membrane, which is an extracellular layer produced by the extra-embryonic parietal endoderm cells, and serves as a barrier between the maternal blood and the developing embryo, and acts as a facilitator of materno-embryonic exchange of nutrients and gases (Williamson et al., 1997), was intact in *Ric-8*$^{-/-}$ embryos (III, Fig. 5). However, the parietal endoderm along with the Reichert´s membrane was disproportionately large in the *Ric-8*$^{-/-}$ embryos compared to the wild-type littermates at E7.5, and on the other side of the Reichert´s membrane the maternal blood cells had accumulated (III, Fig. 3E-H). A critical component of the gastrulation process is the epithelial to mesenchymal transition (EMT) in the primitive streak. Recent studies with chick embryos suggest that the breakdown of the epithelial basement membrane is the first cellular event leading to

EMT during gastrulation. It takes place prior to the breakdown of tight junctions and apical-basal polarity, whereas cadherins shift gradually from epithelial (E-cadherin) to mesenchymal (N-cadherin) type after ingression (Nakaya et al., 2008). Our immunohistochemical analysis of Laminin-1 (basement membrane marker) localization suggested that epithelial to mesenchymal transition might also be interfered in *Ric-8*$^{-/-}$ embryos, but this has to be proven by a more through studies.

The aberrant development of *Ric-8*$^{-/-}$ embryos, e.g. smaller size and interfered alignment of cells suggested the induction of apoptosis. Indeed, we detected a massive apoptosis in the embryonic ectodermal layer of *Ric-8*$^{-/-}$ embryos at E7.5 (III, Fig. 5F). Surprisingly, in other embryonic cell layers of mutant embryos, like embryonic mesoderm and extra-embryonic tissues, apoptotic cells were not detected. It was shown that RIC-8 interacts with the RGS14-$G\alpha_{i1}$-GDP signalling complex to regulate its activation state (Vellano et al., 2010). Like RIC-8, RGS14 is also expressed in the primitive streak and neuroectoderm at gastrulation (E7.5) (Martin-McCaffrey et al., 2005). Reduction of RGS14 level decreased cell proliferation and increased the number of multinucleated cells reflecting aberrant chromosomal segregation (Martin-McCaffrey et al., 2004). Whether such events also occur in the cells of developing *Ric-8*$^{-/-}$ embryos and thereby cause induction of apoptosis, is still unclear and requires further studies.

Although, the role of RIC-8 in mammalian cell division is appreciated (Afshar et al., 2004; Woodard et al., 2010), an intriguing question, how RIC-8 participates in the cell-division mechanisms which are controlled by the G-proteins in mammalian gastrulation, remains still open. The absence of any particular Gα-s (the main targets of RIC-8) was not lethal at the gastrulation stage (Offermanns et al., 1997b; Jiang et al., 1998; Yu et al., 1998) probably due to the compensatory effect of the other Gα isoforms. *ric-8* mutants of *Drosophila* exhibited embryonic lethality and had various defects during gastrulation, which resembled the phenotypes of *Gβ13F* and *Gγ1* (but not *Gαi*) mutant embryos (Schaefer et al., 2001; Hampoelz et al., 2005; Wang et al., 2005). However, the function of Ric-8 in *Drosophila* gastrulation is still obscure. The RIC-8 structure contains *armadillo* motifs, which are known to interact with multiple partners and participate in different cellular functions (Figueroa et al., 2009). Thus, RIC-8 may interact with several protein partners unknown so far and thereby exhibit different cellular functions, which are important for the correct development.

3.4.5. *Ric-8*$^{-/-}$ embryos fail to undergo organogenesis

The majority of *Ric-8*$^{-/-}$ embryos at age E8.5 exhibited several malformations and 59 % of them was resorbed (III, Table 1). Only very few of *Ric-8*$^{-/-}$ embryos survived until to E9.5 (III, Fig. 2F and F`). In the most advanced *Ric-8*$^{-/-}$ embryos the rudiments of the anterior structures and poorly developed trunk

regions still partially formed. However, these mutants exhibited aberrant amalgamation of blood islands and malformed yolk sac vasculature, suggesting the aberrant development of the extra-embryonic mesoderm. Analogously, the *ric-8* mutant embryos of *C. elegans* were mostly composed of a disorganized mass of tissues, wherein some differentiated cell types like the cells of pharynx, gut, hypodermis and body wall muscle also were present (Miller et al., 2000; Miller and Rand, 2000). However, it has to be emphasized that the majority of *Ric-8*$^{-/-}$ embryos were not able to undergo gastrulation and therefore organogenesis could not be initiated.

4. SUMMARY AND CONCLUSIONS

Heterotrimeric G-proteins transmit signals from cell surface receptors to effectors proteins modulating a wide variety of complex physiological and pathological processes. Non-receptor activator of G-proteins, a nucleotide exchange factor (GEF) RIC-8 may also act as a modulator for these G-proteins. RIC-8 is a 63 kDa protein that is conserved in vertebrates and has a GEF activity in the different organisms: *Chaenorhabditis elegans, Drosophila melanogaster* and mammals. It has been suggested to have two different main physiological functions: modulation of signalling through G-proteins in the nervous system and the regulation of cell division in embryogenesis. Although, the importance of RIC-8 in the model organisms, like *C.elegans* and *Drosophila* is appreciated, its function in mammals was largely unexplored.

The main aim of the research presented in this thesis was to elucidate the role of RIC-8 in the mouse development and in the nervous system by using *Ric-8* mutant animals. We characterized the spatial and temporal expression profile of RIC-8 in the development and in the nervous system of adult mouse. We demonstrated that the activity of RIC-8 protein is irreplaceable for the normal gastrulation of mammals. We also proved that RIC-8 plays an important role in the regulation of memory and emotional behaviour in mouse.

