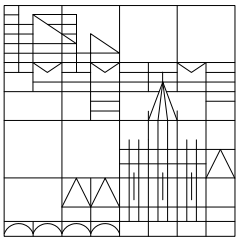

Functional Analysis of Reggie Proteins during Neuronal Differentiation and Axon Regeneration

Dissertation

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Abbreviations

3D	three-dimensional
°C	degree Celsius
aa	amino acid(s)
Ab	antibody
Aβ	*β*-amyloid peptide
APP	amyloid precursor protein
APS	ammonium persulfate
ArgBP2	Arg/Abl-binding protein 2
bp	basepairs
BSA	bovine serum albumin
CA	constitutive active
CAM	cell adhesion molecule
CAP	c-Cbl-associated protein
cDNA	complementary DNA
CNS	central nervous system
ctrl	control
ddH_2O	double-distilled H_2O
DN	dominant negative
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DRG	dorsal root ganglion
DRM	detergent-resistant membrane
dsDNA	double-stranded DNA
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
ESA	epidermal surface antigen
et al.	et alteri
FAK	focal adhesion kinase
F-actin	filamentous actin
g	gram
GAP	growth-associated protein or GTPase-activating protein
GDI	GDP dissociation inhibitors
GDP	guanosine 5'-diphosphate
GEF	GTP exchange factor
GLUT	glucose transporter
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate

h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hh	hedgehog
HRP	horse radish peroxidase
HSP	heat shock protein
i.e.	id est
IGF	insulin-like growth factor
IgG	immunoglobulin G
IRS	insulin receptor substrate
JNK	jun N-terminal kinase
kb	kilobase
kDa	kilodalton
l	litre
m	milli
M	molar
mAb	monoclonal antibody
MAP	mitogen-activated protein
MEM	minimal essential medium
MEK	mitogen-activated protein/extracellular signal-regulated kinase kinase
Mo	morpholino antisense oligonucleotide
mRNA	messenger RNA
MVB	multivesicular body
µ	micro
n	nano
n.a.	numerical aperture
NMR	nuclear magnetic resonance
OD	optical density
Oligo-dT	oligodeoxythymidine
ONS	optic nerve section
p	pico
P-	phospho-
pAb	polylclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	potentia Hydrogenii
PHB	prohibitin
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system

PrP^C	cellular prion protein
PTOV	prostate tumor overexpressed protein
Pyk2	proline-rich tyrosine kinase 2
R1EA	reggie-1EA deletion mutant (aa 184-390)
RNA	ribonucleic acid
rpm	rounds per minute
RT	reverse transcriptase
RGC	retinal ganglion cell
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interfering RNA
SoHo	Sorbin homology
Sos	son of sevenless
SPFH	stomatin/prohibitin/flotillin/HflK/C
ssDNA	single-stranded DNA
TBS	tris-buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGN	trans-Golgi network
Tris	tris(hydroxymethyl)aminomethane
UTR	untranslated region
UV	ultraviolet
V	volt
v/v	volume/volume
Wg	wingless
w/v	weight/volume

Summary

The reggies are scaffolding proteins of membrane microdomains and involved in various cellular processes including neuronal differentiation. Reggie-1 and -2 were originally discovered as proteins upregulated during axon regeneration in retinal ganglion cells (RGCs) after optic nerve injury, suggesting a function of these proteins in axon regeneration. Loss-of-function studies via small interfering RNAs (siRNAs) or morpholino antisense oligonucleotides (Mos) against reggie-1 and -2 were performed to clarify, whether the reggies are causally linked to neuronal differentiation in cell cultures and/or axonal regeneration after optic nerve section (ONS) in zebrafish. Silencing of reggie-1 with siRNAs caused a simultaneous loss of reggie-2 protein by proteasomal degradation in N2a cells. Depletion of both reggies in differentiating N2a cells led to significantly shorter filopodia, more cells with lamellipodia and fewer with neurites, a defect which was rescued by a reggie-1 construct without siRNA binding sites. Furthermore, reggie knockdown strongly perturbed the balanced activation of the Rho family GTPases RhoA, Rac1 and cdc42 and affected activation of MAP kinases p38 and ERK1/2, Ras and focal adhesion kinase (FAK). Downregulation of zebrafish reggie-1a, -2a and -2b expression *in vivo* by application of reggie-specific Mos directly after ONS significantly reduced the capability of zebrafish RGCs to regenerate axons. In an outgrowth assay, the number of re-growing RGC axons *in vitro* from reggie Mo-treated retinae was markedly reduced compared to controls. Moreover, the number of axon regenerating RGCs *in vivo*, identified by insertion of A488-coupled dextran 7d after Mo-application, decreased by 69% in reggie Mo-retinae as opposed to controls. At 10 and 14 d, labeled RGCs decreased by 53 and 33%, respectively, in correlation with the gradual loss of the Mos. Thus, as suggested by their prominent re-expression upon lesion, the reggies represent neuron-intrinsic factors for axon outgrowth and regeneration *in vitro* and *in vivo* by coordinating signal transduction pathways to control cytoskeletal remodeling.

Zusammenfassung

Die Reggie-Proteine bilden die Basiskomponente spezieller Mikrodomänen (Reggie-Mikrodomänen) der Membran und sind an verschiedenen zellulären Prozessen beteiligt, u. a. an der neuronalen Differenzierung und Axon-Regeneration. Reggie-1 und -2 wurden als Proteine entdeckt, deren Expression nach Läsion des optischen Nervs im Goldfisch in retinalen Ganglienzellen (RGZ) hochreguliert wird. Diese Beobachtung implizierte eine Funktion der Reggie-Proteine in der neuronalen Regeneration. Untersuchungen, bei denen ein Funktionsverlust durch Herunterregulation der Expression (= Knockdown) der Reggie-Proteine herbeigeführt wurde, sollen Aufschluss über die ursächliche Beteilung der Reggie-Proteine an der neuronalen Differenzierung an Zellkulturmodellen *in vitro* und der axonalen Regeneration im optischen Nerv des Zebrafisch *in vivo* liefern. Knockdown von Reggie-1 führte zu einem gleichzeitigen Verlust von Reggie-2 durch proteosomalen Abbau von Reggie-2 in neuronalen (N2a) Zellen. Der Verlust von Reggie-1 und -2 führte weiterhin zu kürzeren Filopodien, weniger Zellen mit Neuriten und mehr Zellen mit Lamellipodien. Diese morphologischen Defekte konnten durch die ektopische Expression eines Reggie-1 Konstrukts aufgehoben werden, welches resistent gegenüber dem Knockdown ist. Außerdem führte der Knockdown der Reggie-Proteine zu einer gestörten Aktivierung der kleinen Rho GTPasen RhoA, Rac1 und cdc42, der MAP Kinasen p38 und ERK1/2, Ras und der Proteintyrosinkinase FAK. *In vivo* Knockdown von Reggie-1a, -2a and -2b im Zebrafisch durch das Einbringen von Morpholino Oligonukleotiden unmittelbar nach Durchtrennung des optischen Nervs führte zu einer signifikanten Reduktion regenerierender RGZ Axone in Retina Auswachs-Assays sowohl *in vitro* als auch *in vivo*. Die Anzahl Axon regenerierender RGZs, die durch Einbringung von fluoreszierendem Dextran sieben Tage nach der Morpholino Applikation identifiziert werden konnten, sank um 69% nach Reggie Knockdown im Vergleich zu den Kontrollen. Die gleiche Quantifizierung bei zehn bzw. 14 Tagen nach Morpholino

Anwendung ergab Reduktionen in der Anzahl von RGZs mit regenerierenden Axonen von 53 bzw. 33%, was durch den allmählichen Verlust an Morpholinos bedingt ist.

Demnach stellen die Reggie-Proteine neuron-intrinsische Faktoren für das Wachstum und die Regeneration von Axonen dar, wie bei ihrer Entdeckung vermutet. Diese Funktion der Reggie-Proteine wird durch Beeinflussung/Koordination der Aktivität verschiedener Signaltransduktions-Moleküle und folglich der Restrukturierung des Zytoskeletts hervorgerufen.

1 Introduction

The reggie proteins, as putative neuron intrinsic factors important for neuronal differentiation and regeneration, are subject of investigation in this study. The central question of this work is, whether reggie proteins affect axonal regeneration after optic nerve section (ONS) in zebrafish. At first a comprehensive overview on the reggie proteins is given in the introduction. The second part deals with a brief outline of extrinsic and intrinsic factors determining neural growth, the comparison of fish versus mammalian model systems in the capacity of optic nerve regeneration and insulin growth factor-1 (IGF-1) as neurite inducing factor. At last the connection of reggie proteins to neuronal differentiation and regeneration and links to the actin cytoskeleton are pointed out separately.

1.1 The reggie proteins: highly conserved, enriched in the developing/regenerating nervous system and scaffolding proteins of membrane microdomains

1.1.1 Discovery

The discovery of the reggies is a story told in three acts including different choices of names. Originally, the full length reggie proteins, reggie-1 and -2, were characterized as proteins upregulated during axon regeneration of goldfish retinal ganglion cells (RGCs) in 1997 (Schulte et al., 1997). Reggie-1 and reggie-2 mRNAs as well as proteins could be detected in injured and differentiating but not in mature RGCs. Due to their connection to neuronal *reg*eneration they were called *reg*gies. In the same year, the same two proteins were independently described as proteins enriched in *flo*ating detergent insoluble membrane fractions from bovine brain and murine lung tissue yielding to the names *flo*tillin-1 and -2, corresponding to reggie-2 and reggie-1, respectively (Bickel et al., 1997). Besides these two reports,

already 1994 in a screen for the antigen of mouse monoclonal antibody ECS-1, a truncated version of reggie-1 called ESA (epidermal surface antigen) was found (Schroeder et al., 1994). Later on it was shown that ESA was not the true antigen of ECS-1 (Hazarika et al., 1999). The association of the reggies with neuronal regeneration makes them a highly interesting subject of scientific study.

1.1.2 Reggies are highly conserved during evolution

The reggie protein family includes two highly homologues proteins as mentioned above, reggie-1 and -2, indicating one common ancestor. Bony fish express up to four reggie genes, *reggie-1a* plus *-1b* and *reggie-2a* plus *-2b*, generated by a genome duplication in this group. The zebrafish, as a member of bony fish, maintained *reggie-1a*, *reggie-2a and -2b*, with amino acid identities of 53% between reggie-1a and -2a and 83% within reggie-2 (Malaga-Trillo et al., 2002). Due to a partially duplicated genome of *Xenopus laevis* (Kobel and Du Pasquier, 1986) it carries two copies of *reggie-2*, named *flotillin 1A* and *flotillin 1B*, and one copy of *flotillin 2/reggie-1* (Pandur et al., 2004). Human *reggie-1* gene is located at chromosome 17 and *reggie-2* gene at chromosome 6, the single-copy genes consist of 11 or 13 exons, respectively, and both transcripts are encoded in the minus strand (Schroeder et al., 1994; Edgar and Polak, 2001).

The reggie proteins are remarkably evolutionarily conserved (Malaga-Trillo et al., 2002). Reggie proteins are highly similar within metazoans, whereas bacteria, archaea, plants and fungi exhibit the related reggie-like proteins with lower protein similarities compared to the metazoan homologues (Edgar and Polak, 2001; Borner et al., 2005; Rivera-Milla et al., 2006; Hinderhofer et al., 2009). To get an idea about the high conservation of metazoan reggies: between human and mouse the amino acid sequence identity is 99% and human reggie-1 is 64% identical to fly reggie-1 (Galbiati et al., 1998; Malaga-Trillo et al., 2002). The strikingly high homology of reggies among metazoans as well as the related reggie-like proteins of prokaryo-

tes, fungi and plants suggests an important and basic cellular function of these proteins relying on their structural features.

1.1.3 Structural and functional domains of reggie proteins

In the next paragraphs the characteristic domains of reggie are discussed. Amino acid (aa) positions and molecular masses are referred to human reggie, if not otherwise specified. Reggie-1, consisting of 428 aa, as well as reggie-2, with 427 aa, are proteins with a molecular mass of approximately 47 kDa.

N-terminally the reggies share a protein domain, the SPFH (*S*tomatin/*P*rohibitin/*F*lotillin/*H*flK/C) domain (Figure 1.1A; Tavernarakis et al., 1999) or also called head domain (Stuermer, 2009). Independently this head domain was described with little variations in the amino acid boundaries as prohibitin (PHB) homology domain (Schultz et al., 1998). The head domain encompasses around 45% of each reggie, reaching from residue 7 to 190 in case of reggie-1 and residue 5 to 213 in case of reggie-2 (Rivera-Milla et al., 2006). Thus, the reggies are considered to belong to the SPFH superfamily (Tavernarakis et al., 1999), which contains a variety of protein families spread across kingdoms (Hinderhofer et al., 2009).

Within the head domain, reggie-1 and -2 possess two hydrophobic stretches corresponding to residues 14 - 35 and 134 - 150 in case of reggie-1 or residues 10 - 30 and 134 - 151 in case of reggie-2, respectively (Rivera-Milla et al., 2006). The hydrophobic domains of reggie are not long enough to act as trans-membrane domains, although it was initially suggested that the reggies would traverse the membrane (Bickel et al., 1997; Schulte et al., 1997; Gkantiragas et al., 2001). Afterwards it was shown that the reggies associate with the cytoplasmic face of the plasma membrane (Morrow et al., 2002). A membrane topology model proposes two hairpin-loops within the cytosolic leaflet for the head domain of reggie-1 similar to caveolin (Figure 1.1B; Bauer and Pelkmans, 2006). Furthermore, reggie-1 is

co-translationally myristoylated at glycine residue 2 and post-translationally palmitoylated at cysteines 4, 19 and 20 (Neumann-Giesen et al., 2004), whereas reggie-2 is only palmitoylated in cysteine 34 (Morrow et al., 2002). However, palmitoylation and myristoylation are sufficient to determine membrane association of reggie-1 (Neumann-Giesen et al., 2004; Neumann-Giesen et al., 2007). Different from reggie-1, both hydrophobic stretches of reggie-2, but primarily the second hydrophobic stretch, contribute to its membrane association (Liu et al., 2005). Controversial data exist, whether palmitoylation of reggie-2 is essential for membrane association as well and this is possibly dependent on the cell type (Morrow et al., 2002; Liu et al., 2005).

Reggie-2 is targeted to specialized, detergent-resistant membrane (DRM) microdomains, also called lipid rafts (for definition see section 1.1.5), by its first hydrophobic domain (Liu et al., 2005), while reggie-1 is reported to associate with lipid rafts to some extent via myristoylation and palmitoylation but additional factors like oligomerization are required (Neumann-Giesen et al., 2004). More recently, it was shown that oligomerization of reggie-1 and -2 is not mediated by their head domains but by their C-terminal halves (Solis et al., 2007).

For the head domain of mouse reggie-1 a three-dimensional (3D) nuclear magnetic resonance (NMR) structure is available at the RCSB Protein Data Bank under ID 1win (Miyamoto et al., 2004). The model confirmed an ellipsoidal, globular domain containing six β-sheets and five α-helices for the head domain. Intriguingly, α-helix 4 match with a putative actin-binding site (Rivera-Milla et al., 2006), when compared with other actin-binding proteins, especially their hydrophobic residues within α-helices of proposed binding sites (Dominguez, 2004). The head domain of reggie-1 was shown to interact with filamentous (F-) actin, which is important for plasma membrane localization and stabilization of reggie microdomains (Langhorst et al., 2007). Nevertheless, the exact function of the head domain is still unclear, although all SPFH proteins share a hydrophobic domain at their N-

termini, they form oligomers and most of them are membrane microdomain associated.

C-terminally the reggies contain a unique domain, termed flotillin/tail domain (Stuermer, 2009), with several characteristic repeats of glutamic acid and alanine (EA repeats; Figure 1.1A; Schroeder et al., 1994; Bickel et al., 1997). The tail domain encompasses about 40% of each reggie, reaching from residue 193 to 365 in case of reggie-1 and residue 190 to 362 in case of reggie-2 (Rivera-Milla et al., 2006). Different to the head domain, the 3D structure of the tail domain is not solved to date, but is predicted to form helical structures with significant coiled-coil content, (Figure 1.1C; Rivera-Milla et al., 2006). It has been shown that the C-terminus of reggie is responsible for homo- and hetero-oligomerization (Neumann-Giesen et al., 2004; Solis et al., 2007) with tetramers as basic and very stable structural units (Solis et al., 2007). Hetero-oligomerization might contribute to the stability of reggie proteins. *Drosophila* reggie-2 protein was shown to be unstable in reggie-1 knockdown mutants (Hoehne et al., 2005) and also other SPFH proteins, such as PHB-1 and -2 (Berger and Yaffe, 1998), were reported to be dependent on each other for their stability. Interestingly, reggies exhibit a C-terminal PDZ3-binding motif like the stomatin proteins of the SPFH superfamily, which forms large multiprotein complexes mediated by its PDZ3-binding motif (Hung and Sheng, 2002; Rivera-Milla et al., 2006).

Several putative phosphorylation sites of reggie are known (Bickel et al., 1997; Lang et al., 1998; Neumann-Giesen et al., 2007). Most of them are located in the head domain (Tyr-24, Tyr-27, Thr-119, Tyr-124, Thr-153, Tyr-158, Tyr-163 for reggie-1) and three are located in the tail domain (Tyr-241, Tyr-348, Tyr-351 for reggie-1). Phosphorylation of tyrosine residue 163 was assumed to regulate the cellular localization and thus function of reggie-1 (Neumann-Giesen et al., 2007).

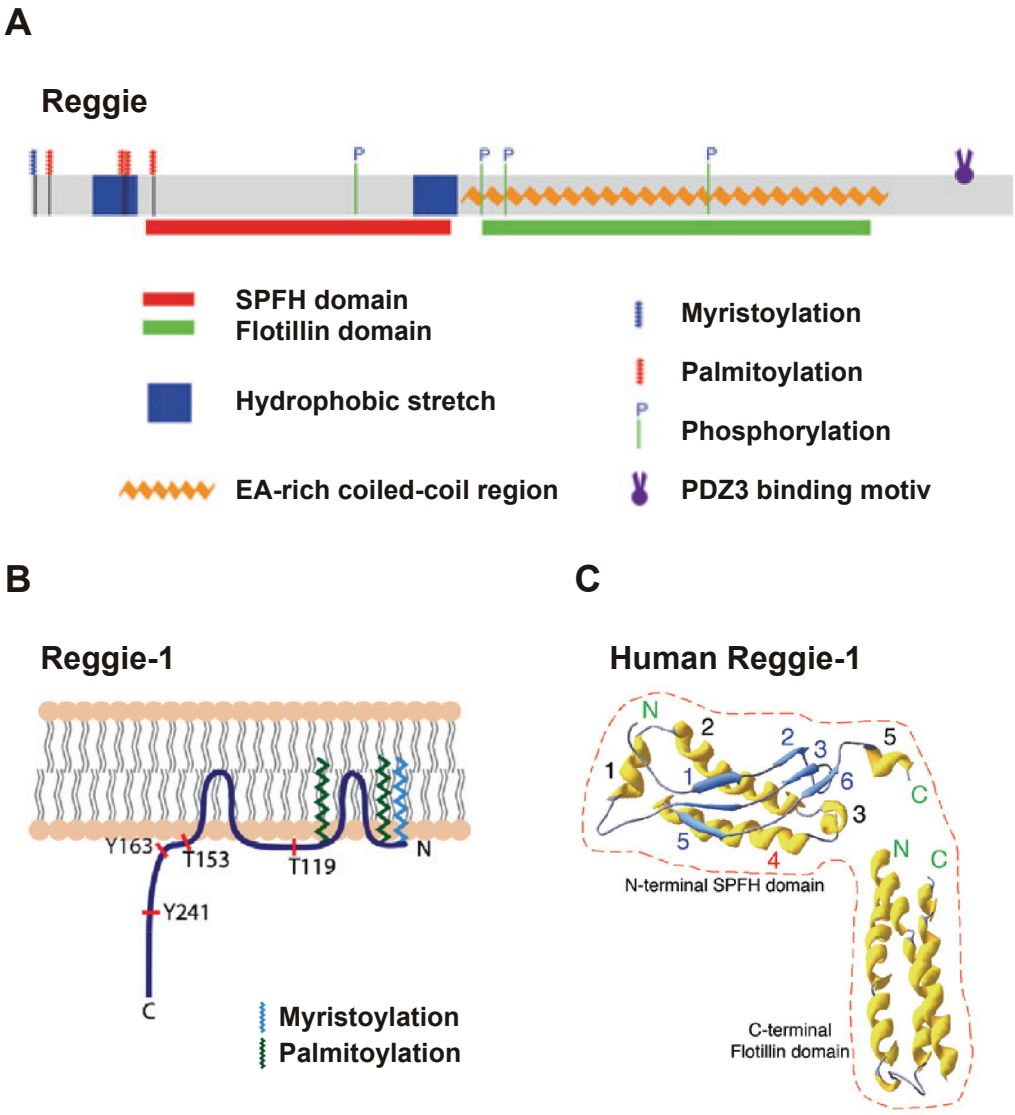

Figure 1.1: Structural features of reggie proteins. A, Consensus model of reggie-1 and -2 shows the relative location of conserved structural and functional protein domains (taken from Rivera-Milla et al., 2006). **B,** One of several putative membrane topologies of reggie-1 contains two membrane-integrated hairpin loops and neighboring phosphorylation sites (red bars) near the lipid bilayer (taken from Bauer and Pelkmans, 2006). **C,** Predicted 3D model of human reggie-1 with α-helices in yellow and β-sheets in blue (taken from Rivera-Milla et al., 2006).

1.1.4 Spatiotemporal patterning of reggie expression: enriched in the developing nervous system

Although the reggies are ubiquitously expressed, and in basically all cells (Volonte et al., 1999; Langhorst et al., 2005; Babuke and Tikkanen, 2007; Stuermer, 2009), they show a distinct spatiotemporal expression pattern.

A comprehensive report on *reggie*-expression during zebrafish development (von Philipsborn et al., 2005) revealed that the *reggies (reggie-1a, -2a* and *-2b*) are first expressed maternally and ubiquitously through blastula and gastrula stages of zebrafish embryos indicating a role of reggies during cell division, migration and differentiation. Upon segmentation, the reggie proteins are present among other structures in all fiber tracts in the developing nervous system and upregulation of reggie expression occurs with axon extension of neurons indicating involvement of both reggies in neuronal differentiation. In the zebrafish visual system, reggie protein expression decreases 3 days post fertilization after the majority of RGC axons formed connections with their targets in the optic tectum (von Philipsborn et al., 2005).