The main results of this dissertation can be concluded as follows:

1. The expression level of RIC-8 in embryonic tissues significantly increases at the beginning of gastrulation. During gastrulation of mouse, RIC-8 is expressed at high level all over the developing embryo, including the embryonic ectoderm, definitive mesoderm and endoderm and also in the extra-embryonic tissues like extra-embryonic ectoderm, visceral endoderm, extra-embryonic mesoderm with primitive blood islets, amnion and allantois.
2. At the later stages of mouse development (starting from E9.5-E12.5) the expression of *Ric-8* is mostly neurospecific. *Ric-8* is expressed in the cranial ganglia, neural tube, sympathetic chain and dorsal root ganglia, in the developing brain, vomeronasal organ, eye and ear.
3. In adult mice *Ric-8* is highly expressed in brain regions involved in the regulation of behaviour, e.g. neocortex, cingulated cortex, *corpus striatum*, hippocampus and cerebellum.
4. Heterozygous *Ric-8*$^{+/-}$ mice reveal no evident morphological or anatomical malformations. They are fertile, but an insufficiency of RIC-8 in *Ric-8*$^{+/-}$ mice leads to the increased anxiety and causes problems with the spatial memory and relearning, suggesting that RIC-8 plays an important role in the regulation of memory and emotional behaviour in mammals.
5. Homozygous *Ric-8*$^{-/-}$ embryos are able to implant in the uterus and to initiate gastrulation, but cannot accomplish gastrulation, which leads to the embryonic lethality at E6.5-E8.5.

6. The development of *Ric-8*$^{-/-}$ embryos is retarded, and their epiblast as well as mesoderm are disorganized. Furthermore, their basement membrane is defective; the folding of amnion and the formation of allantois are interfered, and cavitation aberrant. Moreover, the orientation of the *Ric-8*$^{-/-}$ embryo in the uterus is abnormal.

SUMMARY IN ESTONIAN

Guaniini nukleotiidivahetusfaktor RIC-8 roll ja molekulaarne analüüs hiire arengus ja närvisüsteemis

Loomariigis konserveerunud heterotrimeersed G valgud ja nende poolt vahendatud signalisatsioon on üliolulline mitmetes raku sõlmprotsessides nagu näiteks diferentseerumine, raku jagunemine ja eksotsütoos, mis omakorda on aluseks embrüonaalse arengu, käitumisfüsioloogia ja homöostaasi regulatsioonile. Teadlased on kasutanud erinevaid katsemudeleid, nii *in vitro* kui *in vivo* süsteemides, mõistmaks G valkude ja nende regulaatorite poolt vahendatud signalisatsiooni mehhanisme ja nende füsioloogilist ning patofüsioloogilist rolli organismis. Need teadmised on oluliseks eelduseks mitmesuguste haiguste (nt. neuroloogilised, embrüonaalsed) teraapia välja töötamisel.

Lisaks tavapärasele G valkude ja nendega seotud retseptorite signalisatsioonile on hiljuti avastatud ka alternatiivne, retseptorist sõltumatu G valgu signalisatsiooni süsteem, mis omab olulist funktsiooni rakkude asümmeetrilises/ sümmeetrilises jagunemises ja sünaptilises signaali ülekandes. Üheks oluliseks komponendiks sellise retseptorist sõltumatu G valgu signalisatsiooni aktivatsioonil on valk RIC-8 (tuntud ka nime all Synembryn), mis toimib nukleotiidivahetusfaktorina (GEF) mitmetele Gα subühikutele. RIC-8 seondumine Gα:GDP kompleksile, millest puudub Gβγ, põhjustab kiire GDP vabanemise kompleksist. Järgnevalt saab seonduda Gα-le GTP, vabastades RIC-8 ning aktiveeritud G-valgu. Seega käitub RIC-8 kui mitteretseptoorne GEF võimendades niimoodi G valkude poolt vahendatud signaali rakus. RIC-8 bioloogilist rolli on seostatud närvisüsteemis G valkude poolt vahendatud sünaptilise signaaliülekande regulatsiooniga ja rakkude asümmeetrilise jagunemise kontrollimisega. Varasemad uurimistööd on käsitlenud RIC-8 funktsiooni peamiselt sellistes mudelorganismides nagu nematood *C. elegans* ja äädikakärbes *D. melanogaster*.

Käesoleva doktoritöö peamiseks eesmärgiks oli RIC-8 funktsiooni uurimine imetajates, kasutades mudelorganismina *Ric-8* mutantseid hiiri. Konkreetsemad eesmärgid olid: 1) kirjeldada ajalis-ruumilist *Ric-8* ekspressiooni hiire arengus ja täiskasvanu närvisüsteemis. 2) selgitada RIC-8 roll hiire käitumisfüsioloogias. 3) uurida RIC-8 funktsiooni imetajate arengus, kasutades *Ric-8*$^{-/-}$ nullmutantseid hiiri.

Katsed *Ric-8*$^{lacZ/+}$ embrüotega (*lacZ knock-in* hiireliin, milles *Ric-8* lookusesse on viidud reportergeen *lacZ*, millelt sünteesitava β-galaktosidaasi ekspressioon jäljendab *Ric-8* ekspressiooni) näitasid, et *Ric-8* on ekspresseerunud vanuses E9.5-E12.5 mitmetes neuraalsetes kudedes nagu kraniaalganglionites (V, VII–XII), dorsaalsetes spinaalganglionites, sümpaatilises närvitüves, arenevas peaajus, neuraaltorus, arenevas silmas ja sisekõrvas ning vomeronasaalorganis (I artikkel). Need tulemused olid kooskõlas *Ric-8 in situ* hübridisatsiooni tulemustega metsiktüüpi embrüotes. *Ric-8* ekspressioon E9.5-E12.5 aktiivsetes neurogeneesi piirkondades lubab oletada, et RIC-8-l on oluline

funktsioon imetajate neurogeneesis G valkude poolt vahendatud asümmeetrilise/sümmeetrilise raku jagunemise kujunemisel.

Lähtudes *Ric-8* neurospetsiifilisest ekpressioonimustrist hiire arengus E9.5-E12.5 kirjeldasime *Ric-8* ekpressiooni ka täiskasvanud hiire peaajus (I, II artikkel). *Ric-8* oli avaldunud neokorteksis, vöökäärus (*cingulate cortex*), juttkehas (*cortex striatum*), hippokampuses, väikeajus ning käbinäärmes, mis on käitumuslikult tähtsad piirkonnad peaajus. Tuginedes neile andmetele viisime RIC-8 defitsiitsete heterosügootsete *Ric-8*$^{+/-}$ (*Ric-8*$^{-/-}$ genotüüp on embrüonaalselt letaalne) loomadega läbi ulatusliku käitumiskatsete seeria. Meie tulemused näitasid, et lokomotoorse aktiivsuse, koordinatsiooni ja tasakaalu tunnetuse osas *Ric-8*$^{+/-}$ ja nende metsiktüüpi pesakonnakaaslaste vahel olulisi erinevusi ei olnud. Samal ajal täheldasime *Ric-8* defitsiitsetel loomadel kõrgendatud ärevust ja vähenenud ruumilist taju ning õppimisvõimet. Seega RIC-8-l on oluline osa hiirte ruumilise mälu ja emotsionaalse käitumise regulatsioonis.

Ric-8 välja lülitamine nematoodis *C. elegans* ja äädikakärbses *D. melanogaster* on embrüonaalselt letaalne. Meie katsed homosügootsete *Ric-8*$^{-/-}$ loomadega näitasid, et RIC-8 puudumine põhjustab hiirtel gastrulatsioonis mitmesuguseid arenguhäireid, mis viivad embrüonaalse suremuseni vanuses E6.5-E8.5 (III artikkel). RIC-8 on ekspresseerunud hiire gastrulatsioonis kogu embrüos, nii ekstra-embrüonaalsetes kudedes (ekstra-embrüonaalne mesoderm, – ektoderm ja endoderm; amnion, allantois) kui embrüonaalsetes kudedes (mesoderm, endoderm, ektoderm). *Ric-8*$^{-/-}$ embrüod on kasvult väiksemad ja võrrelduna metsiktüüpi pesakonnakaaslastega arengus maha jäänud. *Ric-8*$^{-/-}$ embrüote fenotüübi täpsemal analüüsimisel leidsime, et nad on võimelised implanteeruma emakasse ja initsieerima gastrulatsiooni (AVE – anterioorne vistseraalne endoderm ja ürgjutt moodustuvad), kuid ei ole võimelised gastrulatsiooni korrektselt läbima ja algatama organogeneesi. *Ric-8*$^{-/-}$ embrüotel esineb mitmeid arengudefekte nagu näiteks häired amnioni ja allantoisi moodustumisel ja kavitatsioonil, basaalmembraani defektid, häired rakkude kinnitumisel ja migratsioonis, indutseeritud apoptoos embrüonaalses ektodermis, embrüo kehatelgede vale paiknemine emaka suhtes jpm. Kokkuvõtvalt näitavad meie tulemused *Ric-8*$^{-/-}$ hiirtega, et GEF RIC-8 on imetajate gastrulatsioonis asendamatu regulaatorvalk.

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REFERENCES

Aaku-Saraste E, Hellwig A, Huttner WB. 1996. Loss of occludin and functional tight junctions, but not ZO-1, during neural tube closure – remodeling of the neuroepithelium prior to neurogenesis. Dev Biol 180:664–679.

Afshar K, Willard FS, Colombo K, Johnston CA, McCudden CR, Siderovski DP, Gonczy P. 2004. RIC-8 is required for GPR-1/2-dependent Galpha function during asymmetric division of C. elegans embryos. Cell 119:219–230.

Afshar K, Willard FS, Colombo K, Siderovski DP, Gonczy P. 2005. Cortical localization of the Galpha protein GPA-16 requires RIC-8 function during C. elegans asymmetric cell division. Development 132:4449–4459.

Arnold SJ, Hofmann UK, Bikoff EK, Robertson EJ. 2008. Pivotal roles for eomesodermin during axis formation, epithelium-to-mesenchyme transition and endoderm specification in the mouse. Development 135:501–511.

Arnold SJ, Robertson EJ. 2009. Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. Nat Rev Mol Cell Biol 10:91–103.

Arnold SJ, Stappert J, Bauer A, Kispert A, Herrmann BG, Kemler R. 2000. Brachyury is a target gene of the Wnt/beta-catenin signaling pathway. Mech Dev 91:249–258.

Barrow JR, Howell WD, Rule M, Hayashi S, Thomas KR, Capecchi MR, McMahon AP. 2007. Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis. Dev Biol 312:312–320.

Blumer JB, Chandler LJ, Lanier SM. 2002. Expression analysis and subcellular distribution of the two G-protein regulators AGS3 and LGN indicate distinct functionality. Localization of LGN to the midbody during cytokinesis. J Biol Chem 277:15897–15903.

Blumer JB, Lord K, Saunders TL, Pacchioni A, Black C, Lazartigues E, Varner KJ, Gettys TW, Lanier SM. 2008. Activator of G protein signaling 3 null mice: I. Unexpected alterations in metabolic and cardiovascular function. Endocrinology 149: 3842–3849.

Bowers MS, McFarland K, Lake RW, Peterson YK, Lapish CC, Gregory ML, Lanier SM, Kalivas PW. 2004. Activator of G protein signaling 3: a gatekeeper of cocaine sensitization and drug seeking. Neuron 42:269–281.

Bowman SK, Neumuller RA, Novatchkova M, Du Q, Knoblich JA. 2006. The Drosophila NuMA Homolog Mud regulates spindle orientation in asymmetric cell division. Dev Cell 10:731–742.