Similar to the expression pattern during zebrafish development both *reggie-2* genes of *Xenopus* are strongly and ubiquitously expressed during early stages of development (Pandur et al., 2004). *Drosophila* reggies are highly expressed in the developing nervous system, but the adult nervous tissue shows a complex reggie expression pattern only in distinct regions including the visual system (Galbiati et al., 1998; Hoehne et al., 2005). Reggie-1 is also involved in the control of the Wingless (Wg) and Hedgehog (Hh) morphogen gradients in *Drosophila* (Katanaev et al., 2008).

The reggies are ubiquitously expressed in adult mammalian organisms with alterations in the expression levels depending on the tissue type. Human and mouse protein-encoding transcriptomes reveal high expression levels of *reggie-1* in bone

marrow, prefrontal cortex, dorsal root ganglia (mouse), thymus, early erythroids, lung, prostate and blood cells whereas *reggie-2* expression levels peak in blood cells, cerebellum peduncles, prefrontal cortex, dorsal root ganglia (mouse), lung, thyroid, cardiac myocytes, pancreatic islet and pituitary (Su et al., 2004). In general, the expression levels of *reggie-1* are higher than the expression levels of *reggie-2*. Additionally, rat reggie-2 was shown to be intensively expressed at synapses (Kokubo et al., 2003).

Upon cell differentiation, reggie expression seems to be upregulated as observed in different cell lines and tissues (Bickel et al., 1997; Volonte et al., 1999; Lopez-Casas and del Mazo, 2003), for example during myoblast differentiation (Volonte et al., 1999). Furthermore upregulation of reggie during axon regeneration is prominent and led to the discovery of both proteins (Schulte et al., 1997; Lang et al., 1998). A twofold upregulation of *reggie-1a* gene expression is reported during optic nerve regeneration in zebrafish (Veldman et al., 2007).

1.1.5 Highly dynamic subcellular localization and trafficking of the reggies

The reggies are mainly localized at the plasma membrane associated with its cytoplasmic leaflet in a variety of cells. Both reggies co-localize to a high extent and are distributed along the plasma membrane in a characteristic punctate pattern at the light microscopic level (Lang et al., 1998). Electron microscopy uncovered stable clusters of ≤ 0.1 μm consisting of reggie-1 and -2, subsequent called reggie microdomains, along the membrane (Lang et al., 1998; Stuermer and Plattner, 2005). At cell-cell contacts or after cross-linking of associated cell surface molecules, for instance the glycosylphosphatidylinositol (GPI)-anchored cellular prion protein (PrP^C) or Thy-1, these microdomains loosely distributed along the plasma membrane become more densely packed (Stuermer et al., 2001; Neumann-Giesen et al., 2004; Stuermer et al., 2004). Due to their abundance in DRM fractions, the reggies

are used as lipid raft markers. Lipid rafts are defined as hypothetical liquid-ordered sterol- and sphingolipid-enriched membrane microdomains (Simons and Toomre, 2000; Pike, 2006). DRMs and associated proteins can be isolated by treatment of cells with cold, non-ionic detergents and subsequent sucrose density fractionation. However, the relationship of DRMs to lipid rafts is under substantial debate (Lichtenberg et al., 2005) as well as the existence of liquid-ordered lipid rafts *in vivo* (Munro, 2003; Yethiraj and Weisshaar, 2007). But ultrastructural and microscopy-based approaches support the hypothesis of existing lipid rafts in living cells and different models of lipid rafts are under discussion (Jacobson et al., 2007). A recent study shows that immuno-isolated secretory vesicles containing raft proteins of yeast exhibited a higher membrane order than the late Golgi membrane and were enriched in sphingolipids and sterols suggesting that lipid rafts do coalesce and concentrate into vesicles budding from the trans-Golgi network (TGN; Klemm et al., 2009). However, existence of specialized membrane microdomains defined by protein scaffolds is not doubted (Zajchowski and Robbins, 2002; Hancock, 2006). Caveolae, small flask-shaped invaginations of the plasma membrane, are described as one special type of those membrane microdomains (Parton, 2003) and caveolin, the principal component of caveolae, is enriched in buoyant, DRM fractions (Kurzchalia and Parton, 1999). Reggies were suggested to reside in caveolae and represent a functional analogue for caveolin in cells lacking caveolin (Bickel et al., 1997; Volonte et al., 1999). But several studies refuted this presumption. Reggies are absent from caveolae (Lang et al., 1998; Souto et al., 2003). Moreover, cell types without caveolin, like neurons and lymphocytes, do not form caveolae-like structures but express reggies, indicating the existence of distinct reggie microdomains (Figure 1.2; Lang et al., 1998; Stuermer et al., 2001).

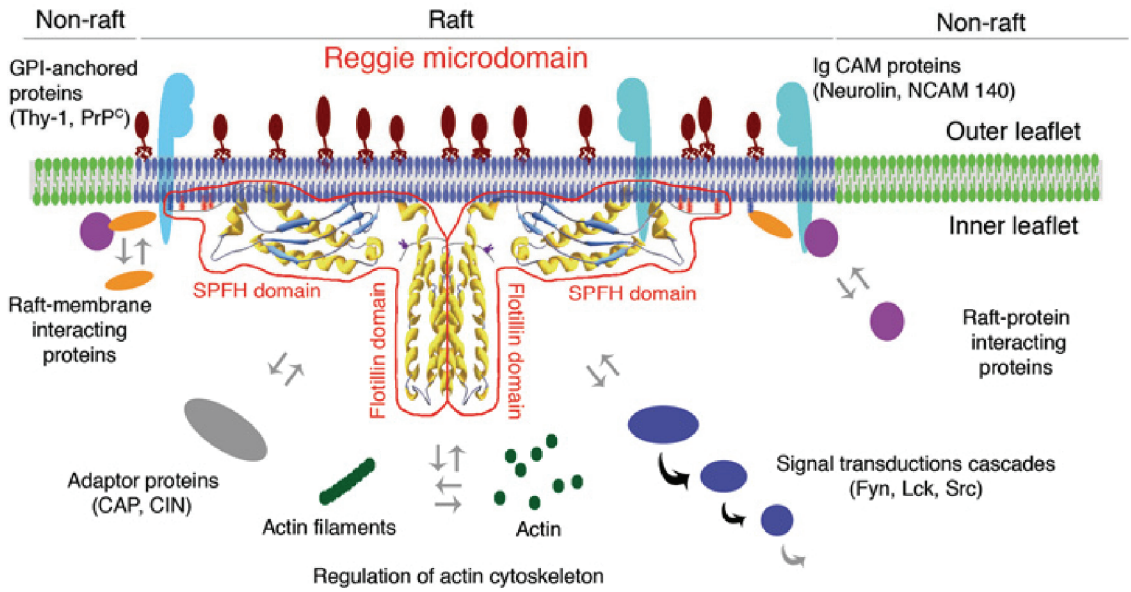

Figure 1.2: Two-dimensional model of the composition of a reggie microdomain. Two oversized reggie molecules (highlighted in red) indicative of hetero-oligomers show the membrane association by the reggie head domain (SPFH domain), whereas the tail domain (flotillin domain) is involved in oligomerization. Proposed interactions of reggie microdomains with membrane and cytosolic proteins are indicated (model taken from Rivera-Milla et al., 2006).

Besides being localized at the plasma membrane, the reggies can be found at vesicles (Table 1.1): late endosomes (Stuermer et al., 2001; Stuermer et al., 2004), recycling endosomes (Gagescu et al., 2000), multivesicular bodies (MVB, de Gassart et al., 2003; Langui et al., 2004; Langhorst et al., 2008), exosomes (de Gassart et al., 2003; Reuter et al., 2004), lysosomes (Stuermer et al., 2004; Langhorst et al., 2008), phago(-lyso)somes (only reggie-2, Dermine et al., 2001; Garin et al., 2001) and the non-vesicular aggresomes (Langhorst et al., 2008). However, localization of reggies to early endosomes was not detected (Langhorst et al., 2005; Langhorst et al., 2008), although they were implied in endocytosis (Glebov et al., 2006; Frick et al., 2007). Reggie-2 seems to be more abundant at intracellular vesicles than at the plasma membrane (Dermine et al., 2001; Liu et al., 2005).

Both reggies localize to the pericentrosomal region in different cell types (Solomon et al., 2002; Langhorst et al., 2005) and lipid droplets (Reuter et al., 2004; Liu et al., 2005). Reggie-2, but not reggie-1, translocates cell-cycle-dependent to the nucleus together with the mitogenic prostate tumour overexpressed protein PTOV-1 (Santamaria et al., 2005).

However, reggie-1 was found on Golgi-associated vesicles but could not be observed in Golgi-stacks or TGN even though deletion mutants of reggie-1 accumulated in the Golgi complex and reggie-1 trafficking was Brefeldin A sensitive, suggesting a Golgi-dependent trafficking of reggie-1 (Langhorst et al., 2008). Whether reggie-2 localizes to the Golgi and travels via a Golgi-dependent pathway to the plasma membrane is not clear (Gkantiragas et al., 2001; Morrow et al., 2002).

The cellular localization and trafficking of reggie-1 and -2 are almost certainly regulated for instance by cell-cell contact formation, growth factor stimulation, cell cycle control, presence of cholesterol and interactions with the cytoskeleton (Langhorst et al., 2008).

Table 1.1: Abundance of reggie-1 and reggie-2 in distinct cellular compartments.

✓ = yes, — = no, **?** = not determined.

Cellular compartment	Reggie-1	Reggie-2
Plasma membrane	✓	✓
Golgi/TGN	?	?
Mitochondria	—	—
Nucleus	—	✓
Endoplasmatic reticulum	—	—
Early endosomes	—	—
Late endosomes	✓	✓
Recycling endosome	✓	✓

MVB	✓	✓
Lysosome	✓	✓
Phagosome	—	✓
Exosome	✓	✓
Aggresome	✓	?
Centrosome	✓	✓

1.1.6 Towards the molecular function of reggie proteins

The reggies or reggie microdomains have been implicated in several cellular functions such as signal transduction, vesicle trafficking, cell proliferation and cytoskeletal dynamics. In addition, they have been associated with some diseases: neurodegenerative diseases, diabetes, cancer and glaucoma.

Meanwhile, a number of interaction partners are known underlying the molecular and cellular functions of the reggies. Examples relevant for this study are presented in the next paragraphs in order to compare and integrate the achieved results with the reported functions. A comprehensive overview on cellular functions and diseases associated with reggie proteins is given in Table 1.2.

1.1.6.1 PrP^C and insulin signaling

Co-localization and co-immunopurification of reggie-1 and -2 with several GPI-linked molecules like Thy-1, PrP^C and F3-contactin hint towards a function in signal transduction of GPI-anchored proteins across the plasma membrane (Lang et al., 1998; Reuter et al., 2004; Stuermer et al., 2004; Stuermer and Plattner, 2005). Reggie accumulation together with PrP^C could be found at PrP^C mediated cell-cell contact sites in S2 cells (Malaga-Trillo et al., 2009). In concert with GPI-anchored

proteins also cell adhesion molecules (CAMs), Src family tyrosine kinases, e.g. Fyn, co-localize and associate with reggie microdomains in several cell types (Figure 1.2; Stuermer et al., 2001; Slaughter et al., 2003; Liu et al., 2005; Kato et al., 2006). For instance, PrP^C accumulation in preformed reggie-caps of T-lymphocytes after antibody cross-linking (Harder et al., 1998) resulted in signal transduction events, e.g. brief elevation of intracellular Calcium concentration, mitogen activated protein (MAP) kinase phosphorylation, local increase of tyrosine phosphorylation and actin polymerization at the reggie-cap (Stuermer et al., 2004). Furthermore, intact reggie microdomains are required for the formation of focal adhesion like structures induced by PrP^C overexpression (Schrock et al., 2009).

The adaptor proteins of the vinexin family, c-Cbl-associated protein CAP/ponsin and relatives, are known to directly interact with reggie-2 via their *So*rbin *Ho*mology (SoHo) domain (Baumann et al., 2000; Kimura et al., 2001; Haglund et al., 2004) and the first hydrophobic stretch of reggie-2 (Liu et al., 2005). The vinexin family includes three members and their main isoforms in parenthesis: Arg/Abl-binding protein ArgBP2 (A, B, neural, gamma), CAP/ponsin (1, 2) and vinculin-binding protein vinexin (alpha, beta, gamma). These proteins possess one SoHo domain at their N-terminus and three SH3 domains at their C-terminus (Kioka et al., 2002). They are ubiquitously expressed, have multiple binding partners, e.g. c-Abl, c-Arg, Sos, c-Cbl, afadin and vinculin, and are involved in cytoskeletal dynamics and signal transduction (Kioka et al., 2002) suggesting a function of reggie-2 in the regulation of signaling events through interaction with SoHo adaptor proteins. For instance, recruitment of CAP in complex with c-Cbl to membrane microdomains of adipocytes after insulin stimulation is mediated by interaction of CAP with reggie-2 and leads to signal transduction (Baumann et al., 2000). This novel insulin signaling pathway over CAP/Cbl/Rho GTPase TC10 as well as the traditional phosphoinositide 3-kinase (PI3K) dependent pathway leads to the actin dependent translocation of the glucose transporter GLUT4 to the plasma mem-

brane and subsequent glucose uptake. In skeletal muscle cells there are two phases of insulin signaling and GLUT4 translocation. The initial step is Caveolin-3/PI3K/protein kinase B (PKB/Akt) dependent and promotes the transfer of the GLUT4 together with reggie-2 towards the plasma membrane. Insulin receptor movement mediates the recruitment of Cbl to reggie-2/GLUT4 containing vesicles and a subsequent reggie-2 mediated Cbl/C3G/TC10-pathway triggers GLUT4 translocation and insertion into the membrane (Fecchi et al., 2006).

Both examples, Prp^C signaling and insulin signaling, are compatible with the reggie microdomain model (Langhorst et al., 2005; Stuermer and Plattner, 2005). This model proposes that reggie proteins are scaffolding proteins and form their own microdomains, which serve as platforms for the assembly of multiprotein signaling complexes (Figure 1.1). A further discussion of the reggie microdomain model will be given in section 5.6.

1.1.6.2 Reggie proteins in G protein-coupled receptor signaling and cell proliferation

In addition to a role of reggie in receptor tyrosine kinase signaling, e.g. insulin signaling, a part in G protein-coupled receptor signaling was suggested. Reggies interact with the trimeric G protein αq (Gαq) which leads to a subsequent activation of p38 MAP kinase (Sugawara et al., 2007). This activation is dependent on Src kinases and lipid raft integrity suggesting that reggies, especially reggie-1, are mediators of Gq-induced p38 activation through Src kinases in lipid rafts (Sugawara et al., 2007). Interactions between reggies and Src kinases are demonstrated by several reports (Stuermer et al., 2001; Liu et al., 2005; Kato et al., 2006). Moreover, reggie-2 was shown to co-immunoprecipitate with a G protein-coupled receptor, the thrombin receptor PAR-1 from melanoma cells (Hazarika et al., 2004). Thus, reggies might act as scaffold proteins in G protein-coupled receptor signaling as well as described above for insulin signaling and PrP^C signaling of T cells.

Reggie-2 translocates to the nucleus after mitogenic stimulation mediated by the PTOV-1 in PC3 cells (Santamaria et al., 2005). Downregulation of both proteins reggie-2 and PTOV-1 leads to a reduction of cell proliferation and overexpression of reggie-2/flotillin-1 constructs results in a stimulation of cell proliferation in presence of PTOV-1. The exact function of reggie in the nucleus is still unclear but the relationship of reggies and cell proliferation is analyzed and discussed below (sections 4.1.7 and 5.4).

1.1.6.3 Reggie-associated diseases

Although no natural mutations of reggies have been identified yet, they have been linked to several diseases, e.g. Alzheimer's disease. The cerebral cortex of dement patients, where senile plaque formation is strongest, showed an enhanced reggie-1 and -2 staining (Kokubo et al., 2000). Reggie-1 was shown to modulate amyloid precursor protein (APP) clustering and endocytosis necessary for processing of APP and generation of the neurotoxic β-amyloid peptide (Aβ) (Schneider et al., 2008). Similar to Alzheimer's disease, in Parkinson's disease reggie-2 was reported to be upregulated in the substantia nigra of Parkinson's patients (Jacobowitz and Kallarakal, 2004). The role of reggie-2 in these cells is unclear.

Changes in reggie expression levels are not only associated with neurodegenerative diseases, but are characteristic for diabetes (James et al., 2001) and cancer (Hazarika et al., 2004; Doherty et al., 2006). A reggie-dependent insulin signaling pathway was suggested for adipocytes and muscle cells (Baumann et al., 2000; Fecchi et al., 2006), but whether upregulation of reggie-2 is causal for diabetes or an adaptive response of it remains to be clarified.

Table 1.2: Summary of putative functions and associated diseases of reggie-1 and reggie-2 including references.

✓ = yes, — = no

Putative functions/ associated diseases	Reggie-1	Reggie-2	Reference
Neuronal regeneration	✓	✓	(Schulte et al., 1997; Lang et al., 1998)
Insulin signaling	—	✓	(Baumann et al., 2000; Fecchi et al., 2006)
Cell proliferation	—	✓	(Santamaria et al., 2005)
Endocytosis	✓	✓	(Glebov et al., 2006; Frick et al., 2007)
Phagocytosis	—	✓	(Dermine et al., 2001; Garin et al., 2001)
IgE receptor signaling	—	✓	(Kato et al., 2006)
PrP^{C} signaling	✓	✓	(Stuermer et al., 2004)
G protein-coupled receptor signaling	—	✓	(Sugawara et al., 2007)
Alzheimer's disease	✓	✓	(Kokubo et al., 2000; Girardot et al., 2003; Langui et al., 2004; Schneider et al., 2008)
Parkinson's disease	—	✓	(Jacobowitz and Kallarakal, 2004)
Cancer	✓	—	(Hazarika et al., 2004; Doherty et al., 2006)
Type2 diabetes	—	✓	(James et al., 2001)
Malaria	✓	✓	(Murphy et al., 2004)
Glaucoma	—	✓	(Joe et al., 2005)

1.2 Intrinsic growth potential of nerve cells: a critical component for neuronal growth, regeneration and plasticity

1.2.1 Intrinsic versus extrinsic factors affecting neuronal growth

The capacity of the adult mammalian central nervous system (CNS) to regenerate is low (Ramón and Cajal, 1928; Fawcett, 2006), whereas axons of the peripheral nervous system (PNS) are able to regenerate (see section 1.2.4; Goldberg and Barres, 2000). There are two reasons for the failure of axon regeneration in the mammalian CNS: a) extrinsic factors of the CNS environment which inhibit axon growth, and b) low intrinsic ability of the adult CNS to regenerate (Fawcett, 2006). An expanding number of growth-inhibitory molecules of the CNS environment (Schwab, 2004; Yiu and He, 2006; Chaudhry and Filbin, 2007) as well as glial scar formation after injury (Fawcett and Asher, 1999; Silver and Miller, 2004) are inhibitory extrinsic factors, which could also influence the intrinsic growth ability of neurons (Plunet et al., 2002). Growth-inhibitory molecules are mostly constitutively expressed and therefore might have further important physiological functions (Schwab et al., 1993; Raisman, 2004; Rossi, 2004). Since abrogation of environmental growth inhibition is not sufficient to induce regeneration itself and distinct populations of central neurons show different growth capabilities, when confronted with a permissive environment, the intrinsic growth potential of neurons represents another crucial factor for neural regeneration (Fawcett, 1992). Central dorsal root ganglion (DRG) axons are able to regenerate into a peripheral nerve implant (Richardson and Issa, 1984) and even across the lesion site into the spinal cord (Neumann and Woolf, 1999; Neumann et al., 2005) in the presence of a peripheral injury, which triggers altered expression of specific gene sets of growth associated proteins (GAPs) in the DRG cell bodies necessary for sustained neurite elongation such as transcription factors, cytoskeletal elements, growth cone components and mediators of signal transduction (Skene and Willard, 1981; Skene,

1989; Fawcett, 2001; Fernandes and Tetzlaff, 2001). These observations showed that nerve cells of the CNS, in principle, can recover from an injury and that the intrinsic growth state of a neuron is important for successful regeneration.

1.2.2 Mammals versus fish: capacity of optic nerve regeneration

The adult CNS of lower vertebrates, fish and amphibians, is able to regenerate axons after injury (Sperry, 1944; Straube and Tanaka, 2006), in contrast to the adult mammalian CNS with little regenerative capabilities (Fawcett, 2006). The adult goldfish and zebrafish visual systems are able to regenerate retinal axons (Stuermer et al., 1992; Becker and Becker, 2007a) and descending brainstem axons (Becker and Becker, 2007b) to functional recovery. Thus, the zebrafish visual system used in this study provides an excellent system to study intrinsic factors contributing to CNS axon regeneration.

After ONS in zebrafish and goldfish, axons re-grow and re-innervate their targets within the optic tectum leading to a functional restoration of vision (Attardi and Sperry, 1963; Meyer, 1980; Matsukawa et al., 2004; McDowell et al., 2004). In the visual system of mammals, however, optic nerve injury results in retrograde degeneration of axons and cell death of RGCs (Chierzi and Fawcett, 2001). The CNS of teleost fish in general, is growth permissive in contrast to the CNS of mammals. Isolated myelin and oligodendrocytes from fish CNS do not inhibit growth of goldfish RGC axons nor rat dorsal root neurons *in vitro* (Bastmeyer et al., 1991; Bastmeyer et al., 1993; Wanner et al., 1995), due to the absence of myelin-associated inhibitors as shown for Nogo-A (Diekmann et al., 2005; Abdesselem et al., in press) and on the other hand expression of growth promoting molecules in the fish CNS environment (Jeserich, 1983; Vielmetter et al., 1991; Bastmeyer et al., 1995; Bernhardt et al., 1996; Petrausch et al., 2000). Thus oligodendrocytes of fish CNS have some characteristics in common with the growth-promoting Schwann cells of the PNS (Bastmeyer et al., 1993; Ankerhold et al., 1998). In addition, the

glial scar of goldfish is growth permissive (Hirsch et al., 1995). Besides those environmental cues, optic nerve lesion induces robust upregulation of growth-associated genes in fish RGCs (Stuermer et al., 1992; Veldman et al., 2007): reggie-1/-2 (Schulte et al., 1997), GAP-43 (Skene et al., 1986), cell adhesion molecules (Vielmetter et al., 1991; Bernhardt et al., 1996; Jung et al., 1997; Lang et al., 2001), transcription factors (Herdegen et al., 1993; Veldman et al., 2007), cytoskeleton regulating proteins (Skene et al., 1986; Schulte et al., 1997; Asch et al., 1998; Bormann et al., 1998; Hieber et al., 1998; Roth et al., 1999; Rodger et al., 2000; Veldman et al., 2007). Other genes are downregulated in response to injury as shown for zebrafish RGCs, e.g. genes involved in ion regulation, cell differentiation or cell fate commitment (Veldman et al., 2007).

1.2.3 Growth factors in neurogenesis: IGF-1 signaling

IGF-1 exerts a key role in the development of the nervous system, affecting different processes comprising cell proliferation, differentiation and cell survival (Russo et al., 2005). Several cell lines, for instance H19-7 rat hippocampal cells or neuroblastoma cells, undergo neuronal differentiation when stimulated by IGF-1 (Morrione et al., 2000; Nielsen et al., 2004). This stimulus is used in this study to induce neuronal differentiation of mouse neuroblastoma N2a cells and to activate signal transduction molecules via two main intracellular signaling pathways: a) Ras/MAPK pathway and b) PI3K/PKB/Akt pathway. The type-1 IGF receptor (IGF-IR) activates in association with the adaptor protein Shc, Grb2 (growth factor receptor-bound protein 2) and guanine nucleotide exchange factor (GEF) Sos, the small GTPase Ras. Ras-GTP stimulates the Raf/MEK/ERK cascade, which regulates numerous transcription factors, cytoskeleton regulating proteins and kinases, resulting in cell proliferation or differentiation dependent on the signaling kinetics, integrations of different signaling pathways and tissue/cell specific downstream effectors (Boulton, 1991; Ebisuya et al., 2005). Ras activates also other signaling

routes via PI3K, phospholipase Cε (PLCε) and Rho-family GTPases (Bar-Sagi and Hall, 2000; Cullen and Lockyer, 2002). The PI3K/PKB pathway is activated by recruitment of IRS (insulin receptor substrate) with subsequent activation of PI3K (phosphatidylinositol-3 kinase) and formation of PIP_3 (phosphatidylinositol triphosphate), which induces the recruitment and activation of PKB. PKB regulates several signaling pathways including anti-apoptotic signals, induction of transcription and translation and cell cycle regulation (Katso et al., 2001). Thus, stimulation with IGF-1 leads to activation of numerous downstream signal transduction molecules (kinases, GTPases) important for neuronal differentiation.

1.3 Reggies in neuronal differentiation and axonal regeneration: a link to actin cytoskeleton?

1.3.1 Cytoskeletal dynamics in neuronal differentiation and regeneration

During development as well as regeneration, axon extension requires assembly and reorganization of the cytoskeleton (Dent and Gertler, 2003). The mobile tip of an axon, the growth cone, is specialized for elongation and navigation with interior cycling filaments, actin filaments and microtubules. Actin filaments are enriched in the peripheral domain of the growth cone including lamellipodia and filopodia. Microtubules of the axon extend in the center of the growth cone and microtubule polymerization into the peripheral domain is necessary for axon elongation. Upon interaction with cell adhesion molecules, a strong connection between surface receptors of the growth cone and the underlying actin meshwork is formed to push the growth cone forward via anterograde actin polymerization (Goldberg, 2003). Despite lots of recent progress more work is necessary to understand the intracellular molecular mechanisms that regulate the axonal cytoskeleton dynamics underlying developmental and regenerative axon elongation and

the differences between both. The Rho GTPases are key signal transduction molecules in the regulation of the actin and microtubule cytoskeleton.

1.3.2 Rho GTPases and neuronal differentiation and regeneration

Rho GTPases act as regulators of the actin and microtubule cytoskeleton and therefore represent important molecules involved in neuronal development and regeneration (Hall, 1998; Govek et al., 2005). They serve as molecular switches of various signaling events by cycling between an active GTP-bound and an inactive GDP-bound state, which is regulated by GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating proteins) and GDIs (guanine nucleotide dissociation inhibitors; Etienne-Manneville and Hall, 2002). Extracellular signals converge on GTPases and through a large number of GEFs and GAPs. By activating distinct effectors, each GTPase regulates a diverse range of downstream pathways (Schwartz, 2004). The best-studied Rho GTPases are RhoA, cdc42 and Rac1. In general Rac and cdc42 activation promotes, whereas Rho activation inhibits neuritogenesis and axon formation (Govek et al., 2005). This generalization is based on a number of studies with constitutive active (CA) or dominant negative (DN) mutants of Rho GTPases and is true for several cell lines and primary culture systems (Kranenburg et al., 1997; Sarner et al., 2000; Da Silva et al., 2003; Aoki et al., 2004; Schwamborn and Puschel, 2004). Inhibitory effects of cdc42 on neurite outgrowth observed in *Drosophila* giant fiber system neurons (Allen et al., 2000), same effects of DN and CA Rac1 mutants in primary chick embryo motor neurons (Kuhn et al., 1998) may be due to the diverse types of species and cells, their age and to the requirement of proper cycling of Rho GTPases. Furthermore, localized activation cycles of RhoA, Rac1 and cdc42 were observed at motile protrusions (Aoki et al., 2004; Kurokawa et al., 2005; Nakamura et al., 2005). Activated Rac and cdc42 interact with several effector molecules, e.g. PAK kinases, N-WASP, WAVE. PAK1 induces neurite outgrowth and the duration of its activity is regulated by Rac1

(Nikolic et al., 1996; Nikolic et al., 1998). PAK5 additionally inhibits RhoA activity besides its neurite promoting effect (Dan et al., 2002). Mutants of another cdc42 effector N-WASP, which are unable to bind cdc42 or activate the actin-nucleating factor Arp2/3, block neurite outgrowth of PC12 cells and rat hippocampal neurons (Banzai et al., 2000). The RhoA pathway is activated in response to spinal cord injury (Dubreuil et al., 2003). Interestingly, there is evidence that interfering with RhoA signaling promotes axonal regeneration and partial functional recovery in axonal regeneration studies *in vivo* (McKerracher and Higuchi, 2006). Inactivation of RhoA was initially reported to generate re-growth of crushed rat optic nerve axons (Lehmann et al., 1999) and later it was also shown to promote axonal regeneration after spinal cord injury in mice (Dergham et al., 2002) and in rats (Fournier et al., 2002; Chang et al., 2005).

However, a fine balanced spatio-temporal regulation of the Rho GTPases by their regulators and the activation of different effector pathways determines a specific cellular response.

1.3.3 Reggies and neuronal differentiation and regeneration

The discovery of the reggies by the Stuermer's group (see also section 1.1.1) connected both reggie proteins from the very beginning with neuronal regeneration (Schulte et al., 1997; Lang et al., 1998). Beside the upregulation of the reggies in goldfish and rat axon regenerating RGCs there are several reports indicating functions of reggie in filopodia and neurite formation (Hazarika et al., 1999; Neumann-Giesen et al., 2004). These results suggest a role for reggie in the reorganization of the actin cytoskeleton particularly important for neuronal differentiation and regeneration. In addition, Haglund et al., 2004 showed that growth factor stimulation of phaeocytochroma PC12 cells resulted in the recruitment of protein tyrosine kinase Pyk2, Cbl and ArgBP2 to membrane microdomains and neurite outgrowth of PC12 cells. The intactness of membrane microdomains is critical for neurite out-

growth and lamellipodia formation at the growth cone mediated by actin cytoskeleton reorganization. Indeed, reggie-2 is able to bind to ArgBP2 and nArgBP2 (Haglund et al., 2004). Based on these findings, it was proposed that ArgBP2 associate via its SH3 domain with Pyk2 and Cbl and via its SoHo domain with reggie-2, thereby translocating Pyk2 and Cbl to membrane microdomains upon growth factor stimulation (Haglund et al., 2004) comparable to reggie-dependent insulin signaling (section 1.1.6.1; Baumann et al., 2000). Activated Cbl recruits Crk and p85 that mediate Rac-dependent changes of the cytoskeleton (Haglund et al., 2004). However, a SoHo domain deletion mutant of ArgBP2 does not completely block translocation of Pyk2 and Cbl to membrane microdomains.

Interference with reggie function during zebrafish development leads to embryos with severe morphological defects of the head with a reduced brain size and malformed tails (Malaga-Trillo et al., 2003). Additionally, overexpression of the DN reggie-1 mutant (R1EA), which interferes with oligomerization and localization of the reggies, impairs the recruitment of the adaptor protein CAP/ponsin to focal contacts and Rho GTPase activation in N2a cells (Langhorst et al., 2008). The R1EA expression inhibits neurite outgrowth in N2a cells and *in vitro* differentiation of primary rat hippocampal cells leading to cells with short and broad membrane protrusions instead of cylindrical neurites. Previously, R1EA was shown to induce mislocalization of the GEF Vav and thus impairment of cytoskeletal rearrangements during stimulation-induced T cell spreading (Langhorst et al., 2006).

Altogether, the reggies seem to play important roles in both neuronal differentiation and regeneration by regulating cytoskeletal dynamics as shown for T lymphocytes, adipocytes and neurons *in vitro*. To gain insights into the molecular functions of reggie proteins via specific loss-of-function approaches particular in neuronal systems *in vitro* and *in vivo* is of great importance with regard to potential therapeutic strategies for neurodegenerative diseases.

2 Aim of this work

This study deals with the function of reggie proteins during neuronal differentiation and regeneration with the central question, whether reggies affect axonal regeneration after ONS of zebrafish. Loss-of-function studies via small interfering RNAs (siRNAs) against reggie-1 and -2 were performed to clarify reggie protein interdependencies, effects on neuronal differentiation and associated signaling pathways in N2a cells in complementation of R1EA overexpression studies presented in the introduction. Supported by the results generated in cell culture, a system, established for brainstem neurons (Becker et al., 2004), was transformed to downregulate the reggies *in vivo* with morpholino antisense oligonucleotides (Mos) in zebrafish RGCs. Two different assays were used to quantify axonal regeneration of RGCs: a) *in vitro* outgrowth assay and b) *in vivo* regeneration assay.

Following questions should be answered by the subsequent described and discussed experiments in chapters 4 and 5.

- **Are the reggie proteins dependent on each other in N2a cells?**

  What type of dependency is present, unidirectional or bidirectional? How is the dependency regulated? Which are the consequences for the experiments in N2a cells concerning neuronal differentiation?

- **Do the reggies affect the morphology of neuronal cells during differentiation upon growth factor stimulation?**

  Which morphological changes can be observed? Which cytoskeletal rearrangements underlie the observed morphological changes? Which reggie is involved in neuronal differentiation, reggie-1 or reggie-2 or both?

- **Do the reggies influence signal transduction in response to growth factor stimulation of neuronal cells?**

Which intracellular pathways and molecules, respectively, are affected by the reggies? Which cellular responses are generated by signal transduction pathways? Do they correspond to the observed morphology of reggie knockdown cells?

- **Do the reggies affect axonal regeneration after optic nerve transection of zebrafish?**

Which reggie is involved? How strong is the effect? Can this effect be verified *in vitro* **and** *in vivo*?

3 Material and Methods

3.1 Antibodies and reagents

The following monoclonal antibodies (mAb) and polyclonal antibodies (pAb) listed in Table 3.1 were used in this work.

Table 3.1: Utilized antibodies.

Antibody (Ab)	Western Blot/ Immuno-fluorescence	Source/company
anti-reggie-1 (ESA) mAb	1:10000/ 1:600	BD Transduction Laboratories
anti-reggie-2 (642) mAb	1:1000/ 1:1500	(Lang et al., 1998)
anti-reggie-1 (722) pAb	1:250-1000/ 1:200-500	(Stuermer et al., 2001)
anti-reggie-1 (Flot) mAb	1:750/ 1:200	BD Transduction Laboratories
anti-GAPDH [6C5] mAb	1:100000	Abcam
anti-cdc42 mAb	1:100	BD Transduction Laboratories
anti-Rac1 mAb	1:1000	BD Transduction Laboratories
anti-RhoA mAb	1:100	Santa Cruz
anti-Ras mAb	1:200	Oncogene/ Calbiochem
anti-FAK mAb	1:1000	BD Transduction Laboratories
anti-PKB mAb	1:1000	Cell Signaling
anti-ERK1/2 pAb	1:1000	Cell Signaling
anti-JNK mAb (rabbit)	1:1000	Cell Signaling
anti-p38 MAP Kinase pAb	1:1000	Cell Signaling
anti-neurolin (zn-5) mAb	–/ 1:500	kindly provided by Wolfgang Driever, Developmental Biology, University Freiburg

Phosphorylation-specific pAbs (phosphorylation sites)		
anti-PKB (Ser473)	1:1000	Cell Signaling
anti-pan-PKC (Ser660 and homologues residues)	1:1000	Cell Signaling
anti-ERK1/2 (Thr202/Tyr204)	1:1000	Cell Signaling
anti-JNK (Thr183/Tyr185)	1:1000	Cell Signaling
anti-p38 (Thr180/Tyr182)	1:1000	Cell Signaling
anti-FAK (Tyr576/577)	1:1000	Cell Signaling
Secondary Abs		
all HRP-, Cy3- and Alexa Fluor®488 coupled Abs	1:20.000/ 1:2000-1:8000	Jackson Immuno Research

The following chemicals and reagents listed in Table 3.2 were used in this work.

Table 3.2: Utilized chemicals and reagents.

Chemical/reagent	**Company**
Alexa Fluor®488 dextran, 10 000MW, fixable	Invitrogen
Alexa Fluor® Phalloidin	Invitrogen
Bromphenol blue	Sigma
$CaCl_2$	Sigma
Complete, mini (EDTA-free protease inhibitor cocktail)	Roche
DTT	Sigma
EDTA	Roth
Ethidium bromide solution	Sigma
F12	Invitrogen
Fetal Bovine Serum (FBS)	Biochrom AG
Gelfoam®	Pharmacia Upjohn
Gibco™ chicken serum	Invitrogen
Gibco™ L-Glutamine	Invitrogen
Gibco™ Penicillin/ Streptomycin	Invitrogen
Gibco™ Pyruvate	Invitrogen
Glycerol	Roth

Glycine	Roth
HEPES, free acid	Invitrogen
Hybond™–c extra	Amersham,
IGF-1	Biomol
KCl	Sigma
Lactacystin	Cayman Chemical
L-15 Leibowitz medium	Invitrogen
MEM	Invitrogen
Methanol	VWR
Methylcellulose	Sigma
$MgCl_2x6H_2O$	Sigma
Mowiol	Calbiochem®, Merck
NaCl	Sigma
PAK 1 PBD (Rac/ cdc42) Assay Reagent (Agarose)	US Biological
PBS	Biochrom AG
Phosphatase Inhibitor Cocktail Set II	Calbiochem®, Merck
Ponceau S	Sigma
Ras Assay Reagent (Raf-1 RBD, Agarose)	Upstate
Rhotekin Rho Binding Domain, Recombinant (Agarose)	US Biological
SDS	Sigma
SuperSignal® West Pico Luminol/Enhancer Solution + stable peroxide solution	Thermo Scientific
TEMED	Serva
Trizma® base	Sigma
Tricine	Sigma
Triton X-100	Sigma
Trizma® acetate	Sigma

The following buffers and media listed in Table 3.3 were used in this work.

Table 3.3: Utilized buffers and media.

Buffer	Components
Lysis buffer	1% SDS in ddH_2O + protease-inhibitors (Complete, mini)
TX-100/SDS lysis buffer	20 mM Trizma®-HCL, pH 7.5 150 mM NaCl 1% TX-100 0.1% SDS + protease-inhibitors (Complete, mini)
Kinase lysis buffer	20 mM Trizma®-HCL, pH 7.5 2 mM EDTA 100 mM NaCl 5 mM $MgCl_2$ x $6H_2O$ 1% (v/v) TX-100 10% (v/v) Glycerol + protease-inhibitors (Complete, mini) + Phosphatase Inhibitor Cocktail Set II (1:100, freshly added)
Ringer's solution, pH 7.2	116 mM NaCl 2.9 mM KCl 1.8 mM $CaCl_2$ 5 mM HEPES
SDS-PAGE running buffer	100 mM Trizma® base 100 mM tricine 1% (w/v) SDS
1x TAE, pH 8.0	40 mM Trizma® acetate 1 mM EDTA pH 8.0
1x TBS, pH 7.4	150 mM NaCl 50 mM Trizma® base
PBS-T	1x PBS 0.1% (v/v) Triton X-100
TBS-T	1x TBS 0.1% (v/v) Triton X-100
Transfer buffer	20 mM Trizma® base 150 mM glycine 25% (v/v) methanol

6x reducing SDS sample buffer	0.7 M Trizma®-HCL, pH 6.8 30% (v/v)Glycerol 10% (w/v) SDS 200 mM DTT Bromphenol blue
F12 complete	0.4 % (w/v) Methylcellulose 10% (v/v) FBS 2% (v/v) Chicken serum 2 mM L-Glutamine 10 U/ml Penicillin 10 µg/ml Streptomycin 84 % (v/v) F12
MEM complete	10% (v/v) FBS 2 mM L-Glutamine 1 mM Pyruvate 10 U/ml Penicillin 10 µg/ml Streptomycin in MEM
MEM starvation medium	2 mM L-Glutamine 1 mM Pyruvate 10 U/ml Penicillin 10 µg/ml Streptomycin in MEM

3.2 Primers, siRNAs, morpholinos

Primers were synthesized by Invitrogen and all morpholinos (Mos) by Gene Tools. R1.0, R2.0 and GL2 siRNAs were from Dharmacon; R1.1 and R1.4 from Ambion.

Table 3.4: Sequences of utilized primers, siRNAs and morpholinos.

Primer	Fragment size	Sequence
Reggie1_sense	929 bp	5′-CAGGTGAAGATCATGACG-3′
Reggie1_antisense		5′-ACCACAATCTCATCGAC-3′
Reggie2_ sense	805 bp	5′-CTTGTGGCCCAAATGAG-3′
Reggie2_antisense		5′-ATCTCCTGCTCCTGCAC-3′
GAPDH_sense	985 bp	5′-GGTCGGTGTGAACGGATTTGGC-3′
GAPDH_antisense		5′-GGAGGCCATGTAGGCCATGAGG-3′
siRNAs		**Target sequence**
R1.0		5′-GTTCATGGCAGACACCAAG-3′
R1.1		5′-GGTGAAGATCATGACGGAG-3′
R1.4		5′-GGTTTATAGGCCTTCTTCC-3′
R2.0		5′-CACACTGACCCTCAATGTC-3′
GL2		5′-CGTACGCCAATACTTCGA-3′
Morpholinos		**Sequence**
re1a_1	Set1	5′-CATTTTCCTTTCTGGACGCCTTTAA-3′
re1a_2	Set2	5′-AAAAAGCGGCTAAAGACCTAATGTT-3′
re2a_1	Set1	5′-TTTTAGACGTTGGCTGAATGATTAG-3′
re2a_2	Set2	5′-CAAGAGACGCAGTAATCCAGCAGCC-3′
re2b_1	Set1	5′-CCTTAATTAGATCGATTGCAGATAT-3′
re2b_2	Set2	5′-TGTGTCAATGGTAAAGAAAACTCGC-3′
Control Mo		5′-ATCCTGCAAAAAGAACAAGTAGCTT-3′
Std control w/ Lissamine		5′-CCTCTTACCTCAGTTACAATTTATA-3′
re1a_2 w/ Lissamine	Set2	5′-AAAAAGCGGCTAAAGACCTAATGTT-3′

3.3 Vectors and constructs

The reggie-1-EGFP rescue construct was kindly provided by Gonzalo P. Solis. It was generated by PCR introducing 12 silent point mutations in the sequences targeted by R1.0 and R1.1 siRNAs (R1.4 siRNA targets a sequence in the 3' UTR). Thus, the reggie-1 cDNA was mutated to `AttTatggcGgaTacGaaA` (R1.0) and `AgtCaaAatTatgacCgaA` (R1.1), confirmed by sequencing, and cloned in frame into EcoRI and BamHI sites of the pEGFP-N1 vector (Clontech). pEGFP-N1 was used as control vector.

3.4 Cell culture

N2a (Neuro-2a) cells, a mouse neuroblastoma cell line (ATCC® Number CCL-131™), were maintained in MEM complete at 37 °C, 5% CO_2. The cells were normally passaged three times a week at a ratio of 1:5. Therefore, the adherent growing cells were treated with 0.25% (w/v) Trypsin, for 3 minutes at 37 °C and resuspended in complete medium. Aliquots of cells were added to new culture flasks with fresh medium.

For differentiation N2a cells were cultured for 24 hours (h) on laminin-coated (0.02 mg/ml) coverslips in starvation medium supplemented with 50 ng/ml IGF-1. Next, the cells were fixated and stained (see section 3.12).

For stimulation of N2a cells with IGF-1, the cells were starved (2 d post transfection) overnight in starvation medium (0% FBS). After 18 h starvation cells were stimulated with IGF-1 (50 ng/ml in starvation medium) for 5 minutes at 37 °C. Afterwards the dishes with cells were placed on ice and washed twice with icecold PBS. Further procedure was dependent on whether extraction of phospho-proteins (P-proteins) (see 3.8.3) or GST-pulldowns for isolation of activated GTPases (see section 3.9) were needed.

3.5 Transfection

Transient transfection of N2a cells was performed with Lipofectamine™ 2000 in a standard or reverse way according to the manufacturer's recommendation. In the reverse transfection method the cells are added directly to the Lipofectamine–DNA/siRNA complexes and transfection occurs while cells are attaching to the well. By contrast, in the standard transfection procedure the Lipofectamine–DNA/siRNA complexes are added after adherence of the cells.