Brennan J, Lu CC, Norris DP, Rodriguez TA, Beddington RS, Robertson EJ. 2001. Nodal signalling in the epiblast patterns the early mouse embryo. Nature 411:965–969.

Bultje RS, Castaneda-Castellanos DR, Jan LY, Jan YN, Kriegstein AR, Shi SH. 2009. Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex. Neuron 63:189–202.

Campbell K, Gotz M. 2002. Radial glia: multi-purpose cells for vertebrate brain development. Trends Neurosci 25:235–238.

Camus A, Perea-Gomez A, Moreau A, Collignon J. 2006. Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo. Dev Biol 295:743–755.

Carver EA, Jiang R, Lan Y, Oram KF, Gridley T. 2001. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. Mol Cell Biol 21:8184–8188.

Chazaud C, Rossant J. 2006. Disruption of early proximodistal patterning and AVE formation in Apc mutants. Development 133:3379–3387.

Cheng NN, Kirby CM, Kemphues KJ. 1995. Control of cleavage spindle orientation in Caenorhabditis elegans: the role of the genes par-2 and par-3. Genetics 139:549–559.

Ciruna B, Rossant J. 2001. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell 1:37–49.

Coleman DE, Berghuis AM, Lee E, Linder ME, Gilman AG, Sprang SR. 1994. Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. Science 265:1405–1412.

Colombo K, Grill SW, Kimple RJ, Willard FS, Siderovski DP, Gonczy P. 2003. Translation of polarity cues into asymmetric spindle positioning in Caenorhabditis elegans embryos. Science 300:1957–1961.

Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B, Robertson EJ. 1994. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 120:1919–1928.

Couwenbergs C, Labbe JC, Goulding M, Marty T, Bowerman B, Gotta M. 2007. Heterotrimeric G protein signaling functions with dynein to promote spindle positioning in C. elegans. J Cell Biol 179:15–22.

Couwenbergs C, Spilker AC, Gotta M. 2004. Control of embryonic spindle positioning and Galpha activity by C. elegans RIC-8. Curr Biol 14:1871–1876.

Crawley J, Goodwin FK. 1980. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13:167–170.

Cuenca AA, Schetter A, Aceto D, Kemphues K, Seydoux G. 2003. Polarization of the C. elegans zygote proceeds via distinct establishment and maintenance phases. Development 130:1255–1265.

David NB, Martin CA, Segalen M, Rosenfeld F, Schweisguth F, Bellaiche Y. 2005. Drosophila Ric-8 regulates Galphai cortical localization to promote Galphai-dependent planar orientation of the mitotic spindle during asymmetric cell division. Nat Cell Biol 7:1083–1090.

Ding J, Yang L, Yan YT, Chen A, Desai N, Wynshaw-Boris A, Shen MM. 1998. Cripto is required for correct orientation of the anterior-posterior axis in the mouse embryo. Nature 395:702–707.

Dobreva MP, Pereira PN, Deprest J, Zwijsen A. 2010. On the origin of amniotic stem cells: of mice and men. Int J Dev Biol 54:761–777.

Du Q, Macara IG. 2004. Mammalian Pins is a conformational switch that links NuMA to heterotrimeric G proteins. Cell 119:503–516.

Du Q, Stukenberg PT, Macara IG. 2001. A mammalian Partner of inscuteable binds NuMA and regulates mitotic spindle organization. Nat Cell Biol 3:1069–1075.

Du Q, Taylor L, Compton DA, Macara IG. 2002. LGN blocks the ability of NuMA to bind and stabilize microtubules. A mechanism for mitotic spindle assembly regulation. Curr Biol 12:1928–1933.

Erickson AC, Couchman JR. 2000. Still more complexity in mammalian basement membranes. J Histochem Cytochem 48:1291–1306.

Fenech C, Patrikainen L, Kerr DS, Grall S, Liu Z, Laugerette F, Malnic B, Montmayeur JP. 2009. Ric-8A, a Galpha protein guanine nucleotide exchange factor potentiates taste receptor signaling. Front Cell Neurosci 3:11.

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

Fujiwara T, Dunn NR, Hogan BL. 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.

Galli M, van den Heuvel S. 2008. Determination of the cleavage plane in early C. elegans embryos. Annu Rev Genet 42:389–411.

Gho M, Bellaiche Y, Schweisguth F. 1999. Revisiting the Drosophila microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development 126:3573–3584.

Gilman AG. 1987. G proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615–649.

Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391:357–362.

Gonczy P. 2008. Mechanisms of asymmetric cell division: flies and worms pave the way. Nat Rev Mol Cell Biol 9:355–366.

Gotz M, Huttner WB. 2005. The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788.

Gotta M, Ahringer J. 2001. Distinct roles for Galpha and Gbetagamma in regulating spindle position and orientation in Caenorhabditis elegans embryos. Nat Cell Biol 3:297–300.

Gotta M, Dong Y, Peterson YK, Lanier SM, Ahringer J. 2003. Asymmetrically distributed C. elegans homologs of AGS3/PINS control spindle position in the early embryo. Curr Biol 13:1029–1037.

Grill SW, Gonczy P, Stelzer EH, Hyman AA. 2001. Polarity controls forces governing asymmetric spindle positioning in the Caenorhabditis elegans embryo. Nature 409:630–633.

Grill SW, Howard J, Schaffer E, Stelzer EH, Hyman AA. 2003. The distribution of active force generators controls mitotic spindle position. Science 301:518–521.

Guo Q, Li JY. 2007. Distinct functions of the major Fgf8 spliceform, Fgf8b, before and during mouse gastrulation. Development 134:2251–2260.

Hampoelz B, Hoeller O, Bowman SK, Dunican D, Knoblich JA. 2005. Drosophila Ric-8 is essential for plasma-membrane localization of heterotrimeric G proteins. Nat Cell Biol 7:1099–1105.