3.5.1 siRNA transfection, standard

One day before transfection, N2a cells were plated in a multi-well plate in order to be 30-50% confluent at the time of transfection. A single siRNA (15 pmol/ml) and appropriate amounts of Lipofectamine were separately diluted with Opti-MEM according to Table 3.5. After 5 minutes incubation at room temperature both mixtures were combined, gently mixed and incubated 20 minutes at room temperature. The siRNA-Lipofectamine complexes were added to each well and removed with change of the growth medium after 4 h of incubation. SiRNA duplexes targeting reggie-1 (R1.0, R1.1 or R1.4), reggie-2 (R2.0) and a non-specific control siRNA targeting luciferase of firefly, referred to as GL2, were used. To block the 20S subunit of the proteasome, lactacystin was applied at a final concentration of 10 μM 8 h post transfection (Fenteany et al., 1995). Cells treated or non-treated with lactacystin were lysed 48 h post transfection for western blot analysis.

Table 3.5: Scaling for standard transfection of N2a cells in different tissue culture formats.

Culture vessel	Volume of plating	siRNA and Opti-MEM	Lipofectamine™ 2000 and Opti-MEM	Cell number of plating
24-well	500 µl	7.5 pmol in 50 µl	0.75 µl in 50 µl	2.5×10^4
12-well	1 ml	15 pmol in 50 µl	0.75 µl in 50 µl	5×10^4
6-well	2.5 ml	37.5 pmol in 50 µl	1.9 µl in 50 µl	1.25×10^5

3.5.2 siRNA transfection, reverse

A mixture of three different siRNAs (R1.0, R1.1 plus R1.4) targeting reggie-1 (3 pmol/ml each one; 9 pmol/ml end-concentration) was used for reverse siRNA transfections of N2a cells (see Table 3.6). GL2 siRNA GL2 served as non-specific control (9 pmol/ml). The pEGFP-N1 (0.8 µg/ml) or R1-EGFP (0.8 µg/ml) rescue constructs were co-transfected. Cells were used for *in vitro* differentiation 24 h post transfection, were lysed 48 h post transfection for western blot analysis or used for P-protein extracts and GST pulldowns, respectively.

Table 3.6: Scaling for reverse transfection of N2a cells in different tissue culture formats.

Culture vessel	Volume of plating	siRNA and Opti-MEM	Lipofectamine™ 2000 and Opti-MEM	Cell number of plating
12-well	1 ml	9 pmol in 50 µl	1 µl in 50 µl	5×10^4
6-well	2.5 ml	22.5 pmol in 50 µl	2.5 µl in 50 µl	1.25×10^5
10 cm	10 ml	135 pmol in 50 µl	15 µl in 300 µl	7.5×10^5

3.6 RNA Isolation and RT-PCR

Knockdown of reggie-1 and -2 on mRNA level was assayed 48 h post transfection. RNA was isolated using the RNeasy Mini Kit (Qiagen) with DNAse digestion step to avoid contamination by genomic DNA according to the manufacturer's recommendation. First strand cDNA was generated using Oligo $(dT)_{12\text{-}18}$ primer and Superscript II Reverse Transcriptase (Invitrogen) as described by the manufacturer.

Concentration of cDNAs was determined by absorbance measurements at 260 nm. An optical density (OD) at 260 nm of 1 equates to 40 µg/ml single-stranded DNA and 50 µg/ml double-stranded DNA, respectively. Purity of cDNA was determined by the proportion of ODs at 260 nm to 280 nm, which should betray 1.8 -2.0 (Sambrook et al., 1989).

The quality of cDNA was controlled by polymerase chain reaction (PCR) with a commercial primer set for actin and clathrin (Invitrogen). Reggie-1, reggie-2 and GAPDH fragments were amplified with specific primer pairs using Taq-Polymerase (GE Healthcare) according to the manufacturer's instruction. Following temperature profiles were used:

	time	**temperature**		
		reggie-1	reggie-2	GAPDH
initial denaturation	1 min	94°C		
denaturation	20 s	94°C		
annealing	30 s	50°C	52°C	55°C
extension	1 min	72 °C		
cycles		28	30	24
final extension	10 min	72 °C		

3.7 Agarose gel electrophoresis

The PCR products of reggie-1, reggie-2 and GAPDH mixed with loading buffer (PeqLab) were separated in a 1% agarose gel at 100V in TAE buffer. To visualize

the DNA, 0.5 µg/ml ethidium bromide were added to the agarose gel and DNA bands were documented under UV with a BioRad Gel Doc 1000. A 1 kb DNA ladder (PeqLab) was used as standard size marker.

3.8 Cell lysates

3.8.1 N2a protein extracts

To analyze knockdown efficacies on protein level 48 h post transfection, the N2a cells were washed with PBS, treated with lysis buffer, sonified for 5 seconds and cleared of insoluble debris by centrifugation at 14000 rpm for 10 minutes. The protein content was determined with Biorad Dc Protein Assay and probes were stored at -20 °C.

3.8.2 Retina protein extracts

One zebrafish retina was added to 200 µl of TX-100/SDS-lysis buffer and homogenized with a 27-gauge syringe needle. The suspension was incubated on ice for 20 minutes and then cleared by centrifugation at 14000 rpm for 10 minutes at +4 °C. The protein content was determined with Biorad Dc Protein Assay and probes were stored at -20 °C.

3.8.3 Phospho-protein extracts

To analyze activation of kinases and GTPases, N2a cells were starved 2 d post transfection for 18 h and starved cells were stimulated with IGF-1 (50 ng/ml starvation medium) for 5 minutes at +37 °C (see also section 3.4). After stimulation of the N2a cells with IGF-1, cells were carefully washed two times with icecold PBS. All following steps for preparing of P-protein extracts were performed in the

coldroom. The cells were scraped off with 100 - 500 µl of kinase lysis buffer per 10 cm tissue culture dish and transferred into a 1.5 ml test tube. The lysates were cleared of insoluble debris by centrifugation for 5 minutes, 14000 rpm and directly used for western blot analysis or GTPase assays. Only fresh prepared P-protein probes were used for subsequent analyses.

3.9 GTPase assays

GTPases assays were performed based on a method first established to precipitate specifically Ras-GTP from cell lysates via a GST (glutathione S-transferase) fusion-protein of the minimal Ras-binding domain (RBD) of Raf coupled to gluthatione agarose (de Rooij and Bos, 1997). Accordingly, Rac1/ cdc42 and RhoA were precipitated with GST fused to residues 67-150 of the effector protein PAK-1 (human) and GST fused to residues 7-89 of mouse Rhotekin Rho binding domain (mouse), respectively. The GTPase assay reagents PAK 1 PBD (Rac/ cdc42) Assay Reagent (Agarose), Ras Assay Reagent (Raf-1 RBD, Agarose) and Rhotekin Rho Binding Domain, Recombinant (Agarose) for GST pulldowns were purchased from US Biological.

All steps were performed at +4 °C. Cleared P-protein extracts (see section 3.8.3) were used for GST pulldowns to precipitate GTP-Rac-1, GTP-cdc42, GTP-RhoA or GTP-Ras, respectively, and incubated with the appropriate GTPase assay reagent for 1-2 h, +4 °C with gentle agitation. Beads were collected by centrifugation at 14000 rpm for 1 minute and washed three times with kinase lysis buffer. Bound proteins were eluted by boiling the agarose beads for 5 minutes in appropriate amounts of 2x reducing SDS sample buffer. Samples were directly used for western blot analysis or stored at -80 °C. Optimal conditions were determined for each GTPase assay using N2a cell extracts as follows:

GTPase assay	amount protein extract	amount 2x reducing SDS sample buffer
Ras	400 µg/10µg agarose	20 µl
Rac1	200 µg/10µg agarose	20 µl
cdc42	2500 -3000 µg/10µg agarose	20 µl
RhoA	1000 µg/30µg agarose	40 µl + 2 µl DTT (1M)

3.10 SDS-PAGE

Separation of (P-) protein lysates and GST-pulldown samples was performed by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970). For GTPase samples, 12% separating gels were used, for all other protein samples separating gels with 10% polyacrylamide were used.

Table 3.7: Composition of 10 und 12% SDS-PAGE separating gels with a 5% stacking gel in front.

	Stacking gel, 5% [ml]	Separating gel, 10% [ml]	Separating gel, 12% [ml]
H_2O	4.1	4.0	3.3
30% Acrylamide/ Bisacrylamide (37.5:1)	1.0	3.3	4.0
0.8% SDS, 1.5M Tris pH 8.8	—	2.6	2.6
0.4% SDS, 1M Tris pH 6.8	0.81	—	—
10% APS	0.06	0.1	0.1
TEMED	0.01	0.01	0.01

Samples were prepared by adding appropriate amounts of SDS sample buffer. After denaturation at +95 °C for 5 minutes, protein samples were loaded onto the

SDS-PAGE gel. Gels were run in vertical Hoefer SE 250 electrophoresis chambers (Amersham Biosciences) in SDS running buffer at 90 - 120 volt (for ca. 90 minutes). Page Ruler™ Prestained Protein Ladder (Fermentas) or Prestained Plus Protein™ Standard All blue (BioRad) were used as standard size protein marker.

3.11 Western blotting

After gel electrophoresis, proteins were transferred onto Amersham Hybond™-C extra nitrocellulose membrane (GE Healthcare) in a tank blot system (Hoefer TE223 transfer chamber) with transfer buffer at +4 °C, 90V for 2h or overnight at 40V. Successful protein transfer was checked by incubating the membrane with Ponceau S solution for 5 minutes (Sigma). After protein transfer, the membrane was blocked with 3% nonfat dry milk in PBS-T or TBS-T, incubated with primary Abs overnight at +4 °C or for 1h at room temperature. After primary Ab incubation, the membrane was washed three times with PBS-T (TBS-T) for 10 minutes each step, incubated with HRP-coupled secondary Abs for 1 h at room temperature and washed three times with PBS-T (TBS-T). Chemiluminescence was detected using West Pico Super Signal chemiluminescence kit (Thermo Scientific) on a Hyperfilm™ ECL (GE Healthcare). Densitometric analysis of western blots was performed with ImageJ (Abramoff et al., 2004).

The following buffers were used for the different antibodies:

	P-specific Abs	**all other Abs**
Blocking solution	3% (w/v) nonfat dry milk in TBS-T	3% nonfat dry milk in PBS-T
Antibody dilution buffer	5% (w/v) BSA in TBS-T	PBS-T (+ 0.02% Thimerosal)
Secondary Antibody	5% nonfat dry milk in TBS-T	3% nonfat dry milk in PBS-T
Washing buffer	TBS-T	PBS-T

3.12 Immunofluorescence

To visualize F-actin of transiently transfected and differentiated N2a cells (see section 3.4 ; 3.5) using phalloidin, cells were fixated with 4% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.1% TritonX-100 for 2.5 minutes at room temperature. Samples were washed three times with PBS and incubated with phalloidin-Alexa Fluor® 568, diluted 1:250 in 1% BSA/PBS for 1 h at room temperature. Samples were washed three times with PBS and once with ddH_2O and embedded in Mowiol. Widefield images were taken on an Axiovert 200M using a 63x/1.4 Plan-Apochromat objective and analyzed with the AxioVision 4.5 software (Carl Zeiss).

To localize reggie-1 and -2, N2a cells were fixated and permeabilized with methanol, -20 °C for 1.5 minutes. Samples were washed three times with PBS and blocked with 1% BSA/PBS for 30 minutes at room temperature. Primary Abs against reggie-1 (pAb 722) and reggie-2 (mAb Flot) were diluted 1:200 in 1% BSA/PBS and added to the cells overnight at +4 °C. Cells were washed three times with PBS and incubated with secondary Abs for 1.5 h at room temperature. Samples were washed three times with PBS and once with ddH_2O and embedded in Mowiol. Staining of axons with pAb 722 and mAb zn-5, diluted 1:500, were performed accordingly.

Retina whole mounts were stained for reggie-1 and -2 as follows: isolated retinae were fixated with methanol, -20 °C for 10 minutes, washed three times with PBS and blocked with 1% BSA/2% goat serum/PBS overnight at +4 °C. Samples were incubated with the primary Abs, 642 (1:1500) or ESA (1:600) in 1% BSA for 1.5 h at room temperature, washed three times with PBS and incubated with secondary Ab (goat anti mouse IgG-A488, 1:8000) for 2 h. Retinae were washed three times and embedded in Mowiol. Images were acquired on a confocal laser-scanning microscope (LSM510 META, Carl Zeiss) with a Plan-Apochromat 40x/1.3 Plan-Neofluar objective.

3.13 Animals, optic nerve sections and morpholino application

For *in vivo* knockdown of reggie proteins, optic nerves of zebrafish (*Danio rerio*), 5-13 months old, were transected under 3-aminobenzoic acid ethylester anesthesia (MS222, 30mg/100ml; Sigma) in compliance with animal welfare legislation. Gelfoam® (Pharmacia & Upjohn) soaked with Mo-solution was applied to the proximal stump immediately after ONS (Becker et al., 2004). For specificity, two different Mo-sets against reggies were tested. Mo-concentrations from 2.5 µg/µl to 100 ng/µl in Ringer's solution were used to determine an effective but non-toxic concentration. In all evaluated experiments, the Gelfoam piece was soaked in 2.5 µl of 312 ng/µl Mos. A lissamine-labeled Mo served to prove retrograde transport into RGCs.

Quantitative outgrowth assay/regeneration assay

Zebrafish retinae, free from pigment epithelium and blood vessel layer, 4 d after ONS/Mo-application, were chopped (MC ILWAIN TISSUE CHOPPER; Mickle Laboratory Engineering Co Ltd) into 200 µm x 200 µm squares (as described by Vielmetter and Stuermer, 1989). Mini-explants (16-24 per retina) were cultured in F12 complete at +28.5 °C on a poly-L-lysine-coated (0.2 mg/ml) 96-well µCLEAR-plate (Greiner). After 24 and 48 h, number of axons per mini-explant was counted (Axiovert 35 microscope; Carl Zeiss) excluding experiments with less than 9 axons per control retina explant (24 h).

In a second assay, the optic nerve of fish after ONS and Mo-treatment was re-sectioned respectively 7, 10 and 14 days later, 2 - 3 mm distal from the first lesion, and Alexa Fluor® 488 (A488) dextran (Invitrogen) was applied to retrogradely label RGCs with regenerating axons. Two days later, the retinae were isolated as above, fixated with 4% paraformaldehyde for 15 minutes at room temperature and mounted. Images were acquired on a confocal laser-scanning microscope (LSM510

META, Zeiss) with a Plan-Apochromat 40x/1.3 Plan-Neofluar objective and the dextran-labeled green RGCs were counted in whole mounts.

4 Results

4.1 siRNA-mediated loss-of-function analysis of reggie-1 in N2a cells

4.1.1 Strategy of siRNA experiments

Three individual siRNAs against reggie-1 and one siRNA against reggie-2 were used to test the silencing efficacy on protein and RNA level of each siRNA (Figure 4.2). These siRNAs differ in their binding sites. R1.0 siRNA binds at nucleotides +615 to +633 in the flotillin domain, R1.1 binds at nucleotides +222 to +240 in the SPFH domain, and R1.4 binds in the 3´UTR region, 825 to 843 nucleotides behind the termination codon, of reggie-1 mRNA (NCBI Accession # NM_001040403). R2.0 siRNA binds at nucleotides +132 to +150 of reggie-2 mRNA (NCBI Accession # NM_008027). N2a cells were transfected with one of the siRNAs against reggie-1 (R1.0, R1.1 or R1.4) or reggie-2 (R2.0). As a negative control siRNA, a commercial siRNA against luciferase of *Photinus pyralis*, referred to as GL2, was used. Optimal siRNA concentrations for lipofection of N2a cells were determined in a concentration row as 15 µM. Two or three days post transfection, protein lysates and RNA extracts were prepared (Table 4.1).

To reduce the risk of off-target effects, instead of single siRNAs a pool of three distinct and effective siRNAs against reggie-1 were used to study morphology and effects on signaling molecules like Rho GTPases, MAP kinases or FAK. This set of siRNAs allowed low concentration (3 µM) of each single siRNA in the mix with no loss of silencing efficacy. Pools of three or more individual siRNAs are known to decrease off-target activity and false positives of single siRNAs (Brown and Samarsky, 2006). To prove the specificity of phenotypes after reggie silencing, further rescue experiments using a reggie-1 rescue construct (R1-EGFP rescue) with

silent mutations in the siRNA binding sites to preserve mRNA degradation were performed. Table 4.1 shows a chronological overview on the main procedures of the distinct siRNA experiments.

Table 4.1: Chronological table of the main procedures of the different siRNA experiments.

✓ = task performed

Days	0	1	2	3
Transfection of siRNAs	✓			
Total RNA Isolation			✓	
Extraction of proteins			✓	✓
IGF-1 stimulation for neurite outgrowth		✓		
Fixation of cells after stimulation			✓	
Starvation of cells 17 h before GTPase assays			✓	
IGF-1 stimulation of cells for 5 minutes (immediately before GTPase assay)				✓
Extraction of phospho-protein lysates/GTPase assay				✓

4.1.2 Downregulation of reggie-1 protein leads to a simultaneous loss of reggie-2 protein

In order to test the different siRNAs against reggie-1 (R1.0, R1.1 or R1.4) and against reggie-2 (R2.0), RNA and protein levels of siRNA-treated N2a cells were examined via RT-PCR and western blotting (Figure 4.1). Reggie-1 or reggie-2 siRNA treatment of N2a cells lead to a specific and strong reduction of the corresponding mRNA, *reggie-1* or *reggie-2*, respectively, compared to unspecific siRNA GL2. No difference on reggie mRNA levels between GL2 and control transfected

cells with Lipofectamine only could be recognized (Figure 4.1A). As expected, all reggie-1 siRNA treatments lead to a strong depletion of reggie-1 protein ranging from 66% to 84% depletion as quantified via densitometric analysis of the western blot signal intensities normalized to GL2 of three independent experiments (Figure 4.1B and C). Interestingly, reggie-2 protein was also depleted with every reggie-1 siRNA treatment, up to 83% (Figure 4.1B and D). This cross silencing effect on reggie-2 protein with reggie-1 siRNA was not present on mRNA level as shown by RT-PCR in Figure 4.2A and thus, is not due to a direct interaction of reggie-1 siRNA with reggie-2 mRNA. Reggie-2 siRNA treatment on the other hand induced a reduction of 63% of reggie-2 protein as shown in Figure 4.1B and D, whereas reggie-1 protein and mRNA were unaffected (Figure 4.1A, B and C). This implies that reggie-2 protein is dependent on reggie-1 protein but not vice versa. Reasons for this unilateral dependency of reggie-2 could be either translational problems or effects on protein stability.

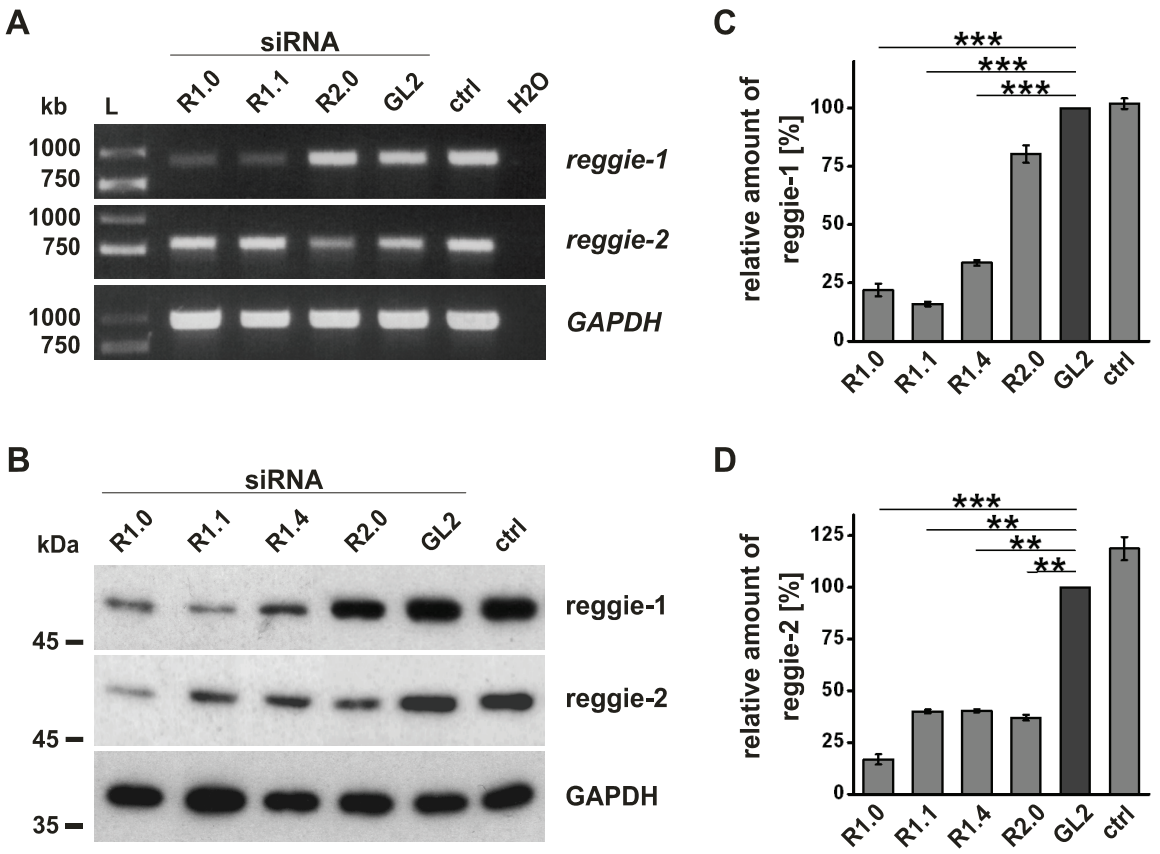

Figure 4.1: Depletion of reggie-2 protein after reggie-1 silencing in N2a cells. N2a cells transiently transfected with siRNAs against reggie-1 (R1.0, R1.1, R1.4) and reggie-2 (R2.0) or with a non-specific siRNA GL2, and control N2a cells treated with Lipofectamine only (ctrl) were analyzed. **A,** RT-PCRs of total mRNA extracts 48h post siRNA transfection showed specific downregulation of *reggie-1* mRNA with reggie-1 siRNA treatment and downregulation of *reggie-2* mRNA with reggie-2 siRNA-treatment. *GAPDH* mRNA was used as loading control. **B,** All reggie-1 siRNAs led to a strong reduction of reggie-1 protein as well as reggie-2 protein analyzed by western blotting, whereas reggie-2 siRNA specifically induced loss of reggie-2 protein. Protein levels of GAPDH were used as a loading control. **C, D** Quantification of protein signals on western blots by densitometric analysis using ImageJ (n = 3 independent experiments). **C**, Amounts of reggie-1 relative to GL2 (= 100%) were determined with siRNAs as indicated: R1.0 = 22 ± 3%; R1.1 = 16 ± 1%; R1.4 = 34 ± 1%; R2.0 = 80 ± 4%; ctrl = 102 ± 2%. **D**, Amounts of reggie-2 relative to GL2 (= 100%) were determined with siRNAs as indicated: R1.0 = 17 ± 2%; R1.1 = 40 ± 1%; R1.4 = 40 ± 1%; R2.0 = 37 ± 1%; ctrl = 119 ± 6%. Mean ± SEM; $p^{**} < 0.01$, $p^{***} < 0.001$, Student's *t* test.