Hess HA, Roper JC, Grill SW, Koelle MR. 2004. RGS-7 completes a receptor-independent heterotrimeric G protein cycle to asymmetrically regulate mitotic spindle positioning in C. elegans. Cell 119:209–218.

Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W. 2000. Requirement for beta-catenin in anterior-posterior axis formation in mice. J Cell Biol 148:567–578.

Inman KE, Downs KM. 2006. Localization of Brachyury (T) in embryonic and extraembryonic tissues during mouse gastrulation. Gene Expr Patterns 6:783–793.

Izumi Y, Ohta N, Hisata K, Raabe T, Matsuzaki F. 2006. Drosophila Pins-binding protein Mud regulates spindle-polarity coupling and centrosome organization. Nat Cell Biol 8:586–593.

Jiang M, Boulay G, Spicher K, Peyton MJ, Brabet P, Birnbaumer L, Rudolph U. 1997. Inactivation of the G alpha i2 and G alpha o genes by homologous recombination. Receptors Channels 5:187–192.

Jiang M, Gold MS, Boulay G, Spicher K, Peyton M, Brabet P, Srinivasan Y, Rudolph U, Ellison G, Birnbaumer L. 1998. Multiple neurological abnormalities in mice deficient in the G protein Go. Proc Natl Acad Sci U S A 95:3269–3274.

Kerr DS, Von Dannecker LE, Davalos M, Michaloski JS, Malnic B. 2008. Ric-8B interacts with G alpha olf and G gamma 13 and co-localizes with G alpha olf, G beta 1 and G gamma 13 in the cilia of olfactory sensory neurons. Mol Cell Neurosci 38:341–348.

Kimple RJ, Kimple ME, Betts L, Sondek J, Siderovski DP. 2002. Structural determinants for GoLoco-induced inhibition of nucleotide release by Galpha subunits. Nature 416:878–881.

Kimura-Yoshida C, Nakano H, Okamura D, Nakao K, Yonemura S, Belo JA, Aizawa S, Matsui Y, Matsuo I. 2005. Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm. Dev Cell 9:639–650.

Kinder SJ, Tsang TE, Quinlan GA, Hadjantonakis AK, Nagy A, Tam PP. 1999. The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. Development 126:4691–4701.

Klattenhoff C, Montecino M, Soto X, Guzman L, Romo X, Garcia MA, Mellstrom B, Naranjo JR, Hinrichs MV, Olate J. 2003. Human brain synembryn interacts with Gsalpha and Gqalpha and is translocated to the plasma membrane in response to isoproterenol and carbachol. J Cell Physiol 195:151–157.

Kleppisch T, Voigt V, Allmann R, Offermanns S. 2001. G(alpha)q-deficient mice lack metabotropic glutamate receptor-dependent long-term depression but show normal long-term potentiation in the hippocampal CA1 region. J Neurosci 21:4943–4948.

Knoblich JA. 2008. Mechanisms of asymmetric stem cell division. Cell 132:583–597.

Knoblich JA. 2010. Asymmetric cell division: recent developments and their implications for tumour biology. Nat Rev Mol Cell Biol 11:849–860.

Konno D, Shioi G, Shitamukai A, Mori A, Kiyonari H, Miyata T, Matsuzaki F. 2008. Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. Nat Cell Biol 10:93–101.

Kosodo Y, Roper K, Haubensak W, Marzesco AM, Corbeil D, Huttner WB. 2004. Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. Embo J 23:2314–2324.

Kraut R, Chia W, Jan LY, Jan YN, Knoblich JA. 1996. Role of inscuteable in orienting asymmetric cell divisions in Drosophila. Nature 383:50–55.

Kriegstein A, Alvarez-Buylla A. 2009. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149–184.

Kruegel J, Miosge N. 2010. Basement membrane components are key players in specialized extracellular matrices. Cell Mol Life Sci.

Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL. 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13:424–436.

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Lee CY, Wilkinson BD, Siegrist SE, Wharton RP, Doe CQ. 2006. Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. Dev Cell 10:441–449.

Lee SE, Simons SB, Heldt SA, Zhao M, Schroeder JP, Vellano CP, Cowan DP, Ramineni S, Yates CK, Feng Y, Smith Y, Sweatt JD, Weinshenker D, Ressler KJ, Dudek SM, Hepler JR. 2010. RGS14 is a natural suppressor of both synaptic plasticity in CA2 neurons and hippocampal-based learning and memory. Proc Natl Acad Sci U S A 107:16994–16998.

Li HS, Wang D, Shen Q, Schonemann MD, Gorski JA, Jones KR, Temple S, Jan LY, Jan YN. 2003. Inactivation of Numb and Numblike in embryonic dorsal forebrain impairs neurogenesis and disrupts cortical morphogenesis. Neuron 40:1105–1118.

Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. 1999. Requirement for Wnt3 in vertebrate axis formation. Nat Genet 22:361–365.

Lu CC, Brennan J, Robertson EJ. 2001. From fertilization to gastrulation: axis formation in the mouse embryo. Curr Opin Genet Dev 11:384–392.

Lu CC, Robertson EJ. 2004. Multiple roles for Nodal in the epiblast of the mouse embryo in the establishment of anterior-posterior patterning. Dev Biol 273:149–159.

Manabe N, Hirai S, Imai F, Nakanishi H, Takai Y, Ohno S. 2002. Association of ASIP/mPAR-3 with adherens junctions of mouse neuroepithelial cells. Dev Dyn 225:61–69.

Marthiens V, ffrench-Constant C. 2009. Adherens junction domains are split by asymmetric division of embryonic neural stem cells. EMBO Rep 10:515–520.

Martin-McCaffrey L, Hains MD, Pritchard GA, Pajak A, Dagnino L, Siderovski DP, D'Souza SJ. 2005. Differential expression of regulator of G-protein signaling R12 subfamily members during mouse development. Dev Dyn 234:438–444.