4.1.3 Reggie-2 is degraded by the proteasome in the absence of reggie-1

The proteasome is an important system for the cell to eliminate misfolded, short-lived and unneeded proteins (Ciechanover, 2005). To analyze if the proteasome is involved in the loss of reggie-2, when reggie-1 is silenced, cells were treated with lactacystin, a specific inhibitor of the 20S proteasome core particle. Treatment of N2a cells with 10 μM lactacystin prevented the degradation of reggie-2 in the absence of reggie-1 in contrast to the siRNA treatment without lactacystin (Figure 4.2A). Under reggie-1 siRNA treatment, the protein signal intensities quantified via densitometry showed that without lactacystin, reggie-1 and reggie-2 were downregulated to the same extent, 84% and 73%, respectively, as before (Figure 4.1B-D). In contrast, reggie-1 siRNA plus lactacystin did not affect reggie-1 silencing, but reggie-2 depletion was prevented. The reduction of reggie-2 by 7% for R1.1 and 29% for R1.0 in presence of lactacystin was not statistically different to GL2 siRNA treatment (Figure 4.2B). Comparing each siRNA treatment with or without lactacystin, R1.0 as well as R1.1 showed strong differences in the amount of reggie-2, varying from 51% (R1.0) to 61% (R1.1), which were highly statistically significant ($p^{***} < 0.001$, Student's t test). GL2 treatment did not affect reggie-2 with or without lactacystin (Figure 4.2B). This result indicates that reggie-2 needs the presence of reggie-1 for its stability, because reggie-2 is otherwise degraded by the proteasome system. Translational problems induced by reggie-1 silencing can be excluded, because reggie-2 is properly synthesized in the presence of lactacystin.

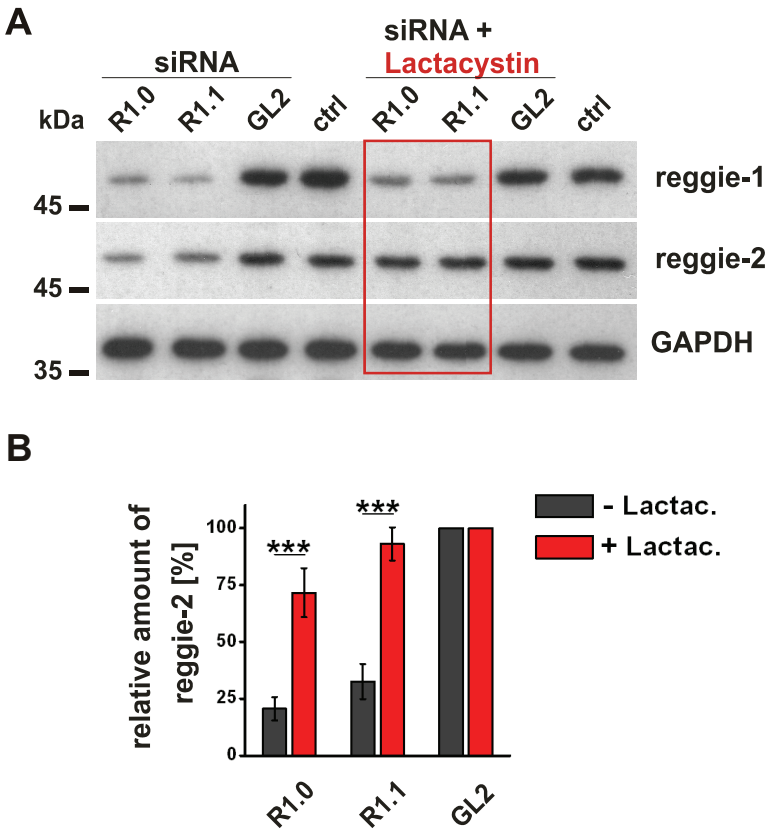

Figure 4.2: Reggie-1 silencing induces proteasomal degradation of reggie-2 protein in N2a cells. A, Western blot analysis of siRNA-mediated reggie-1 knockdown showed a depletion of reggie-1 and reggie-2 proteins with both siRNAs against reggie-1 (R1.0 and R1.1). Treatment with 10 µM of lactacystin, a specific proteasome inhibitor, abolished the loss of reggie-2 protein in the absence of reggie-1. Reggie-1 silencing was not affected by lactacystin. GAPDH was used as a loading control. **B,** Densitometric analysis of protein signal intensity relative to GL2 demonstrated the strong differences in the concentration of reggie-2 with or without lactacystin in reggie-1 siRNA treated cells (n = 3 independent experiments). Amounts of reggie-2 relative to GL2 (= 100%) after siRNA knockdown were: R1.0 = 21 ± 5%; R1.0 + lactacystin = 72 ± 10%; R1.1 = 33 ± 8%; R1.1 + lactacystin = 93 ± 7%. Mean ± SEM; $p^{***} < 0.001$, Student's *t* test.

4.1.4 Localization of reggie-1 and -2 in the cell body, along neurites and at tips of neurites in N2a cells

To analyze the localization of reggie-1 and reggie-2 in differentiated N2a cells, immunostainings of cells 24h after IGF-1 stimulation were performed. IGF-1 application is a well known procedure to stimulate the differentiation of N2a cells and other cell types (Ivankovic-Dikic et al., 2000; Kim et al., 2004; Langhorst et al., 2008). Double immunostainings with a mAb against reggie-2 and a pAb against reggie-1 revealed appearance of both reggies in all characteristic structures of a neuronal cell: at the cell body, on vesicles and at the plasma membrane, along neurites and at the tips of neurites as shown for PC12 cells (Stuermer et al., 2001); (Figure 4.3A, B). The merged pictures indicated a high degree of co-localization of immunofluorescent signals (yellow) of reggie-1 (green) and reggie-2 (red) in (Figure 4.3C–I). The association of the reggies with intracellular compartments on the one hand and with the plasma membrane in the typical punctate distribution on the other hand is shown in (Figure 4.3D–F). Filopodia are also stained for reggies and so are the tips of neurites as shown in a high magnification in (Figure 4.3G–I). Neurites and growth cones are special sites for process growth, elongation and guidance. Thus, the reggies are available at important and strategic sites of N2a and neuronal cells in general. Therefore, experiments were performed to analyze the functions of the reggies in N2a cells via siRNA-mediated knockdown.

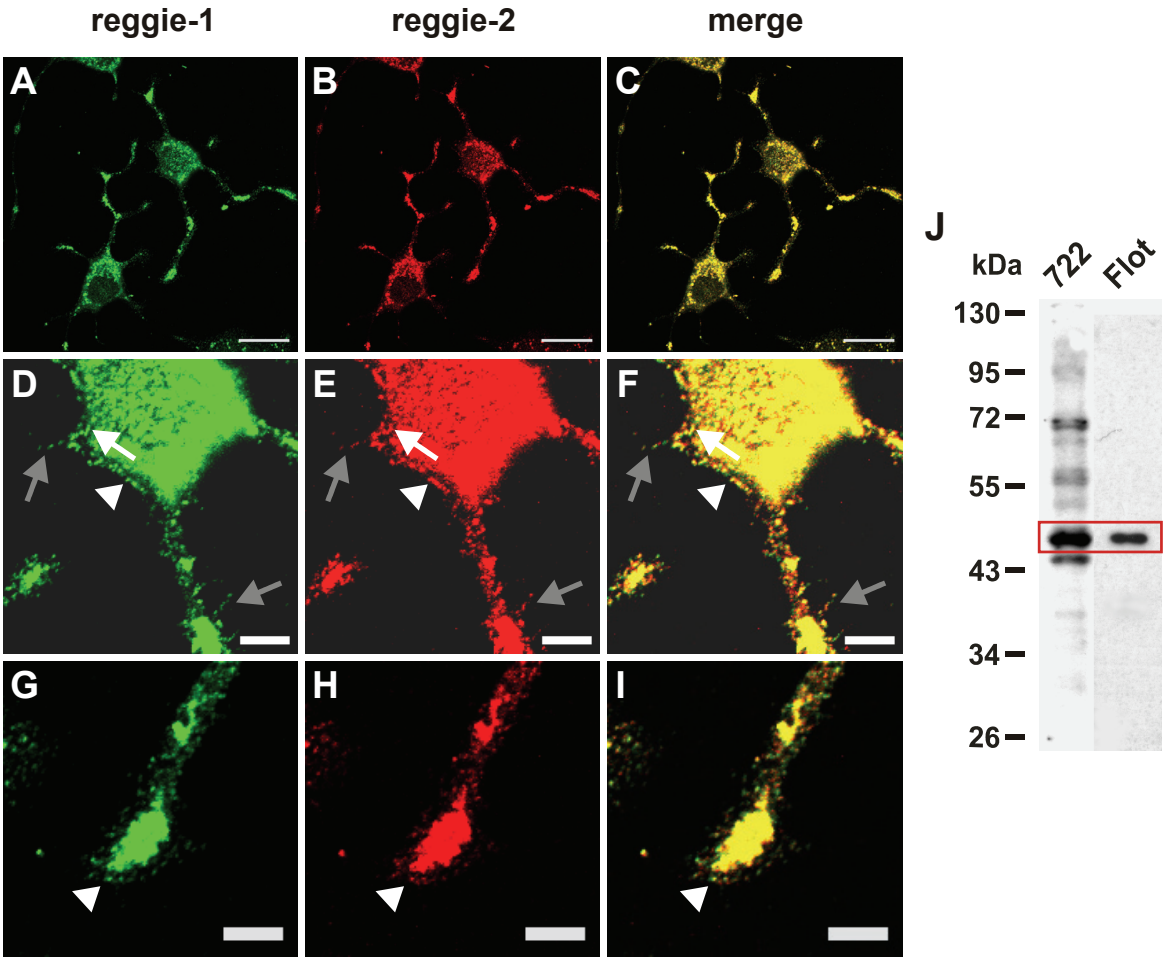

Figure 4.3: Co-localization of reggie-1 and reggie-2 in differentiated N2a cells. Confocal sections through N2a cells stimulated for 24 h with IGF-1 showed reggie-1 (green) and reggie-2 (red) staining of cell bodies and along the entire neurites including their tips/growth cones (**A, B**). Higher magnifications indicated the existence of reggie-1 (**D**) and reggie-2 (**E**) at intracellular compartments (arrow) and at the plasma membrane (arrowhead) in the typical punctate reggie pattern. Filopodia (grey arrow) at the neurite are also stained for reggie-1 and -2. Reggie was present at the tips of neurites shown in high magnifications in **G** and **H**. Overlay of both channels indicated in yellow a high co-localization of reggie-1 and -2 (**C, F, I**). **J**, Reggie-1 pAb 722 and reggie-2 mAb Flot, also used for reggie immunostainings of N2a cells, detected specific signals at 47 kDa (red box) on western blots with N2a protein lysates. However, pAb 722 also produced unspecific signals with lower intensities than the specific reggie-1 band. Scale bars **A–C**, 20 μm; scale bars **D–I**, 5 μm.

4.1.5 Silencing of reggie-1 impairs process formation in N2a cells

To determine whether reggie knockdown induces a loss-of-function phenotype, N2a cells were transfected with a mixture of three siRNAs against reggie-1 (R1mix), which leads to a loss of both reggie proteins (Figure 4.5) as shown for the single siRNAs against reggie-1 in Figure 4.2 and Figure 4.3. This siRNA mixture was used to circumvent sequence-specific off-target effects allowing low individual concentrations. Further, a reggie-1 rescue construct (R1-EGFP rescue) with mutations in the siRNA binding sites was expressed in presence of reggie-1 siRNAs. This resulted in the rescue of reggie-1 and in consequence of endogenous reggie-2, too (Figure 4.4). The rescue of reggie was later used to verify the specificity of silencing phenotypes.

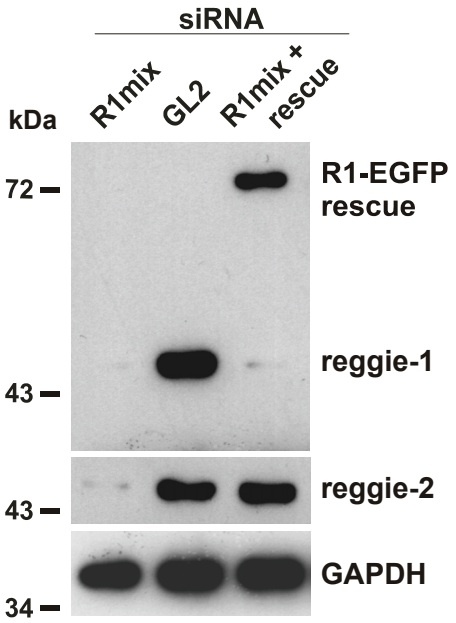

Figure 4.4: Knockdown of reggie-1 and reggie-2 protein, mediated by the reggie-1 siRNA mixture (R1mix), can be rescued by expression of a reggie-1 rescue construct (R1-EGFP rescue). N2a cells transfected with a mix of siRNAs against reggie-1 (R1mix), a non-specific siRNA GL2, and with R1mix plus R1-EGFP rescue construct (R1mix + rescue) were analyzed. R1mix led to a strong reduction of reggie-1 as well as reggie-2 protein compared with GL2 control as shown by western blotting. Additional expression of R1-EGFP rescue (75 kDa) in presence of R1mix led to the rescue upon silencing of endogenous reggie-1 and in consequence of reggie-2 protein. Protein levels of GAPDH were used as a loading control.

To follow morphological changes induced by reggie silencing, N2a cells were transfected with siRNAs/rescue constructs as indicated. One day after transfection the cells were stimulated with IGF-1 to induce neurite formation for one more day. At the second day post transfection, cells were fixed and stained with phalloidin for the actin cytoskeleton. Co-expression of EGFP alone or recombinant as a reggie-1-EGFP rescue construct allowed to discriminate between transfected and untransfected cells. N2a cells transfected with the unspecific control siRNA GL2 differentiated well after IGF-1 stimulation (Figure 4.5B) and showed neurite growth similar to untreated cells (Figure 4.6A). In contrast, loss of reggie-1 and -2 by R1mix treatment led to significantly fewer cells with neurites (74 ± 5%, n = 250

cells, Figure 4.5C, E) compared to GL2 (91 ± 2%, n = 197 cells, Figure 4.5B, E). The percentage of R1mix cells with lamellipodia (73 ± 3%, n = 211 cells, Figure 4.5C, F) was increased by 30% compared to GL2 control cells (44 ± 4%, n = 270 cells, Figure 4.5B, F). Filopodia length was changed as well: reggie-1 siRNA-treated cells showed filopodia with a mean length of 4.3 ± 0.07 μm (n = 130 cells), whereas control cells possessed filopodia with mean length of 5.6 ± 0.08 μm (n = 144 cells). However, reggie-2 downregulation alone did not change the percentage of neurite-bearing cells (85 ± 4%, n = 67) and cells with lamellipodia (42 ± 9%, n = 67 cells) significantly compared to GL2 control cells. The observed effects on the morphological changes of N2a cells in response to reggie-1 siRNA were abrogated in reggie-1 and -2 rescue experiments. R1-EGFP rescue construct was co-transfected with the R1mix. This population of cells showed numbers of neurite-bearing cells of 95 ± 3% (n = 118 cells, Figure 4.5D, E) and cells with lamellipodia of 48 ± 3%, (n = 118 cells, Figure 4.5D, F) which were not statistically different from GL2 cells (Figure 4.5D, F). These experiments demonstrate, that the reggies regulate process formation like lamellipodia formation, filopodia extension and neurite growth during differentiation of N2a cells.

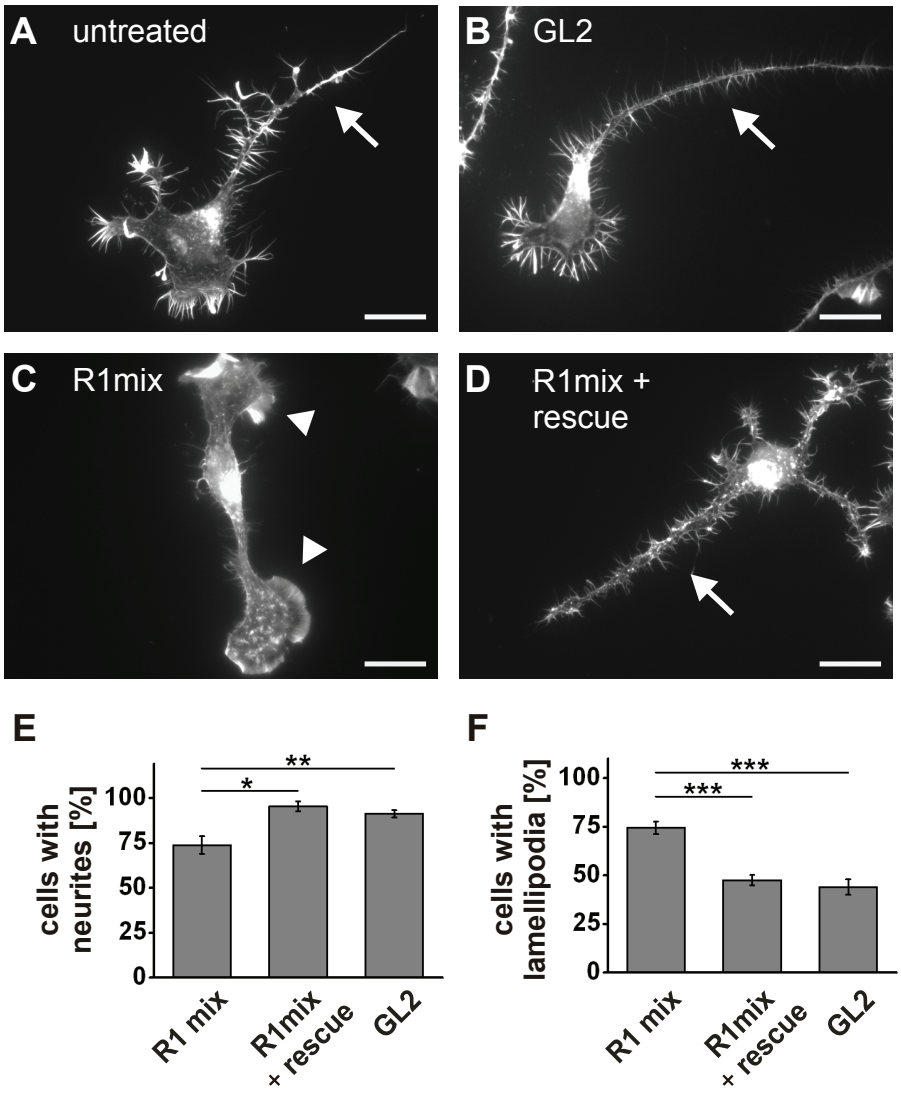

Figure 4.5: siRNA-mediated reggie-1 knockdown affects process formation in N2a cells. Cells, co-transfected with either control siRNA GL2 or a mix of reggie-1 specific siRNAs (R1mix) plus pEGFP, were stimulated with IGF-1, fixed 24 h after stimulation and stained with phalloidin to visualize F-actin and cell morphology. Untreated and GL2 siRNA-transfected cells produced numerous filopodia (**A**, **B** indicated by arrows), whereas reggie-1 downregulation (R1mix) led to the formation of large lamellipodia (**C, F**, marked by arrowheads) and significantly fewer cells formed neurites after reggie-1 siRNA transfection (**E**). **D,** Rescue of the reggie knockdown-induced N2a cell phenotype. N2a cells were simultaneously transfected with reggie-1 siRNA and R1-EGFP rescue (R1mix + rescue) without siRNA binding sites. A large proportion of the cells no longer exhibited the lamellipodia-rich phenotype (**D**, **F**) but had instead many filopodia and neurites (arrow, **D**, **E**) much as control cells (**A**, **B**). Mean ± SEM; ***$p < 0.001$, **$p < 0.01$, Student's t test. Scale bars, 20 μm.

4.1.6 Reggie-1 downregulation affects Rho GTPase activation in N2a cells

The phenotypes of reggie knockdown cells suggest an effect on cytoskeletal remodeling. Therefore, signaling pathways known to be involved in cytoskeleton dynamics, like the Rho family of small GTPases as central regulators of the cytoskeleton and neuronal development, were analyzed in reggie-silenced cells (Hall, 1998; Govek et al., 2005). To examine activation patterns of the small GTPases Rac1, cdc42 and RhoA, GST-pulldown assays were performed with minimal binding domains of effector proteins specific for the GTP-bound form of the GTPase (de Rooij and Bos, 1997) and further analyzed by western blotting and bands were quantified via densitometry. Upon IGF-1 stimulation, activation of Rac1 increased 3.8 times compared to unstimulated GL2 control cells (= control, Figure 4.6A). Reggie siRNA-treated cells showed elevated levels of activated Rac1 with and even without IGF-1, 4.7 times more than in control cells. Upon IGF-1 application, this level of Rac1-GTP further increased to 6.9 times above control (Figure 4.6A). In contrast, cdc42 activity decreased by 83% upon IGF stimulation in control cells, whereas activated cdc42 in reggie-knockdown cells remained at low levels (with or without IGF-1) which were, however, higher than in the stimulated control (Figure 4.6A). The remaining cdc42-GTP level of R1 siRNA cells was 55% and 66%, respectively, relative to the control (Figure 4.6B). RhoA activation of reggie knockdown cells was slightly enhanced by a factor of 1.5 without IGF-1 and 1.8 with IGF-1, respectively, compared to control cells. The activation of RhoA by IGF-1 in GL2 control cells was low (Figure 4.6C). Thus, knockdown of reggie leads to an imbalanced activation of small GTPases of the Rho-family. Resulting alterations in downstream signaling of Rho GTPases, for example actin polymerization and microtubule stability, would explain the phenotype of reggie-silenced N2a cells with defects in neurite formation, altered lamellipodia formation and filopodia extension (shown in section 4.1.5).

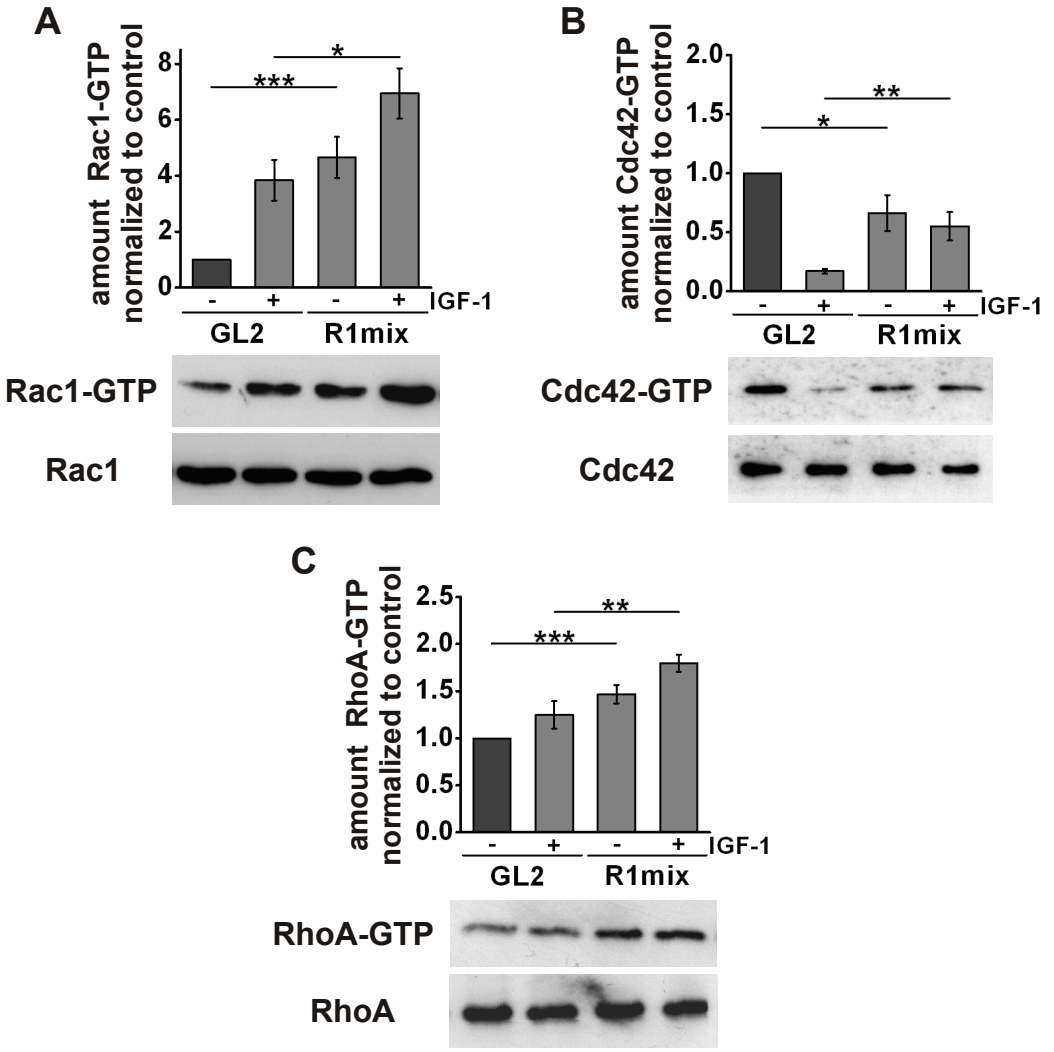

Figure 4.6: Reggies interfere with Rho GTPase activation in N2a cells. N2a cells, control (GL2) or reggie-siRNA (R1mix) transfected, were starved overnight, stimulated with 50 ng/ml IGF-1 for 5 min and assayed for GTP-loading of small GTPases. Changes in the intensity (relative to control) of protein bands were quantified by densitometry of the respective western blots (n = 4 independent experiments), which are exemplified below each histogram. Mean ± SEM; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, Student's *t* test. Total Rac, cdc42, RhoA and Ras of crude lysates were used as loading controls. **A**, **B,** Reggie-1-silenced cells exhibited significant alterations in the activation patterns of Rac1 and cdc42. IGF-1 application increased the activation of Rac1 in control-transfected cells, whereas R1mix-treated cells showed an elevated level of activated Rac1 without IGF-1 treatment which increased further after IGF-1 application. In contrast, cdc42 activity decreased upon IGF-1 application in control-transfected cells and remained at low levels in R1mix-treated cells with or without IGF-1. **C,** RhoA activation increased upon IGF-1 stimulation in control cells and further increased in reggie-1 knockdown cells.

4.1.7 Reggie silencing inhibits activation of Ras and cell growth in N2a cells

Not only the Rho GTPases Rac-1, cdc42 and RhoA, but also stimulation of Ras was observed in reggie-silenced and control cells. Ras stimulation by IGF-1 increased 1.9 times over the level of activated Ras in control transfectants. This stimulation was absent in reggie siRNA-treated cells with Ras-GTP levels of 63% without and 72% with IGF-1 stimulation, when compared to non-stimulated controls (Figure 4.7A). As Ras is known to influence cell growth (Chang et al., 2003), the behavior of reggie knockdown cells regarding cell growth was analyzed. N2a cells (5×10^4 cells per condition) were transfected and incubated for 2 days in serum to allow growth. Cell number increased by 4.8 times in the GL2 control cells, i.e. to 24.1×10^4 cells, whereas the cell number increased just by 3.2 times to 16.2×10^4 cells in reggie knockdown cells (Figure 4.7B). Accordingly, reggie silencing caused a reduction of cell growth by 33% compared to GL2 control cells. GL2 transfected cells and cells treated with Lipofectamine only showed no significant difference in cell growth. The number of dying cells assessed by trypan blue staining was also unchanged. Thus, the 37% reduction of activated Ras along with the reduction of cell growth in reggie siRNA-treated cells suggests a function of the reggies in signaling pathways regulating cell proliferation. Therefore, MAP kinases as critical regulators of cell proliferation (Katz et al., 2007) were analyzed for changes in their activation state caused by reggie knockdown in the next section 4.1.8.

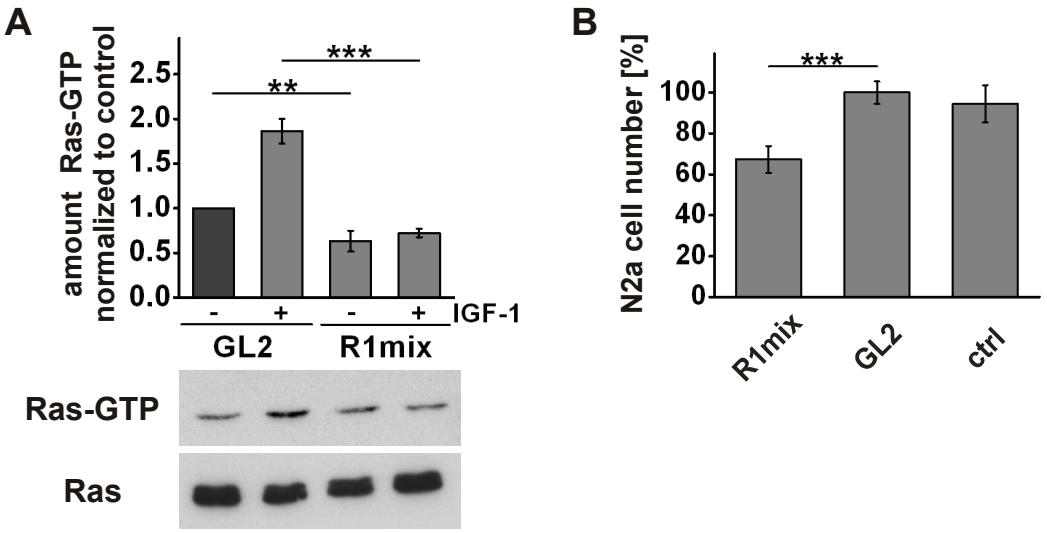

Figure 4.7: Knockdown of reggie inhibits Ras activation and cell growth of N2a cells. N2a cells were starved overnight, stimulated with 50 ng/ml IGF-1 for 5 min and assayed for GTP-loading of small GTPases. Densitometric analysis of the respective western blots (n = 4 independent experiments), which are exemplified below each histogram, were performed. Mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t* test. Total Ras of crude lysates was used as loading controls. **A**, Stimulation of Ras by IGF-1, as shown for control cells transfected with GL2 siRNA was absent in reggie siRNA (R1mix)-treated cells. Levels of Ras-GTP were amounted to 63% (basal) and 72% (stimulated) normalized to control in R1mix-treated cells. **B,** Reggie knockdown affected cell growth of N2a cells. Two days after transfection R1mix cells exhibited a reduction of 33% in cell number compared to GL2 transfected cells. No significant difference in cell number was observed between GL2 siRNA and Lipofectamine only treatment (ctrl).

4.1.8 Reggie silencing affects the phosphorylation of mitogen-activated protein kinases ERK1/2 and p38 and the focal adhesion kinase

MAP kinases are key signaling molecules for cell proliferation (Chang and Karin, 2001). As reggie silencing caused a reduction in N2a cell growth, the activation pattern of the MAP kinases ERK1/2, p38 and JNK and in addition phosphorylation of other signaling proteins like FAK, PKB and C were analyzed using phospho-specific Abs in comparison to the corresponding control situations with and with-

out IGF-1 treatment. In reggie siRNA-treated cells, MAP kinase ERK1/2 phosphorylation decreased by 25% in unstimulated conditions and by 36% when stimulated with IGF-1 (Figure 4.8A). Interestingly, activation of p38 was drastically reduced in reggie-silenced cells by 74% in unstimulated conditions and by 86% in stimulated conditions, when compared to corresponding control cells (Figure 4.8B). In addition, tyrosine phosphorylation of FAK at residues 567 and 577 was strongly reduced after reggie knockdown by 30% in non-stimulated and 39% in IGF-1 stimulated conditions (Figure 4.8C). No effects were seen on the IGF-induced phosphorylation of the MAP kinase JNK and the phosphorylation of PKB and PKC (Figure 4.8D). These results suggest, that blockage of reggie function interferes with signaling pathways regulating cell proliferation, cell substrate interaction and cell differentiation.

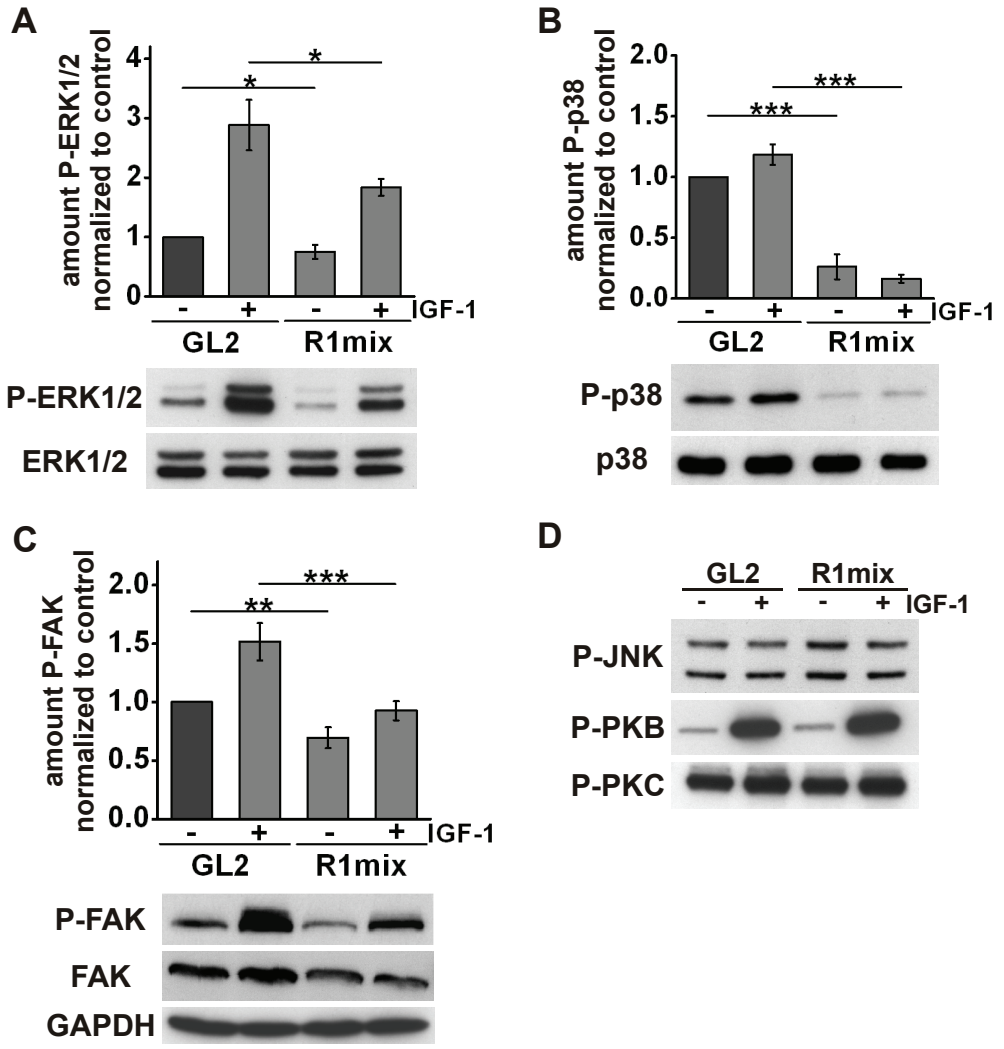

Figure 4.8: Knockdown of reggie inhibits ERK1/2, p38 and FAK phosphorylation in N2a cells. N2a cells were starved over night, stimulated with 50 ng/ml IGF-1 for 5 min and cell lysates were analyzed by western blotting with phospho-specific Abs. Densitometric analysis of the respective western blots (n = 4 independent experiments), which are exemplified below each histogram, were performed. Mean ± SEM; ***p < 0.001, **p < 0.01, *p < 0.05, Student's t test. Total ERK1/2, p38, FAK and GAPDH served as loading controls. **A,** Basal ERK1/2 activation decreased by 25% compared to control and upon IGF-1 treatment by 36% in response to reggie siRNA (R1mix) compared to stimulated GL2 control. **B,** Activation levels of p38 were reduced by 80% in R1mix-treated cells compared to control cells, independent of IGF-1 stimulation. **C,** Basal phosphorylated FAK (P-FAK) showed a significant increase upon IGF-1 stimulation and these activation levels were reduced in both conditions, basal and stimulated, by R1mix-treatment. Total FAK increased upon IGF-1 stimulation but was decreased in R1mix-treated cells. Therefore GAPDH was used as loading control. **D,** JNK, PKC and PKB phosphorylation was not affected by reggie siRNA-treatment.

4.2 Morpholino-mediated *in vivo* loss-of-function analysis of reggie-1 and -2 in the zebrafish visual system

4.2.1 Strategy of morpholino downregulation

Expression of reggies in zebrafish RGCs was downregulated *in vivo* by application of a Gelfoam piece soaked with Mos directly onto the proximal optic nerve stump after ONS (Becker et al., 2004) for retrograde Mo-transport to RGCs. Since zebrafish possesses the three reggie genes *reggie-1a*, *reggie-2a* and *-2b* (Malaga-Trillo et al., 2002), a Mo cocktail against all reggies was applied to the nerve stump on the right side. The left optic nerve stump of the same fish obtained a control Mo without target sequences in the zebrafish genome. To compare Mos and their effects another set against the three reggie genes was used with different binding sites on the reggie mRNAs. In another experiment, instead of knocking down expression of all reggie genes, single Mos were used to understand the roles of each reggie in axon growth.

Two different assays were performed: an axon outgrowth assay *in vitro* and axon regeneration assay *in vivo* (Figure 4.9). In the outgrowth assay, reggie was downregulated *in vivo* as explained above. After 4 days the retinae were prepared and chopped into squares and placed on poly-L-lysine coated cell culture plates where the RGCs regenerate their axons. In the regeneration assay, downregulation was performed as before, but now the number of regenerating axons crossing the lesion site *in vivo* was determined by retrograde tracing with A488 dextran, applied to the axons 7 d, 10 d or 14 d after Mo-application 3 mm distal from the Mo-application site. Counts of green RGCs, which had regenerated their axons across the first lesion, in control and reggie Mo-treated retinae were compared.

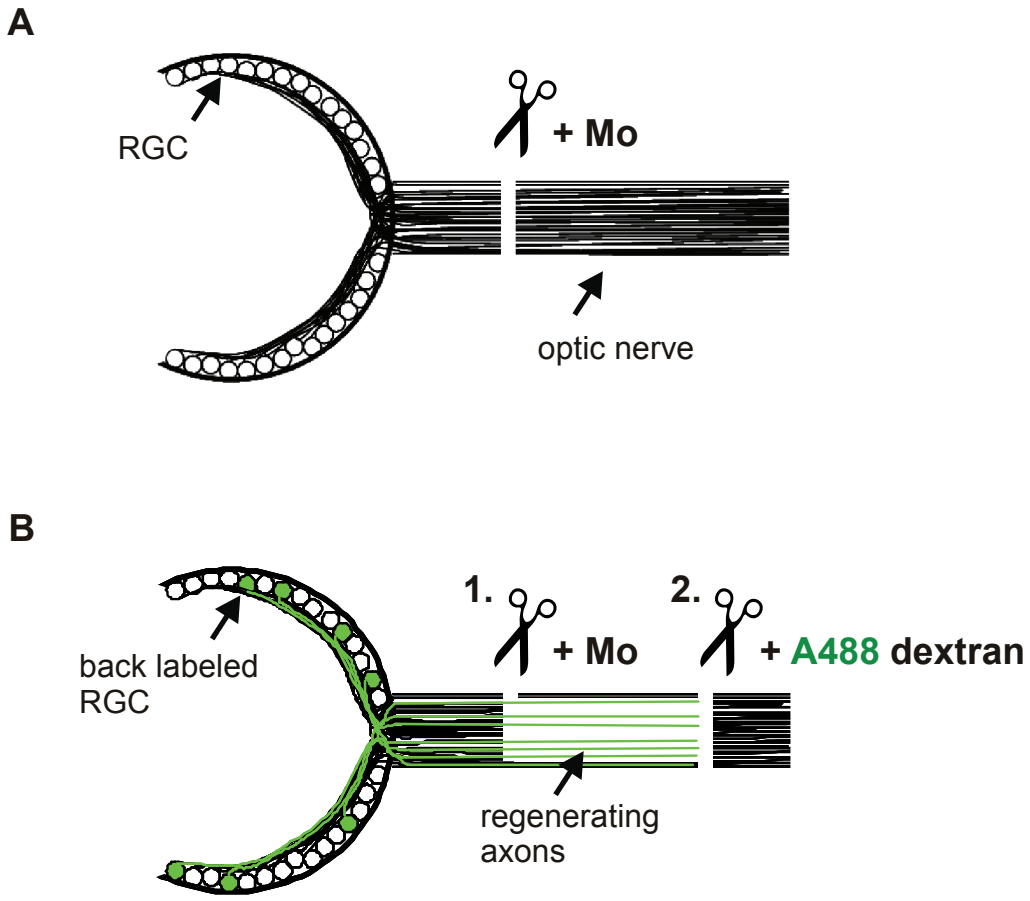

Figure 4.9: Schematic representation of the surgery conditions at the optic nerves of zebrafish. A, In the outgrowth assay the optic nerve was cut to immediately apply Mos soaked in Gelfoam. Mos are retrogradely transported by the axons into the RGCs of origin. After 4 days retinae were isolated and retina mini-explants cultivated for axon outgrowth. **B,** In the *in vivo* regeneration assay the optic nerve was cut to insert the Mos (red labeled) as in **A**. 7 to 14 days later the nerve was cut distal from the first lesion and A448 dextran was applied as described. Thus, RGCs of origin could be counted in retina whole mounts, which is a measure of the number of regenerating axons crossing the A488 dextran application site.

4.2.2 Retrograde transport of morpholinos to retinal ganglion cells leads to *in vivo* downregulation of reggies

The retrograde transport of the Mos from the application site in the optic nerve to the RGCs in the retina was verified by using a Lissamine-labeled Mo (Figure

4.10A–D). RGCs filled with the labeled Mo to different extents were counted. Labeled axons running above the RGC-layer were observed, too (Figure 4.10A). To judge approximately how many of the RGCs received Mo, quantification with Hoechst 33342 nuclear staining was performed. This DNA dye was chosen, because it is membrane permeable without permeabilization treatments, which would cause loss of Mos. Maximum intensity projections of Z-stacks of the RGC-layer were used to quantify cells labeled with Lissamine (red, Figure 4.10B) and cells with Hoechst staining (blue, Figure 4.10C). Cells with both labels appear purple in the merged image (Figure 4.10D). On average, 67 ± 7% of the cells in the RGC-layer contained both, Mo-Lissamine and Hoechst staining, 33 ± 7% showed only Hoechst staining (n = 3434 total cells analyzed from 6 regions of 2 retinae, Figure 4.10E). Retina 1 and retina 2 differed in the percentage of cells containing Mo-Lissamine (81% in retina 1 versus 52% in retina 2) and in addition, the distinct regions (a–c) on each retina altered in the percentage of Lissamine-labeled cells. Variations in the number of cells with Mo-labeling between retinae and in the intensity of Mo-labeling between RGCs were probably a result of different quality of the ONS and quantity of uptake and transport of Mos by the cut axons.