Martin-McCaffrey L, Willard FS, Oliveira-dos-Santos AJ, Natale DR, Snow BE, Kimple RJ, Pajak A, Watson AJ, Dagnino L, Penninger JM, Siderovski DP, D'Souza SJ. 2004. RGS14 is a mitotic spindle protein essential from the first division of the mammalian zygote. Dev Cell 7:763–769.

Mesnard D, Filipe M, Belo JA, Zernicka-Goetz M. 2004. The anterior-posterior axis emerges respecting the morphology of the mouse embryo that changes and aligns with the uterus before gastrulation. Curr Biol 14:184–196.

Mesnard D, Guzman-Ayala M, Constam DB. 2006. Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. Development 133:2497–2505.

Miller KG, Emerson MD, McManus JR, Rand JB. 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 KG, Rand JB. 2000. A role for RIC-8 (Synembryn) and GOA-1 (G(o)alpha) in regulating a subset of centrosome movements during early embryogenesis in Caenorhabditis elegans. Genetics 156:1649–1660.

Morin X, Jaouen F, Durbec P. 2007. Control of planar divisions by the G-protein regulator LGN maintains progenitors in the chick neuroepithelium. Nat Neurosci 10:1440–1448.

Morris RG, Garrud P, Rawlins JN, O'Keefe J. 1982. Place navigation impaired in rats with hippocampal lesions. Nature 297:681–683.

Murai K, Qiu R, Zhang H, Wang J, Wu C, Neubig RR, Lu Q. 2010. Galpha subunit coordinates with ephrin-B to balance self-renewal and differentiation in neural progenitor cells. Stem Cells 28:1581–1589.

Nagai Y, Nishimura A, Tago K, Mizuno N, Itoh H. 2010. Ric-8B stabilizes the alpha subunit of stimulatory G protein by inhibiting its ubiquitination. J Biol Chem 285:11114–11120.
Nakao K, Ikegaya Y, Yamada MK, Nishiyama N, Matsuki N. 2002. Hippocampal long-term depression as an index of spatial working memory. Eur J Neurosci 16:970–974.
Nakaya Y, Sheng G. 2008. Epithelial to mesenchymal transition during gastrulation: an embryological view. Dev Growth Differ 50:755–766.
Nakaya Y, Sukowati EW, Wu Y, Sheng G. 2008. RhoA and microtubule dynamics control cell-basement membrane interaction in EMT during gastrulation. Nat Cell Biol 10:765–775.
Neves SR, Ram PT, Iyengar R. 2002. G protein pathways. Science 296:1636–1639.
Nguyen-Ngoc T, Afshar K, Gonczy P. 2007. Coupling of cortical dynein and G alpha proteins mediates spindle positioning in Caenorhabditis elegans. Nat Cell Biol 9:1294–1302.
Nipper RW, Siller KH, Smith NR, Doe CQ, Prehoda KE. 2007. Galphai generates multiple Pins activation states to link cortical polarity and spindle orientation in Drosophila neuroblasts. Proc Natl Acad Sci U S A 104:14306–14311.
Nishimura A, Okamoto M, Sugawara Y, Mizuno N, Yamauchi J, Itoh H. 2006. Ric-8A potentiates Gq-mediated signal transduction by acting downstream of G protein-coupled receptor in intact cells. Genes Cells 11:487–498.
Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR. 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136–144.
Noctor SC, Martinez-Cerdeno V, Kriegstein AR. 2007. Contribution of intermediate progenitor cells to cortical histogenesis. Arch Neurol 64:639–642.
Noctor SC, Martinez-Cerdeno V, Kriegstein AR. 2008. Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28–44.
Norris DP, Robertson EJ. 1999. Asymmetric and node-specific nodal expression patterns are controlled by two distinct cis-acting regulatory elements. Genes Dev 13:1575–1588.
Offermanns S, Hashimoto K, Watanabe M, Sun W, Kurihara H, Thompson RF, Inoue Y, Kano M, Simon MI. 1997a. Impaired motor coordination and persistent multiple climbing fiber innervation of cerebellar Purkinje cells in mice lacking Galphaq. Proc Natl Acad Sci U S A 94:14089–14094.
Offermanns S, Toombs CF, Hu YH, Simon MI. 1997b. Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183–186.
Pawlisz AS, Mutch C, Wynshaw-Boris A, Chenn A, Walsh CA, Feng Y. 2008. Lis1-Nde1-dependent neuronal fate control determines cerebral cortical size and lamination. Hum Mol Genet 17:2441–2455.
Perea-Gomez A, Camus A, Moreau A, Grieve K, Moneron G, Dubois A, Cibert C, Collignon J. 2004. Initiation of gastrulation in the mouse embryo is preceded by an apparent shift in the orientation of the anterior-posterior axis. Curr Biol 14:197–207.
Perea-Gomez A, Meilhac SM, Piotrowska-Nitsche K, Gray D, Collignon J, Zernicka-Goetz M. 2007. Regionalization of the mouse visceral endoderm as the blastocyst transforms into the egg cylinder. BMC Dev Biol 7:96.
Perea-Gomez A, Rhinn M, Ang SL. 2001. Role of the anterior visceral endoderm in restricting posterior signals in the mouse embryo. Int J Dev Biol 45:311–320.
Perea-Gomez A, Vella FD, Shawlot W, Oulad-Abdelghani M, Chazaud C, Meno C, Pfister V, Chen L, Robertson E, Hamada H, Behringer RR, Ang SL. 2002. Nodal

antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. Dev Cell 3:745–756.
Petersen PH, Zou K, Krauss S, Zhong W. 2004. Continuing role for mouse Numb and Numbl in maintaining progenitor cells during cortical neurogenesis. Nat Neurosci 7:803–811.
Pfister S, Steiner KA, Tam PP. 2007. Gene expression pattern and progression of embryogenesis in the immediate post-implantation period of mouse development. Gene Expr Patterns 7:558–573.
Rasin MR, Gazula VR, Breunig JJ, Kwan KY, Johnson MB, Liu-Chen S, Li HS, Jan LY, Jan YN, Rakic P, Sestan N. 2007. Numb and Numbl are required for maintenance of cadherin-based adhesion and polarity of neural progenitors. Nat Neurosci 10:819–827.
Reynolds NK, Schade MA, Miller KG. 2005. Convergent, RIC-8-dependent Galpha signaling pathways in the Caenorhabditis elegans synaptic signaling network. Genetics 169:651–670.
Rhyu MS, Jan LY, Jan YN. 1994. Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell 76:477–491.
Rivera-Perez JA, Mager J, Magnuson T. 2003. Dynamic morphogenetic events characterize the mouse visceral endoderm. Dev Biol 261:470–487.
Rodriguez TA, Srinivas S, Clements MP, Smith JC, Beddington RS. 2005. Induction and migration of the anterior visceral endoderm is regulated by the extra-embryonic ectoderm. Development 132:2513–2520.
Rolls MM, Albertson R, Shih HP, Lee CY, Doe CQ. 2003. Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. J Cell Biol 163:1089–1098.
Russ AP, Wattler S, Colledge WH, Aparicio SA, Carlton MB, Pearce JJ, Barton SC, Surani MA, Ryan K, Nehls MC, Wilson V, Evans MJ. 2000. Eomesodermin is required for mouse trophoblast development and mesoderm formation. Nature 404:95–99.
Sanada K, Tsai LH. 2005. G protein betagamma subunits and AGS3 control spindle orientation and asymmetric cell fate of cerebral cortical progenitors. Cell 122:119–131.
Schade MA, Reynolds NK, Dollins CM, Miller KG. 2005. Mutations that rescue the paralysis of Caenorhabditis elegans ric-8 (synembryn) mutants activate the G alpha(s) pathway and define a third major branch of the synaptic signaling network. Genetics 169:631–649.
Schaefer M, Petronczki M, Dorner D, Forte M, Knoblich JA. 2001. Heterotrimeric G proteins direct two modes of asymmetric cell division in the Drosophila nervous system. Cell 107:183–194.
Schaefer M, Shevchenko A, Knoblich JA. 2000. A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in Drosophila. Curr Biol 10:353–362.
Schneider SQ, Bowerman B. 2003. Cell polarity and the cytoskeleton in the Caenorhabditis elegans zygote. Annu Rev Genet 37:221–249.
Schober M, Schaefer M, Knoblich JA. 1999. Bazooka recruits Inscuteable to orient asymmetric cell divisions in Drosophila neuroblasts. Nature 402:548–551.

Shawlot W, Deng JM, Behringer RR. 1998. Expression of the mouse cerberus-related gene, Cerr1, suggests a role in anterior neural induction and somitogenesis. Proc Natl Acad Sci U S A 95:6198–6203.
Shen CP, Jan LY, Jan YN. 1997. Miranda is required for the asymmetric localization of Prospero during mitosis in Drosophila. Cell 90:449–458.
Siderovski DP, Willard FS. 2005. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci 1:51–66.
Siegrist SE, Doe CQ. 2005. Microtubule-induced Pins/Galphai cortical polarity in Drosophila neuroblasts. Cell 123:1323–1335.
Siller KH, Cabernard C, Doe CQ. 2006. The NuMA-related Mud protein binds Pins and regulates spindle orientation in Drosophila neuroblasts. Nat Cell Biol 8:594–600.
Siller KH, Doe CQ. 2008. Lis1/dynactin regulates metaphase spindle orientation in Drosophila neuroblasts. Dev Biol 319:1–9.
Siller KH, Doe CQ. 2009. Spindle orientation during asymmetric cell division. Nat Cell Biol 11:365–374.
Simon MI, Strathmann MP, Gautam N. 1991. Diversity of G proteins in signal transduction. Science 252:802–808.
Smith LJ. 1985. Embryonic axis orientation in the mouse and its correlation with blastocyst relationships to the uterus. II. Relationships from 4 1/4 to 9 1/2 days. J Embryol Exp Morphol 89:15–35.
Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M, 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.
Spana EP, Kopczynski C, Goodman CS, Doe CQ. 1995. Asymmetric localization of numb autonomously determines sibling neuron identity in the Drosophila CNS. Development 121:3489–3494.
Srinivas S. 2006. The anterior visceral endoderm-turning heads. Genesis 44:565–572.
Srinivas S, Rodriguez T, Clements M, Smith JC, Beddington RS. 2004. Active cell migration drives the unilateral movements of the anterior visceral endoderm. Development 131:1157–1164.
Srinivasan DG, Fisk RM, Xu H, van den Heuvel S. 2003. A complex of LIN-5 and GPR proteins regulates G protein signaling and spindle function in C elegans. Genes Dev 17:1225–1239.
Sun X, Meyers EN, Lewandoski M, Martin GR. 1999. Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. Genes Dev 13:1834–1846.
Zhang XM, Ramalho-Santos M, McMahon AP. 2001. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. Cell 106:781–792.
Zhou X, Sasaki H, Lowe L, Hogan BL, Kuehn MR. 1993. Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. Nature 361:543–547.
Tall GG, Gilman AG. 2004. Purification and functional analysis of Ric-8A: a guanine nucleotide exchange factor for G-protein alpha subunits. Methods Enzymol 390:377–388.
Tall GG, Gilman AG. 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 GG, Krumins AM, Gilman AG. 2003. Mammalian Ric-8A (synembryn) is a heterotrimeric Galpha protein guanine nucleotide exchange factor. J Biol Chem 278:8356–8362.

Tam PP, Beddington RS. 1987. The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. Development 99:109–126.

Tam PP, Loebel DA. 2007. Gene function in mouse embryogenesis: get set for gastrulation. Nat Rev Genet 8:368–381.