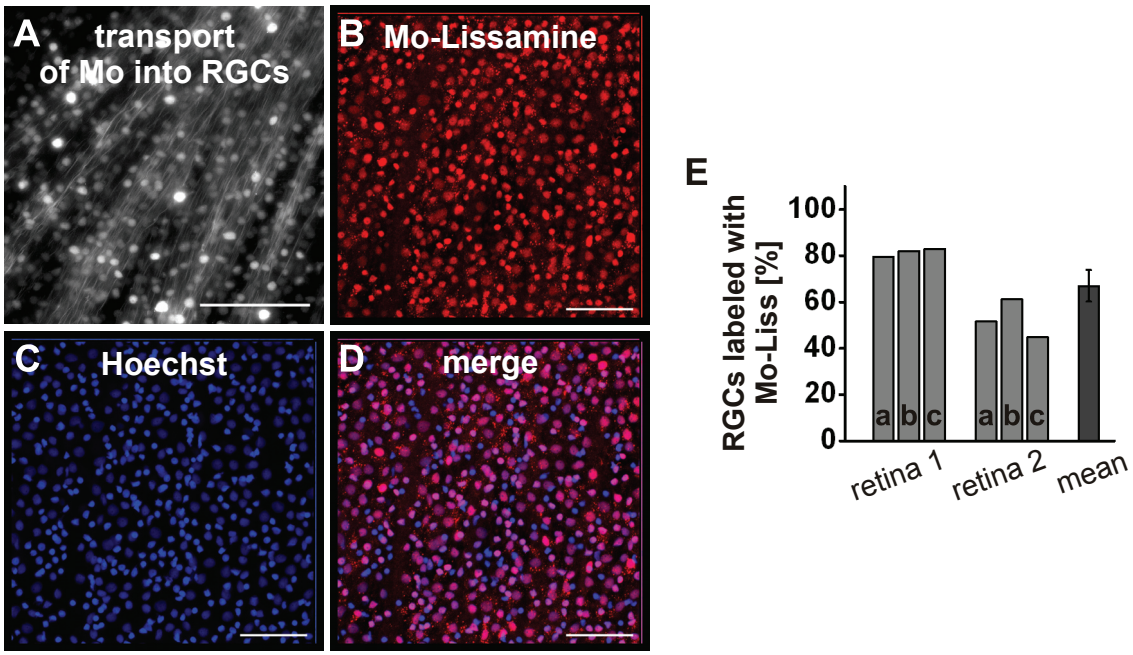

Figure 4.10: Successful retrograde transport of Mo to zebrafish RGCs. A, Mos labeled with Lissamine were applied immediately after ONS. A maximum intensity projection of a deconvoluted Z-stack from a retina whole-mount, 3 d after ONS, illustrates that RGCs and axons were labeled by retrograde Mo-transport. **B–E**, Quantification of retrograde Mo-transport. Lissamine-labeled Mos were applied as in A, retina whole mounts were stained with Hoechst 33342 and Z-stacks of the RGC-layer of different retina regions (a–c) were acquired. Lissamine-labeled RGCs (**B**), Hoechst stained nuclei (**C**) and cells with both stainings in purple (**D**) were quantified by scan^R Analysis (Olympus Soft Imaging Solutions). On average, 67 ± 7% of cells contained both stainings; n = 3434 total cells from 6 regions of 2 retinae (**E**). Retina 1 and 2 with the distinct regions (a–c) differ in the mean percentage of Mo-labeled RGCs (a–c). Mean ± SEM; scale bars, 50 µm.

To know if the transported Mo were functional and efficient enough in the RGCs, downregulation of reggie was assessed by immunostainings with Abs against reggie-1 and -2 on isolated retinae. Since all neurons (and not only RGCs) in the zebrafish retina express reggie, protein re-expression over time cannot be determined in western blots. A cocktail of Mos against all reggies was applied to the nerve stump on the right side, which caused *in vivo* downregulation of reggie-1 and -2, while application of control Mo to the left side of the same fish did not alter intensity of immunostainings (Figure 4.11A, B and not shown). 67% of fish (6 of 9)

showed downregulation of reggie expression as judged by reggie immunostainings on isolated retinae. In 33% of retinae downregulation failed probably because of inefficient Mo-uptake and/or transport.

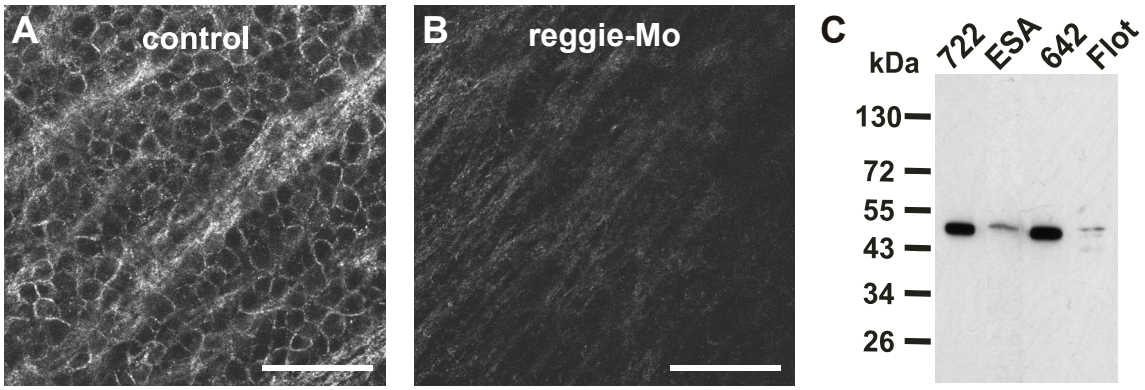

Figure 4.11: Downregulation of reggie expression *in vivo* by retrogradely transported reggie-Mos to RGCs. A, Reggie-2 immunostaining by primary mAb 642 is present in control Mo-treated retinae of zebrafish. **B**, Reggie Mos reduced reggie-2-Ab staining of RGCs 7 d after ONS (confocal images were taken with the same microscope settings). **C**, reggie-1 pAb 722 and reggie-1 mAb ESA detected both one specific band at 47 kDa much as reggie-2 mAb 642 and Flot on western blots with zebrafish retina lysates. Scale bars, 50μm.

4.2.3 Reggie downregulation impairs axon outgrowth *in vitro*

To gain insights into the consequence of reggie downregulation, axon outgrowth assays were performed. Immunostainings of re-growing axons and growth cones with a reggie pAb of retina mini-explants were performed to confirm reggie expression. As the immunostainings of N2a cells suggested, reggie-1 and reggie-2 (not shown) appeared not only in the cell body of RGCs, but all along the re-growing axons (Figure 4.12A–D). A counterstaining against neurolin was used as a RGC axon marker (Figure 4.12B). In a higher magnification of a growth cone, a dotted distribution of reggie-1 into the filopodia can be recognized (Figure 4.12D).

The retrogradely transported reggie Mos partially block reggie protein expression as shown in section 4.2.2. The potential for axon regeneration of RGCs treated with a mix of Mos against all reggies was assessed by counting axons from retina mini-explants, obtained 4 d after ONS and after 48 h in culture. Right reggie-Mo-treated and left control Mo-treated retinae of the same fish were analyzed for axon outgrowth in at least 16 mini-explants per retina. 14 retina pairs of a total of 21, showed reduced axon outgrowth from the reggie-Mo-treated retina explants, which was statistically significant in 11 retina pairs (Figure 4.12D, E). In the remaining 7 retina pairs, axon outgrowth from reggie Mo-treated explants was similar to controls. This number might correspond to the one third of retinae from separate Mo-treated fish, which showed no reggie downregulation when analyzed with reggie-specific Abs. Averaged over all 21 fish, including the ones without effects, reggie-Mo-treatment reduced the number of axons per explants significantly from 51 of control retinae to 38 of reggie-Mo-treated retinae, which corresponds to a reduction of 26.9 ± 2.3% ($p < 0.001$, Student's t test) (Figure 4.12E). This number would increase to 44 ± 2% if retinae without effects were subtracted. Control Mos did not reduce axon outgrowth compared to buffer controls, confirming that the Mo-concentrations had no unspecific effects.

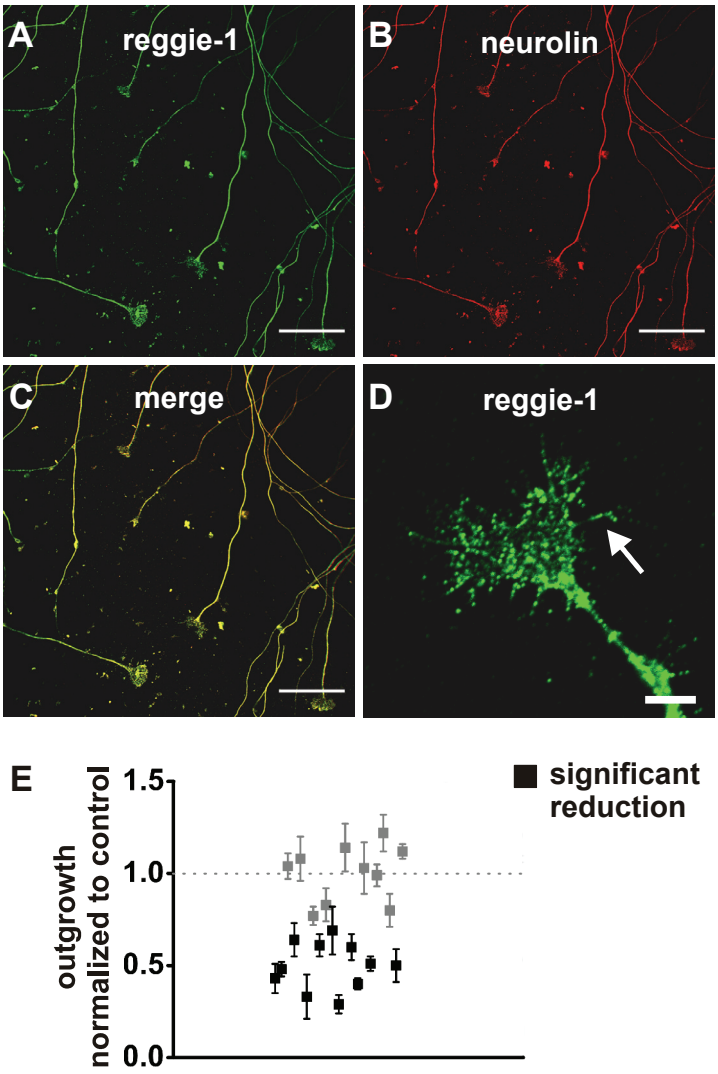

Figure 4.12: Simultaneous downregulation of reggie-1a, -2a and -2b reduces axon outgrowth from zebrafish retina mini-explants. A–D, Localization of reggie-1 in regrowing axons of retina mini-explants. RGC axons exhibited reggie-1 staining with pAb 722 along the entire axon into the growth cones (**A**) much as neurolin staining with mAb zn-5, used as a marker for regenerating axons (**B**), as visualized by an overlay in **C**. A higher magnification of a growth cone with the dotted distribution of reggie-1 staining, into its filopodia is shown in **D**. Scale bars, 50 µm in **A–C**, 5 µm in **D**. **E, F,** Axon outgrowth was assessed by quantifying axons from mini-explants cultivated for 48 h and isolated 4 d after ONS. 21 retina pairs (control- and reggie-Mo-treated eyes from the same fish) were analyzed. Relative outgrowth efficiencies for each reggie-Mo-treated retina normalized to control retina is shown in **E.** Reggie Mos reduced the mean number of axons per explants, averaged over 21 fish, by 26.9 ± 2.3% compared to control. Mean ± SEM; ***$p < 0.001$, Student's *t* test).

Instead of using a mixture of Mos, single Mos specific for each *reggie* gene were analyzed in outgrowth assays as described before. Two different Mos from two sets against each reggie were used separately to compare their effectiveness. Five out of six morpholinos displayed a reduction in axon outgrowth, however, to different extents (Figure 4.13). Reggie-1a downregulation by Mo re1a_1 caused a reduction of 11%; using a second Mo against Reggie-1a, named Mo re1a_2, an outgrowth-reduction of 15% was observed. With Mo re2a_1 against reggie-2a a reduction of 12% was achieved, while Mo re2a_2 reduced outgrowth by 52%. Knockdown of reggie-2b via Mo re2b_1 caused no reduction, whereas Mo re2b_2 reduced outgrowth by 25%. It seems that the Mos out of set 2 have a greater potential to reduce axon outgrowth, probably due to a greater effectiveness of blocking gene expression. Moreover, the Mos of Set 2 performed better with 20% non-effected retinae on average compared to 38% of Set 1. These experiments indicated an involvement of all reggies in the complex process of axon re-growth. For further *in vivo* experiments with reggie-Mos, those of set 2 were used.

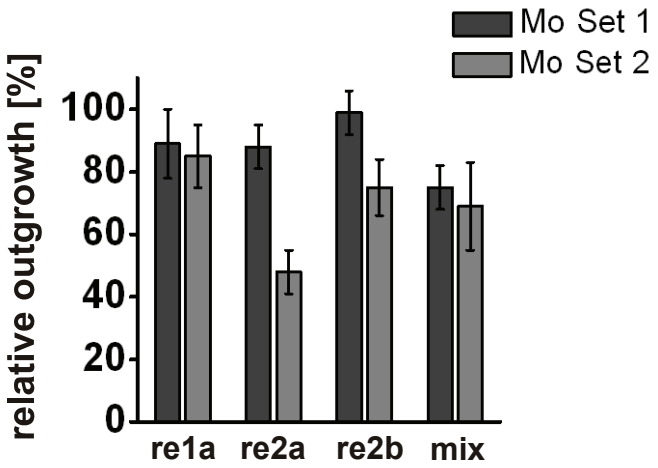

Figure 4.13: Separate downregulation of reggie1a, -2a as well as -2b reduces axon outgrowth from zebrafish retina mini-explants. Axon outgrowth was assessed by quantifying axons cultivated for 48 h from retina mini-explants isolated 4 d after ONS. Two Mos against every *reggie* gene out of two sets were compared. Relative outgrowth efficiencies for each Mo relative to the control are shown**.** Five out of six Mos caused a reduction in axon outgrowth, while Mos of Set 2 performed better. Mean ± SEM; n = 6 - 12 fish per Mo.

4.2.4 Reggie downregulation impairs axon regeneration *in vivo*

To corroborate the results of the outgrowth assays in section 4.2.3, an *in vivo* assay for regenerating axons under reggie knockdown conditions was performed. Surgery and Mo-application of the reggie Mo mix of Set 2 and A488 dextran application were performed as explained in section 4.2.1. Counts of green back-labeled RGCs with A488 dextran application at 7 d post ONS (Figure 4.14A, C) on the reggie Mo-treated side showed a significant reduction of 69% (Figure 4.14D) in comparison to the number of green cells on the control side (on average 31.4 ± 2.5 cells versus 9.8 ± 1.2 cells, $p < 0.001$, Student's t test). A488 dextran application at 10 d post ONS yielded to 17.6 ± 2.2 back-labeled RGCs on reggie Mo-treated side compared to 37.1 ± 7.0 on control side corresponding to a reduction of 52% (Figure 4.14E, G, H; $p < 0.05$, Student's t test, n = 4). However, A488 dextran application

14 d post ONS lead to a remaining reduction of 33% back-labeled RGCs of the reggie Mo-treated retinae compared to control side (Figure 4.14I,K,L, on average 43.3 ± 5.3 cells versus 64.5 ± 6.0 cells, $p < 0.05$, Student's *t* test, n = 6). Reggie downregulation affected axon regeneration *in vivo* in a time-dependent manner of A488 dextran application. Over an examined period of 14 days back-labeled RGCs increased by 36%. Contemporary with an increase in back-labeled RGCs a decrease in the amount of Mos in RGCs could be observed. It has been described before that Mo abundance in RGCs decays with time (Diekmann and Stuermer, 2009, for zebrafish neurolin) allowing protein production to resume. Therefore, decrease in fluorescence intensity of the Mo-associated Lissamine tag over retinae was evaluated at 7, 10 and 14 d after ONS, respectively. Lissamine fluorescence decreased significantly about 76% from day 7 to day 14 ($p < 0.001$, Student's *t* test, Figure 4.14 B, F, J) which correlated with the increase in regenerating axons assessed by A488 dextran application at 10 and 14 d after ONS. Thus, recovery of reggie expression due to a decreasing reggie-Mo concentration in RGCs allows progressively more RGCs to regenerate their axon. Reggie downregulation significantly impaired RGC axon regeneration *in vivo* implying reggie as an intrinsic factor for axonal regeneration affecting signal transduction pathways and cytoskeletal dynamics as shown in sections 4.1.5 - 4.1.8.

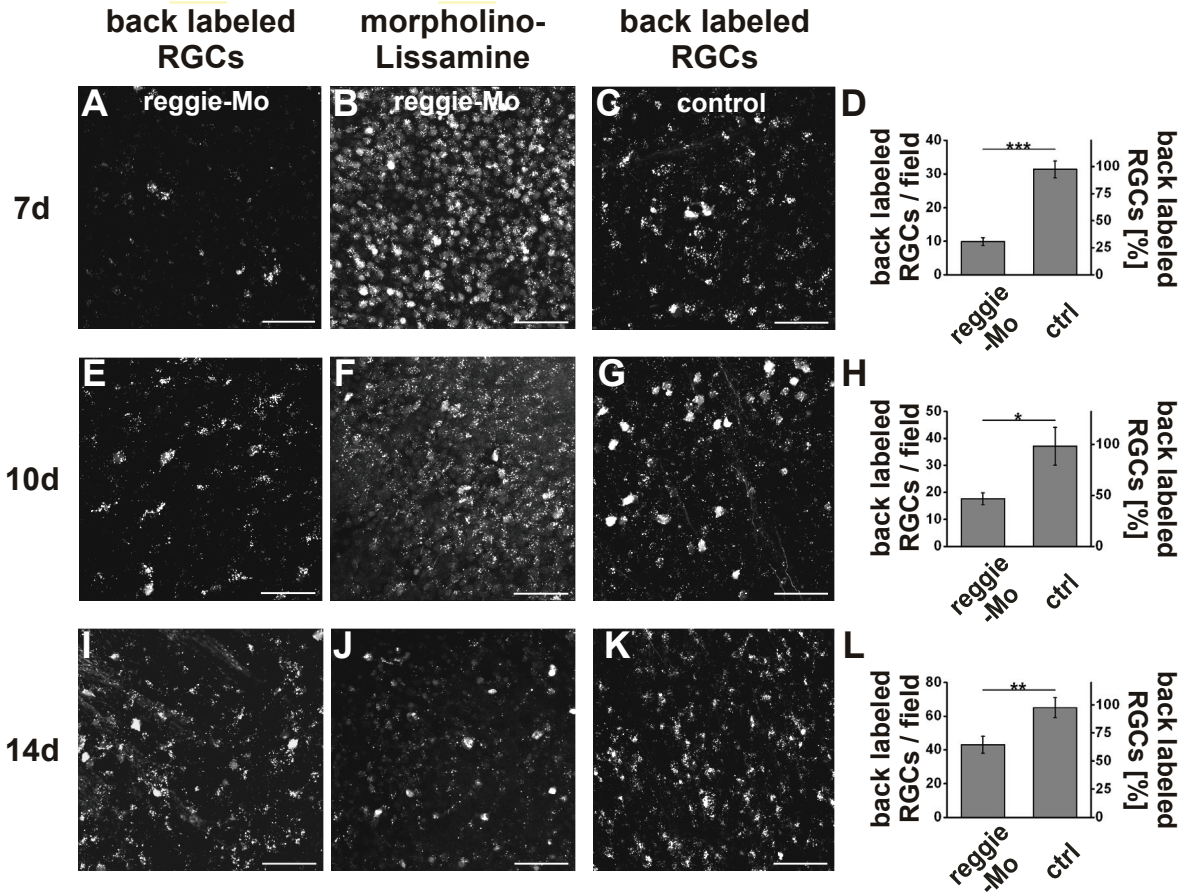

Figure 4.14: Mo-mediated downregulation of reggie in RGCs caused a reduction of regenerating axons. A mix of Mos was applied to the zebrafish optic nerves as described. A488 dextran was inserted into a second lesion site 2-3 mm distal from the first one at 7d (**A, B, C, D**), 10d (**E, F, G, H**), and 14d (**I, J, K, L**), respectively. Counts of retrogradely labeled RGCs (**A,E,I**) showed reduced numbers in the reggie Mo-receiving RGCs (**B, F, J**) as compared to the control Mo-containing RGCs (**C, G, K**): by 69% at 7d, by 52% at 10d and by 33% at 14d after ONS. The quantification of A488 back-labeled cells (with regenerating axons) compared to control retinae is shown in **D. H, L.** Mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Student's t test. Scale bars, 50 μm.

5 Discussion

The fact that the reggies proteins are upregulated in RGCs after ONS suggested that they might play a role as neuron-intrinsic factors in axon regeneration. This study showed that high level reggie expression is indeed causally linked to neurite outgrowth *in vitro* and axon regeneration *in vivo*. Downregulation of reggie impaired axon regeneration in the zebrafish visual system, affected process formation in N2a cells and – in a continuation of this study - prevented neurite extension of primary hippocampal neurons (Munderloh et al., 2009). Accordingly, reggie knockdown in N2a cells led to an imbalanced activation pattern of Rho GTPases necessary for process extension. Furthermore, p38 and FAK activation was impaired, thus connecting focal adhesion dynamics with the reggie proteins. In conclusion, reggies regulate axon regeneration and growth *in vivo* and *in vitro* by coordinating signal transduction pathways to control cytoskeletal remodeling.

5.1 Reggie downregulation impairs axon outgrowth *in vitro* and axon regeneration *in vivo*

The retrograde transport of Mos to RGCs was successful. On average, 67% of RGCs were labeled with Mos, although the percentage of labeled RGCs varied from retina to retina and over its regions. Furthermore, RGCs showed differences in fluorescence intensities which may indicate variations in Mo concentrations across RGCs. The efficacy of Mos *in vivo* was proven by reggie immunostainings of isolated retinae. 67% of retinae showed a reduction of reggie staining, so that Mos were functional *in vivo*. Thus, the 33% of retinae showing no effects on reggie-expression could reflect inefficient Mo uptake and transport due to suboptimal surgery and positioning of the Mo-soaked piece of Gelfoam.

Axon growth from reggie Mo-affected RGCs was strongly impaired: 69% inhibition of axon regeneration at 7 days post lesion *in vivo* and 30% inhibition of axon

outgrowth *in vitro*. Not all retinae subjected to reggie Mo application responded with a reduced number of outgrowing axons *in vitro*. One third of reggie Mo-treated retinae exhibited a mean number of axons per explant similar to controls, which correlates with the proportion of retinae showing no effect on reggie-expression after reggie Mo-treatment. This is attributed to technical difficulties, as explained above.

The discrepancy of axon re-growth inhibition of 30% in the *in vitro* outgrowth assays versus 69% in the *in vivo* quantification of RGCs might be explained by the different assay conditions. Axon re-growth *in vitro* takes place outside of the optic nerve and its myelin debris, glial cells and macrophages in growth-supporting media. Moreover, axons are cut again during the production of retina mini-explants close to the cell body known to stimulate axon re-growth. In contrast, axon regeneration takes place *in vivo* and axons are cut once distal to the cell body of origin. A disadvantage of applying Mos *in vivo* is their gradual inactivation in the target cells, e.g. by dilution, incorporation into vesicles or exocytosis, leading to gradual protein re-expression (Diekmann and Stuermer, 2009). By following the fluorescence intensities of the Mo-coupled lissamine-tag, the decrease of active Mo inside the RGCs was estimated to 76% over 7 days in agreement with an increase of axon regenerating RGCs. This implies a partial Mo-mediated blockage of reggie expression with time. A conceivable improvement would consist in a renewed Mo application, which would involve re-sectioning of the optic nerve and thus would not give any useful information. Application of Mos via the eye in the hope that RGCs can take it up, would be one way to try in future assays.

In conclusion, lesion-induced upregulation of the reggies is needed for the regeneration of axons since blockage of reggie expression strongly impaired axon re-growth *in vitro* and *in vivo*.

5.2 Silencing of reggie impairs process formation of N2a cells

Downregulation of reggie-1 and -2 by reggie-1 siRNAs impaired the differentiation of N2a cells into a neuronal shape resulting in fewer cells with neurites and more cells with lamellipodia. This implies that reggie proteins are important for the initiation and/or elongation of neurites. Since reggie-1 depletion leads to simultaneous loss of reggie-2, the effect of reggie-1 downregulation alone can not be measured. The dependency of reggie-2 protein on reggie-1 is further discussed in section 5.3. Downregulation of reggie-2 at least did not impair neurite formation. This speaks for a functional independency of reggie-1 in N2a cell differentiation, or the reggie-2 function can be rescued by other proteins or pathways. In contrast, each single Mo against zebrafish *reggie-1a* as well as *reggie-2a* and-*2b* showed effects on axon outgrowth *in vitro* (chapter 4) indicating that each reggie is functional in axon outgrowth of zebrafish RGCs. This divergence between N2a cells and zebrafish RGCs can be attributed to different processes between neuronal differentiation and axon re-growth and/or the different cell/tissue culture systems.

In primary hippocampal neurons, silencing of the reggies caused short and stubby protrusions instead of a long, thin axon and branched dendrites (Munderloh et al., 2009). The axon often failed to elongate and ended in bulbs, a feature recognized before in hippocampal neurons transfected with the dominant-negative R1EA construct (Langhorst et al., 2008). R1EA prevents reggie-1 and -2 from associating with plasma membrane microdomains but does not impair the protein expression of reggie-1 or reggie-2 (Langhorst et al., 2006; Langhorst et al., 2008). Impaired neuronal differentiation after siRNA-mediated reggie downregulation suggests that expression levels of reggie-1 and -2 are important for neurite extension of N2a cells and axon growth of hippocampal neurons (Munderloh et al., 2009) and RGCs of zebrafish (assessed by Mo-knockdown). The earlier experiments with R1EA indicate that a proper localization of reggie is crucial for neuronal differentiation. Formation of reggie microdomains at the plasma membrane may be a critical de-

terminant which is in agreement with the restoration of reggie microdomains in regenerating RGC axons including growth cones (this study, Lang et al., 1998).

5.3 The stability of reggie-2 depends on the presence of reggie-1: reggie-2 is degraded by the proteasome in the absence of reggie-1

That silencing of reggie-1 in N2a cells causes loss of reggie-2 protein, but not mRNA, indicates that reggie-2 protein needs the presence of reggie-1 for its stability. This kind of reggie-2 regulation was also observed in *Drosophila* null mutants of reggie-1 suggesting a conserved mechanism among metazoans (Hoehne et al., 2005). But this issue is not yet solved for reggie proteins of zebrafish and is there, because of duplicated *reggie-2* genes, more complex than in mammals or flies. The dependency of reggie-2 protein on reggie-1 is unidirectional character: reggie-1 protein is not affected by knockdown of reggie-2 in N2a cells. Furthermore, the proteasome-inhibitor lactacystin was able to block almost completely (93%) the degradation of reggie-2 in the absence of reggie-1. Intracellular proteolysis is a tool for the cell to remove abnormal proteins, to generate active protein fragments or to rapidly adjust the concentrations of short-lived or conditionally short-lived proteins depending on the state of the cell (Varshavsky, 1997). Many proteins, including SPFH proteins, are known to be stable and long-lived in complexes or oligomers, but not as free subunit/monomer (Nijtmans et al., 2002; Kasashima et al., 2006). One principle of regulated proteolysis is conserved from bacteria to mammals known as the N-end rule. This rule states, that the *in vivo* half-life of a protein is related to the nature of its N-terminal aa residue. In general, destabilizing residues are larger (Phe, Leu, Trp, Tyr, Ile) than stabilizing ones (Gly, Val, Met, Ser) (Varshavsky, 1997). In eukaryotes the N-end rule pathway is part of the ubiquitin-proteasome system so that substrates are finally degraded by the proteasome

(Varshavsky, 2000). Analyzing the primary sequence, reggie-2 contains a destabilizing amino acid phenylalanine at position 2 (and 3) after the initial methionine. The initial methionine is often excised co-translationally by methionine aminopeptidases. Proteins in which the second aa has a short side chain (such as Gly, Ala, Pro, Ser, Thr) are more likely to lose their methionine (Frottin et al., 2006). In the case of N-terminal methionine excision, reggie-2 would be a substrate for the N-end rule pathway, which would comply with the observation that reggie-2 is proteasomally degraded in the absence of reggie-1. Expression of the reggie-1 rescue construct (R1-EGFP rescue) rescued reggie-2 from degradation (see Figure 4.4), even if the expression level of R1-EGFP rescue was low (data not shown).

Interestingly, some SPFH domain containing proteins show similar dependencies. Loss of PHB-1, by knockdown and knockout experiments, leads to loss of PHB-2 on the protein level, and vice versa (Tatsuta et al., 2005; Kasashima et al., 2006). In addition, HflK and HflC of *E. coli* also showed such a bidirectional interdependency (Kihara and Ito, 1998). Hetero-oligomerization seems to stabilize the interaction partners in all these cases. But reggie-1 is stable without hetero-oligomerization, but is involved in homo-oligomerization. This raises the question, whether reggie-1 has got cellular functions without reggie-2 and if so, which function. What is the function of reggie-2 and which cellular events require hetero-oligomers of the two proteins? Since reggie-1 and reggie-2 were reported to be located separately in subcellular compartments of specific cells (Garin et al., 2001; Liu et al., 2005; Santamaria et al., 2005), it remains to be determined how the stability of reggie-2 is regulated in these cells and whether requirement of hetero-oligomerization is temporally and/or spatially regulated.

5.4 Reggie-1 downregulation affects activation of Rho GTPases and reduced N2a cell growth

The morphological abnormalities after reggie knockdown in N2a cells are evoked by defects in cytoskeletal rearrangements, which was visualized by F-actin staining. Rho GTPases are central regulators of actin dynamics and also affect the microtubules (Hall, 1998; Burridge and Wennerberg, 2004). The regulated activation and interactions of different Rho GTPases and their downstream effectors are crucial for neurite outgrowth, axon formation, axon pathfinding and dendritic spine formation (see section 1.3.2; Govek et al., 2005). The phosphorylation of actin-depolymerizing factor cofilin and the formation of the actin-regulating cortactin/N-WASP/Arp2/3 complex were shown in prolongation of this study to be impaired by reggie knockdown (Munderloh et al., 2009). Cofilin-dephosphorylation means its activation is linked to neurite outgrowth in PC12 cells (Zhang et al., 2006) and the components Arp2/3, N-WASP and cortactin are all reported to be involved in neuritogenesis (Banzai et al., 2000; Suetsugu et al., 2002; Korobova and Svitkina, 2008; Mingorance-Le Meur and O'Connor, 2009). Activation of actin-binding proteins is controlled upstream by balanced interactions of Rho GTPases (see section 1.3.2). This study showed that activation of the Rho GTPases RhoA, cdc42 and Rac1 was significantly disturbed in reggie knockdown cells: whereas RhoA and Rac1 activation was enhanced, cdc42 activity was reduced in reggie-silenced cells compared to control cells. IGF-1 stimulation increased the effects on RhoA and Rac1 but led to no significant change in the case of cdc42. It is reported that RhoA activation inhibits neurite formation (Kranenburg et al., 1997; Sebok et al., 1999; Da Silva et al., 2003; Schwamborn and Puschel, 2004), whereas cdc42 and Rac1 activation stimulates neurite outgrowth (Sarner et al., 2000; Aoki et al., 2004; Schwamborn and Puschel, 2004). Yet, Rho GTPase activities are tightly regulated in space and time with repetitive activation/inactivation cycles at motile protrusions and different locations of their peak activity (Aoki et al., 2004; Kurokawa et

al., 2005; Nakamura et al., 2005). A recent study, on the relations between local morphological changes and spatio-temporal Rho GTPase activities showed that morphological changes at the leading cell edge preceded Rac1 and cdc42 activities (Tsukada et al., 2008). This result suggests that the cell first change its shape possibly by random fluctuations and then uses localized Rac activation to sustain the morphological change. Various hypotheses were presented to account for this phenomenon, including parallel signaling pathways to regulate extensions and signaling crosstalk to regulate timing of extension and retraction (Tsukada et al., 2008). Besides local and temporal activation, the duration and amplitude of GTPase activity controlled by Rho GTPase regulators (Arakawa et al., 2003; Govek et al., 2005), convergent and divergent signaling pathways mediated by different Rho GEFs (Ng and Luo, 2004) as well as GTPase feedback loops (Arimura and Kaibuchi, 2007) are important determinants of the outcome of GTPase activity. Thus, a balanced and localized activation of Rho GTPases and a fine-tuned interplay of the Rho GTPases and/or their regulators and effectors determine successful neurite extension.

Disturbances in the activation pattern of Rho GTPases also occurred in R1EA overexpressing cells but differ from this study in so far as the two mechanisms of interference with reggie functions are different in the two systems. R1EA overexpression leads to mislocalization but not depletion of the reggies as with siRNAs. Therefore, the differences in the activation pattern of Rho GTPases are not unexpected, even though the resulting morphologies of the cells are comparable.

In addition to the Rho-family GTPases an impaired activation pattern of Ras and phosphorylation of its downstream target the MAP kinases ERK1/2 was observed after reggie knockdown. This correlates well with the observed reduction in cell growth of reggie-depleted N2a cells. In agreement, Santamaria et al., 2005, showed that reggie-2 may be required for normal cell growth in PC-3 cells which suggests that reggie-2 is required for normal N2a cell growth, too. An effect on cell growth

was not observed in R1EA treated cells as expected since reggie-2 expression levels are not affected by R1EA.

5.5 Reggie silencing affected the phosphorylation of MAP kinase p38 and FAK

Interestingly, phosphorylation of the MAP kinase p38 was drastically suppressed after reggie depletion in serum-starved or IGF-1 stimulated N2a cells. Similar effects of reggie knockdown were shown in G protein-mediated MAP kinase activation in HeLa cells (Sugawara et al., 2007). In HeLa cells, downregulation of reggie-1 but not reggie-2 resulted in an attenuation of G protein-coupled receptor (GPCR)-mediated activation of p38 providing another hint for a functional independency of reggie-1 (see section 5.2). Furthermore, p38 phosphorylation was dependent on Src family kinases and lipid raft integrity. According to Sugawara et al., 2007, reggies bind to the G protein Gαq independent of its activation state so that the authors suppose that the reggies recruit Gαq to reggie microdomains rather than controlling the GTPase cycle of Gαq (Sugawara et al., 2007). Several reports have described interactions between reggie-1 and reggie-2 with the Src-kinases Lyn, Lck and Fyn (Stuermer et al., 2001; Slaughter et al., 2003; Liu et al., 2005; Kato et al., 2006). Thus, reggie-mediated recruitment of Gαq and Src kinases to reggie microdomains seems to be crucial for p38 activation during Gαq-coupled GPCR signaling and supports the model of reggie microdomains as centers for protein complex formation and signal transduction (Stuermer et al., 2004; Stuermer and Plattner, 2005; Schrock et al., 2009). It remains to be determined whether Gαq directly or indirectly stimulates the activity of Src kinases and which member of the Src family of kinases is involved in Gαq-mediated p38 activation. Reggies seem to be important for p38 phosphorylation not only in GPCR signaling but also in serum-starved N2a cells as shown in section 4.1.8. The involvement of Src

kinases and/or adaptor proteins in reggie-dependent p38 activation remains to be clarified.

Beside the conventional tasks of p38 (stress response, inflammation, apoptosis) diverse biological functions of p38 emerged, e.g. cell differentiation, proliferation, survival and endocytosis in various cell types including neurons (Nebreda and Porras, 2000; Takeda and Ichijo, 2002). Previous studies demonstrated the involvement of p38 activation in neurite outgrowth of PC12 and neuroblastoma cells (Morooka and Nishida, 1998; Iwasaki et al., 1999; Ishii et al., 2001; Monaghan et al., 2008). Paxillin, as a substrate of p38 MAP kinase, is one possible link to focal adhesions (Huang et al., 2004). Paxillin is an adaptor protein of focal adhesions and is involved in NGF-induced neurite outgrowth of PC12 cells, possibly by regulating focal adhesion turnover/organization (Huang et al., 2004). Phosphorylation of paxillin was shown to facilitate its interaction with Pyk2, a nonreceptor tyrosine kinase of the FAK family, which is involved in neurite outgrowth and focal adhesion turnover (see below, Ivankovic-Dikic et al., 2000; Park et al., 2000; Taniyama et al., 2003). On the other hand, integrin-linked kinase (ILK) signals via the p38 pathway and is a candidate mediator of the integrin-induced neurite outgrowth (Ishii et al., 2001). Downstream signaling of p38 can regulate actin dynamics by phosphorylation of the heat shock protein 27 (HSP27), acting as an actin capping protein (Guay et al., 1997). This pathway was shown to regulate cell migration in neurons (Allen et al., 2002) and axonal growth *in vitro* (Williams et al., 2005). However, the reggie-dependent downstream signaling pathways that lead to activation of p38 and regulate neurite outgrowth remain to be elucidated.

Interestingly, silencing of reggie also disturbed activation of FAK. FAK showed a significant activation during IGF-1 stimulation of N2a cells and this stimulation was reduced in reggie knockdown cells. FAK is required to link signals from integrins (focal adhesions) via paxillin, Src kinases, PI3K, GRAF and ASAP1 to small GTPases of the Rho and Arf family and to support growth factor stimulation of the

MAP kinase pathway (Parsons, 2003). Inhibition of FAK or Pyk2 blocked neurite outgrowth in EGF-stimulated PC12 cells indicating that FAK and Pyk2 regulates neurite outgrowth by signal integration of growth factor receptor and integrin complexes in neurons (Ivankovic-Dikic et al., 2000). More recently, life time imaging of neurons revealed that impaired FAK activity led to reduced rates of neurite outgrowth due to a decreased filopodial turnover, impaired point contact formation and downstream phospho-tyrosine signaling (Robles and Gomez, 2006). FAK activity was also implied in process formation and focal adhesion dynamics in a PrP^C dependent manner (Schrock et al., 2009). The integrin- and vinculin-binding protein paxillin was found to mediate signals downstream of FAK and Pyk2 to regulate neurite outgrowth (Ivankovic-Dikic et al., 2000). Binding of the cytoskeleton-associated proteins Src and PI3K to tyrosine 397-phosphorylated FAK was crucial for growth factor- and integrin-induced neurite extension in human neuroblastoma cells (Ivankovic-Dikic et al., 2000). Tyrosine 397-dependent activation of FAK and subsequent recruitment of Src have also been implicated in tyrosine phosphorylation of additional sites of FAK, e.g. residues 576 and 577 important for downstream signaling (Owen et al., 1999).

In summary, reggies seem to control neurite outgrowth by regulating diverse signaling molecules: small GTPases of the Rho family, MAP kinase p38 and FAK, resulting in cytoskeletal remodeling.

5.6 Implications for the reggie microdomain model

This study suggests that reggie microdomains at the plasma membrane of neurons are necessary units for neurite outgrowth (N2a cells) and axon regeneration (zebrafish). It complies with the hypothesis that reggie microdomains represent centers for multiprotein complex formation and signal transduction, e.g. to the cytoskeleton (Stuermer et al., 2004; Stuermer and Plattner, 2005; Schrock et al., 2009).

The involvement of reggies in the regulation of Rho GTPases was also suggested in earlier reports. Reggies were implicated in insulin signaling in adipocytes and skeletal muscle cells by allowing the recruitment of CAP/Cbl/C3G resulting in activation of the atypical small GTPase TC10 (see also 1.1.6.1; Baumann et al., 2000; Fecchi et al., 2006). In T-Lymphocytes, reggie-dependent localization of the GEF Vav is essential for actin remodeling after T-cell activation (Langhorst et al., 2006). Interference with reggie function/localization by overexpression of R1EA strongly perturbed the balanced activation of Rac1 and cdc42 and accordingly *in vitro* differentiation of hippocampal neurons and N2a cells (Langhorst et al., 2008). Moreover, reggie-1 is able to bind F-actin (Langhorst et al., 2007) on top of being involved in regulating cytoskeletal dynamics.

Reggies are also involved in controlling p38 and FAK activity. FAK is known to integrate signals from integrin and growth factors via the SoHo domain containing adaptors vinexin, CAP and ArgBP2 (Kioka et al., 2002; Parsons, 2003; Mitsushima et al., 2004). Indeed, reggie was shown to mediate the recruitment of CAP to focal contacts (Langhorst et al., 2008) and to be involved in focal adhesion dynamics (Schrock et al., 2009). Thus, the reggies may be crucial for the integration of growth factor and focal adhesion signaling by recruiting the adaptor protein CAP. In addition, reggie-mediated recruitment of Gαq and Src kinases seems to be crucial for p38 activation during GPCR signaling.

The reggie proteins and their associated signaling pathways are one example of the importance of microdomains in coordinated signal transduction. Different scenarios of recruitment are possible.

1. “Outside in” mechanism: Upon stimulation (growth factors, extracellular matrix, GPCR substrates etc.) signaling molecules are recruited to reggie microdomains through interaction with reggie-1 and/or reggie-2 (e.g. CAP/Cbl). Signaling cascades are activated regulating, e.g. cytoskeleton dynamics, cell adhesion, cell

proliferation. Thus reggies at the plasma membrane seem to recruit proteins and to transduce signals into the cytoplasm.

2. “Inside out” mechanism: Due to a stimulus reggie(-2) vesicles loaded with a specific cargo (e.g. GLUT4) are recruited to the plasma membrane, possibly by interacting with reggie microdomains, to fulfill a function at the cell surface. Reggie recruits signaling molecules and thus acts as a scaffold protein at the vesicle to activate signals (to the cytoskeleton) necessary for the translocation to the plasma membrane, docking and/ or fusion with the plasma membrane.

Insulin signaling of skeletal muscle cells is an example for the “inside out” mechanism, where also two different microdomain proteins act in cooperation to regulate the same signaling pathway within the same cell type (Fecchi et al., 2006). In agreement with the “inside out” mechanism, Langhorst et al., 2008, showed that the reggies exhibits a regulated vesicular cycling near to the plasma membrane. During axon growth and regeneration involvement of the “outside in” and also the “inside out” mechanism is possible. Reggie downregulation was shown to impair cytoskeletal rearrangements necessary for neurite outgrowth in response to growth factor stimulation. Moreover, the localization of reggie-1 and -2 at the plasma membrane seems to be crucial for axon growth (Langhorst et al., 2008). Nevertheless, it is likely that reggie-dependent recruitment of specific cargos (e.g. growth receptors, adhesion molecules, guidance receptors) from vesicles to the plasma membrane and their selective localization in reggie microdomains is involved in axon growth. Selective recruitment of building blocks, transmembrane and extracellular secreted proteins as well as membrane components, is important for axon growth and regeneration (Goldberg, 2003).

5.7 Outlook

Although insights into in the molecular and cellular functions of reggie proteins lately increased, more work is needed to elucidate the function of the reggies. The present work contributes to understanding the role of reggies in neurite growth and axon regeneration. Still, more biochemical data is needed to clarify the reggie-associated signaling pathways contributing to neuronal differentiation and axon regeneration. Which adaptor proteins are recruited to regulate the distinct signaling pathways to control cytoskeletal reorganization, focal adhesion dynamics or cell proliferation? Which regulators and effectors of the different Rho GTPases are involved in the reggie-dependent signaling pathways? Cell-based imaging techniques that combine information of the activation state of a given target and its cellular localization will shed light on the spatial (and temporal) regulation in the observed cellular signaling pathways. Further putative candidates involved in signal transduction necessary for axon growth are GPI-anchored proteins, e.g. PrP^C, Thy1 or F3, which clusters in reggie microdomains after activation (Stuermer et al., 2001; Stuermer et al., 2004) together with Src kinases, adaptor proteins (e.g. CAP, vinexin) and cytoskeleton regulating proteins. Interference with function or association of GPI-anchored proteins and reggie microdomains might reveal their contribution to axon regeneration in future. PrP^C co-immunoprecipitates and promotes signal transduction in association with the reggie-T cell cap (see 1.1.6.1). Thy-1 was shown to associate with FAK and Src family kinases in a lipid raft-dependent manner and to modulate Src kinase and FAK phosphorylation via its GPI-anchor in fibroblasts (Rege and Hagood, 2006). In addition, Thy-1 is upregulated in fish RGCs after ONS comparable to the reggies (Schulte et al., 1997; Lang et al., 1998) and possibly contributes to axon regeneration via a reggie-associated signal transduction pathway.

The present study showed the relevance of reggie function in axon regeneration of zebrafish. Whether reggies are necessary for axon growth in mammals, too, is one

open question of great impact. One hint in this direction is the observation, that graft-assisted regeneration of rat RGC axons correlates with upregulation of *reggie* expression only in those RGCs which regenerate an axon into the graft (Lang et al., 1998). Another interesting question would be whether growth cone guidance is affected by acute inactivation of reggie in neurons possibly through chromophore/fluorophore-assisted laser inactivation CALI/FALI.

6 Literature

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