Tanaka J, Nakagawa S, Kushiya E, Yamasaki M, Fukaya M, Iwanaga T, Simon MI, Sakimura K, Kano M, Watanabe M. 2000. Gq protein alpha subunits Galphaq and Galpha11 are localized at postsynaptic extra-junctional membrane of cerebellar Purkinje cells and hippocampal pyramidal cells. Eur J Neurosci 12:781–792.

Thomas CJ, Tall GG, Adhikari A, Sprang SR. 2008. Ric-8A catalyzes guanine nucleotide exchange on G alphai1 bound to the GPR/GoLoco exchange inhibitor AGS3. J Biol Chem 283:23150–23160.

Thomas P, Beddington R. 1996. Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. Curr Biol 6:1487–1496.

Thomas PQ, Brown A, Beddington RS. 1998. Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. Development 125:85–94.

Tremblay KD, Dunn NR, Robertson EJ. 2001. Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. Development 128:3609–3621.

Tsang TE, Shawlot W, Kinder SJ, Kobayashi A, Kwan KM, Schughart K, Kania A, Jessell TM, Behringer RR, Tam PP. 2000. Lim1 activity is required for intermediate mesoderm differentiation in the mouse embryo. Dev Biol 223:77–90.

Wang H, Ng KH, Qian H, Siderovski DP, Chia W, Yu F. 2005. Ric-8 controls Drosophila neural progenitor asymmetric division by regulating heterotrimeric G proteins. Nat Cell Biol 7:1091–1098.

Wang SC, Lai HL, Chiu YT, Ou R, Huang CL, Chern Y. 2007. Regulation of type V adenylate cyclase by Ric8a, a guanine nucleotide exchange factor. Biochem J 406:383–388.

Varlet I, Collignon J, Robertson EJ. 1997. nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. Development 124:1033–1044.

Watson N, Linder ME, Druey KM, Kehrl JH, Blumer KJ. 1996. RGS family members: GTPase-activating proteins for heterotrimeric G-protein alpha-subunits. Nature 383:172–175.

Vellano CP, Shu FJ, Ramineni S, Yates CK, Tall GG, Hepler JR. 2010. Activation of the regulator of G protein signaling 14-Galphai1-GDP signaling complex is regulated by resistance to inhibitors of cholinesterase-8A. Biochemistry 50:752–762.

Wilkie TM, Kinch L. 2005. New roles for Galpha and RGS proteins: communication continues despite pulling sisters apart. Curr Biol 15:R843–854.

Williamson RA, Henry MD, Daniels KJ, Hrstka RF, Lee JC, Sunada Y, Ibraghimov-Beskrovnaya O, Campbell KP. 1997. Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. Hum Mol Genet 6:831–841.

Winnier G, Blessing M, Labosky PA, Hogan BL. 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9:2105–2116.

Wiser O, Qian X, Ehlers M, Ja WW, Roberts RW, Reuveny E, Jan YN, Jan LY. 2006. Modulation of basal and receptor-induced GIRK potassium channel activity and neuronal excitability by the mammalian PINS homolog LGN. Neuron 50:561–573.

Von Dannecker LE, Mercadante AF, Malnic B. 2005. Ric-8B, an olfactory putative GTP exchange factor, amplifies signal transduction through the olfactory-specific G-protein Galphaolf. J Neurosci 25:3793–3800.

Von Dannecker LE, Mercadante AF, Malnic B. 2006. Ric-8B promotes functional expression of odorant receptors. Proc Natl Acad Sci U S A 103:9310–9314.

Woodard GE, Huang NN, Cho H, Miki T, Tall GG, Kehrl JH. 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.

Yamamoto M, Beppu H, Takaoka K, Meno C, Li E, Miyazono K, Hamada H. 2009. Antagonism between Smad1 and Smad2 signaling determines the site of distal visceral endoderm formation in the mouse embryo. J Cell Biol 184:323–334.

Yamamoto M, Saijoh Y, Perea-Gomez A, Shawlot W, Behringer RR, Ang SL, Hamada H, Meno C. 2004. Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo. Nature 428:387–392.

Yingling J, Youn YH, Darling D, Toyo-Oka K, Pramparo T, Hirotsune S, Wynshaw-Boris A. 2008. Neuroepithelial stem cell proliferation requires LIS1 for precise spindle orientation and symmetric division. Cell 132:474–486.

Yu F, Cai Y, Kaushik R, Yang X, Chia W. 2003a. Distinct roles of Galphai and Gbeta13F subunits of the heterotrimeric G protein complex in the mediation of Drosophila neuroblast asymmetric divisions. J Cell Biol 162:623–633.

Yu F, Kuo CT, Jan YN. 2006. Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. Neuron 51:13–20.

Yu F, Morin X, Cai Y, Yang X, Chia W. 2000. Analysis of partner of inscuteable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscuteable apical localization. Cell 100:399–409.

Yu F, Morin X, Kaushik R, Bahri S, Yang X, Chia W. 2003b. A mouse homologue of Drosophila pins can asymmetrically localize and substitute for pins function in Drosophila neuroblasts. J Cell Sci 116:887–896.

Yu S, Yu D, Lee E, Eckhaus M, Lee R, Corria Z, Accili D, Westphal H, Weinstein LS. 1998. Variable and tissue-specific hormone resistance in heterotrimeric Gs protein alpha-subunit (Gsalpha) knockout mice is due to tissue-specific imprinting of the gsalpha gene. Proc Natl Acad Sci U S A 95:8715–8720.

PUBLICATIONS

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Scientific work

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Scientific publications

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

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

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 239:3404–3415.

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2006 Laboratory course on introduction to *C. elegans*, University of Tartu, Estonia

Peamised uurimisvaldkonnad

G valkude guaniini nukleotiidivahetusfaktor RIC-8 funktsiooni uurimine imetajate arengus (gametogeneesis, implantatsioonil, gastrulatsioonis, neurogeneesis) ja närvisüsteemis.

Publikatsioonid

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

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

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 239:3404–3415.

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